Differentiation 92 (2016) 10–23

Contents lists available at ScienceDirect

Differentiation

journal homepage: www.elsevier.com/locate/diff

Poly (ADP-ribose) polymerase1 regulates growth and multicellularity in *D. discoideum*

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ARTICLE INFO

Article history:
Received 24 November 2015
Received in revised form
9 March 2016
Accepted 15 March 2016
Available online 26 March 2016

Keywords:
ADPRT1A
Overexpression
Cell cycle
Cell death
Development
D. discoideum

ABSTRACT

Poly (ADP-ribose) polymerase (PARP)-1 regulates various biological processes like DNA repair, cell death etc. However, the role of PARP-1 in growth and differentiation still remains elusive. The present study has been undertaken to understand the role of PARP-1 in growth and development of a unicellular eukaryote, *Dictyostelium discoideum. In silico* analysis demonstrates *ADPRT1A* as the ortholog of human PARP-1 in *D. discoideum*. The present study shows that *ADPRT1A* overexpression (A OE) led to slow growth of *D. discoideum* and significant population of AOE cells were in S and G2/M phase. Also, AOE cells exhibited high endogenous PARP activity, significant NAD^+ depletion and also significantly lower ADPRT1B and ADPRT2 transcript levels. Moreover, AOE cells are intrinsically stressed and also exhibited susceptibility to oxidative stress. AOE also affected development of *D. discoideum* predominantly streaming, aggregation and formation of early culminant which are concomitant with reports on PARP's role in *D. discoideum* development. In addition, under developmental stimuli, increased PARP activity was seen along with developmentally regulated transcript levels of *ADPRT1A* during *D. discoideum* multicellularity. Thus the present study suggests that PARP-1 regulates growth as well as the developmental morphogenesis of *D. discoideum,* thereby opening new avenues to understand the same in higher eukaryotes.

1. Introduction

Poly (ADP-ribose) polymerase (PARP)-1 is a multifunctional nuclear protein belonging to the transferase family that catalyzes the formation of both linear and branched poly ADP-ribose polymers (PAR) on target proteins by utilizing NAD^+ as its substrate (Ame et al., 2004). In higher eukaryotes, PARylation is reversible through the action of PAR glycohydrolases (PARG) (Uchida et al., 1993). Poly (ADP-ribosyl) ation is a widely used post translational modification in eukaryotes and the presence of PARPs has been reported in all major eukaryotic groups (Citarelli et al., 2010; Perina et al., 2014). PARP-1 and PARylation impacts a variety of biological processes like DNA repair, transcriptional regulation, cell growth, differentiation and programmed cell death (Hottiger et al., 2010; Messner and Hottiger, 2011; Quenet et al., 2009; Mir et al., 2012). PARP-1 influences ~60–70% of genes controlling important processes like cell cycle and transcription (Chaitanya et al., 2010). The role of PARP-1 is majorly identified as NAD^+ dependent modifying enzyme that mediates important steps in DNA damage response, transcription etc., however, its role during development and differentiation is yet to be fully understood. PARP homologs have been identified in plants, metazoans, protists and filamentous fungi with the notable exception of unicellular fungi, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Citarelli et al., 2010).There are a few reports suggesting that PARP-1 regulates critical gene transcription and cellular events during development. Regulated expression of prpA (PARP ortholog) was reported for *Aspergillus nidulans* asexual development (Semighini et al., 2006). Although, PARP-1 is constitutively expressed, its enzyme activity is also reported to be developmentally regulated (Ji and Tulin, 2010). Moreover, regulated expression of PARP was found to be essential for conidiospore development (Semighini et al., 2006). Also maximum accumulation of pADPr was observed at the pre-pupal stage of Drosophila development (Kotova et al., 2009). Thus the absence of PARP in unicellular eukaryote, yeast and above reports on essentiality of PARP for development connotes plausible role of PARP in multicellularity and development.

Dictyostelium discoideum is the simplest studied eukaryote that exhibits multicellularity (Raper, 1984) and it has eight potential *parp* genes (Kawal et al., 2011). As per studies from our lab and Couto et al. (2013); out of the eight isoforms, three are reported to

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http://dx.doi.org/10.1016/j.diff.2016.03.002
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0301-4681/

be active (Rajawat et al., 2011; Couto et al., 2013). However, all eight isoforms show structural features for poly ADP-ribosylation but no studies show presence of mono ADP-ribosyltransferase activity (Fey et al., 2004; Citarelli et al., 2010). *D. discoideum,* being at the transition point from unicellular to multicellular forms, offers to be an excellent model system to study the role of PARP in growth and multicellular development. We have investigated the role of PARP in stress induced cell death in *D. discoideum* (Rajawat et al., 2007). We have also showed PARP's role in *D. discoideum* development by inhibiting its basal PARP activity with Benzamide (PARP inhibitor), delayed development was observed. We have also demonstrated that constitutive *parp* down-regulation did not affect the growth of *D. discoideum,* nevertheless its development was found to be blocked at initial aggregation stage (Rajawat et al., 2011). Delayed development due to UV-C stress was also found to be rescued in *D. discoideum* via PARP inhibitor (Mir et al., 2015). Thus, our reports suggest that PARP plays an important role in *D. discoideum* development and cell death. We are interested to further explore the role of PARP-1 in *D. discoideum* growth and development.

In the present study, we have analyzed the role of PARP-1 ortholog viz., *ADPRT1A* in growth and development of *D. discoideum. In silico* analysis of *ADPRT1A* showed that it possesses all major domains that define PARP-1. Our results substantiate that *ADPRT1A* is essential for the growth of *D. discoideum* and its regulated expression is essential for the proper cellular metabolism. We have also demonstrated *ADPRT1A* activity during *D. discoideum* development and substantiate its role in multicellularity.

2. Materials and methods

2.1. D. discoideum culturing

D. discoideum (Ax-2 strain) cells were grown in HL5 medium, pH 6.5 with 150 rpm shaking at 22 °C and also maintained on nutrient agar with *Klebsiella aerogenes* and harvested using standard procedures (Watts and Ashworth, 1970). All the experiments were carried out with the *D. discoideum* cells at mid-log phase at a cell density of 2×10^6 cells/ml with $>$ 95% viability (tested with Trypan blue exclusion).

2.2. Sequence similarity, domain and phylogenetic analysis

The *ADPRT1A* protein was aligned by Clustal W. The aligned sequences were analyzed by the maximum likelihood method (Dereeper et al., 2008). The treefile was constructed by TreeView. Domain analyses were done using Pfam and Prosite and the alignment of *ADPRT1A* from *D. discoideum* and similar PARP-1 proteins in other model organisms were made using ClustalW.

2.3. Generation of ADPRT1A overexpression construct

Full length *ADPRT1A* (3046 bp) was amplified from the genomic DNA and cloned in act15/Acg-Eyfp vector using *ADPRT1A* specific primers. Purified PCR product digested with *Sac*I and *BamH*I was inserted in to act15/Acg-Eyfp (Saran and Schaap, 2004) from which the Acg was replaced by *ADPRT1A*. The positive constructs were then transformed into *D discoideum* Ax-2 cells by electroporation and selected till 100 μg/mL G418 and were labeled as *ADPRT1A-Eyfp OE* (AEOE).

A second construct lacking EYFP (as EYFP interferes with FITC tagged antibodies used in our cell death studies) was made by restriction digestion of PCR product and act15/Acg-Eyfp with enzymes *Sac*I and *Xba*I. The positive constructs were then transformed into Ax-2 cells by electroporation and selected till 100 μg/mL of G418 and were labeled as *ADPRT1A OE* (AOE).

2.4. Functional characterization of ADPRT1A overexpression

ADPRT1A overexpression was confirmed by monitoring gene specific expression of *ADPRT1A* by Real time-PCR with *RNLA* as an internal control.

2.4.1. Cellular localization of ADPRT1A

To determine cellular localization of *ADPRT1A*, AEOE (YFP-tagged) was stained with DAPI and images were captured under 63X by Zeiss confocal laser scan fluorescence-inverted microscope (LSM 710; Carl Zeiss). Data are representative image of three independent experiments.

2.4.2. Estimation of NAD^+ levels

Intracellular levels of NAD^+ were determined by enzymatic recycling method by Bernofsky and Swan (1973) using alcohol dehydrogenase to reduce NAD^+ to NADH. NAD^+ levels were estimated at 570 nm and protein concentration was estimated by Lowry method (Lowry et al., 1951).

2.5. Growth profile analysis of ADPRT1A-Eyfp OE and ADPRT1A OE

The growth profile of AEOE and AOE were studied by inoculating mid log phase cells at the density of 0.6×10^6 cells/ml in HL5 medium. Cell viability was checked after every 2 h initially till 12 h and after 12 h interval thereafter. The cell suspension was mixed with trypan blue solution [0.4% (w/v) in Phosphate Buffer] in the ratio of 2:1 and cell count was taken using haemocytometer (Kosta et al., 2001).

Similar growth profile studies were also done in presence of Benzamide, a PARP inhibitor (1 mM) and NAC, an antioxidant (2.5 mM).

2.5.1. Cell cycle analysis

Cell cycle was analyzed by Flow cytometry using Propidium Iodide. Mid log phase cells were fixed with drop wise addition of 70% ethanol and incubation at 4 °C overnight. Fixed cells were resuspended in staining solution (TritonX-100, DNase free RNase and Propidium Iodide) and incubated for 30 min followed by FACS analysis (Chen et al., 2004). Quantification was done by flow cytometry using FACS ARIA III (BD Biosciences) and data were analyzed with FACSDiva software.

2.5.2. Induction of oxidative stress

Oxidative stress was induced in *D. discoideum* cells by exogenous addition of cumene H_2O_2 (Sigma). Log phase cells at a density of $\sim 2.5 \times 10^6$ cells/ml were exposed to paraptotic (0.03 mM) and necrotic (0.05 mM) doses of cumene H_2O_2 as described in (Rajawat et al., 2014) in HL-5 medium at 22 °C in a sterile flask.

2.6. ROS estimation (Degli Esposti, 2002)

In order to observe the formation of reactive oxygen species, a fluorescent dye 2′,7′ dichlorodihydrofluorescein diacetate (H2DCFDA-AM) was used. Oxidation of H2DCFDA by ROS converts the molecule to 2′,7′ dichlorodihydrofluorescein (DCF), which is highly fluorescent. Upon stimulation, the resultant production of ROS causes an increase in fluorescence over time.

2×10^6 cells were harvested and washed with 1X KK2 twice. DCFDA (50 nM) was added to cells and was incubated for 15 min at 22 °C with shaking, followed by two washes with 1X SB. Fluorescence was measured by fluorimeter (F7000, Hitachi, Japan) using 200 μl sample diluted 5 times using KK2 buffer. Excitation (λ_{ex})

and emission (λ_{em}) wavelengths used for fluorimetric studies were 480 and 525 nm respectively.

2.6.1. Monitoring stress induced DNA damage by immunofluorescence (Minami et al., 2005)

Phospho-Histone H2AX (S139) antibody at a concentration of 2 ug/ml (R&D systems) and anti-mouse IgG (whole molecule) TRITC conjugate (Sigma) at a dilution of 1:400 were used to study DNA damage. Cells were pelleted and washed once with phosphate buffered saline (PBS) pH 7.4, fixed in 70% chilled methanol for 10 min at −20 °C and then washed with blocking solution (1.5% BSA with 0.05% Tween 20 in PBS), incubated for 8 h in primary antibody. After incubation the cells were washed 2–3 times with blocking solution and further incubated for 1 h with TRITC labeled secondary antibody. Followed by 2 PBS washes, cells were observed for fluorescence which was monitored under 63X by Zeiss confocal laser scan fluorescence-inverted microscope (LSM 710; Carl Zeiss).

2.7. PARP activation

PARP was assayed by indirect immunofluorescence (Cole and Perez-Polo, 2002) using anti-PAR mouse mAb (10H) (Calbiochem) at a concentration of 0.5 μg/ml and anti-mouse IgG (whole molecule) FITC conjugate (Sigma) at a dilution of 1:200. Cells were observed for fluorescence which was monitored under 63X by Zeiss confocal laser scan fluorescence-inverted microscope (LSM 710; Carl Zeiss).

2.7.1. Evaluation of mitochondrial membrane potential

Potential sensitive dye $DiOC_6$ (3,3′-dihexyloxacarbocyanine iodide) (Sigma) was used to evaluate changes in mitochondrial membrane potential (MMP) (Koning et al., 1993). $\sim 2.0 \times 10^6$ cells were pelleted and washed twice with 1X SB. Cells were stained with $DiOC_6$ (400 nM) for 15 min in dark and then washed once with 1X SB and fluorescence was monitored under 63X by Zeiss confocal laser scan fluorescence-inverted microscope (LSM 710; Carl Zeiss).

MMP was also and quantitated by flow cytometry using a FACS ARIA (BD Biosciences). Data were analyzed with FACSDiva software.

2.7.2. Assessment of cell death by AnnexinV-FITC/PI dual staining

To differentiate between apoptotic and necrotic cell death, dual staining with Annexin V-FITC/PI (Miller, 2004) was performed using apoptosis detection kit (Molecular Probes). $\sim 2.0 \times 10^6$ cells were pelleted and washed twice with 1X Sorenson's buffer (SB). *D. discoideum* cells were then suspended in binding buffer provided in the kit and incubated with Annexin V for 10 min and then with PI for 5 min in dark at 22 °C. Fluorescence was monitored under 63X by Zeiss confocal laser scan fluorescence-inverted microscope (LSM 710; Carl Zeiss).

2.7.3. Development

For development, mid log phase cells were washed twice and resuspended in 1X SB at a density of 1×10^8 cells/ml. This cell suspension was spotted on 2% non-nutrient agar and incubated at 22 °C (Sussman, 1987). Images were taken every 2 h initially till 12 h and then at 12 h interval. Development was synchronized by incubating the cells at 4 °C for 4–5 h and then transferring them to 22 °C for further development.

2.7.4. Transcript analysis of ADPRT1A during development

Total RNA samples extracted from *D. discoideum* Ax- 2 cells during the growth and developmental phases were used for *ADPRT1A* transcript analysis using *ADPRT1A* specific primers by Real time PCR. Amplification of *RNLA* was carried out as an internal control. Fold change in transcript levels in developmental phases ($2^{-\Delta\Delta Ct}$) as compared to growth (vegetative) phase is shown graphically.

3. PARP activation on initiation of development

Cells were subjected to development as explained above followed by collection of cells at 0, 2, 4 and 6 h of development. PARP activity was monitored by indirect immunofluorescence (Cole and Perez-Polo, 2002) using anti-PAR mouse mAb (10H) (Calbiochem) at a concentration of 0.5 μg/ml and anti-mouse IgG (whole molecule) FITC conjugate (Sigma) at a dilution of 1:200. Cells were observed for fluorescence which was monitored under 63X by Zeiss confocal laser scan fluorescence-inverted microscope (LSM 710; Carl Zeiss). Mean density of fluorescence was plotted for quantification.

3.1. Data analysis and statistics

Flow cytometry and colorimetric assay experiments were repeated at least three times. Data were analyzed according to mean fluorescence intensity or optical density and plotted on histograms or on graphs. Statistical analysis was performed by *t* test for experiments with single comparisons.

4. Results

4.1. Sequence similarity, domain and phylogenetic analysis

ADP-ribosylation is a reversible post-translational modification that is involved in many cellular processes, including various signaling cascades, DNA repair, gene regulation and cell death (Gibson and Kraus, 2012). However, studies on its roles in growth and differentiation remain elusive. Several aspects of the life cycle of *D. discoideum* make it an attractive model to investigate the possible physiological role(s) of ADP-ribosylation in growth and differentiation. PARP-1 contributes to 80% of PARylation in cells. Thus, with this aim to identify the PARP-1 isoform in *Dictyostelium*, protein sequences of known PARPs in *Dictyostelium* were obtained from *D. discoideum* database by searching for proteins containing the PARP catalytic domain. Eight protein sequences were retrieved from dictyBase and the phylogenetic tree was constructed by maximum likelihood method to identify the isoform closest to human PARP-1. Of the eight isoforms identified, *ADPRT2*, *ADPRT1A* and *ADPRT1B* displayed maximum similarity to human PARP-1 (Fig. 1A). Further, domain-wise analysis was carried out to identify the closest ortholog of human PARP-1 in *D. discoideum* using the Pfam and Prosite. Unlike *ADPRT2* and *ADPRT1B*, the predicted domain analysis of *ADPRT1A* shows presence of a 2 zinc fingers, pADPR1 domain, BRCT domain, WGR domain, PARP regulatory domain; which are the key features of human PARP-1 (Fig. 1B). Also, multiple alignments of *ADPRT1A* with PARP-1 from *Homo sapiens, Drosophila melanogaster, Arabidopsis thaliana, Mus musculus* by Clustal W indicate that the protein encoded by *ADPRT1A* transcript has the definitive features of human PARP-1 i.e., the metal binding residues of zinc finger 1 (Eustermann et al., 2011), WGR motif, D loop residues (Wahlberg et al., 2012), K893 for poly ADP initiation (Simonin et al., 1993) and E988, the catalytic active site except a canonical caspase cleavage site (DEVD) [Fig. 1C]. *ADPRT1A* is thus identified as human PARP-1 ortholog in *D. discoideum*.

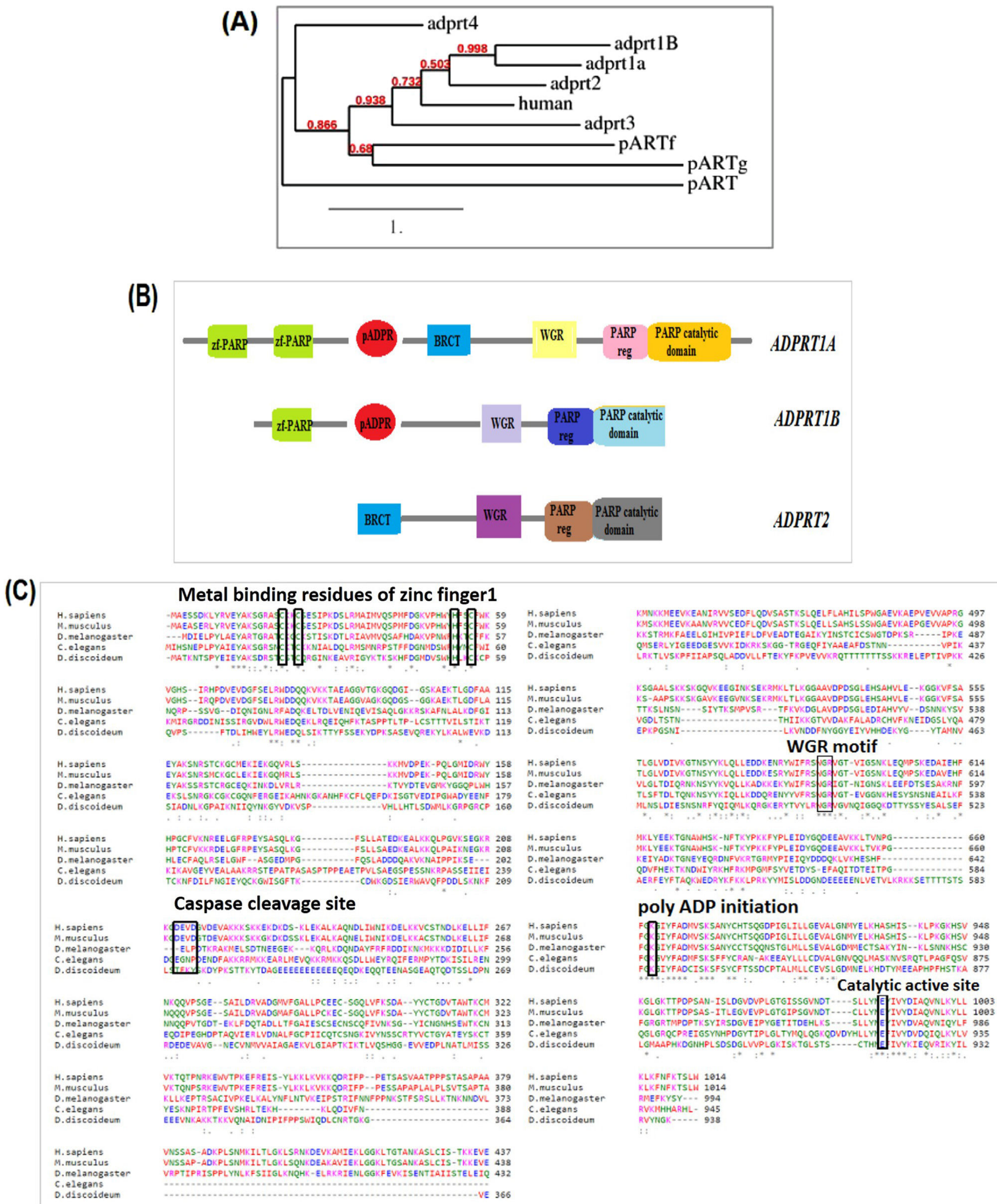

Fig. 1. *In silico* analysis of PARP1 ortholog in *D. discoideum.* (A) Phylogenetic analysis of PARP isoforms in *D. discoideum* and human PARP by maximum likelihood method. (B) Domain organization of ADPRT1A, ADPRT1B and ADPRT2 by Pfam and Prosite. ADPRT1A shows all domains like human PARP1 protein. (C) Multiple alignment of protein sequences of PARP1 protein from human and its orthologs from *M. musculus*, *D. melanogaster*, *C. elegans* and *D. discoideum* (ADPRT1A). ADPRT1A displays key features – CCHC– type zinc finger, WGR motif, poly ADP-initiation residues K893 and the catalytic active site.

4.2. Functional characterization and localization of ADPRT1A over expressing cells

In order to unravel the novel role of ADPRT1A in growth and multicellularity, we studied the effect of overexpressed *ADPRT1A* in AEOE cell lines. Semi quantitative Reverse Transcriptase PCR (Fig. 2A) showed significantly higher *ADPRT1A* transcript levels in AEOE cells which were further confirmed by qPCR studies wherein 61.10 ± 7.354 fold higher expression of *ADPRT1A* transcripts were observed in AEOE cells as compared to control (Fig. 2B). ADPRT1A

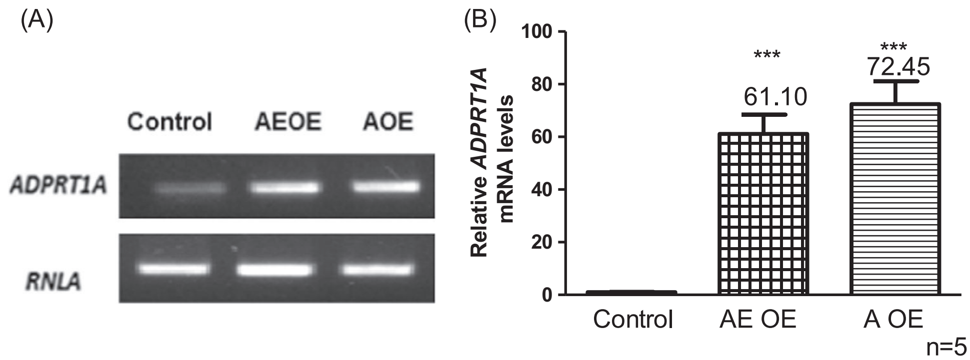

Fig. 2. Functional characterization of ADPRT1A overexpression AE OE and A OE. (A) Semi-quantitative RT-PCR of *ADPRT1A* in AE OE and AOE. AE OE and AOE exhibited increased ADPRT1A transcript levels as compared to control. (B) Real time analysis in AE OE and A OE cells showed 61.10 ± 7.354 and 72.45 ± 8.678 fold overexpression of *ADPRT1A* transcripts as compared to control. Data is a representation of SEM values of three independent experiments.

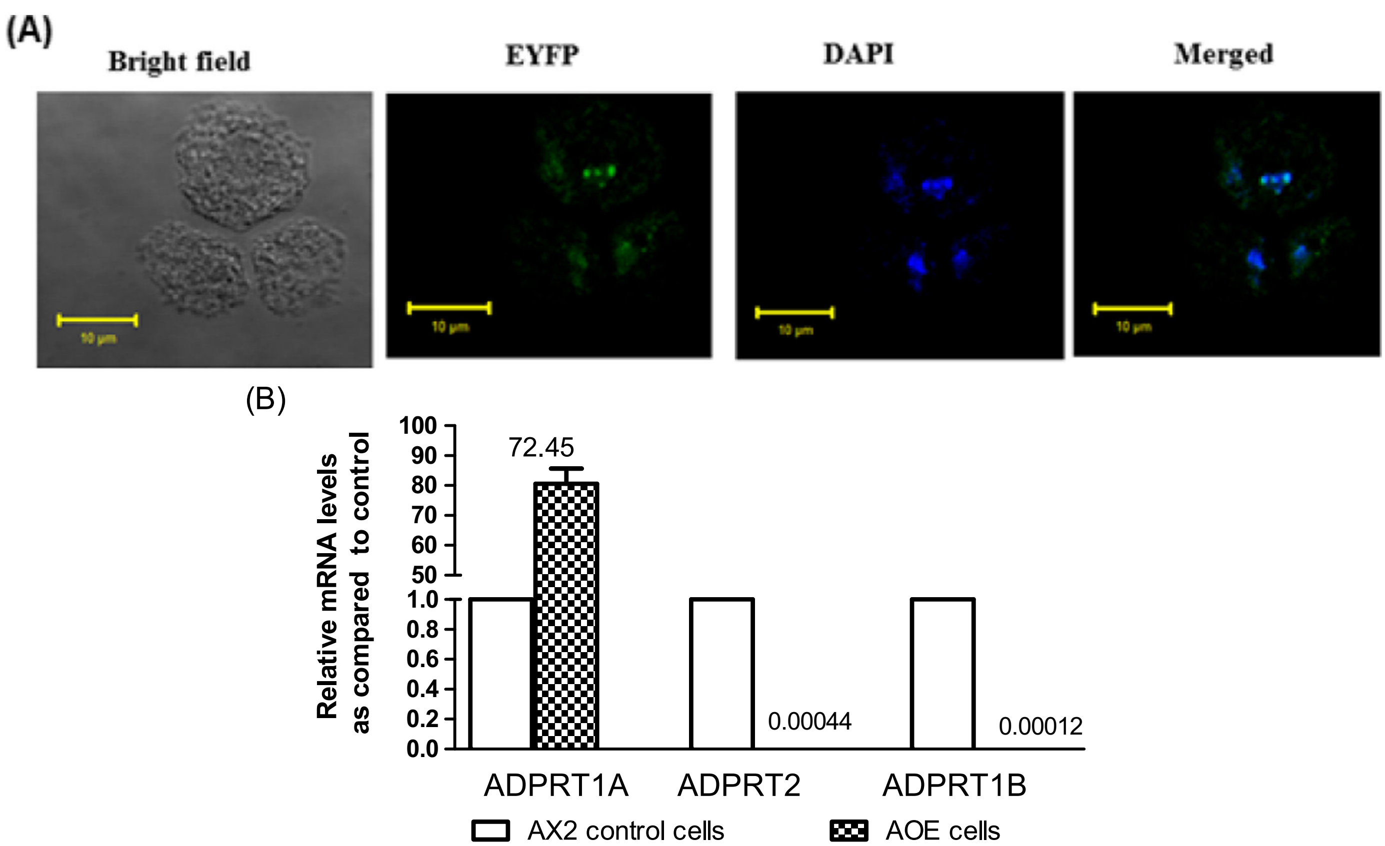

Fig. 3. Localization of ADPRT1A-EYFP in *D. discoideum* cells and transcript analysis of ADPRT isoforms in A OE. (A)Localization of ADPRT1A-EYFP in *D. discoideum* cells: EYFP merges with the DAPI stain demonstrating ADPRT1A to be a nuclear protein. Scale: 10 μm. Data are representative of three independent experiments (Photographs were taken at 63X objective). (B) Relative mRNA levels of PARP isoforms – *ADPRT1A*, *ADPRT2* and *ADPRT1B* in AOE cells as compared to their respective levels in control cells. Data is a representation of SEM values of three independent experiments.

OE (untagged- AOE) also showed 72.45 ± 8.678 fold higher *ADPRT1A* transcript levels. AOE was used for all our fluorescence based studies in order to avoid interference of YFP tag. In addition, the localization of cloned ADPRT1A was confirmed to be nuclear as YFP was colocalized with that of DAPI indicating that *ADPRT1A* is a nuclear localizing protein (Fig. 3A). As per *in silico* analysis, ADPRT2 and 1B also show homology to human PARP-1. Hence, to ensure that only ADPRT1A was overexpressed, ADPRT2 and 1B transcript levels were compared to their respective levels in control cells. Fig. 3B shows that ADPRT1A transcript levels are 72.45 fold higher in AOE cells as compared to control cells. On the contrary, there is significant decrease in ADPRT2 and 1B transcripts in AOE cells as compared to their respective expression in control cells. Thus, re-establishing that only ADPRT1A is overexpressed.

4.3. NAD^+ estimation in ADPRT1A over expression cell line

Further, it was essential to confirm the activity of the over-expressed ADPRT1A. Activation of PARP can lead to depletion of cellular NAD^+ pools which it uses as its substrate (Szabó and Dawson, 1998). Interestingly, the overexpressing cells show 60% reduction in NAD^+ levels in AEOE as well as AOE as compared to control cells, confirming the ADPRT-1 activation in ADPRT1A overexpressing *D. discoideum* cells. (Fig. 4).

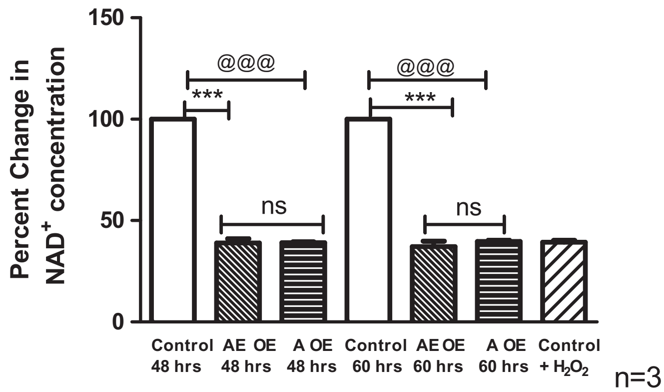

Fig. 4. Percent change of NAD$^+$ concentration in control and AE OE and AOE cells using Bernofsky's Enzymatic recycling method. Data are representative of three independent experiments. ***p value for AE OE < 0.001 as compared to control, @@@p value for AOE < 0.001 as compared to control.

4.4. *Effect of ADPRT1A overexpression on growth and cell cycle of D. discoideum*

Followed by NAD$^+$ estimation, the effect of *ADPRT1A* overexpression on cellular proliferation of *D. discoideum* cells was studied. AEOE *D. discoideum* cells were significantly slow growing with doubling time of approximately 18.48 ± 1.65 h while AOE cells showed a doubling time of approximately 17.84 ± 1.45 h (Fig. 5A) while control cells and EYFP vector control cells divided at approximately every 12.41 ± 0.5166 h and 12.89 ± 1.126 h respectively. Thus, ADPRT1A overexpression significantly reduced cellular proliferation in *D. discoideum* cells.

Since PARP-1 is known to be involved in regulation of cell-cycle (Yang et al., 2013), we studied its effects on cell growth and cell cycle using Propidium Iodide staining. We found that AEOE *D. discoideum* cells exhibited predominant population of cells in S and G2/M phases as compared to population of cells in G2/M phase in control cells at 48 h and 60 h (log-phase cells) respectively (Fig. 5B). Similar growth pattern and cell cycle profile was exhibited by AOE *D. discoideum* cells. These results suggest that PARP-1 homeostasis is indispensable for proper cell-cycle regulation and maintenance.

Since the redox status of the cell, influences various cellular activities including growth and cell death, the decreased cellular proliferation in AOE cells may be a consequence of ROS generation (Suzuki et al., 1997). Our studies showed higher endogenous ROS in AOE cells as compared to that of control (Fig. 6A). Interestingly we found that presence of Benzamide, a PARP inhibitor, alone rescued the delay in growth of AOE cells while N-Acetyl Cysteine

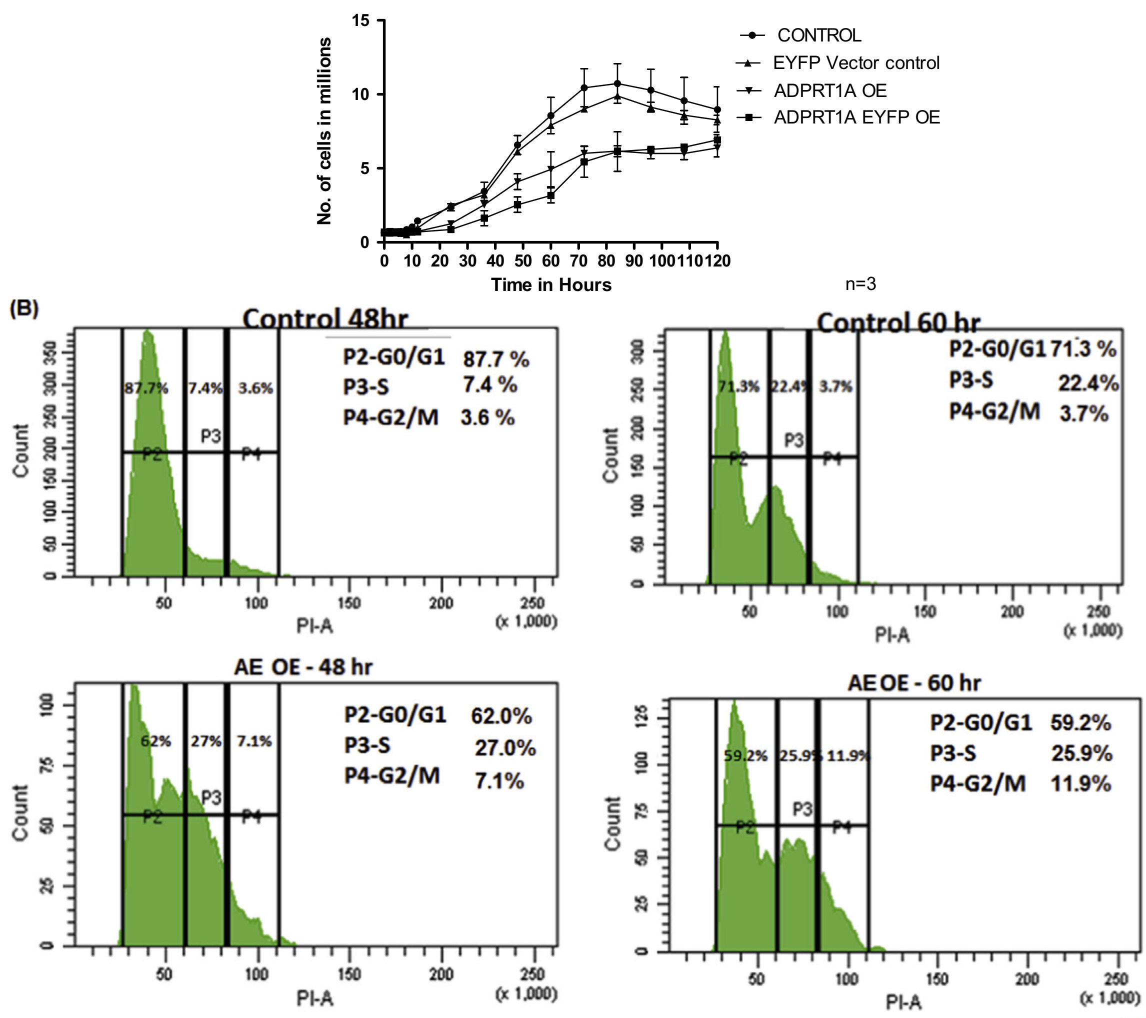

Fig. 5. Growth analysis of Control, AE OE , AOE and EYFP vector control cells. (A) Doubling Time in exponential phase for control cells was 12.41 ± 0.5166 h, EYFP vector control cells showed 12.89 ± 1.126 h while that of AE OE cells and A OE were 18.48 ± 1.65 h and 17.84 ± 1.45 h respectively. Data are representative of three independent experiments. (B) Analysis of cells cycle by FACS using Propidium Iodide. AE OE cells show marked population of cells in S and G2/M phase as compared to control, both at 48 and 60 h of growth. Data are representative of three independent experiments.

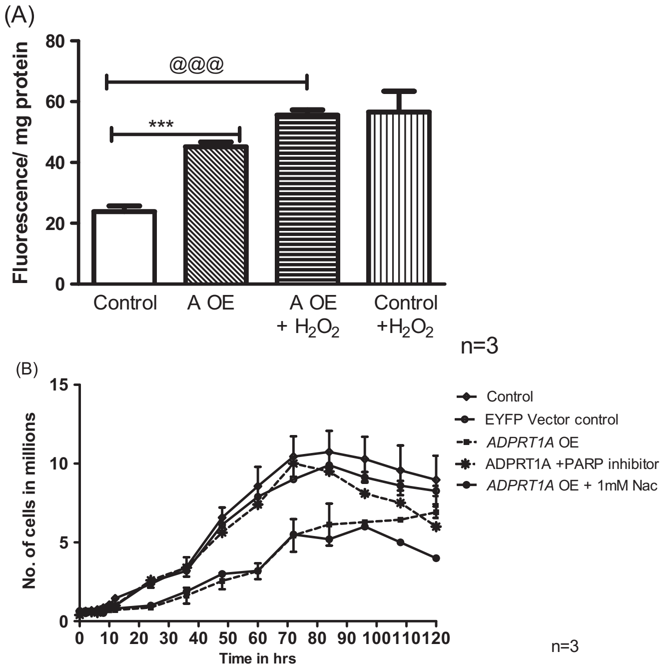

Fig. 6. ROS and PARP inhibition in AOE. (A) Fluorimetric analysis of ROS levels using DCFDA dye in control and AOE cells. AOE shows significantly higher ROS levels as compared to control. Data are representative of three independent experiments. ***p value for AOE <0.001 as compared to control, @@@p value for A OE+H_2O_2<0.001 as compared to control. (B) Rescue in slow growth of AOE on treatment with PARP inhibitor benzamide as compared to untreated AOE and control cells. However no change was seen in growth when AOE were treated with antioxidant N-acetyl cysteine NAC as compared to untreated AOE and control cells. Data are representative of three independent experiments.

(NAc), an antioxidant did not have any significant effect on growth of AOE cells, thus indicating that decrease in NAD^+ levels or the accumulation of poly-ADP-ribose product could account for the phenotypes observed in *ADPRT1A* overexpressing cells (Fig. 6B).

4.5. Effect of ADPRT1A overexpression on cell death of D. discoideum

Depending on the signal, PARP-1 either favors cell survival or triggers cell death during extensive DNA damage, thus deciding cell fate. We were interested to study the effect of overexpressed ADPRT1A on cell survival during oxidative stress.

4.6. ROS estimation and DNA damage

ROS generation causes DNA lesions; the most abundant being base modification and phosphorylation of gamma H2AX protein (Minami et al., 2005). ROS levels in AOE cells peaked within 10 min after 0.03 mM H_2O_2 treatment (Fig. 7A) while in control cells the peak was obtained at 30 min. The effect of ROS on DNA damage in treated and untreated AOE cells was confirmed using pH2AX (Fig. 7B). AOE cells treated with 0.03 mM H_2O_2 showed significant DNA damage as compared to untreated control. However, it was lesser as compared to control cells treated with similar H2O2 dose.

4.7. PARP activation under oxidative stress

Subsequent to ROS estimation, PARP activation was checked after 0.03 mM H_2O_2 treatment at 2 min, 5 min and 10 min in AOE cells as well as control cells. AOE cells showed higher basal levels of poly ADP-ribosylation activity (Fig. 7C) as compared to control without any oxidant treatment. On subjecting cells to oxidative stress, the significant increase in fluorescence indicating PARP activation was seen within 5 min (Fig. 7C). However, in control cells highest PARP activation signals were observed at 10 min (Fig. 7C). It was thus observed that endogenous PAR levels were higher in AOE cells. Moreover, under oxidative stress AOE cells showed faster PARP activation as opposed to control cells.

4.8. Mitochondrial membrane potential

Mitochondrial membrane potential (MMP) loss is a characteristic feature of cell death and as PARP-1 has a major role to play in cell death, overexpression of *ADPRT1A* was hypothesized to change the MMP in *D. discoideum* cells. MMP loss was monitored using $DiOC_6$ dye after subjecting AOE cells to oxidative stress of 0.03 mM H_2O_2 dose after 3 h and 5 h. AOE cells showed marked decrease in fluorescence within 3 h (Fig. 8A) suggesting significant loss of mitochondrial membrane potential while in control cells loss of MMP was observed at 5 h of 0.03 mM H_2O_2 dose as opposed to PARP inhibitor treated cells wherein there was no significant loss of mitochondrial membrane potential even at 5 h.

MMP changes were also quantified using $DiOC_6$ dye by FACS. Population of cells showing MMP loss post 3 h of 0.03 mM H_2O_2 dose in AOE cells were observed to be ~81% (P1+P2), out of which 40.1% (P1: lowest fluorescence) cells (Fig. 8B) had very less fluorescence intensity suggesting significant mitochondrial membrane potential loss. Control cells after 3 h of 0.03 mM H_2O_2 dose did not show complete mitochondrial membrane potential loss while PARP inhibitor treated control showed no significant loss in MMP.

4.9. Annexin V FITC-PI

PARP-1 on over activation causes severe NAD^+ and ATP depletion resulting in energy crisis and thus results in necrotic cell death (Edinger and Thompson, 2004). However moderate PARP-1 activation favors programmed cell death or apoptosis. Above cell death parameters illustrated ADPRT1A OE cells to demonstrate an oxidative stress sensitive phenotype. Nevertheless, mode of cell death under ADPRT1A overexpression background remains to be studied. AOE and control cells after 0.03 mM H_2O_2 treatment exhibited both Annexin V-FITC and Propidium Iodide (PI) staining, however, AOE cells showed early Annexin V-FITC staining (due to early exposure of phosphatidylserine) as well as early PI staining (1 h and 3 h respectively) (Fig. 9). Control cells, on the other hand, showed Annexin V-FITC and PI staining at 3 h and 12 h respectively (Fig. 9). Together, these results indicate that AOE cells are susceptible to oxidative stress as 0.03 mM H_2O_2 dose led to paraptotic cell death in control cells nevertheless, AOE cells exhibited necrotic cell death at the same dose.

4.10. Effect of ADPRT1A overexpression on development of D. discoideum

Morphogenesis during developmental program involves large scale changes in gene expression. PARP-1 is reported to regulate gene expression via transcriptional control thereby controlling growth and differentiation (Ji and Tulin, 2010). To pin down the role of *ADPRT1A* during *D. discoideum* development, control and AOE cells were subjected to starvation and then developmental morphogenesis was studied. Interestingly, AOE cells showed delay at aggregation stage. Streaming started in these cells at around 8 h thereby forming loose aggregates at 10 h and tight aggregates at 14 h, tipped aggregates at 18 h followed by slug formation at 22 h. However, AOE cells remained in the slug stage until 28 h and also showed much delay in fruiting body formation at 38 h as opposed

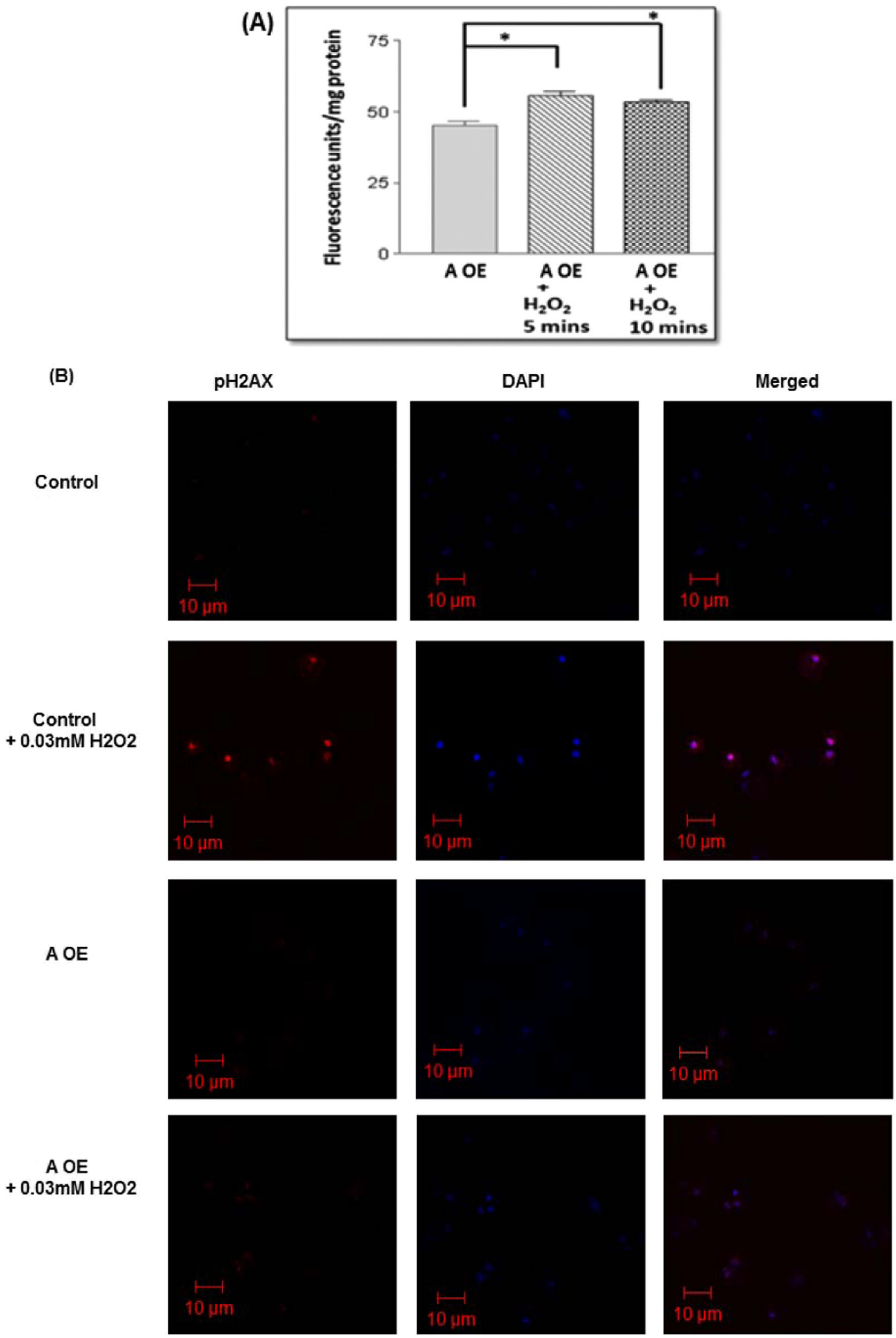

Fig. 7. ROS estimation, DNA damage and PARP activation post 0.03 mM cumene H_2O_2 treatment in control and ADPRT1A OE (A OE). (A) Fluorimetric analysis of ROS levels using DCFDA dye in control and ADPRT1A OE (A^{OE}) cells. Data are representative of three independent experiments. **p* value < 0.05 as compared to control. (B) DNA damage was observed in control and A OE 5 min post 0.03 mM cumene H_2O_2 stress by immunofluorescence using antibody against H2AX. Data are representative of three independent experiments. Scale: 10 μm. (C) PARP activation post 0.03 mM cumene H_2O_2 treatment using anti-PAR antibodies in control, PARP inhibitor control and A OE cells by confocal microscopy at 63X magnification. Peak PARP activity was observed at 10 min post 0.03 mM cumene H_2O_2 stress in control cells as opposed to 5 min in A OE cells. Data are representative of three independent experiments. Scale: 10 μm.

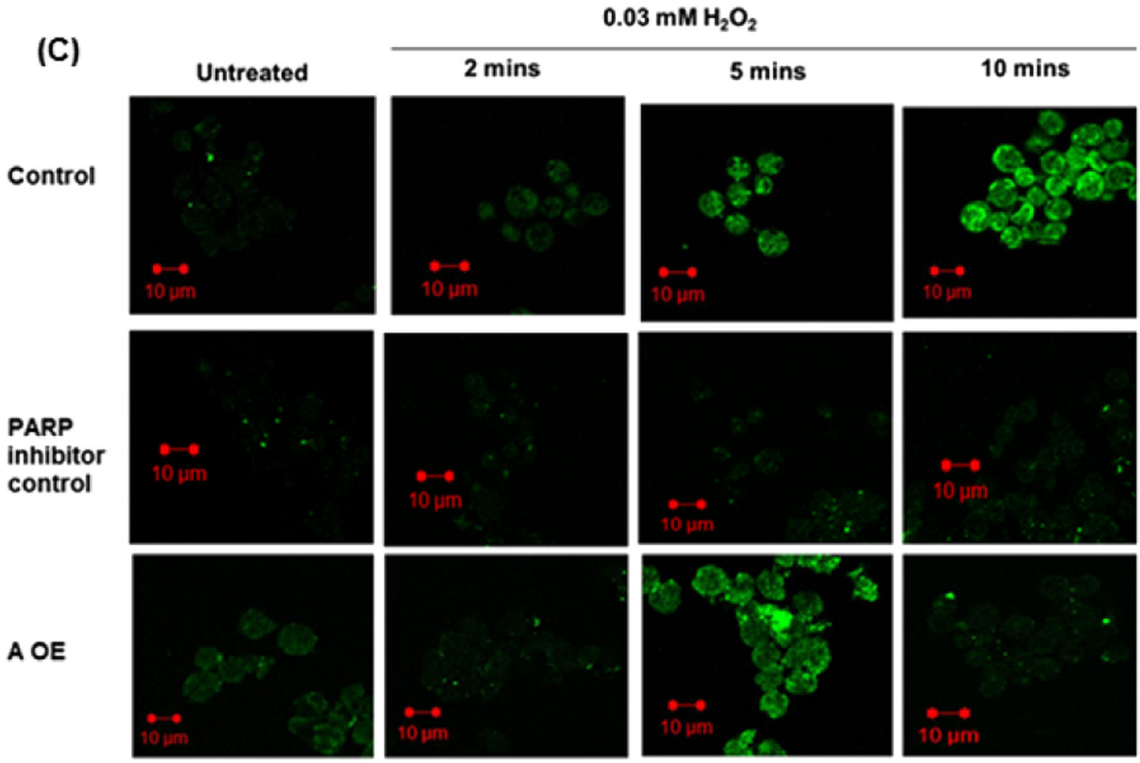

Fig. 7. (*continued*)

to 24 h in control cells (Fig. 10A). Thus the aggregation stage seems to be affected in *ADPRT1A* OE cells, hence these cells required prolonged time to form the culminants.

4.11. ADPRT1A transcript levels and PARP activation during D. discoideum development

Additionally, to understand whether ADPRT1A is developmentally regulated in *D. discoideum,* the expression pattern of *ADPRT1A* during the growth and developmental phases were monitored by Real Time PCR. Interestingly, *ADPRT1A* transcript levels were highest at the loose aggregate stage followed by significant reduction at the tight aggregate stage after which an increase was seen at the migrating slug stage. The fruiting body of *D. discoideum* showed the lowest transcript levels of *ADPRT1A* (Fig. 10B). However, during development there is a significant decrease in *ADPRT1A* mRNA levels as compared to vegetative stage. Although the expression was seen to drop at the onset of development, PARP activity was found to be significantly higher in cells subjected to nutrient starvation from 2 h till 6 h than the vegetative stage implicating a strong role of PARP during developmental morphogenesis of *Dictyostelium* and specifically during aggregation (Fig. 10C). Also, these results are consistent with the above results wherein aggregation stage seems to be affected more due to ADPRT1A overexpression. These results along with existing literature suggest ADPRT1A homeostasis may be essential for the aggregation process in *Dictyostelium.*

In conclusion, the above results illustrate a definite role of *ADPRT1A* in *D. discoideum* growth and multicellularity.

5. Discussion

PARP-1 is an abundant and ubiquitous nuclear enzyme involved in many diverse functions like DNA repair, chromatin modulation, transcription regulation, cell death etc. In addition, recent reports suggest a novel function of PARP-1 in multicellularity and differentiation as well. PARP deletion mutants in Drosophila develop only up to larval stages due to defects in chromatin remodeling and regulation of gene expression (Tulin and Allan, 2003), suggesting that poly (ADP-ribosyl)ation is essential for normal development. In addition, overexpression of *PrpA* leads to increased spore production and fluffy colonies largely consisting of aerial hyphae, reflecting PARP's role in regulating the expression and/or activity of proteins essential in its development (Semighini et al., 2006). Interestingly, our earlier reports are also suggestive of a role of PARP-1 in *D. discoideum* multicellularity since downregulation of PARP using antisense against catalytic domain resulted in stalled development at aggregate stage of *D. discoideum* (Rajawat et al., 2007). Thus, an understanding of how *ADPRT1A*, an ortholog of human PARP-1 in *D. discoideum*, functions in growth and multicellularity of *D. discoideum* would help us identify some of its unknown functions that still remain obscure.

The *Dictyostelium* genome encodes eight PARP proteins (Kawal et al., 2011). The *in silico* analysis by phylogenetic tree construction and domain analysis identified ADPRT1A as the PARP-1 ortholog in *D. discoideum*. This analysis is in accordance with *ADPRT1A* domains defined by Couto et al. (2011) using InterProScan. In addition, multiple alignment studies clearly confirm that *D. discoideum* may be a suitable model system to study PARP proteins (Fig. 1C).

It is already known that PARP activation results in drop of NAD^+ levels upon excessive DNA damage (Berger, 1985) and hence triggering NAD^+ turnover (Houtkooper et al., 2010). Similar results were obtained in ADPRT1A overexpressed cells wherein there was significant NAD^+ depletion (Fig. 4). This suggests that PARP is indeed activated in ADPRT1A overexpressed cells. This result is consistent with our PARP activation results where basal level of PARP activation was higher as compared to control cells (Fig. 7A). Higher basal levels of PAR were observed during hPARP overexpression in transfected hamster cell lines thus indicating PARP activation (Van Gool et al., 1997). Also, it was seen that

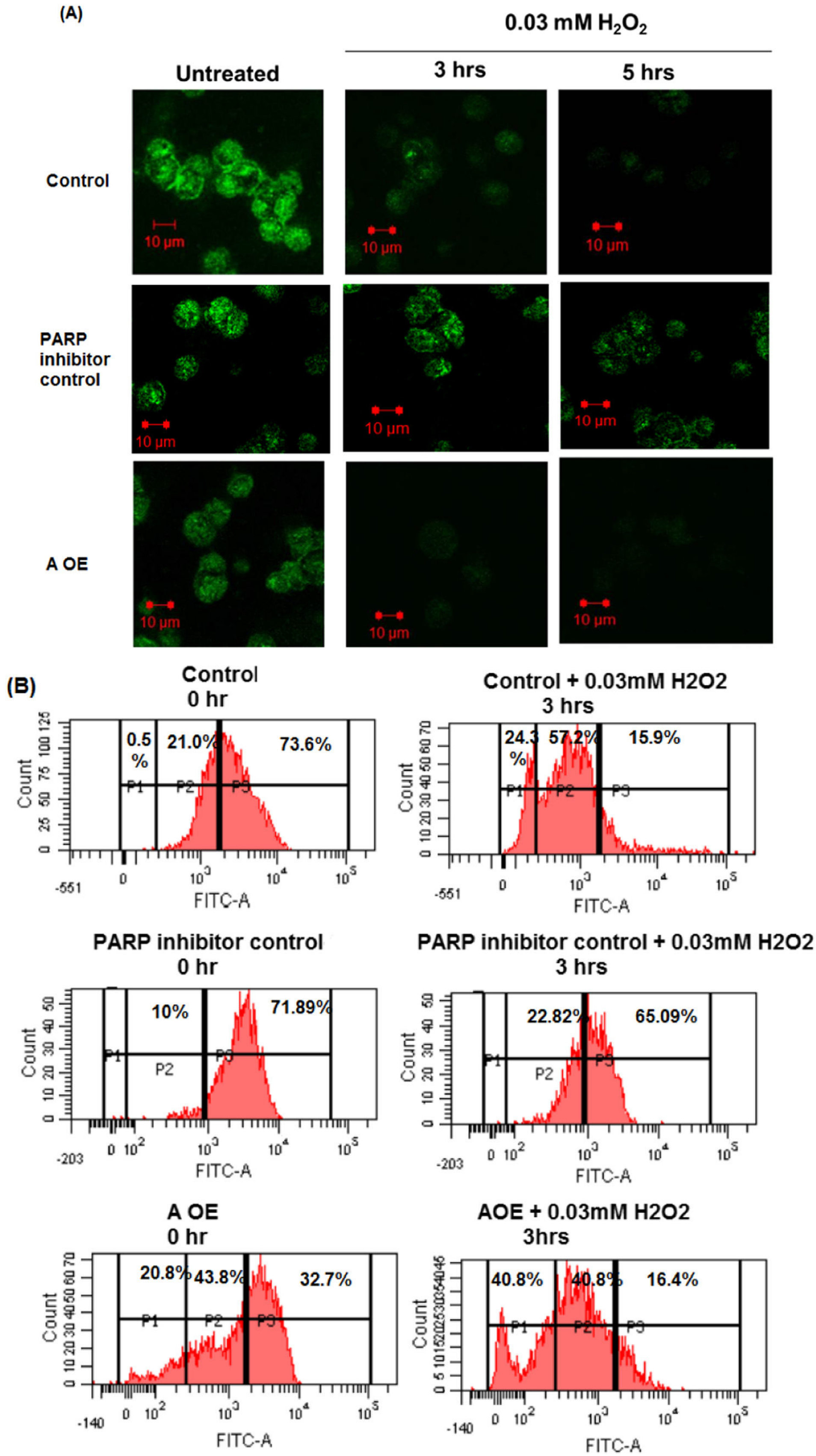

Fig. 8. Mitochondrial membrane potential changes in control, PARP inhibitor treated and ADPRT1A OE (AOE) cells at 3 h and 5 h post 0.03 mM cumene H_2O_2 treatment. (A) Mitochondrial membrane potential changes by $DiOC_6$ staining in control, PARP inhibitor treated cells and ADPRT1A OE (A OE) cells by confocal microscopy at 63X magnification. Data are representative of five independent experiments. Scale: 10 µm. (B) FACS analysis of mitochondrial membrane potential change in A OE and control cells post 3 h of 0.03 mM cumene H_2O_2 treatment using $DiOC_6$ dye Data are representative of three independent experiments.

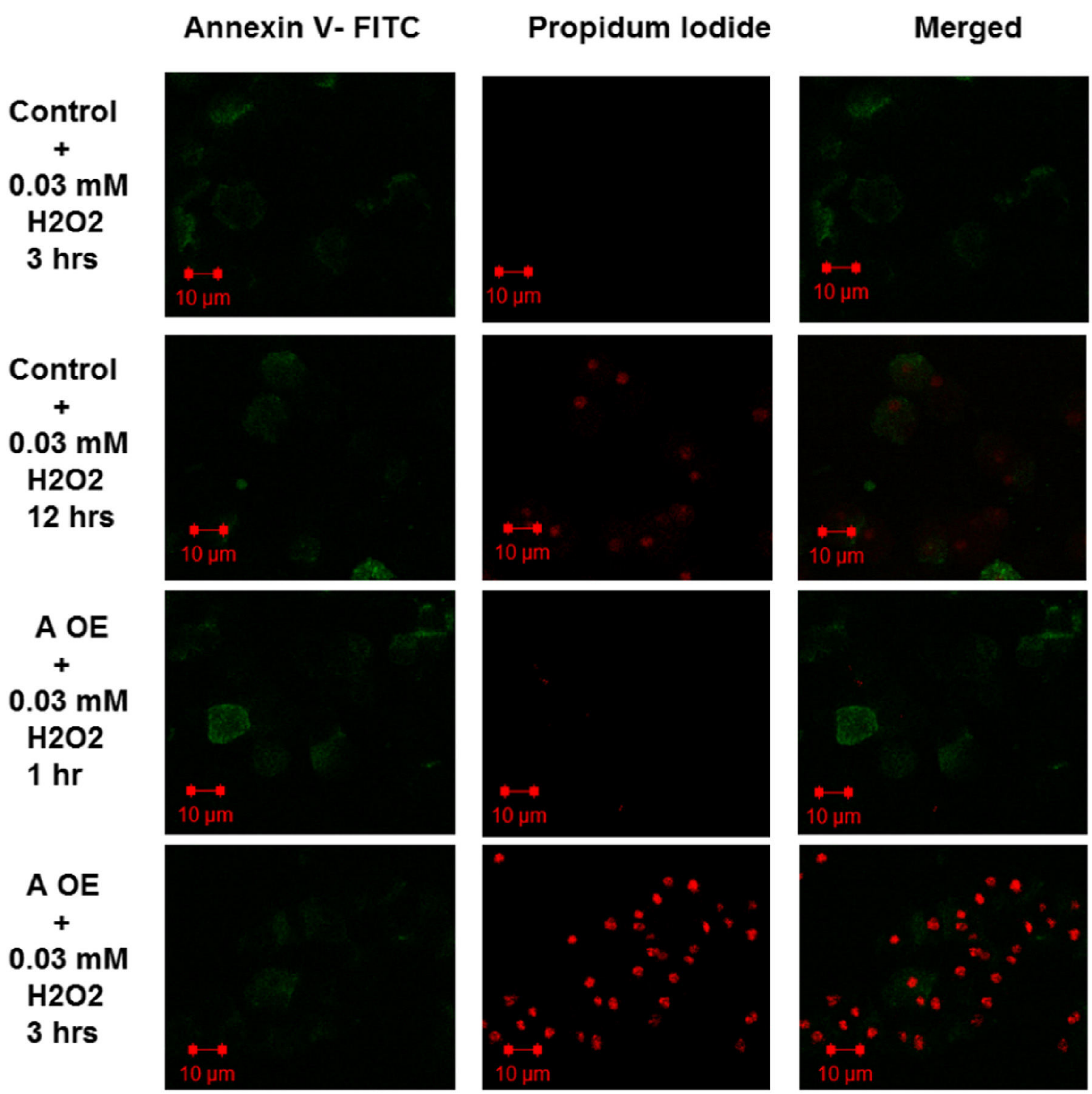

Fig. 9. Phosphatidylserine (PS) exposure (Annexin V-FITC Staining) and PI staining in control and ADPRT1A OE (AOE). Cells were by confocal microscopy at 63X magnification. A OE cells showed both PS (green - Annexin V FITC staining) and PI (red) positive cells as early as 3 h post 0.03 mM cumene H_2O_2 treatment indicating necrotic mode of cell death while control cells showed only PS (green) positive cells at 3hrs indicating paraptotic mode of cell death under the same oxidative stress. Data are representative of three independent experiments. Scale: 10 μm.

overexpression of ADPRT1A led to downregulation of the other PARP isoforms ADPRT1B and ADPRT2 (Fig. 3B). This decrease in ADPRT2 and 1B levels could be the cells' mechanism to circumvent the further reduction of the common substrate for all the isoforms i.e NAD^+, thereby keeping the cell viable. This is supported by the growth profile of the A OE cells wherein in spite of the high NAD^+ consumption, the cells are viable but show delayed growth. There are reports wherein overexpression of the Na^+/K^+ATPase α2 elicited downregulation of the other isoform α1 isoform in a manner that preserved total levels of $Na^{+/}K^+$ATPases (Correll et al., 2014).

NAD^+ and ATP depletion can be seen as a result of PARP activation in response to DNA damage due to ROS (Cantó et al., 2013). Our results are consistent with the fact that despite having high ROS levels (Fig. 6A), AOE cells showed negligible cell death. This could be due to the presence of more PARP molecules in ADPRT1A overexpressed cells as compared to control, making the cells more equipped to combat the increased DNA damage caused by ROS as seen in Fig. 7B wherein untreated AOE do not show significant DNA damage. Reports suggest that ADPRT1A is involved in double stranded break repair while ADPRT1B and ADPRT2 are required for tolerance to single stranded breaks (Tulin and Allan, 2003). Couto et al., also explain that in absence of ADPRT2, ADPRT1A signals single strand DNA lesions to promote resistance of cells to DNA damage (Couto et al., 2013). Our results clearly indicate lower ADPRT1B and ADPRT2 levels in AOE cells as compared control cells (Fig. 3B). Hence, overexpressed ADPRT1A may compensate for the other isoforms. Together, these results suggest that ADPRT1A homeostasis is very important, and any disruption/change in ADPRT1A levels and activated PARP levels may disturb normal functioning of the cell.

Higher NAD^+ and ATP reduction along with high basal ROS thus justify increased doubling time (~18 h) in *ADPRT1A* overexpressed cells (Fig. 5A). Therefore, decreased energy availability, would result in slow growth of *ADPRT1A* overexpressed cells. Apart from NAD^+ and ATP reduction, cell cycle analysis by FACS also revealed that population of cells in S and G2/M phase was higher in *ADPRT1A* OE cells as compared to control (Fig. 5B). Thus ADPRT1A also seems to affect cell-cycle regulation in *ADPRT1A* OE cells. This goes in accordance with reports suggesting that PARP-1

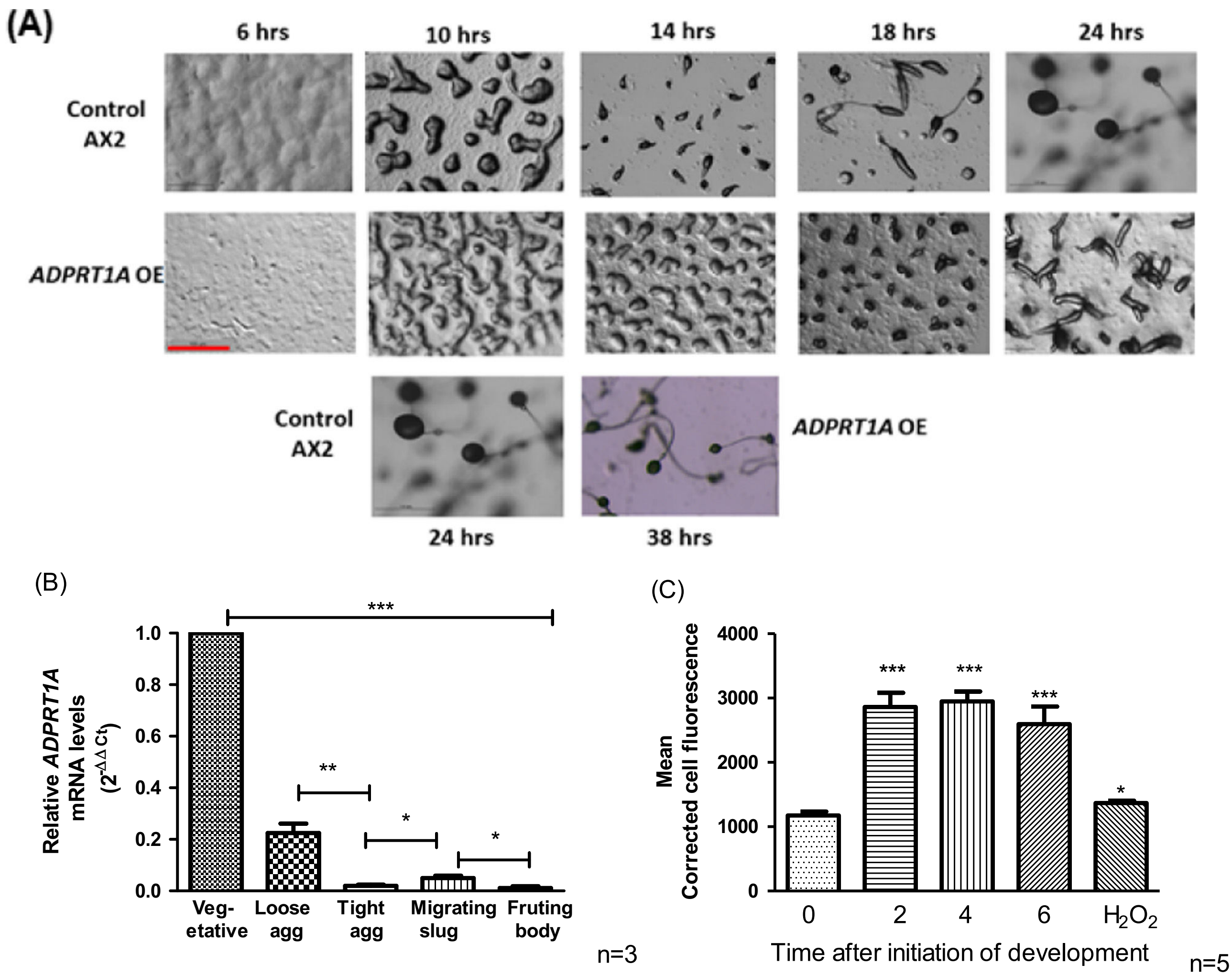

Fig. 10. Role of ADPRT1A in *D. discoideum* development. (A) Development of Control Ax-2 and *ADPRT1A*-OE cells were plated on nutrient-free agar and allowed to develop at 22 °C followed by synchronization. Photographs were taken at various time points. Scale: 100 μm. Data are representative of three independent experiments. (B) Developmental expression pattern of *ADPRT1A*. Change in gene expression was analyzed by Real time PCR using total RNA samples extracted from *D. discoideum* Ax-2 cells in the growth and developmental phases as templates. Relative mRNA levels ($2^{-\Delta\Delta Ct}$) as compared to vegetative phase *ADPRT1A* transcript levels were plotted graphically. Data are representative of three independent experiments. (C) Densitometric analysis of PARP activation up to 6 h of nutrient starvation. Mean corrected fluorescence was plotted for confocal microscopic pictures of PARP activity checked using FITC labeled Anti-PAR antibodies. ***p value < 0.001, *p value < 0.05 as compared to control. Data are representative of five independent experiments.

over-expression results in an increase in the number of cells in the diploid population (Bhatia et al., 1996). Moreover, PARP-1 is critical for the induction of G1 arrest and is also involved in the regulation of G2 arrest (Masutani et al., 1995). PARP-2 is reported to regulate cell cycle-related genes independently of poly (ADP-ribosyl)ation (Liang et al., 2013). The present study thus provides the first report in *Dictyostelium* wherein ADPRT1A, ortholog of PARP-1 is involved in cell cycle control via poly (ADP-ribosyl)ation.

PARP-1 is a well-established mediator of necrotic cell death (Edinger and Thompson, 2004; Rajawat et al., 2014). It is known that in *D. discoideum*, high oxidative stress causes DNA damage and PARP activation (Fey et al., 2004). PARP-1 depletes cellular NAD^+ and ATP levels leading to necrotic cell death instead of apoptosis (Ha and Snyder, 1999). Similarly *ADPRT1A* overexpressed *D. discoideum* cells showed susceptibility to oxidative stress during 0.03 mM H_2O_2 insult resulting in necrosis (Fig. 9).

In addition to PARP's role in growth, evidences also suggest that it might be playing a key role in development and differentiation. PARP-1 and PARP-2 double knockouts in mice exhibit embryonic lethality (Henrie et al., 2003) suggesting the role of PARP in development. Constitutive *PARP* downregulation inhibits development at aggregate stage (Rajawat et al., 2007). Interestingly, *ADPRT1A* OE cells showed delayed streaming, loose aggregate stage and early culminant stage (Fig. 10A). Aggregation defective phenotypes of *D. discoideum* display disruption in cAMP signaling. (Sawai et al., 2007; Bader et al., 2006). PARP-1 is known to interact with histones and other chromatin modifying enzymes to control their activity at target gene promoters, eventually influencing gene expression (Frizzell et al., 2009).

Thus, our results show that analysis of endogenous *ADPRT1A* transcript level in *D. discoideum* cells subjected to starvation display highest *ADPRT1A* transcript levels at aggregation stage (Fig. 10B). Furthermore, the developmental stimulus of nutrient starvation not only causes increased transcript levels but also a significant increase in PARP activity in the aggregation stages (Fig. 10C). Masutani et al., also report significant change in PARP mRNA expression throughout the developmental stages of *Sarcophaga peregrina* (Masutani et al., 2004). Overall, it could be concluded that *ADPRT1A* has a definite role in development and may affect the cAMP signaling which is quintessential for aggregation. Our results for the first time reveal that there is increased PARP activity and developmentally regulated transcript profile of *ADPRT1A* during *D. discoideum* development. Thus, it would be of great interest to further explore how ADPRT1A affects *D. discoideum* developmental morphogenesis.

6. Conclusion

The current study shows that PARP-1 is essential for differentiation and its function may be linked to multicellularity. This new finding will give us an insight into the role of PARP-1 in differentiation and developmental cell death in higher complex organisms. It would add one more feather to the multitasking functions of PARP-1 thereby igniting new ideas to understand the manner in which this multifunctional protein can be tapped for therapeutic purposes.

Acknowledgment

Infrastructure facilities provided by The Maharaja Sayajirao University of Baroda, Vadodara and DBT-MSUB ILSPARE are gratefully acknowledged. RB thanks the Department of Biotechnology, New Delhi (BT/PR4383/BRB/10/1014/2011) and Council of Scientific and Industrial Research (38(1383)/14/EMR-II) for research support; and TJ thanks the UGC-DRS (Grant number: F.3 -13/2012(SAP-II)) for awarding JRF.

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Received: 9 March 2016 | Accepted: 4 May 2016
DOI: 10.1111/cpr.12268

REVIEW ARTICLE

WILEY Cell Proliferation

The PARP family: insights into functional aspects of poly (ADP-ribose) polymerase-1 in cell growth and survival

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Abstract

PARP family members can be found spread across all domains and continue to be essential molecules from lower to higher eukaryotes. Poly (ADP-ribose) polymerase 1 (PARP-1), newly termed ADP-ribosyltransferase D-type 1 (ARTD1), is a ubiquitously expressed ADP-ribosyltransferase (ART) enzyme involved in key cellular processes such as DNA repair and cell death. This review assesses current developments in PARP-1 biology and activation signals for PARP-1, other than conventional DNA damage activation. Moreover, many essential functions of PARP-1 still remain elusive. PARP-1 is found to be involved in a myriad of cellular events *via* conservation of genomic integrity, chromatin dynamics and transcriptional regulation. This article briefly focuses on its other equally important overlooked functions during growth, metabolic regulation, spermatogenesis, embryogenesis, epigenetics and differentiation. Understanding the role of PARP-1, its multidimensional regulatory mechanisms in the cell and its dysregulation resulting in diseased states, will help in harnessing its true therapeutic potential.

1 | INTRODUCTION

Poly (ADP-ribose) polymerase (PARP) enzymes are a family of proteins involved in a number of cellular processes including gene regulation, chromatin remodelling, DNA repair and apoptosis.[1] These enzymes are present in all eukaryotes except yeast.[2] PARPs can either transfer a single unit of (ADP-ribose) or more than one (ADP-ribose) moieties from NAD^+onto substrates yielding poly (ADP-ribose) (PAR) chains, which can be of varying length and branch content. ADP-ribosyltransferase D-type 1 (ARTD-1 or PARP-1) falls in the latter category.[1,3] The PAR polymers are rapidly degraded by poly (ADP-ribose) glycohydrolase (PARG)[4] possessing both endoglycosidic and exoglycosidic activities,[5] and PAR hydrolase (ARH3), which also shares catalytic domain similarity with PARG.[6] However, ARH3 does not hydrolyse ADP-ribose-arginine, -cysteine, -diphthamide or -asparagine bonds.[6] Another set of enzymes known as macro domain-containing proteins and NUDIX hyrolases have also been reported to be involved in PAR degradation.[7,8] There are 17 different homologues of PARP that have a conserved catalytic domain with various domains like zinc finger, BRCT, SAM, SAP, ankyrin and macro domain.[9] Though PARP-1 has been demonstrated as a key player in DNA repair and cell death, many of its equally vital cellular functions have been overlooked. In this review, we discuss the distribution of PARP homologues across all organisms and the role of PARP-1 in various cellular functions like transcription, spermatogenesis, epigenetics and the most novel in differentiation and multicellularity.

2 | THE PARP FAMILY

Based on new proposed nomenclature by Hottiger et al.,[10] the human PARP (hPARP) family is classified into three groups depending on their motifs and functions: (1) PARP 1-5: have a conserved glutamate residue (Glu988); (2) PARP 6-8, 10-12 and 14-16: are putative mono-(ADP-ribose) polymerases and (3) PARP 9 and 13 which do not have PARP signature motif that binds NAD^+nor do they have Glu988 implying that they are inactive.[10] PARP superfamily can be subdivided into six clades which are shown in Table 1.

Out of 17 members, PARP-1 (113 kDa) was the first characterized and extensively studied enzyme recognized to play an essential role in DNA repair.[11] PARP-1 and PARP-2 share ~69% homology in the catalytic domain and they are documented as vital proteins in DNA repair system,[2] while PARP-3 is reported to be a mono-ADP-ribosylating enzyme by Loseva and group.[12] PARP-2 and PARP-3 were considered as a subgroup of PARP-1 as they all carry out synthesis of branched polymers.[13] PARP-4 also known as Vault PARP, is a ribonucleoprotein complex having PARylation activity and it is thought to be involved in multidrug resistance of tumour and intracellular transport.[14] Tankyrase-1 (TRF-1-interacting ankyrin-related ADP-ribose polymerase-1), also known as PARP5a, is identified to enhance telomere elongation by telomerase.[15] Other PARP homologues show structural and functional differences. Tankyrase-2 lacks N-terminal HPS (His-Pro-Ser) domain, but it may share some overlapping functions with tankyrase-1.[16] Other PARP family members like tiPARP, PARP-12 and PARP-13 share PARP catalytic, WWE and CX8CX5CX3-like zinc finger domains.[2] PARP-13 has been reported to be an important regulator of cellular mRNA via regulation of miRNA activity.[17] The next subgroup which includes PARP-9/BAL1, PARP-14/BAL2/CoaSt6 and PARP-15/BAL3 are macro-PARPs, characterized by macro domains positioned before the PARP domain. This domain is found to be involved in transcriptional repression and X-chromosome inactivation, suggesting it as a transcription factor.[18] The RNA recognition motif (RRM) and the Gly-rich domain of PARP-10 are known to help in binding of RNA with proto-oncoprotein c-Myc.[2] Other PARP family members such as PARP-6, PARP-8, PARP-11 and PARP-16 have been identified but their functions are still elusive, though PARP-8 and 16 have been recently shown to be involved in assembly or maintenance of membranous organelles.[19]

3 | DISTRIBUTION OF PARP ACROSS LIFE

3.1 | PARP in lower life forms

3.1.1 | PARP in bacteria

Numerous PARP-like proteins are detected in several bacterial genomes.[20,21] Till now, around 28 PARP homologues have been suggested across 27 bacterial species.[22] However, only a few bacteria possess the entire machinery required for PARP metabolism. Some also show the conserved histidine-tyrosine-glutamate (H-Y-E) catalytic triad which is essential for its activity.[21] PARP from *Herpetosiphon aurantiacus* has been reported to have conserved catalytic triad having the same characteristics as human PARP-1 enzyme.[21]

3.1.2 | PARP in archaea

Archaea do show the presence of PARP homologues. PARP-like thermozymes have been identified from *Sulfolobus solfataricus*. This PARP-like protein shows oligo (ADP-ribosyl) transferase activity and DNA-binding activity.[23]

3.1.3 | PARP in viruses

PARP-like proteins have also been identified in a few double-stranded DNA viruses [24] such as *Aeromonas* phage—Aeh1, *Anticarsia gemmatalis* nucleopolyhedro virus, invertebrate iridescent virus 6 and cellulophagaphage phi4:1. All these viral PARPs have been found to possess the conserved catalytic triad H-Y-E with an exception of one which has an aspartate instead of glutamate suggesting that these PARPs are active ADP-ribosyl transferases. Some viruses such as Herpes simplex virus and Epstein-Barr virus have also been reported to use PAR metabolism for their replication.[25,26]

3.2 | PARP in higher eukaryotes

PARPs are found in a divergent group of eukaryotes.[9,10] PARP expression has been identified in nearly all eukaryotic cells ranging from plants to vertebrates.[27] PARP-1 was long assumed to be the single enzyme with PARylation function until two PARP isoforms were discovered in plants.[28] Citarelli et al.[29] investigated at least two more PARP proteins in the last common extant ancestor of eukaryotes.

In conclusion, it is clear that the complexity of PARP proteins is augmented with the evolutionary level of the species. Vyas et al.[19] evidently illustrated that this domain complexity confers the diversity in functions to the PARP family.

PARP-1 is best studied out of this 17-member family of hPARPs. PARP has been implicated in development and cell differentiation from lower life forms to higher eukaryotes.[30] However, it is involved in a plethora of functions and many of its functions in spermatogenesis, epigenetics and differentiation remain unclear. Thus, understanding PARP-1 and its role in the above processes is the focus of this review.

4 | PARP-1: STRUCTURE, ACTIVATION SIGNALS AND ITS DIVERSE CELLULAR ROLES

4.1 | Gene organization of PARP-1 and its modifications

PARP-1 (EC 2.4.2.30) is a prominent member of the PARP family. It is a nuclear enzyme with approximately 10^6 molecules per cell [31] and accounts for 80%–90% of total cellular PARylation. Gene structure of PARP-1 mainly consists of DNA binding, an auto modification and a catalytic domain (Fig. 1). (1) The N-terminal DNA-binding domain has three zinc fingers and a nuclear localization sequence (NLS). The two homologous zinc finger proteins (Zn1 and Zn2) are characterized by a CCHC ligand pattern.[32,33] (2) The auto modification domain has BRCA1 C terminus (BRCT) motif and it is involved in protein-protein interaction.[2,10,34] (3) The catalytic domain at C terminus comprises of PARP signature motif (six β-strands and one α-helix) that binds to $NAD^+$and glutamate residue at its 988 position.[2]

TABLE 1 Distribution of PARP. PARP has been divided into six clades depending on the domains present[22,29] and [30]

Clade	Clade sub group	Class	Key features
Clade 1	Clade 1A	Amoebozoa (*Dictyostelium*) Opisthokonta (Fungi) Chromalveolates	Ankyrin repeats, WGR PRD, PARP catalytic domains.
	Clade 1B	Opisthokonta (animals and *Choanoflagellata*) and the Excavata (the *Heterolobosea* member Naegleria)	three N-terminal zinc fingers that contribute to DNA binding, a BRCT domain and a PADR1 domain in addition to WGR, PRD and the catalytic domain
	Clade 1C	Oomyocete Phytophtora species (within the Excavata) and one basal animal.	WGR, PRD and PARP catalytic domains and mostly do not contain other functional domains.
	Clade1D	Opisthokonta, the animals *Xenopus laevis* (Q566G1) and *Schistosoma japonicum* (Q5DAZ0) and the fungus *Batrachochytrium dendrobatidis* and Plantae (land plants) as well as ciliate members of the Chromalveolates.	WGR, PRD and PARP catalytic domains and mostly do not contain other functional domains.
	Clade 1E	most of the fungal members of Clade 1	BRCT domains N-terminal to WGR, PRD and PARP catalytic domains.
	Clade 1F	the Excavata	—
	Clade 1G	Opisthokonta (both animals and the Choanoflagellate *Monosiga brevicollis*)	only WGR, PRD and PARP catalytic domains
	Clade 1H	Two *Caenorhabditis elegans (C. elegans)* proteins	PADR1, WGR, PRD and PARP
Clade2	Clade 2A	—	an N-terminal WWE domain, the PARP signature and a C-terminal extension
	Clade 2B	—	only the PARP signature and the C-terminal extension
Clade 3	Clade 3A	—	RRM RNA-binding domain, a glycine-rich region (GRD), and a UIM domain
	Clade 3B	*Trichoplax adhaerens*	Macro domain N-terminal to their C-terminal catalytic domain
	Clade 3C	—	Macro domain N-terminal to their C-terminal catalytic domain
	Clade 3D	two *Dictyostelium discoideum* and four *Tetrahymena thermophila* proteins	—
	Clade 3E	—	one to two WWE domains, alone or in combination with zinc fingers (either CCCH or CCCH types) in front of their PARP catalytic domains
	Clade3F	—	PARP9
Clade 4	Clade 4	—	15–18 ankyrin repeats followed by a sterile alpha motif (SAM) and the PARP catalytic domain
Clade 5	Clade 5A	Opishthokonts (animals)	the PARP signature is found in the middle of the protein, rather than at the C terminus
	Clade 5B	Amoebozoa	—
Clade 6	Clade 6A	Opisthokonts (animals and fungi), Excavates (Parabasalids and Heterolobosa), and Plantae (chlorophyta and bryophytes)	N termini with no known functional domains and C-terminal extensions beyond the PARP catalytic domain of varying lengths
	Clade 6B		PfamB_2311 domains as well as the PARP catalytic domain
	Clade 6C		PfamB_2311 domain and a PARP catalytic domain
	Clade 6D	Deuterostomes with the exception of the mollusc *Lottia gigantean*	PfamB_2311 domain and the PARP catalytic domain
	Clade 6E	seven proteins encoded by *Trichomonas vaginalis*	PfamB_2311 domain and the PARP catalytic domain

The next important component of this enzyme is the PARP signature motif (PSM). It has two sites, acceptor site for adenosine and donor site for nicotinamide wherein ADP residues from $NAD^+$are transferred to target site.[35] His-862 and Glu-988 play important role in NAD^+binding.[36] In addition to this, WGR domain also contains highly conserved amino acid sequence i.e. Trp, Gly and Arg, but its role

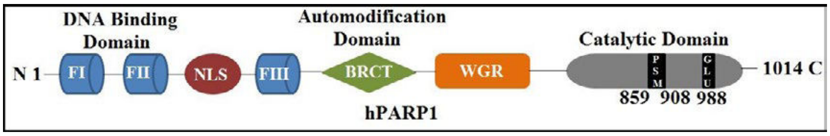

FIGURE 1 Structural gene organization of human PARP-1 (hPARP-1). It is characterized by FI, FII: Zinc finger motifs, FIII: Zinc ribbon domain (1–333 bp); NLS: Nuclear localization sequence; BRCT: BRCA1 C terminus motif (386–464 bp); WGR domain (549–634 bp) and the most conserved catalytic domain with PARP signature motif (PSM) between 859–908 bp and glutamate (Glu) at 988 position

TABLE 2 Post-translational Modifications of Poly (ADP-ribose) polymerase 1

Modification in PARP-1	Source	Residue modified	Activator	Result	References
Auto modification Poly (ADP-ribosylation)	PARP	K498, K521 and K524	Intact and damaged DNA	Regulation of PARP activity	Altmeyer et al.[38]
Mono-ADP-ribosylation	SIRT6	K521	dsDNA damage	Enhances double-strand break repair under oxidative stress	Mao et al.[42]
Sumolyation small ubiquitin-related modifier (SUMO)	SUMO-2 SUMO 3	K203, K486 and K512	Heat shock intact DNA	Transcriptional co-activator of hypoxia-responsive genes and promotes induction of the heat shock-induced HSP70.1 promoter	Zilio et al.[43]
Acetylation	p300/CREB-binding protein	K498, K505, K508, K521 and K524	Inflammatory stimuli	NF-κB-dependent gene activation	Hassa et al.[40]
Phosphorylation	ERK1/2 Protein Kinase C	S372 and T373	DNA damage	Neuronal cell death Decreased PARP-1 DNA-binding and catalytic activity	Kauppinen et al.,[41] Beckert et al.[39]

is yet to be identified.[2,34] However, Langelier et al.[37] showed that Zn3 along with Zn1 and WGR domain of PARP-1 together bind to the DNA damage leading to structural changes eventually abridging DNA damage site to its catalytic domain.

Other than auto modification by PARylation, PARP-1 itself undergoes various other modifications enlisted in Table 2 that has various cellular effects.[38–43]

4.2 | Mechanism of PARP-1 activation

The enzymatic activity of PARP-1 is stimulated significantly in the presence of a range of activators like damaged DNA, non-B-DNA structures, nucleosomes and various protein-binding partners.[1,44–46] Lonskaya et al.[47] reported that DNA bent, cruciform DNA or stably unpaired DNA regions can also stimulate PARylation. The activation signal for PARP-1 is DNA damage although several reports illustrate that PARP-1 may also be activated in the absence of DNA damage. The best characterized ligands for PARP-1 are single-strand and double-strand breaks (SSBs and DSBs).

There are reports suggesting that PARP-1 activation by SSBs requires presence of both the zinc fingers while only Zn1 is required for DSBs.[33] Zn1 has been demonstrated to relay the signals to the catalytic domain for formation of PAR molecules,[33,48] while Zn2 has been shown to be majorly involved in DNA binding as compared to Zn1 due to its higher affinity to DNA.[49] Eustermann et al.[50] have demonstrated very recently how the two zinc fingers recognize SSBs and coordinate domain folding in PARP-1 to control the activity of the C-terminal catalytic domain. PARP-1 has been reported to have affinity for intact DNA structures and recognizes specific octamer motif “RNNWCAAA” found in various gene promoters.[51]

Another mode of alternative DNA-independent mode of PARP-1 activation is based on kinase cascades. Phosphorylated ERK2 has been shown to significantly enhance and maximize PARP-1 catalytic activity in the presence and absence of damaged DNA.[41,52] Interaction between PARP-1 and a pre-phosphorylated kinase has also been shown to mediate PARP-1 activation.[52,53] Likewise, phosphorylation by activated calcium-dependent protein kinase (CaMKII) is also capable of activating PARP-1 enzyme during neuronal development thereby promoting the nuclear export of its negative regulator KIF4.[54] Moreover, overexpression of protein phosphatase 5 (PP5) led to increase in PARP-1 enzymatic activity in response to double-stranded DNA breaks.[55] Nuclear nicotinamide mononucleotide adenylyl transferase 1 (NMNAT1), an enzyme involved in NAD^+synthesis, also associates with PAR to enhance PARP-1 enzyme activity.[56] Other proteins regulating PARP-1 activity include Ku,[55] histone variant macroH2A1.1[57] and KIF4.[58] Protein-protein interactions also seem to activate PARP-1. Mao et al.[42] have demonstrated that SIRT6 activates PARP-1 by mono-ADP-ribosylating it in position Lys521. Developmental or environmental stimuli induce PARP-1 activation and the PAR-dependent nucleosome loosening leading to histone stripping and hence opening of the chromatin structure. This process allows

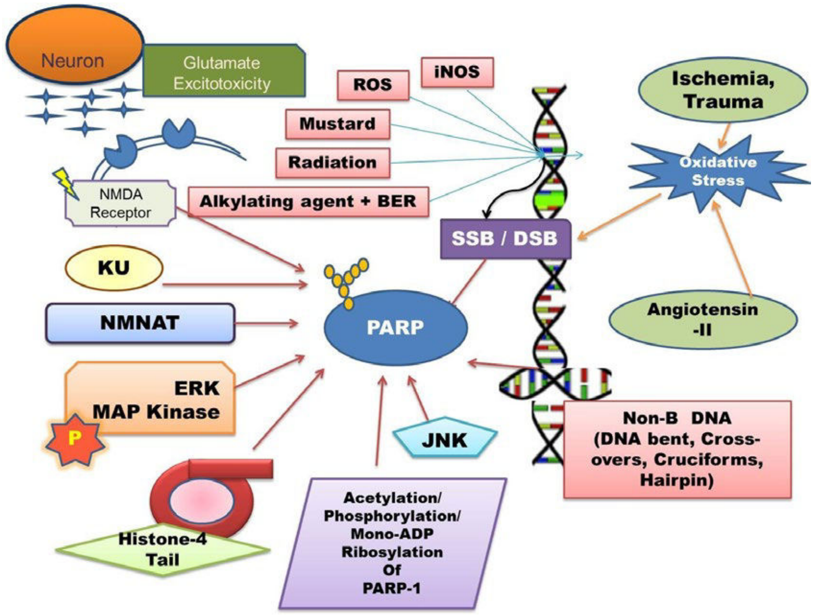

FIGURE 2 Mechanism of PARP-1 Activation. The nuclear enzyme PARP-1 can bind to DNA breaks resulting in the activation of the enzyme. DNA breaks are caused either by ROS, RNS or radiation or indirectly by DNA repair machinery where breaks are introduced into the DNA strands as in the case of alkylating DNA damage. Binding to special non-B-DNA structures such as bent or cruciform DNA or four-way junctions may culminate into PARP-1 activation. Protein-protein interactions or covalent modifications (e.g. mono-ADP-ribosylation, acetylation or phosphorylation) have also been described as activation mechanisms for PARP-1 which are DNA-independent. Other proteins activating PARP include nuclear NMNAT, Ku and phosphorylated ERK2 and Histone-4 tail

transcriptional activation. This PAR-mediated chromatin loosening phenomenon is detected at larval salivary-gland polytene chromosome puffs.[59] Hence, PARP-1 can be activated by DNA-dependent and -independent manner which is summarized in Fig 2.

4.3 | PARP-1: single protein with varied roles

4.3.1 | PARP-1 in DNA repair

ADP-ribosylation activity of PARP-1 is an instantaneous biochemical response to DNA damage induced by ionizing radiations, alkylations etc. At low levels of DNA damage, it detects DNA damage followed by repair and cell survival, whereas at high levels of DNA damage, it activates the cell death pathway.[60] Upon DNA damage, PARP-1's zinc finger FI/Zn1, FII/Zn2 and FIII/Zn3 motifs have been reported to relay binding signal to catalytic domain followed by the recruitment of proteins involved in repair mechanism such as base excision repair (BER), single-strand breaks (SSBs) and double-strand breaks (DSBs) repair.[1,61] It is also indicated to act as a DNA damage sensor [62] and help in chromatin remodelling at DNA damage sites.[63] A variety of proteins like ALC1, histone mH2A1.1, scaffold attachment factor SAFB1 have been illustrated to be recruited to DNA damage sites via PARP-1 thus proving its indispensable role in DNA repair.[64–66] Evidences show presence of PAR-binding zinc finger motifs in DNA damage response and checkpoint regulation proteins.[67,68] PARP-2 was also shown to be involved in the later steps of BER/single-strand break repair.[69] In nucleotide excision repair, PARP-1 inhibition or depletion has also shown to cause low efficiency of removal of UV-induced DNA damage.[70] Among mammalian DNA repair pathways, PARP-1 has been also implicated in homologous recombination[71] and non-homologous end-joining pathways.[72] PARP-1 has been reported to interact with replication fork protein (Timeless) in a PAR-independent manner thereby allowing its recruitment to DSB sites to promote homologous recombination.[73] Thus, it is clearly illustrated that PARP-1 plays a vital role in DNA damage response.

4.3.2 | PARP-1 in cell death

Under normal physiological conditions, cell morphology, numbers, pattern and injury are taken care of by the process of apoptosis.[74] The mode of cell death depends on the extent of DNA damage. Low DNA damage can activate PARP-1 resulting in cell survival via DNA repair mechanisms. At moderate levels of DNA damage, cell undergoes apoptosis and PARP-1 activation results into cleavage of PARP-1 by caspases-3 and -7 into two fragments (89 kDa and 24 kDa)[75] which is believed to be a key feature of apoptosis.[76] N-terminal 24 kDa fragment remains in nucleolus and other 89 kDa fragment translocates from nucleus to cytosol wherein it acts as a target for autoimmunity.[77] Severe DNA damage leads to programmed necrotic cell death through over-activation of PARP-1.[78] Ring finger protein 146 (RNF146), a cytoplasmic E3-ubiquitin ligase, acts as a direct interactor of PARP-1 during this process and elicits release of PARP-1 from the nucleus. This has been demonstrated during myocardial ischaemia-reperfusion injury.[79] On the other side, in caspase-independent cell death, it plays an important role in the release of apoptosis-inducing factor (AIF) from mitochondria to nucleus. Yu et al.[80] have studied the dependence of PARP-1 and AIF in caspase-independent cell death which is termed as 'parthanatos'. PARP-1 has been reported to play a very crucial role in initiation and regulation of this type of cell death.[81] Parthanatos has been detected in many disease conditions like stroke, Parkinsons, diabetes, etc.[82] Upon PARP-1 activation stimulated with various DNA-damaging agents like NMDA, H_2O_2, etc., AIF translocates from mitochondria to nucleus and finally culminates into cell death.[83–85] On the contrary, Mir et al. showed that staurosporine-induced cell death did not involve PARP.[86]

PARP-1 is also reported to be involved in autophagy induced by DNA damage.[87] PARP-1 via autophagy displays a cytoprotective role in oxidative stress-induced necrotic cell death.[88] Moreover, Son et al.[89] have also reported that cadmium-mediated ROS generation leads to PARP-1 activation and energy (ATP) reduction, eventually culminating into autophagy in skin epidermal cells. Wyrsch et al.[90] have found that PARP-1 and PARP-2 control cytosolic Ca^{2+}shifts from extracellular and intracellular sources during oxidative stress. The different Ca^{2+}signals arise from the transient receptor potential melastatin 2 (TRPM2) channels located in the cellular and lysosomal membranes. This Ca^{2+}overload induces specific stress kinase response which leads to autophagy or cell death. Under mild oxidative stress conditions, PARP-1 operates as an autophagy suppressor after oxidative stress leading to cell death by activating downstream of extracellular signal-regulated kinase 1/2 (ERK1/2) and AKT. Under severe oxidative conditions, PARP-2 induces Ca^{2+}shifts from lysosomes, while PARP-1 becomes completely inactive. The cytosolic Ca^{2+}overload leads to phosphorylation of p38, stress-activated protein kinase/Jun amino-terminal kinase (SAPK/JNK), and cyclic AMP response element-binding protein (CREB) with its activating transcription factor (ATF-1), further activating autophagy markers leading to cell survival.

PARP-1 and related PARP family members are at the intersection of conversing stress signalling pathways. Oxidative stress causes disruption in redox potential that extends to the ER, causing accumulation of misfolded proteins, finally stimulating the unfolded protein response (UPR).[91] It would be interesting to know if PARP-1 has a role in ER stress-mediated cell death as it is upstream to autophagy, where PARP-1 is demonstrated to play an essential role. Hence, it is clear that PARP-1 is an essential regulator in many of the cell death pathways and this has been demonstrated in many tissues. However, a very interesting work by Jog and Caricchio[92] illustrates a characteristic difference in PARP-1-mediated necrosis in males and females. Male mice were shown to be prone to PARP-1-mediated necrosis while female mice showed PARP-1-independent cell death.[92] Understanding the role of PARP-1 in different stress conditions and even in different sexes would help us dissect out pathomechanisms of various disease conditions.

4.3.3 | PARP-1 and epigenetics

The poly (ADP-ribosyl)ation of histones leading to open chromatin conformation at DNA damage sites was the first indication to the function of PAR as an epigenetic modification.[2] Recent evidence has shown that PAR has an important role in the epigenetic regulation of chromatin structure and in gene expression under physiological conditions wherein DNA integrity is maintained.[93] Lodhi et al.[94] have demonstrated PARP-1 as a genome-wide epigenetic memory mark in mitotic chromatin. They report that PARP-1 establishes stable epigenetic marks at the transcription start sites in metaphase chromatin and these marks are a prerequisite for transcriptional restart after mitosis. Moreover, PARP-1 activity epigenetically regulates mitochondrial DNA repair and transcription.[95] PARP-1 also associates with genome-wide epigenetic regulatory elements suggesting a functional interplay between PARP-1 and DNA methylation.[96] Previous studies have shown that PARP-1 can affect the genomic DNA methylation pattern via DNA methyl transferase, Dnmt1, both by regulating its expression as well as activity.[93,97] Furthermore, the role of PARP-1 in DNA methylation events has been explored in induced pluripotent stem cells (iPSCs).[98] Recently, PARP-1 has been shown to be associated epigenetically with Tet2 (a methyl cytosine dioxygenase) during somatic cell reprogramming which leads to transcriptional induction at the pluripotency loci.[99] PARP-1 has also been demonstrated to interact with TIP5 via non-coding RNA, thereby playing a role in maintenance of silent rDNA chromatin in mid-late S phase.[100] Though, these studies suggest the possible epigenetic involvement of PARP-1; its mechanistic role in epigenetic control is still elusive and remains to be an area of great interest to researchers.

4.3.4 | PARP-1 as a chromatin modulator

Chromatin consists of genomic DNA, linker histones (H1), core histones (H2A, H2B, H3 and H4) and other chromatin-associated proteins. Early reports have shown that purified PARP-1 could ADP-ribosylate chromatin proteins (e.g. mainly H1), by decondensation of chromatin and destabilization of nucleosomes.[101] Also proven in recent reports, PARP-1 binding to chromatin can change the conformation and composition of nucleosome.[32,102] In addition, it has also been demonstrated that PARP-1 interacts with core histone variants resulting in the recruitment and integration of histone variants to specific sites in the genome.[57] Local chromatin loosening by PARP-1 has also been demonstrated well at the puff loci in *Drosophila* facilitating transcription and eventually helping chromatin remodelling during development.[59] Nalabothula et al.[96] discussed the possible mechanisms of chromatin structure remodelling by PARP-1 as: a) it binds between entry and exit sites between nucleosomes and linker DNA, b) it PARylates histones, linker histone H1, etc. thus modifying chromatin architecture and c) it competes with histone H1 for nucleosome binding. All the above reports strengthen the role of PARP-1 in chromatin remodelling.

4.3.5 | PARP-1 in transcription

It is well studied that PARP-1 behaves as chromatin modifier at transcriptional level with a number of in vitro and in vivo experiments. Electrostatic repulsion between DNA and histones due to transfer of negatively charged PAR molecules onto accepter proteins promotes transcription by recruiting transcriptional machinery.[103] PARP-1 is observed to be more localized at the promoter regions of most actively transcribed genes.[104] The transcriptional regulatory roles of PARP-1 are manifested mainly through two processes, modulating chromatin structure and acting as a part of enhancer/promoter-binding complexes. Based on the cell type, it can enhance transcription with co-activators or inhibit transcription by repressors.[105] Chromatin-dependent gene expression is controlled by

PARP-1 interacting with histones at promoter.[104] The type of histone modification (acetylation, phosphorylation and methylation) is very essential for interaction between PARP-1 and DNA because it can add structural changes into histones.[106] Phosphorylation of histone variant, H2Av, promotes activity of PARP-1 in *Drosophila* at specific promoter regions.[107] PARP-1 is also found to be localized at DNA repair sites after binding to other histone variant, macroH2A.[108] Also, macroH2A1-stimulated H2B acetylation was seen in cancer progression which was PARP-1-dependent.[109] Depletion of PARP-1 activity resulted into ineffective loading of RNA polymerase II transcriptional machinery implying its role in gene regulation.[110]

Recent studies suggest that PARP-1 functions as a co-activator, which upregulates the transcription of Nrf2, promoting the interaction among Nrf2 and ARE (antioxidant response elements).[111] Reduced expression of CCN2 was found in tubular epithelial cells of kidney upon knockdown of PARP-1.[112] In addition to this, PARP-1 also functions as an insulator that organizes the genome into distinct regulatory units by controlling the effects of enhancers on promoters, or by preventing the spread of heterochromatin.[113] In vivo and in vitro binding studies of PARP-1 and transcription factor Yin Yang 1 (YY1) suggested that PARP-1 plays a promoter regulatory role and inhibits the transcription of Cxcl12. In addition, changes in PARP-1-CTCF interactions due to serum shock induced recruitment of circadian loci to the lamina leading to transcriptional attenuation.[114] PARP-1 is also known to be acting as an exchange factor thereby controlling transcription. Recently, it has been demonstrated that PARP-1 functions in remodelling of promoter-associated nucleosomes by replacing H2A.Z by H2A from *FOS* promoter to allow transcriptional activation in response to ERK signalling.[115] Thus, the underlying mechanism of PARP-1-mediated transcriptional regulation is very complex and extensive and hence more studies are required to explore the transcriptional role of PARP-1.

4.3.6 | PARP and spermatogenesis

Both PARP-1 and PARP-2 have been found to have a significant role in spermatogenesis.[116] It has been observed that there is significant PARP expression during the earlier stages of spermatogenesis and its transcription declines during late stages of maturation.[117,118] The levels of PARP-1, PARP-2 and PARP-9 were found to be increased in mature sperms as compared to immature sperms [116] and interestingly PARP-1 was also found to be down-regulated during the haploid stage of meiosis.[119] The presence of PARG in the nuclei of rat primary spermatocytes also suggests that the levels of poly (ADP-ribose) in these germ cells are highly regulated.[117] Moreover, Meyer-Ficca et al.[120] reported the presence of PAR polymerization by PARP-1 and PARP-2 in rat spermatids, highest during the phase of chromatin condensation.

Studies demonstrating an increase in DNA strand breaks in all population of elongating spermatids in human testis [121] and the presence of higher levels of PARP-1, PARP-2 and PARP-9 in ejaculated sperm from fertile men compared to infertile men indicate a possible relationship between PARP expression and male infertility.

4.3.7 | PARP-1 in cell differentiation/multicellularity

Out of the various roles of PARP-1, its role in cell differentiation and multicellularity has yet to be unravelled. However, accumulating reports in different model systems suggest a definite role of PARP-1 in growth and multicellularity. For example, Drosophila PARP has been shown to act in ectodermal specification and neural crest development in zebrafish.[122] Our laboratory studies are indicative of PARP's role in *D.discoideum* development wherein its down-regulation led to arrested development.[123] Recent studies from our laboratory show PARP-1 involvement in *D. discoideum* growth and multicellularity by ADPRT1A (PARP-1 orthologue) overexpression which led to delayed growth and developmental morphogenesis.[124] We have also reported that PARP may be essential in combating stress conditions in *D. discoideum*.[83–85,125,126] Genetic studies on PARP-1 orthologues in fungus demonstrated defective development and decreased life span.[127–129] As we move to the higher life forms like plants, it was seen that AtPARP-1 and/or AtPARP2 knockdown reported to alter *Arabidopsis* development [130] and AtPARP2 orthologue in oilseed rape (*Brassica napus*) did not affect its development.[131] However, further work is mandatory to explore the role of PARP in plant development. In addition, studies in Drosophila also suggest importance of PARP in chromatin loosening at ecdysone-inducible regions thereby inducing purparium formation and metamorphosis.[59,132] These results are also substantiated by mice studies wherein PARP-1 and PARP-2 double-mutant mice were found to be not viable and die at the onset of gastrulation, establishing the importance of both the PARPs during early embryogenesis.[133] Recently, Hamazaki et al.[134] have shown that PARP inhibition caused inhibition of DNA demethylation of the *IL17d* promoter region at the two-cell stage leading to down-regulation of genes essential for early embryogenesis. Thus it is clear from the above that a strong association of PARP-1 exists in differentiation and multicellularity, which is yet to be explored in detail.

4.3.8 | PARP-1 in metabolic regulation

PARP-1 has been known for its role in DNA repair as discussed in above sections. However, recent data suggest a role for PARP-1 in metabolic regulation by influencing mitochondrial function and oxidative metabolism. Mouse knockout studies showed that PARP-1 deletion led to increased food intake.[135,136] PARP-1^{-}/$^{-}$ mice showed an increased metabolic rate.[137] PARP-1 has also been associated with reduction in the glycolytic rate which has been linked to a reduction in NAD^{+}availability over the years.[138] Over-activation of PARP activity can lead to metabolic perturbations through reduction in ATP, NAD^{+}/NADH levels, which is enough to impair carbohydrate metabolism.[139] It also changes the flow of glycolytic metabolites into Krebs cycle and thereby compromised energy production in mitochondria.[140] However, recent evidence indicates that PARP-1 may be responsible for reduction in hexokinase activity and hence affects the

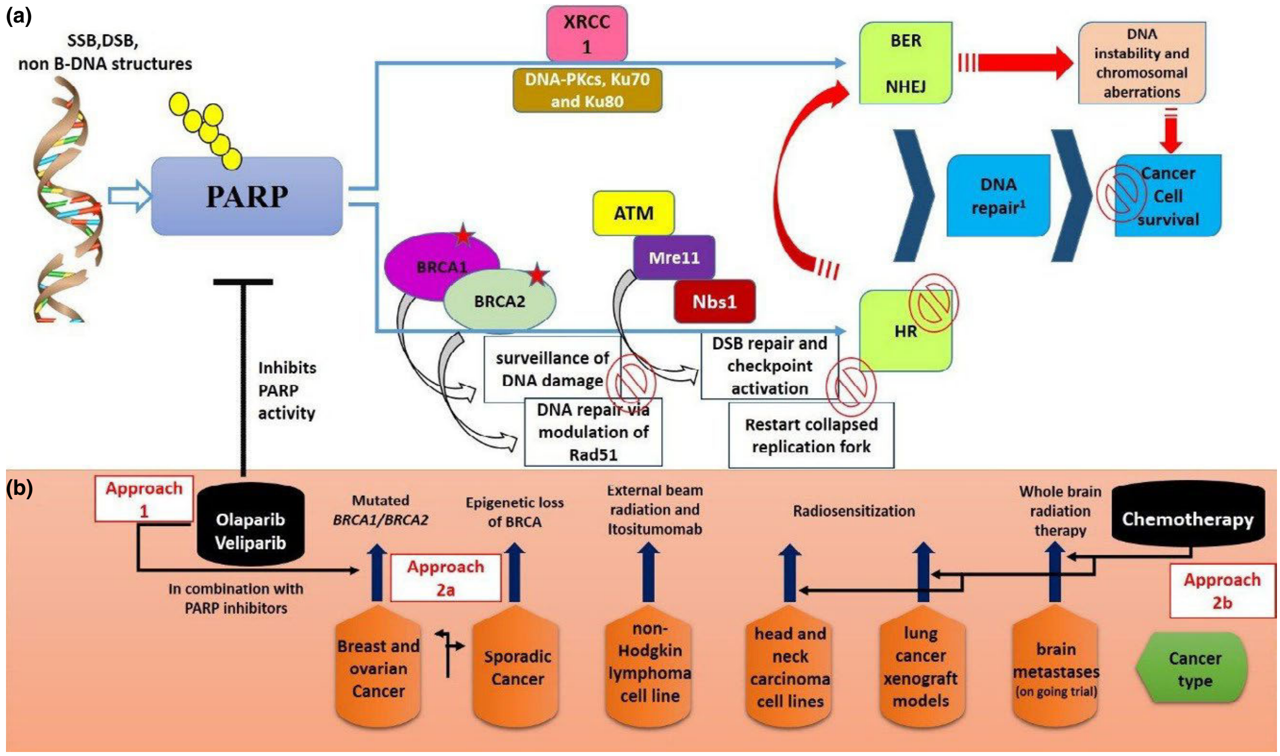

FIGURE 3 PARP-1 and cancer therapy. (a) In normal cells, upon DNA damage like SSB, DSB and non-B-DNA structures, PARP-1 gets activated and thereby aids in the recruitment of DNA repair proteins such as the scaffolding protein XRCC1 to sites of SSBs through BER, whereas DNA-PKcs, Ku70 and Ku80 to sites of DSBs through NHEJ. It also aids HR via recruitment of factors like ATM, Mre11 and Nbs1 to sites of DSBs. Another very essential process of HR repair involves localization of BRCA-1 and BRCA-2 to sites of double-stranded DNA damage. In cancer cells bearing BRCA1/2 mutations or deficiency (red star), cells are rendered faulty in HR repair (red no symbol) and thus there is complete dependence on NHEJ (error-prone) for DSB DNA repair and SSB for BER (red arrows); both of which are PARP-1-dependent. Thus, PARP inhibition serves as an excellent approach for therapy. BRCA1/2 mutations or deficiency along with PARP-1 inhibition leads to amplification of DNA instability due to impairment in BER-, NHEJ- and ATM-mediated HR repair and chromosomal aberrations results in cell death. (b) PARP-1 inhibitors like Olaparib, Veliparib, etc. have been promising therapeutic candidates in case of breast cancer and ovarian cancer—Approach 1. Approach 2a uses PARP-1 inhibitors in case of epigenetic modulation or artificial inactivation of BRCA pathway in case of sporadic cancers, whereas approach 2b involves use of chemotherapy and radiation along with PARP-1 inhibitor depending on the cancer type

cellular glycolytic rate via poly (ADP-ribosyl)ation of hexokinase directly.[141] PARP-1 and PARP-2 activation have also been demonstrated to affect mitochondrial activity negatively.[142] Hence, PARP inhibition arises as therapeutant to treat mitochondrial dysfunction.

In addition, PARP-1 also plays a crucial role in the circadian entrainment and regulates feeding behaviour. Asher et al.[143] demonstrated that CLOCK (Circadian transcription factor)–BMAL1-dependent gene expression was altered in PARP-1-knockout mice, in response to changes in feeding times. In contrast, the deletion of PARP-2 did not affect food intake or daily behaviour.[135] Moreover, both PARP-1^{-}/$^{-}$ and PARP-2^{-}/$^{-}$ mice displayed enhanced energy expenditure.[135,138] PARP-1^{-}/$^{-}$ mice showed an increased mitochondrial content in their brown adipose tissue (BAT),[138] which physiologically renders them to be able to maintain their body temperature during cold exposure. Interestingly, PARP-2 deletion does not influence mitochondrial biogenesis in BAT.[138] Furthermore, it has been suggested that PARP-1 acts as a positive regulator of adipogenesis and adipocyte function resulting in fat deposition.[144] Studies have confirmed that PARP-1 regulates adipogenic gene expression and is required selectively for adipocyte function.[145] PARP-1^{-}/$^{-}$ and PARP-2^{-}/$^{-}$ mice also displayed an increased glucose clearance [135,138] suggesting the increased insulin sensitivity. Thus, these reports suggest the metabolic involvement of PARP-1 and PARP-2; however, more studies are needed to confirm these findings and to explore new metabolic regulatory functions of PARP.

4.3.9 | PARP-1 and cancer

Errors in replication process, production of ROS and UV radiations result in DNA damage which includes single-strand breaks (SSBs), double-strand breaks (DSBs), etc. Cells then signal DNA repair pathways such as nucleic acid excision repair (NER), base excision repair (BER), mismatch repair (MMR), non-homologous end-joining (NHEJ) and homologous recombination (HR) resulting into cell survival with an exception of tumour cells. PARP-1 and PARP-2 are key regulators for the function of DNA repair mechanisms; however, genetic disorders, such as BRCA1 and BRCA2 mutations, prevent

TABLE 3 PARP inhibitors—Olaparib and Veliparib in various disorders

Mode of administration	Disease/disorder	Nature of disorder	Clinical trial status and results	Dosage (mg)	Side effects/toxicity	Reference
(a) PARP inhibitor Olaparib in various cancers						
Oral, monotherapy	Ovarian cancer	Recurrent; with *BRCA1* or *BRCA2* mutation	Phase II; positive	200; Twice a day	Anaemia and Vomiting	Kaye et al.,[167] Mateo et al.[168]
				300; Twice a day	Anaemia	Mateo et al.[168]
				400; Twice a day	Fatigue, nausea, vomiting and decreased appetite	Gelmon et al.,[169] Kaye et al.,[167] Ledermann et al.,[170] Kaufman et al.[171]
		Advanced; without *BRCA1/2* mutation	Phase II; negative	400; Twice a day	—	Gelmon et al.[169]
	Triple-negative breast cancer	Advanced; with *BRCA1* or *BRCA2* mutation	Phase II; positive	100; Twice a day	Fatigue and nausea	Tutt et al.[172]
	Breast cancer	Advanced; with *BRCA1* or *BRCA2* mutation	Phase II; positive	400; Twice a day	Fatigue, nausea and vomiting	Tutt et al.[172]
Oral, Combination therapy with Cediranib	Ovarian cancer	Recurrent; with *BRCA1* or *BRCA2* mutation	Phase I; positive	200/400 mg twice daily plus Cediranib 30 mg daily	Bowel obstruction, fatigue, hypertension, thrombocytopenia	Liu et al.[173]
Oral, Combination therapy with Cediranib	Ovarian fallopian tube, or primary peritoneal cancer	Recurrent; platinum-sensitive, or with *BRCA1*/2 mutations	Phase II; Positive compared to monotherapy (400 mg)	200 mg twice daily plus Cediranib 30 mg daily vs 400 mg Olaparib	Fatigue, diarrhoea and hypertension	Liu et al.[174]
Oral, Combination therapy with Cediranib	Triple-negative breast cancer	Metastatic	Phase I; Negative	200/400 mg twice daily plus Cediranib 30 mg daily	—	Liu et al.[173]
Oral, Triple combination therapy with carboplatin and paclitaxel	Ovarian cancer	Advanced, with or w/o *BRCA1* or *BRCA2 mutations*, relapsed	Phase Ib/II; Positive	200 mg twice daily plus carboplatin and paclitaxel, followed by olaparib 400 mg	Neutropenia and anaemia	Oza et al.,[175] Rivkin et al.[176]
Oral, Triple combination therapy with carboplatin+paclitaxel and Chemo therapy	Ovarian cancer	Advanced, with or without *BRCA1* or *BRCA2 mutations*, relapsed	Phase I; Positive	200 mg twice daily plus carboplatin and paclitaxel, followed by olaparib 400 mg	Alopecia, nausea, neutropenia, diarrhoea, headache, peripheral neuropathy and dyspepsia	Oza et al.[175]
Oral; with Chemo therapy	Ewing sarcoma	Refractory	Phase II; Negative	400; Twice a day	Anaemia and thrombocytopenia	Choy et al.[177]
Oral, monotherapy	Pancreatic cancer	With germline BRCA1/2 mutation	Phase II; Positive	400; Twice a day	Fatigue, nausea, anaemia and vomiting	Kaufman et al.[171]
Oral, monotherapy	Prostate cancer	With germline BRCA1/2 mutation	Phase II; Positive	400; Twice a day	Fatigue, nausea, anaemia and vomiting	Kaufman et al.[171]
Intravenous; Combined with cetuximab (cet) and intensity modulated radiation therapy	Head and neck squamous cell carcinoma	Heavy smokers, locally advanced	Phase I; Uncertain/ ongoing	400 mg/m^2+cet 250 mg/m^2 IV.	Mucositis, dermatitis, clinically insignificant lymphopenia, and hypomagnesaemia	Waxweiler et al.[178]

TABLE 3 (continued)

Mode of administration	Disease/disorder	Nature of disorder	Clinical trial status and results	Dosage (mg)	Side effects/toxicity	Reference
Oral; with temozolomide	Glioblastoma	Relapsed	Phase I; Positive penetration	400	—	Chalmers et al.[179]
Oral; with topotecan	Advanced solid tumours	—	Phase I; Negative	100 mg twice daily	— events	Samol et al.[180]
(b) PARP inhibitor Veliparib in various cancers						
Oral, monotherapy	Epithelial ovarian cancer	Recurrent or persistent; With germline BRCA1/2 mutation	Phase II; Negative; discontinued	400	Grade 3-fatigue, nausea, leukopenia, neutropenia, dehydration, and ALT. Grade 2 events-nausea, fatigue, vomiting and anaemia	Coleman et al.[181]
Oral; monotherapy	Serous ovarian cancer	Without BRCA1/2 mutation	Phase I; Negative	400	Nausea/vomiting, fatigue and leukopenia	Pahuja et al.[182]
Oral; monotherapy	Triple-negative breast cancer	Without BRCA1/2 mutation	Phase I; Negative	400	—	Pahuja et al.[182]
Oral; monotherapy	Castration-resistant prostate cancer	BRCA2-mutated metastatic	Phase I; Positive	400	—	Pahuja et al.[182]
Oral; with irinotecan	Triple-negative breast cancer	Without BRCA1/2 mutation With BRCA1/2 mutation	Phase I; Negative Phase I; positive	40 mg+irinotecan 100 mg/m^2	Leukopenia, neutropenia, nausea, diarrhoea, fatigue, anaemia and vomiting	LoRusso et al.[183]
Oral; Combined with Cisplatin and etoposide	Small cell lung cancer	Previously untreated	Phase I; positive	100+Cisplatin 75 mg/m^2+etoposide 100 mg/m^2	Dehydration, diarrhoea, fatigue, febrile neutropenia, heart failure, leukopenia, lymphopenia, nausea, neutropenia, respiratory failure and thrombocytopenia	Owonikoko et al.[184]
Oral, Combined with carboplatin and paclitaxel	Squamous (Sq) non-small cell lung cancer	Untreated advanced/ metastatic	Phase III;	120 mg+carboplatin AUC 6 mg/mL/m IV and paclitaxel 200 mg/m^2 IV	—	McKee et al.[185]
Oral; with metronomic Cyclophosphamide	Solid tumours and lymphomas	Advanced	Phase I; Positive	60 mg+cyclophosphamide 50 mg	—	Kummar et al.[186]
Oral; with cisplatin and gemcitabine	Pancreas adenocarcinoma	Potential BRCA/PABL2 mutated	Phase Ib; Positive	80 mg+C 25 mg/m^2 IV, G 600 mg/m^2 IV	Anaemia, neutropenia, thrombocytopenia, haematologic toxicity and fatigue	O'Reilly et al.[187]
Oral; With Temozolomide (TMZ)	Castration-resistant prostate cancer	Docetaxel-pretreated patients with metastatic	Phase I; positive	40 mg+TMZ 150 mg/m^2	Thrombocytopenia, anaemia, fatigue, neutropenia, nausea and constipation	Hussain et al.[188]

DNA repair mechanism and increase the risk of malignancies.[146] Inhibition of DNA repair process may lead to cell death and this brings PARP-1 as a perfect target for anti-cancer therapy. PARylation of targeted proteins by PARP-1 on activation by SSBs and DSBs facilitates the recruitment of DNA repair proteins such as XRCC1 to sites of damage.[147,148] PARP-1 may also facilitate HR via recruitment of factors like ataxia telangiectasia-mutated (ATM, Ataxia Telangiectasia Mutated), Nijmegen breakage syndrome 1 (Nbs1) and mitotic recombination 11 (Mre11) to sites of DSBs.[149] However, major role in HR repair involves localization of BRCA-1 and BRCA-2. BRCA-1 plays an essential role in the surveillance of DNA damage and transduction of DNA repair responses, while BRCA-2 is directly involved in double-stranded DNA repair, *via* modulation of Rad51 by HR.[150]

PARP-1 inhibition does not cause cell lethality by itself, as the cell has an intact HR pathway for DNA repair. Cells that have a mutated *BRCA1* or *BRCA2* genes as in the case of breast cancer or those that are deficient in BRCA1 or BRCA2 proteins like sporadic cancers are found to be defective in their ability to repair DNA through HR and henceforth depend on error-prone NHEJ. This results in amplification of DNA instability and chromosomal aberrations eventually causing cell death (Fig 3a). This synergistic effect has been very well demonstrated by Arun et al.,[151] wherein PARPi AZD2281 showed more promising results in BRCA1- and BRCA2-bearing mutants *via* induction of autophagy. This concept of synthetic lethality has been implemented upon in cancer therapeutics. In cases of breast and ovarian cancer, treatment with PARP-1 inhibitors Olaparib and Veliparib (Approach A) has found positive clinical results.[152] Epigenetic modulation or artificial inactivation of BRCA pathway (Approach 2a) in cases of sporadic cancer along with the use of PARPi plays a key to therapeutics. This synergistic inhibition of DNA repair poses as a double-hit mechanism for cancer cell death. PARPi can also be used in combination with chemotherapy and radiation (Approach 2b) to render the cells prone to cell death under enhanced damaged conditions as in cases of non-Hodgkin lymphoma cell line, use of PARPi in combination with both external beam radiation and ^{131}I-tositumomab; radio sensitization with veliparib in head and neck carcinoma cell lines and lung cancer xenograft models; or with niraparib in neuroblastoma cell lines, and whole brain radiation in cases of brain metastases [153] (Fig 3b). In addition, Table 3 compiles various drug combinations with Olaparib (Table 3a) and Veliparib (Table 3b) which are being currently extensively used in various cancers along with its side effects.

The transcriptional role of PARP-1 in cancer includes chromatin modulation of tumour suppressor and oncogene function, regulation of the metastatic processes, alteration of cell survival and adaptation. For example, in liver cancer, ATPases activity of ALC1 (amplified in liver cancer 1) was found to be dependent upon both PARP-1 and NAD^+.[154] Furthermore, various tumour cell lines exhibited overexpression of PARP-1 with malignancy progression.[155] One of the recent studies indicated that following irradiation, PARP-1 activation plays a critical role in prostate cancer cell lines (LNCaP and DU145).[156]

PARP-1 is also thought to be an important modulator of tumour suppressor gene, p53.[157] In addition, PARP-1 is known to regulate organ site-specific tumour suppressors as explained by tumour

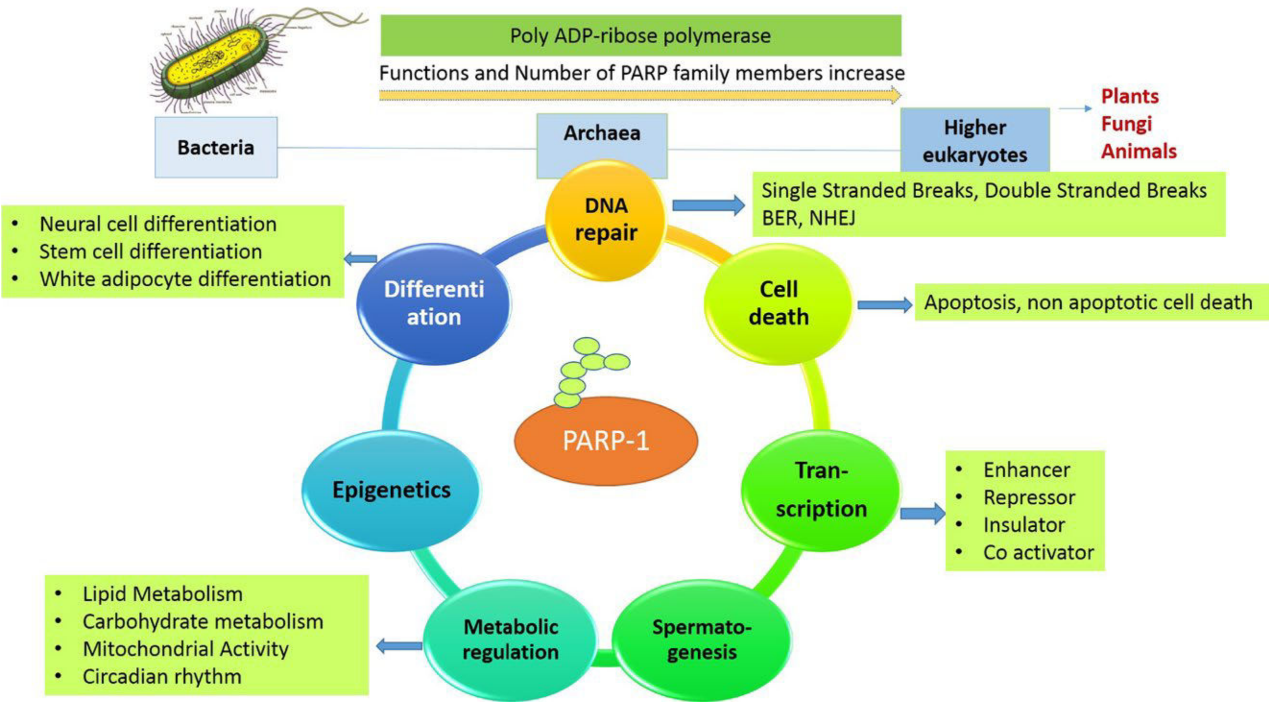

FIGURE 4 PARP-multifunctional protein. Poly ADP-ribose polymerase family of proteins are detected from prokaryotes to eukaryotes. The number of PARP family members and their involvement in various cellular processes increases with the complexity of the organism. PARP-1, the most studied PARP family member is involved a wide range of processes like DNA repair, cell death, transcription, spermatogenesis, metabolic regulation, epigenetics and differentiation

suppressor gene, APC (adenomatous polyposis coli). The loss of this gene was associated with sporadic colorectal cancer (CRC). Collective reports suggest that PARP-1 controls activity of T-cell factor (TCF)/lymphoid enhancer factor (LEF), i.e. TCF/LEF complex in CRC with higher expression levels of PARP-1.[158,159] In addition, Schiewer et al. [160] showed that PARP-1 controls androgen receptor (AR) association and functions with chromatin using in vitro and in vivo systems. In particular, reduction in AR activity was correlated with significant anti-tumour response to PARP-1 inhibition, indicating the dependence of prostate cancer on PARP-1 activity.[160] Thus, these studies suggest that inhibition of PARP-1 has potential as a cancer therapeutic through at least two mechanisms: (1) by potentiating chemotherapeutic agents that damage DNA and increasing tumour sensitivity; and (2) by inducing "synthetic lethality" in cells that are highly dependent on PARP-1, due to deficiency in homologous recombination such as BRCA1 mutants.

4.3.10 | Clinical implications of PARP-1 in other diseases

Dysfunctional PARP-1 has been linked to the onset and progression of myriad of diseases including cancer, ageing, diabetes, neurological diseases, etc. Several evidences point out the role of PARP-1 in cancer. In addition, PARP-1 has also been associated in neuronal pathology. PARP-1 inhibition has been proven to play a protective role in Parkinsons and Alzheimer's disease.[161] Moroni et al. also illustrated PARP-1 inhibitor HYDAMTIQ to be very effective in conferring neuroprotection post stroke.[162] In addition, PARP-1 activation plays a role in diabetic nephropathy, neuropathy and retinopathy. Studies in experimental models reflect the role of PARP-1 in inflammatory responses by promoting inflammation-relevant gene expression. Moreover, activation of NF-κB, AP-1 and heat shock factor protein-1 transcription factors, classically known to signal inflammatory gene expression are mediated by PARP-1.[163,164] PARP-1 also controls immunosuppressive function of regulatory T cells by destabilizing Foxp3.[165] Also, an increase in Foxp3$^+$T regulatory cells has been observed in PARP-1 deficiency.[166] PARP-1 has thus emerged as a very important therapeutic target not only in cancer but also in several other diseases which can be further probed for its therapeutic potential.

5 | CONCLUSION

The current research in PARP-1 biology unravels the role of PARP-1 beyond DNA repair and its involvement in several biological/cellular processes, such as epigenetics, transcriptional regulation, spermatogenesis, differentiation, etc. (Fig 4). The role of PARP-1 as a transcriptional regulator has shed light on the broader aspect of PARP-1 in the cell. Recent studies have also highlighted the multifaceted role of PARP-1 in transcriptional regulation and provided new insights into how PARP-1 plays a very important role in signalling pathways in the cell. In addition, PARP-1's potential in therapeutics for diverse disease conditions require more animal-based clinical studies. Much work needs to be done to understand how PARP-1 works in conjunction with the other PARP family members. Moreover, PARP-1 inhibitors have been a promising therapeutic for a wide range of pathological conditions. Inhibiting PARP activity uncovers potential of PARP inhibitors as promising candidates for cancer therapy, particularly in BRCA1/2-mutated cancers, alone or in combination with cytotoxic drugs. p53-deficient breast cancer cells treated with a PARP inhibitor happen to lose resistance to an apoptosis promoting, clinically active anti-tumour agent called doxorubicin. However, these PARP inhibitors have several side effects that are toxic to the cell as the reports clearly show PARP-1's role in physiological conditions. Hence, to harness the therapeutic potential of PARP-1, studies are required to find out new inhibitors with least side effects. Thus, PARP-1 has now opened new avenues for researchers to understand PARP-1's multifunctional role in the cell which would eventually aid to further expand the utility of PARP family and its inhibition in therapeutics.

ACKNOWLEDGEMENTS

RB thanks Council of Scientific and Industrial Research, New Delhi (38 (1383)/14/EMR-II) Department of Biotechnology, New Delhi (BT/PR4383/BRB/10/1014/2011) and Department of Science and Technology, New Delhi (SR/SO/BB-03/2010) and for financial support. TJ thanks UGC-SAP DRS for fellowship. RB thanks Dr. Mitesh Dwivedi for his critical suggestions. It is to be noted that none of the authors have any conflict of interest.

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Seminars in Cell & Developmental Biology xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Seminars in Cell & Developmental Biology

journal homepage: www.elsevier.com/locate/semcdb

Review

Poly ADP-ribose polymerase-1: Beyond transcription and towards differentiation

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A R T I C L E I N F O

Article history:
Received 29 June 2016
Accepted 27 July 2016
Available online xxx

Keywords:
PARP-1
DNA independent activation
Gene regulation
Differentiation
Therapeutics

A B S T R A C T

Gene regulation mediates the processes of cellular development and differentiation leading to the origin of different cell types each having their own signature gene expression profile. However, the compact chromatin structure and the timely recruitment of molecules involved in various signaling pathways are of prime importance for temporal and spatial gene regulation that eventually contribute towards cell type and specificity. Poly (ADP-ribose) polymerase-1 (PARP-1), a 116-kDa nuclear multitasking protein is involved in modulation of chromatin condensation leading to altered gene expression. In response to activation signals, it adds ADP-ribose units to various target proteins including itself, thus regulating various key cellular processes like DNA repair, cell death, transcription, mRNA splicing etc. This review provides insights into the role of PARP-1 in gene regulation, cell differentiation and multicellular morphogenesis. In addition, the review also explores involvement of PARP-1 in immune cells development and therapeutic possibilities to treat various human diseases.

Contents

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http://dx.doi.org/10.1016/j.semcdb.2016.07.027
1084-9521/

1. Introduction

Innumerable studies over decades have illustrated the most fundamental concepts in gene regulation [1,2]. Cell growth and differentiation involve cellular transition from one type to the other, a process that is tightly modulated by gene regulatory networks that involve activation and recruitment of specific transcription factors, chromatin remodeling, histone regulation, post translational modifications, alternative splicing, epigenetics etc. Multiple molecular systems involving an array of gene regulatory machinery initiate, promote and execute various processes involved in maintaining these complex cellular signaling pathways. Poly (ADP-ribose) polymerase-1 (PARP-1) is a ubiquitously expressed highly conserved protein in mammalian tissues with predominant nuclear localization. Exploration of several molecular events governed by PARP-1 to regulate gene expression, cell differentiation and development pathways may lead to new therapeutic possibilities for several human diseases. The article focuses on the unaccounted roles of PARP-1 in gene regulation, differentiation, development, morphogenesis, and multicellularity along with its therapeutic potential in immune cell development.

2. Poly ADP-ribose polymerases (PARP-1)

One of the emerging families of proteins involved in gene regulation for development and differentiation is the Poly ADP-ribose polymerase family. Poly ADP-ribose polymerase belongs to the ADP-ribosyltransferase (ART) family [3,4]. These transferases utilize nicotinamide adenine dinucleotide (NAD^+) as its substrate for the synthesis of mono or poly (ADP-ribose) polymers on target acceptor proteins and this process is termed as poly (ADP-ribosylation) or PARylation [5,6]. The negatively charged ADP-ribose units are degraded by poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribosylhydrolase 3 (ARH3) [7,8]. However, macro domain containing proteins and NUDIX hydrolases have also been shown to degrade ADP-ribose polymers [9]. In the due process of PARylating target proteins, PARP-1 itself becomes a prime target for PARylation- this process is called as auto- modification. PARylation and PARP proteins have been identified across various domains of life from plants to animals as well as lower life forms like bacteria, double-stranded DNA viruses etc. [10,11]. The human PARP family comprises of 17 members with either mono- or poly-ADP-ribosyltransferase activity possessing a conserved PARP signature motif in their catalytic domain [3]. PARP-1, recently termed as ADP-ribosyltransferase diphtheria toxin-like (ARTD-1), is one of the most abundant nuclear enzymes responsible for ~90% of the PARylation activity [12,13]. Although PARP-1 is one of the well-studied and characterized members of the PARP protein family, yet many of its features still remain unexplored. PARP-1 is a 116-kDa nuclear protein with well characterized structural domains [3,4] viz., (1) the N-terminal DNA binding domain comprising of three zinc finger motifs, a nuclear localization signal (NLS) and a zinc binding domain [15,14] (2) an auto-modification domain with a BRCA1 C-terminus (BRCT) motif, for protein–protein interactions [4] (3) a WGR (Trp-Gly-Arg) motif with a plausible role in nucleic acid binding, and (4) a C-terminal catalytic domain comprising of the PARP regulatory domain (PRD), and the highly conserved PARP signature motif with acceptor site for adenosine and donor site for nicotinamide [4]. PARP-1 acts as a DNA damage sensor molecule and participates in various DNA repair processes involved in protection against cell death [16,17]. Conversely, a hyper-activated PARP-1 is destructive [18]. Accumulating evidences demonstrate that the activity of PARP-1 is also linked to cellular signaling pathways wherein they regulate gene expression, interactions of RNA/protein and protein/protein, recruitment of proteins and the location and activity of proteins involved in signaling responses [19].

3. PARP-1 in regulation of gene expression

The regulation of gene expression by PARP-1 is a crucial aspect for cell differentiation and development in many organisms. The underlying mechanisms for PARP-1 dependent gene expression or transcriptional regulation are well established. However, its activation and signaling are prerequisites to execute these important processes.

3.1. Activation of PARP-1: Kicking signals up a notch

Varied endogenous and exogenous genotoxic stress cues have been implicated in PARP-1 activation. Genotoxic stress can arise due to; (a) Endogenous agents such as reactive oxygen and nitrogen species that are generated during various metabolic activities and cellular processes [20,21]. Conditions including hypoxia [22], elevated extracellular glucose concentration [23,24] and angiotensin II [25,26] are known to produce rampant reactive oxygen species (ROS) within the cell endangering the cellular genomic integrity. (b) Exogenous agents involving different environmental factors that can be harmful for the genome. (c) non B-DNA structures, DNA breaks like cruciform, protein-protein interactions, nuclear nicotinamide mononucleotide adenylyl transferase 1 (NMNAT1), hormone secretion etc. [27–31] are also known to activate PARP-1. Auto-modification domain of PARP-1 is implicated in protein-protein interactions along with PARP-1 homodimerization [32], an indispensable factor for its higher enzymatic activity. As per other reports, N-terminal region of PARP-1 is important for its dimerization and activation [33]. However, the basis and importance of PARP-1 dimerization for its activity is yet unclear.

Previous research established that formation of a compact PARP-1–DNA complex is imperative for its catalytic activity [15]. The helical subdomain in the catalytic domain of PARP-1 is autoinhibitory in nature and regulates productive binding of NAD^+ [34]. In addition, the N-terminal tail of histone H4 can also induce PARP-1 activation to a higher degree than damaged DNA [35]. Upon genomic insult, maximal PARP-1 activation is achieved by phosphorylation of PARP-1 by the mitogen-activated protein kinase (MAPK) and extracellular-signal-regulated kinase (ERK) 1/2 [36]. This in turn stimulates transcription of genes implicated in cell development and proliferation through phosphorylation of transcription factor, Elk1 [37]. Also, NMNAT-1 modulates PARP-1 by binding to auto-modified PARP-1, triggering its enzymatic activity and allosterically regulating both the PAR synthesis activity and PAR chain elongation of PARP-1. NMNAT-1 is shown to stimulate PARP-1 in the absence of ongoing NAD^+ biosynthesis, suggesting that NAD^+ synthesis and PARP-1 activation are two separable biochemical functions displayed by NMNAT-1 [31]. Furthermore, kinase-PARP-1 interaction is crucial for gene regulation during cellular differentiation. Reports have proposed that calcium-dependent protein kinase, CamKIIδ stimulates PARP-1 activation through its phosphorylation during neuronal development [38]. This cross talk between PARP-1 and kinase is important for fibroblast growth factor (FGF)-stimulated neuronal differentiation of embryonic stem cells and cell death (JNK, p38, Akt, Erk etc.). Another post-translational modification that activates PARP-1 is acetylation; upon coactivation of NF-κB by interaction with subunits of NF-κB (p65 and p50) and p300 [39]. In addition, developmental or environmental (exogenous) cues, such as steroids or thermal shock also stimulate PARP-1. dPARP (*Drosophila* homolog of PARP-1) plays a pivotal role in chromatin decondensation during heat shock of polytene chromatin [40,41]. Upon heat shock, PARP-1 activation occurs at heat-shock-responsive loci which alters or loosens nucleosome assembly in a PAR-dependent manner to enhance RNA polymerase II dependent transcription [40,42]. SIRT6, a member of another NAD^+ utilizing enzyme *i.e* Sirtuins, possesses either mono-ADP-ribosyltransferase, or deactylase activity and is identified to activate PARP-1 by mono-ADP-ribosylating PARP-1 in response to DSB's [43]. SIRT1 is also reported to inhibit PARP, and in the absence of SIRT1, hyperactivated PARP leads to AIF mediated cell death [44]. Also, Kinesin superfamily protein 4 (KIF4) interacts with PARP-1 via its C terminal domain, and keeps it inactive. Thus, it is evident that DNA damage alone does not induce PARP-1 activation but environmental, developmental cues and certain post-translational modifications can also activate PARP-1, thereby supporting the multifunctional roles of PARP-1 in different aspects of cell biology.

3.2. Signaling of PARP-1: Sensing the nicks

PARP-1 identifies the genomic lesions such as SSBs, DSBs, non-BDNA structures etc., gets activated, and recruits itself at the deformed site, forming homodimers and triggering the transfer of an ADP-ribosyl moiety from NAD^+ to aspartate or glutamate residues of its target substrates in the chromatin, mainly linker histone H1. This leads to structural changes involving unwinding of the highly condensed chromatin that renders the DNA easily accessible to transcription and repair enzymes [45,46,40]. This entire process commences with NAD^+ which is synthesized from nicotinic acid (NA). NA phosphoribosyltransferase (NAPRT) initiates the reaction, which uses phosphoribosyl pyrophosphate (PRPP) to form phosphoribosyl pyrophosphate (NAMN). Together with ATP, NAMN is then converted into (NA adenine dinucleotide) NAAD by the NMNAT1-3 enzymes. Finally, NAAD is transformed to NAD^+ through an amidation reaction catalyzed by the NAD^+ synthetase (NADSYN) enzyme. PARPs catalyze the polymerization of ADP-ribose units from donor NAD^+ molecules on accepter proteins, resulting in the addition of linear or branched polymers. Covalently attached PAR gets hydrolyzed by PARG and ARH3, both of which possess both endoglycosidic and exoglycosidic activities [47].

The different mechanisms of PARP-1 dependent regulation of gene expression are explained here.

3.3. PARP-1 dependent gene expression by chromatin remodeling: Revamping the DNA

Several *in vivo* and *in vitro* studies have revealed the transcriptional regulatory mechanism of PARP-1 for shaping the chromatin architecture. Binding of PARP-1 to nucleosomes facilitates compaction or loosening of the chromatin structure and regulates gene expression at both splicing and transcriptional levels [48,49]. PARP-1 interacts with chromatin based on nucleosome complexity such as histone variants and modifications of histones. It has the ability to recruit chromatin architectural/remodeling proteins, histones or histone variants by PARylating them [50,51] or it can compete with histone H1 to bind to nucleosomes [52].

The histone H2A variant, macroH2A, inhibits PARP-1, leading to silencing of inactive X chromosome [53]. In *Drosophila*, the conformational changes of core nucleosome due to replacement of H2A with variant H2Av assist the binding of PARP-1 to H3 and H4 [54] through C-terminal domain of PARP-1 for chromatin structure rearrangement [35]. H4 activates whereas H2A completely inhibits PARP-1 enzymatic activity. Acetylation of H2A at 5th lysine residue activates PARP-1 by removing the inhibitory effects of H2A (Fig. 1). Phosphorylation of H2Av histone variant at Ser137 by JL1 kinase in *Drosophila* activates PARP-1 and increases the interaction between PARP-1 and H4 causing H4 to get polyADP-ribosylated (Fig. 1). This in turn induces JL-1 dependent phosphorylation of H3Ser10 causing nucleosomal shifts and conformational changes leading to chromatin loosening and transcriptional initiation [55].

Local chromatin loosening by PARP-1 has also been reported at the puff loci in *Drosophila* which assists transcription and chromatin remodeling during development [40]. Interaction of nucleosome remodeling or histone modifying enzymes with PARP-1 can modulate their localization and activities. SMARCA5, the catalytic subunit of ISWI chromatin remodeling complex, gets recruited to DNA damage site in PARP-1 dependent manner, which then interacts with RNF168, E3 Ubiquitin ligase. This implies that spatial organization of PARylated RNF168-driven response to DNA damage depends on the activity of PARP-1 [56]. Previous reports by Meyer-Ficca et al., have shown that PARP inhibition results in aberrant chromatin condensation and histone retention by removing histone H1 during spermiogenesis, linking PAR metabolism with the integrity of mice sperm chromatin [57,58]. The same group further revealed that PARP-1 and PARP-2 activities modulate the chromatin structure through DNA Topoisomerase II beta (TOP2B) during spermiogenesis in mice [59]. Recently, it is reported that PARP-1 may act as a chaperone of histone molecules, recruiting or directly binding to histones or other proteins. PARylation of PARP-1 itself can bind to free histones to assist assembly of nucleosomes [60]. This association of PARP-1 and histones is summarized in Fig. 1.

In addition to this, PARP-1 can bind between linker DNA and nucleosome to open nucleosome structure, allowing regulatory proteins to access DNA [61,62]. It has been demonstrated that lipopolysaccharide (LPS) stimulation enhances PARP-1 enzymatic activity and PARylation of histones at nucleosome-occupied promoters which increases the promoter accessibility to facilitate NF-κB recruitment followed by gene transcription in macrophages [63]. Izhar et al., [64] also discovered amyotrophic lateral sclerosis (ALS) factor-TAF15 that gets recruited at the DNA lesion in a PARP-1 dependent manner thus emphasizing the critical role of PARP-1 in mediating DNA damage response.

3.4. PARP-1 dependent gene expression by splicing (alternative/pre-mRNA/co-transcriptional): Sp(li)cing up the genome

Another indispensible function of PARP-1 is alternative splicing by PARylating splicing factors or modulating RNAs. PARP-1 has been found in association with mRNA, human pre-mRNA 3′-end-processing complex factor and noncoding pRNA (promoter-associated RNA) [65,66], chromatin and splicing factors suggesting its role in co-transcriptional splicing [67]. The components involved in PARP-1 mediated splicing are shown in Fig. 2. In addition, heterogeneous nuclear ribonucleoproteins (hnRNPs) have been identified to be PARylated by PARP-1 during alternative splicing [68]. This is substantiated by presence of conserved PAR binding motif in

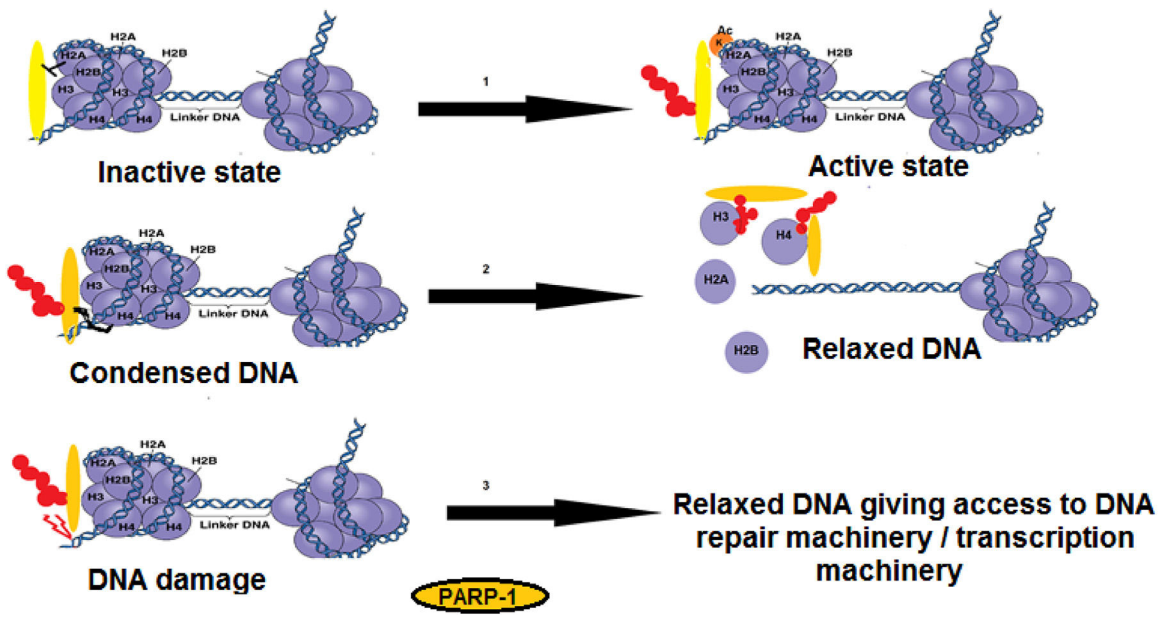

Fig. 1. PARP-1 and histones: (1) Under normal physiological state, histone H2A interacts with PARP-1 inhibiting PARP-1 activity. However, acetylation of the 5th lysine residue of H2A relieves the inhibition allowing functioning of PARP-1 (2) DNA-independent PARP-1 activation. Developmental or environmental cues cause changes in the "histone core" and consequently the N-terminal tail of histone H4 is exposed followed by H4-dependent PARP-1 activation. (3) Stress-dependent PARP1 activation: The N-terminal domain of PARP-1 protein serves as a sensor of the damaged DNA. Upon binding to such structures, it mediates conformational changes leading to disruption of interaction with histones and subsequently relaxed DNA giving access to DNA repair proteins or transcription machinery.

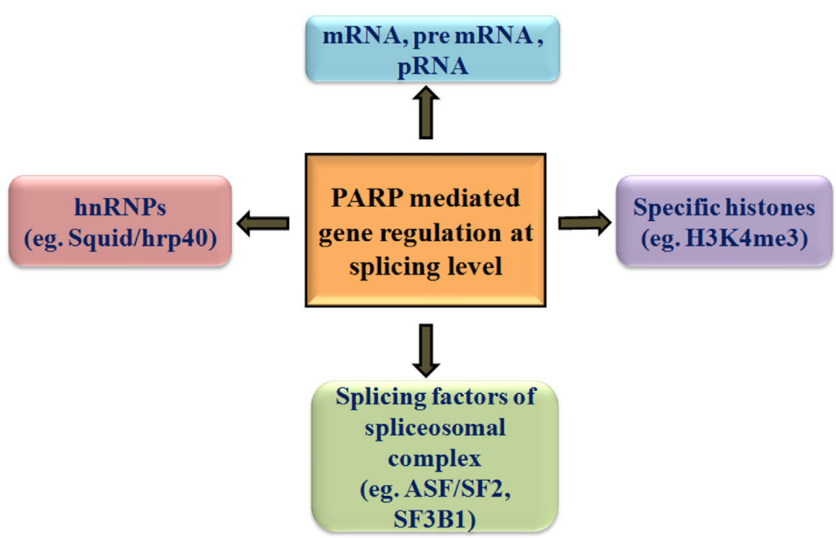

Fig. 2. Components of PARP mediated regulation of splicing: PARP-1 can interact or form a complex with mRNA, pre mRNA, noncoding pRNA (promoter-associated RNA), hnRNPs and splicing factors by PARylating or modulating them, proving its importance in splicing for regulation of gene expression.

human hnRNPs such as A1, G, H, K etc. [69]. PARylation of *Drosophila* hnRNPs (Squid/hrp40 and Hrb98DE/hrp38) decreases their RNA-binding activity subsequently altering their alternative splicing activities [68]. Assembly of Cajal Bodies (CB) involved in pre-mRNA splicing, spliceosome formation and RNPs maturation [70], are maintained by PARP-1 autoPARylation too. During splicing, the PAR moieties interact with coilin and fibrillarin and get localized in Cajal Bodies (CBs) in a PARP-1 dependent manner. Not only PARP-1 but PAR molecules also behave as a regulator of gene expression. PAR molecules form a complex with alternative splicing factor/splicing factor 2 (ASF/SF2) to control topoisomerase I mediated gene regulation [71]. Collectively, all these findings strongly support the regulatory role of PAR and PARP-1 in alternative splicing and co-transcriptional mechanism. Further studies are needed to decipher the gene regulatory mechanism of PARP-1.

3.5. *PARP-1 dependent gene expression through transcriptional regulation: Managing the chromatin dynamics*

PARP-1 can stimulate transcription positively with co-activators or negatively with repressors [72], on the basis of DNA binding transcription factors' sequence specificity [49]. Activated PARP-1 gets auto-PARylated followed by its dissociation from nucleosome assembly which in turn decondenses the chromatin to initiate/enhance transcription [35]. Actively transcribed sites (promoters) are found to be enriched with PARP-1 [49]. PARP-1 acts as a co-activator for the Nrf2 genes, enhancing their expression through interaction with antioxidant response elements (ARE) [73]. Recently, it has been investigated that stimulation-induced Erk-PARP-1 synergism in chromatin of cerebral neurons is essential for immediate early gene expression involved in long-term memory [74]. Repression of transcriptional activities by incorporation of PARP-1 into chromatin structure leads to compaction of chromatin and inhibition of transcription [75,14]. In an Amish population study, nucleosome positioning was shown to be influenced by PARP-1 leading to heterozygous mutation in microsomal epoxide hydrolase (mEH) gene. The transcription of mEH was inhibited through PARP-1 binding to the mEH proximal promoter, impacting chromatin structure [76] . Kotova et al., [75] have disclosed PARP-1 induced silencing of retro-transposable genes and formation of heterochromatin structures in *Drosophila.* Pharmacological inhibition of PARP-1 with Olaparib results in global gene deregulation through polycomb repressive complex 2 (PRC2) member, EZH2 in a lymphoblastoid B cell line [77]. Recent studies are drawing attention towards rDNA silencing by PARP-1 in cell division. TIP5 (component of NoRC chromatin remodeling complex) combines with PARP-1 to maintain rDNA silencing after replication and represses transcription in a chromatin dependent manner [78].

3.6. PARP-1 dependent gene expression by DNA methylation: Silencing the genome

Methylation is one of most key post-translational modifications of histone proteins which can alter chromatin structure and thereby regulate gene expression. PARP-1 and PAR molecules play an important role in maintaining DNA methylation patterns by regulating the activity as well as expression levels of DNA methyl transferases (Dnmt)1 [79–81]. Functional interaction of PARP-1 with methylcytosine dioxygenase, Tet2 stimulates formation of 5-hydroxymethylcytosine (5hmC) from 5-methylcytosine (5mc). This interaction plays an important role in epigenetic programming of somatic cells during their transition to pluripotency [82]. Demethylase activity of histone lysine demethylase was shown to be maintained by PARP-1, PARylating it and hence inhibiting binding of KDM5 to chromatin, influencing alterations in chromatin structure. This study explains the co-ordination of PARP-1 with histone modification for controlling transcription [83]. CTCF (CCCTC-binding factor) is considered to be a vital protein through which PARylation conserves the unmethylated pattern of a few DNA sequences [84]. Irrespective of DNA damage, CTCF stimulates PARylation activity of PARP-1. Moreover, CCCTC-binding factor (CTCF) loses its nuclear localization due to PAR depletion eventually resulting in changes in the chromatin arrangement. This work underlined the link between CTCF and PARylation to uphold chromatin architecture and DNA methylation patterns [85] as also shown in Fig. 3 .

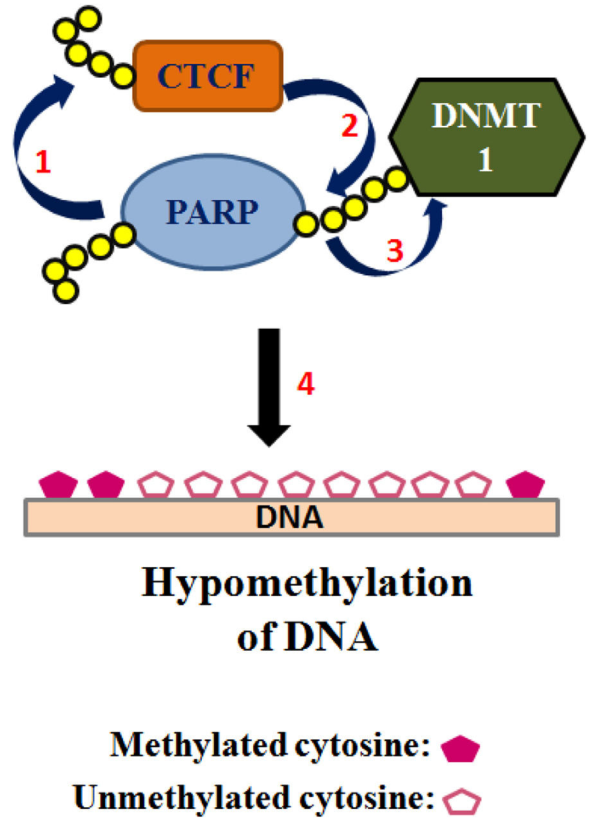

Fig. 3. DNA methylation control by PARP-1: PARP-1 PARylates N-terminal end of transcription factor, CTCF, which in turn stimulates auto-PARylation of PARP-1. This CTCF-PARP-1 interaction inhibits the catalytic activity of DNA methyl transferase 1 (DNMT1) through non covalent binding between PAR-DNMT1 due to which DNA methylation is inhibited thereby maintaining the methylation pattern.

4. PARP-1 in development: From the cradle to the grave

4.1. PARP-1 in embryonic development

Accumulating evidences suggest a role of PARP-1 in driving cell differentiation and cell fate commitment. PARylation by PARP-1 is developmentally regulated. PARP-1 and PARP-2 have been established to play an important role in mouse embryogenesis and development. de Murcia et al. (2003) reported that PARP-1 and PARP-2 double mutant mice died at the onset of gastrulation [86]. Moreover, female embryonic lethality is found to be associated with specific X-chromosome instability in *PARP* knock out (KO) mice [86]. An early post implantation embryonic lethality is observed in mouse having double mutation in both ATM and PARP-1 genes at embryonic day 8.0. The synergistic effect of these genes may be responsible for either monitoring or repairing DNA damage during embryo development [87]. PARP-1–Ku80 interaction is vital for early developmental stage, as suggested by Henrie et al., [88]. They show that PARP-1$^{-/-}$Ku80$^{-/-}$ embryos fail to repair DNA damage in early embryogenesis developmental stage generating an embryonic lethal phenotype of these double KO mice. The Wnt signaling pathway is considered to be a central pathway for embryogenesis wherein T-cell factor-4 (TCF-4) combines with nuclear B-catenin to form a complex [89]. Earlier studies show that transcriptional activity of TCF-4 and B-catenin complex is enhanced by interaction with PARP-1 [90]. The same group further revealed the competitive interaction between PARP-1 and Ku70 to control TCF-4 and B-catenin complex mediated gene trans-activation [91]. Moreover, the loss of SSBR/BER and DSB repair pathways' component led to massive apoptosis of the embryo as a result of defect in DNA-damage signaling. *DYX1C1* is a known candidate gene for dyslexia susceptibility. PARP-1 is also reported to be essential in migration of neurons during embryogenesis, forming a complex with TFII-I and SFPQ proteins to control transcription of *DYX1C1* [92]. *Drosophila* has been used as a model system to illustrate the role of PARP-1 during larval development. Higher PARP enzymatic activity is found at the prepupal stage of development in *Drosophila* [93]. These reports are indicative of role of PARP-1 in embryogenesis *via* different pathways wherein DNA repair is identified to be PARP-1's primary role.

4.2. PARP-1 in germline development

Germ cells play an important role in reproduction; development and evolution and therefore transfer of conserved genetic information from these cells to the next progeny is a matter of prime importance. PARP-1 *via* its multifunctional roles in DNA repair, gene transcription, chromatin remodeling and epigenetic modification proves to be an important candidate in germline development. The temporal and spatial expression of few PARP-1 target genes, along with their molecular interactions, is ultimately responsible for germ cell differentiation [94]. PARylation regulates the key events in epigenetics, chromatin structure modulation and transcription in germ line differentiation and development. It was reported that PARP inhibition in mice ovaries increases oogenesis and folliculogenesis by regulating expression of transforming growth factor (TGF) super-family members and other genes involved in oogenesis pathways [95]. *PARP-1* KO was found to be more sensitive to genotoxic stress due to its compromised DNA repair ability. PARP1$^{-/-}$ mice embryonic fibroblast displayed tetraploid population with an unstable genome [96] and immortalized embryonic fibroblasts showed hyperploidy in *PARP-1* KO conditions [97]. Partial KO of PARP (*PARP-1*$^{+/-}$) and complete KO of PARP-2$^{-/-}$ was found to be associated with X chromosome instability conferring specifically female lethality [86]. PARP-1 null oocytes exhibit defects in meiosis like persistent H2AX phosphorylation, suggesting a role of PARP-1 in chromatin modification during oogenesis [98]. The expression of PARP-1 is restricted to the peripheral cellular layer which contains proliferating spermatogonia, whereas the PARP-2 is evenly distributed all over the seminiferous tubules, suggesting a crucial role of PARP for different aspects of spermatogenesis [32]. Dantzer et al., [99] have reported a decline in fertility potential of males in PARP-2 deficient mice that relates to defects in spermiogenesis and meiosis-I. GATA3 regulates cell growth and tumorigenesis by favoring G1/S transition and transcriptional regulation of the CCND1 (coding Cyclin D1 protein). PARP-1, a transcriptional coactivator for GATA3, interacts with the later and this interactome in turn transactivates the CCND1 gene [100]. Mouse PARP-1 is found to be enriched and is suggestive of a role in gene silencing of inactive X chromosome [53]. Thus, it will be interesting to further probing the roles of PARP-1 in X chromosome inactivation and germ cell development.

4.3. Mechanism of gene regulation for development/differentiation by PARP-1

PARP-1 has been shown to regulate growth and development of variety of cells [37,30] through transcriptional regulation [101]. Although PARP-1's role in differentiation had been detected long back, only recently it has grabbed the interest of researchers. It has been demonstrated that mouse myeloid leukemia cells treated with purified poly (ADP-ribose) differentiated into mature macrophages and granulocytes [102]. ADP-ribosylation mediates formation of a complex between PARP-1 and PPARγ at the promoter regions of target genes, a process that increases ligand binding and aids ligand induced co-factor exchange. ADP-ribosylated PARP-1 then stabilizes the PPAR ligand binding. Torsional stress created during TopoII cleavage further recruits and activates PARP-1. The modified PARP-1 interacts with PPARγ and stabilizes PPARγ ligand binding, which permits an exchange of the NCoR1 co-repressor with the p300 co-activator at PPARγ promoter sites. Further, the PAR formation favors ligand binding and p300 recruitment to PPARγ response elements (PPREs) and thus leading to PPARγ dependent gene expression [103].

PARP-1 is also reported to have role in differentiation of neuroectoderm. Yoo et. al., discovered that FGF-ERK1/2 orchestrates neural specification via regulating PARP-1 activity, thus reinforcing role of PARP-1 in neural induction from human embryonic stem cells [104,36,37]. Recently, PARP-1 has been reported to regulate osteoclastogenesis *via* inhibition of recruitment of p65/RelA to the IL-1β promoter subsequently affecting IL-1β expression [105].

The transforming growth factor beta (TGFB) signaling pathway is another pathway in which PARP-1 plays a crucial role. TGFB signaling pathway is implicated in both the adult organisms and the developing embryo. The TGFB regulates cellular proliferation, differentiation, and migration during embryonic development and adult tissue homeostasis. Smad proteins mediate signals by the TGFB family of morphogens. PARP-1 has been shown to dissociate the Smad complexes from DNA and attenuate Smad mediated transcription [106]. However, Huang et al., [107] demonstrated PARP-1 to be a requisite for TGFB1 induced Smad3 activation. The TGFB1 promotes PARylation of Smad3 resulting in enhanced Smad-Smad binding element [SBE] complex formation and increased DNA binding activity and eventually resulting in transcription of its target genes.

Recent report have further illustrated that PARP-2 assists PARP-1 in negatively regulating Smad function while PARG is a positive regulator [108]. Reports indicate that PARP-1 can act either as a negative regulator of responses to TGFB, as seen in epithelial cells [106] and CD4^{+}T cells [109], or as a positive regulator of TGFB responses, as seen in vascular smooth muscle cells [107] to TGFB signaling. Poly ADP-ribosylation has also been strongly implicated to regulate TGF super family members during oogenesis and folliculogenesis in mice [95]. Moreover, reports also illustrate role of PARP-1 in white adipocyte differentiation and lipid metabolism by regulating PPARγ dependent gene expression [110,111].

Switching on and off of gene expression in response to temporal and spatial patterns is essential in normal development. PARP-1 has been demonstrated to play such a pivotal role in neuronal differentiation. Activated PARP-1 as a part of the groucho/TLE-co-repressor complex drives the dismissal of the poly ADP-ribosylated components of the co-repressor complex from HES1 regulated promoters followed by CAMKIIδ phosphorylating HES1 thereby permitting neurogenic gene activation [38]. PARP-1 has also been recently associated with HIF [Hypoxia inducible factors] in hypoxic response. HIFs control induction of genes involved in cellular metabolism, cell growth, metastasis, apoptosis, and others [112]. PARP-1 is also suggested as an essential co-regulator for retinoic acid [RA] induced gene expression *in vivo* and thus a requisite of RA dependent growth and developmental pathways [113]. In addition, role of poly (ADP-ribosyl)ation by the PARP family member, tankyrase has also been established in the regulation of Wnt-β-catenin signaling pathway which plays a fundamental role during embryonic development and adult tissue homeostasis [114]. On similar lines, PARP-1 also regulates embryonic stem cell differentiation *via* PARylation of SOX2 protein levels and regulation of *FGF4* expression [115]. However, poly ADP-ribosylation of the target proteins finally leading to gene regulation may not be the only mode of regulating gene expression by PARP-1. PARP-1 is reported to regulate *Nrf2* transcription by neither PARylating Nrf2 nor physically interacting with it. Rather, PARP-1 interacts directly with small Maf proteins and ARE of Nrf2 target genes, which amplifies ARE-specific DNA-binding of Nrf2 and enhances the transcription of Nrf2 target genes thereby maintaining intracellular redox homeostasis [73]. Rajawat et al., also showed impediment of development of *Dictyostelium discoideum* when PARP was down-regulated, showcasing its importance during developmental program of the organism [116]. In view of the above literature, it is evident that PARP-1 through its modes of transcriptional regulation as discussed above regulates many signaling pathways in the cell thereby controlling important cellular processes like differentiation which are summarized in Fig. 4.

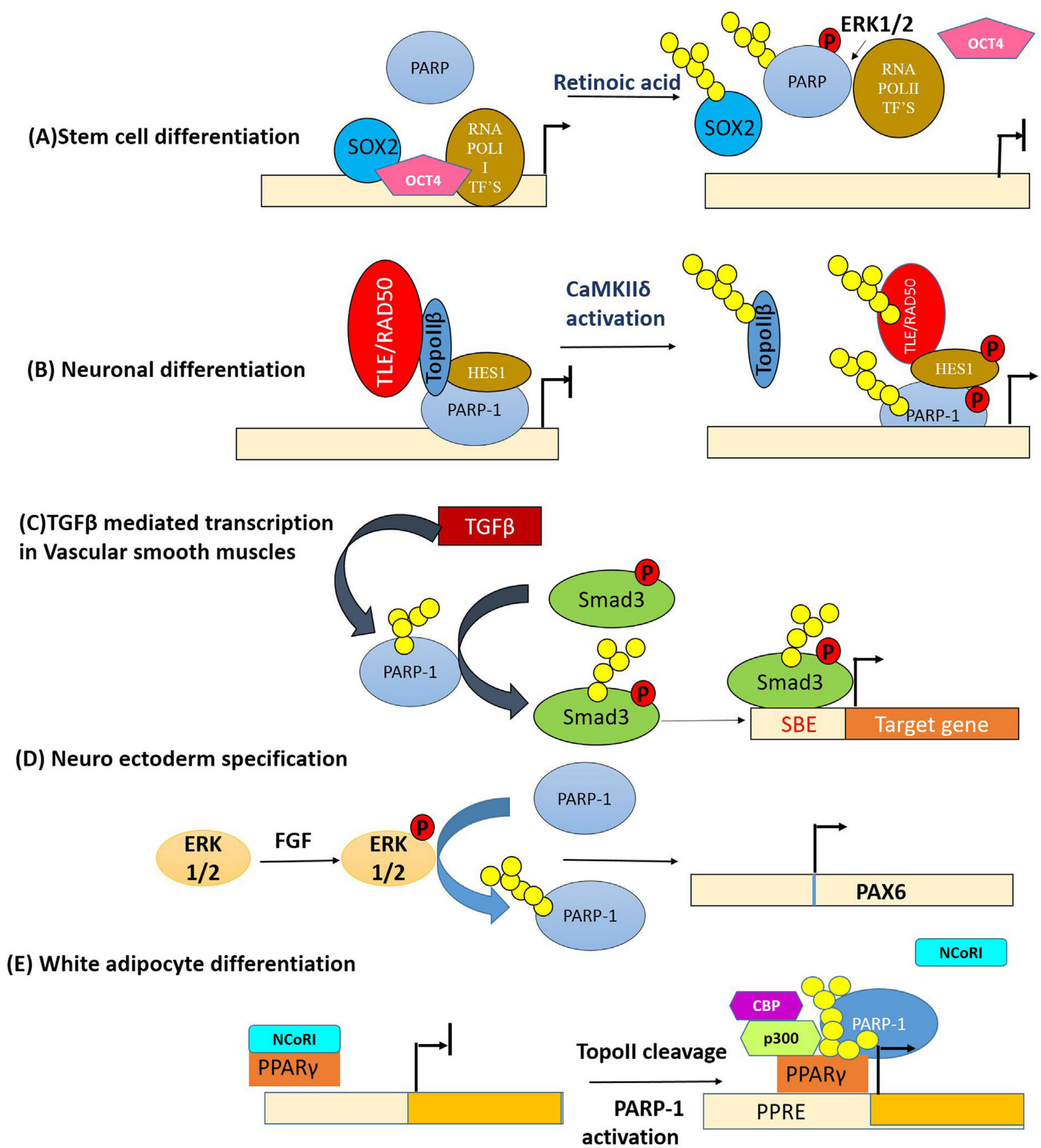

Fig. 4. PARP-1 in differentiation: (A) PARP-1 in regulation of stem cell differentiation: Phosphorylation of PARP-1 by kinase ERK1 leads to auto-modification of PARP-1. PARylated PARP-1 abolishes SOX2 DNA-binding and dimerization with OCT4 leading to transcription inhibition of target genes involved in differentiation. (B) PARP-1 in neuronal differentiation: Treatment of neuronal stem cells with PDGF (platelet derived growth factor) induces activity of the kinase CaMKIIδ. Activated CaMKIIδ leads to phosphorylation of PARP-1, which in turn leads to PARylation resulting in disruption of co-repressor complex including TLE, RAD50, TopoIIβ, PARP-1 and HES1 at promoter. Auto-modified PARP-1 and HES1 recruit histone acetyl-transferase CBP, and also subsequent phosphorylation of repressor protein HES1 by CaMKIIδ initiates transcription. (C) PARP-1 in TGFβ mediated transcription: TGFβ promoted PARylation of Smad3 ensuing into stronger Smad-Smad binding element [SBE] complex formation and increased DNA binding activity and eventually transcription of its target genes (D) PARP-1 in neuro ectoderm specification: FGF-ERK1/2 coordinates PARP-1 activation which eventually facilitates transcription of genes involved in neural differentiation from human embryonic stem cells. (E) PARP-1 in white adipocyte differentiation: ADP-ribosylated PARP-1 stabilizes PPAR ligand binding. Torsional stress created during TopoII cleavage leads to PARP-1 activation. Modified PARP-1 interacts with PPARγ and stabilizes PPARγ ligand binding, which allows an exchange of the NCoR1 co-repressor with the p300 co-activator at the PPARγ response elelments (PPREs) and thereby ensured continuous expression of PPARγ target genes.

5. PARP-1 in cell differentiation/multicellularity in different organisms: Translating time

Cell growth and differentiation processes involve transcriptional activation of a variety of genes in response to specific developmental and environmental stimuli. PARP-1 has also been reported to engage in transcriptional control during development as well [93]. Evidences across domains of life substantiate the role of PARP-1 from unicellular to multicellular forms [117]. The differentiation from preadipocytes to adipocytes triggered by the nuclear receptor PPARγ is an illustration of the involvement of PARP-1 [111] in cellular differentiation. Many such recent accumulating evidences enlisted below propose a strong association of PARP-1 in differentiation and multicellularity.

5.1. PARP-1 in *Dictyostelium discoideum* development

D. discoideum is a unicellular eukaryote exhibiting multicellularity upon starvation [118] and has eight potential *PARP* genes [119]. Our previous study showed that *D. discoideum* exhibits basal PARP activity and inhibition of PARP by benzamide delays the development [120]. Our further study demonstrated PARP's role in development by down-regulation and no effect is observed on the growth profile of unicellular amoebae. On the contrary, development was blocked at the initial aggregation stage, signifying the involvement of PARP-1 during development of *D. discoideum* [116]. In addition, PARP-1 has also shown to be involved in stress responses like oxidative stress via proteases, UV-C stress *via* nitric oxide and DNA repair in *D. discoideum* whereas it has been shown to play no role in staurosporine induced cell death [121–125]. Moreover, our recent lab report shows association of ADPRT1A (PARP-1 ortholog in *D. discoideum*) with cell survival and development [126]. In addition, *ADPRT1A* knockout resulted in altered levels of genes essential for cAMP production and signaling as well as markers of differentiation (Unpublished data). These findings clearly establish the importance of ADPRT1A in *D. discoideum* life cycle.

5.2. PARP-1 in fungus development

The presence of PARP in only multicellular eukaryotes is suggestive of the association between PARP function and a multicellular lifestyle. It has been reported that PARP homologs exist in fungi that have multicellular hyphae and organized developmental structures and lack yeast-like unicellular growth pattern. However, PARP homologs are notably absent in *S. cerevisiae* and *S. pombe* while they are found in all other eukaryotes [127]. Semighini et al., [128] reported that the disruption of the *PrpA* gene (PARP-1 ortholog) in filamentous fungus *Aspergillus nidulans* was found to be lethal in haploid strains, whereas *PrpA* overexpression led to fluffy phenotype caused by defect in conidiation while generation of *PrpA*$^{-}/^{-}$ failed.

Interestingly, PARP overexpression in *P. anserina* led to reduced number of ascospores and fruiting body and also decreased the growth rate and life span whereas complete deletion failed thereby suggesting a vital role of PARP-1 in growth and development [129]. Furthermore, Neurospora PARP-1 orthologue [NPO] mutant strain also exhibited an accelerated aging in mycelia [130]. All these reports are suggestive of PARP-1 role in multicellularity and how its regulated expression is quintessential in the correct development and cellular processes.

5.3. PARP-1 in plant development

In plants, the roles of PARP-1 orthologs in development remain obscure. Unlike in mammals, plants lack the variety of PARP super-family members. However, plants encode a group of PARP-like proteins belonging to the SRO family. Plants PARP family members and/or poly [ADP-ribosyl] ation have been linked to various processes like DNA repair, innate immunity, mitosis, and stress responses. In addition, SRO family members have been also shown to be essential for normal sporophyte development [131]. Moreover, PARP super-family members have been found to be crucial for stress responses in *Arabidopsis thaliana* [131]. In *Arabidopsis*, AtPARP3 is reported to be expressed specifically in seed development signifying its potential role [132]. In addition, AtPARP-1 and/or AtPARP2 knockdown were reported to alter *Arabidopsis* development [133]; however, AtPARP2 ortholog in oilseed rape (*Brassica napus*) did not affect its development [134]. These reports suggest that further research is necessary to establish the role of PARP in plant development.

5.4. PARP-1 in *Drosophila melanogaster* development

Role of PARP-1 in differentiation and development is well studied in the model organism *Drosophila melanogaster*. In *Drosophila*, PARP activity is found to be developmentally regulated [135]. Also, the expression levels of PARP in *Drosophila* are shown to be the highest in embryos whereas reduced expression was seen in larvae, pupae, and adults [136]. Moreover, PARP overexpression hampers the cytoskeletal organization (F-actin) and disrupts tissue polarity, suggesting its participation in *Drosophila* development [137].

Previous literature strengthens the importance of PARP as a transcriptional co-activator [138], implicating that PARP may regulate the expression of a few genes involved in determining tissue polarity or cytoskeleton in *Drosophila*. Miwa et al., [139] reported that *PARP* deletion mutant failed to grow into an adult fly suggesting PARP's involvement in larval metamorphosis since 50% of mutant larvae were arrested at this stage. In addition, PARP activation is a prerequisite for viability and organization of chromatin, heterochromatin, and other sequences during development [140]. Studies have been shown PARP to be vital for purparium formation and metamorphosis [40,141]. Thus, PARP-1 also plays an important role during the *Drosophila* development.

5.5. PARP-1 in mice development

PARP-1 and PARP-2 have been implicated in playing an important role in mouse embryogenesis and development over the past couple of decades. In particular, PARP-1 and PARP-2 double mutations in mice were found to be lethal as the embryos died at the onset of gastrulation [86]. This result is further supported from ATM and Ku80 deficient mice in PARP-1 knockout background [142,88]. It has been suggested that both PARP-1 and ATM act synergistically during mouse development [142]. Moreover, the loss of SSBR/BER and DSB repair pathway components lead to massive apoptosis of the embryo as a result of defect in DNA-damage signaling. PARP-1 is involved in germline development wherein PARP-1 deficient oocyctes were found to exhibit defective meiosis [98]. In addition, PARP-1 knockout mice also demonstrate altered gene expression of cytoskeletal elements [143]. Furthermore, PARP-1 has been reported to contribute to DNA demethylation in pre-implantation embryos of mice [144]. Authors report that the mean life span of *PARP-1*$^{-/-}$ knockout mice was significantly reduced while these mice also showed accelerated aging of the reproductive function as compared to wild type mice. PARP-1 and PARP-2 have been reported to be a requisite for the differentiation of mouse embryonic carcinoma F9 cells into endodermal cells via recruitment of the transcriptional intermediary factor (TIFIβ) for the expression of the endoderm specific gene during endodermal differentiation [145]. Similarly, adipogenic differentiation is reduced in adipose derived stromal cells isolated from *PARP-1* knockout mice. Recently it has been reported that PARP-1 deficiency notably reduce bone mass in mice as a result of increased osteoclast differentiation [105]. Zhou et al., report that PARP-1 inhibition by PJ-34 induces angiogenesis in diabetic and ischemic conditions. Thus, PARP-1 plays a very important role in cell differentiation and development in mice [146].

5.6. PARP-1 in immune cells development

Recently, growing body of reports indicate that PARP-1 engages in inflammatory/immune responses as well. PARP-1 controls cell functions in many types of immune cells, including dendritic cells, macrophages, and T and B lymphocytes [147–150]. It has been sug-

Please cite this article in press as: T. Jubin, et al., Poly ADP-ribose polymerase-1: Beyond transcription and towards differentiation, Semin Cell Dev Biol (2016), http://dx.doi.org/10.1016/j.semcdb.2016.07.027

gested that *PARP-1* KO T cells stimulated in the absence of antigen presenting cells exhibit a lower cellular proliferation rate [151]. Moreover, V(D)J recombination deficient T cells can be partially rescued by knocking out PARP-1, implicating its role in T-cell maturation too. Several studies demonstrate that *PARP-1* KO cells display a reduced ability to differentiate in Th2 cells. In immune cell differentiation, PARP-1 genetic ablation decreases specifically Th2 cell differentiation and enhances regulatory [$CD4^+CD25^+FoxP3^+$] T cells population [152,153]. PARP-1 is also reported to play a role in differentiation to plasma cells *via* transcriptional repression of Bcl-6 [154].

Apart from erratic environmental genomic insults, certain cellular processes such as lymphocyte development and antibodies production involve spontaneous DNA damage wherein PARylation and PARPs/ARTDs play a crucial role in combating the insults. B lymphocytes undergo a course of rigorous genomic rearrangements and recombination along with somatic mutations, to generate B cells that produce related antibodies possessing varying degrees of specificity and diverse functions due to class switching and affinity/maturation. Thus, during the B cell development, differentiation, and maturation, the genome in question undergoes lesions and damages, that eventually activates the DNA repair machinery and cell cycle check points to initiate genomic repair. Moreover, NF-κB signaling plays a crucial role in immature B cell development and differentiation and mature B cell survival. Earlier evidences suggest a crosstalk between intrinsic NF-κB activity of B cells and spontaneous DNA damage/repair for proper B cell function. Stilmann et al., demonstrate the importance of PARylation by PARP1/ARTD1 in bridging the nuclear sensing of DNA damage with cytoplasmic activation of the IKK (IκB kinases) complex [155]. Upon DNA damage, the PAR- modified PARP-1 dissociates from damage sites, and orchestrates the assembly of transient multiprotein PARP1/ARTD1-IKKγ–PIASy-ATM signalosome complex which in turn trigger stimulation of IKKγ via its sumoylation by PIASy (protein inhibitor of activated STAT superfamily), leading to IKKγ induced NF-kB activation of survival pathways [155,156]. These findings suggest the importance of PARP-1 in immune cells differentiation and maturation, implicating PARP as a therapeutic target for several immune mediated diseases.

6. PARP-1 and immune mediated diseases:Diss(ease)ing the alter ego

PARP-1 is expressed in almost all cells and plays a central role in inflammation and immunity [157]. Its enzymatic inhibition confers protection in several models of immune-mediated diseases, mostly through an inhibitory effect on NF-κB (and NFAT) activation [153]. PARP inhibitors ameliorate immune mediated diseases in several experimental models, including colitis, rheumatoid arthritis, allergy and experimental autoimmune encephalomyelitis [158]. Previously, *PARP-1* KO mice have been suggested to display a decreased severity of rheumatoid arthritis due to lower IL-1β and MCP-1 expression which led to decreased destruction of bone and cartilage in arthritic joints [159]. Rosado et al., [158] demonstrate that PARP-1 have involvement in the differentiation of $Foxp3^+$ regulatory T (Treg) cells, suggesting it's role in tolerance induction. Also ARTs is involved in regulating Treg cell homeostasis by promoting Treg cell apoptosis during inflammatory responses, thus rendering it a crucial target for immune suppressive therapy.

Several studies show that PARP-1 deficiency and PARP inhibition confer resistance to inflammatory bowel disease (IBD) in rodent models of dinitrobenzene/trinitrobenzene sulphonic acid (DNBS/TNBS)-induced colitis by dampening AP-1 and NF-κB activation, inflammatory cytokine production and apoptosis, with consequent reduction of colon damage [160,161]. Together, these findings show that PARP-1 plays a pivotal role in the regulation of immune responses and may represent a good target for new therapeutic interventions in immune-mediated diseases.

PARP-1 also changes the responsiveness of dendritic cells, T-cell activation and antibody production via alteration in gene regulation and secretion of various cytokines and adhesion molecules [150]. PARP-1 inhibition targeting the enzymatic site reduces the secretion of pro-inflammatory cytokines and ameliorates various autoimmune and inflammatory diseases like heart and brain inflammatory processes, rheumatoid arthritis, inflammatory bowel disease, colitis, autoimmune encephalomyelitis etc. [162–168]. Furthermore, PARP-1 acts as a crucial mediator in maintaining the balance between pro-inflammatory/effector and anti-inflammatory/regulatory responses, suggesting possible therapeutic perspectives of PARP inhibition [150]. Studies demonstrate that PARP-1 sustains NF-kB induced transcription of several genes involved in inflammation, including cytokines, such as TNFα, IL1-B, IL6, IFN and CCL3, and inducible nitric-oxide synthase (iNOS) [169,170]. In view of these studies, PARP inhibitors can be proposed as a potent future therapeutant for various autoimmune disorders. Moreover, role of PARP-1 has been suggested in adaptive immune response as it gets stimulated during T-cell activation. In addition,PARP-1 regulates NFAT function as well, which is involved in T-cell differentiation and functions [171,172], and modulates the expression of Th1/Th2 cytokines [151].

The promising results of various studies including animal models emphasize the urgency of introducing PARP inhibitors into clinical trials for various autoimmune diseases exhibiting cytokine imbalance, upregulation of adhesion molecules, dysregulation in antibody production etc., implicating the multi targeting effects of PARP-1 in attenuation of such diseases.

7. Conclusion

A wealth of reports over the past decades have drawn the attention of researchers towards PARP-1 from being just a 'DNA damage sensor', to a multitasking protein in the cell with prime roles such as gene regulation, chromatin remodelling, differentiation etc. Structural evidences now explain the multidomain structure of PARP-1 and the process of poly ADP-ribosylation in response to various stimuli. PARP-1 is well demonstrated to be activated not just by damaged DNA but also by various developmental and environmental cues. PARP-1 PARylates numerous chromatin-remodeling factors, resulting in chromatin modification and regulation of gene expression. In addition, PARP-1 also regulates splicing and DNA methylation processes. All these functions of PARP-1 in gene regulation are mirrored in its association with developmental and differentiation processes in the cell, including embryogenesis, germline development and cell differentiation. Thus, instead of just being merely treated as a DNA damage sensing protein, PARP-1 should be explored for its role in differentiation that has been highlighted in the present review. Nonetheless, the role of PARP in gene regulation, cell differentiation and development also makes it suitable and promising target for therapeutic armenatrium to treat several human diseases.

Acknowledgements

RB thanks the Department of Biotechnology, New Delhi (BT/PR4383/BRB/10/1014/2011) and Council of Scientific and Industrial Research (38 (1383)/14/EMR-II) for financial support. TJ thanks UGC-SAP DRS for fellowship. MS thanks CSIR for fellowship. RB also thanks DBT-MSUB ILSPARE. It is to be noted that none of the authors have any conflict of interest.

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Cell Proliferation

Cell Prolif., 2015, **48**, 363–374 doi: 10.1111/cpr.12182

Response of *Dictyostelium discoideum* to UV-C and involvement of poly (ADP-ribose) polymerase

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Received 2 June 2014; revision accepted 5 December 2014

Abstract

Objectives: Radiation and chemical mutagens are direct DNA-damaging agents and ultraviolet (UV) radiation is frequently used in biological studies. Consequent to ozone depletion, UV-C could become a great challenge to living organisms on earth, in the near future. The present study has focused on the role of poly (ADP-ribose) polymerase (PARP) during UV-C-induced growth and developmental changes in *Dictyostelium discoideum,* a phylogenetically important unicellular eukaryote.

Materials and methods: *Dictyostelium discoideum* cells were exposed to different doses of UV-C and PARP activity, and effects of its inhibition were studied. Expression of developmentally regulated genes *yakA*, *car1*, *aca*, *csA*, *regA*, *ctnA*, *ctnB*, *gp24*, *hspD* and *dsn* were analysed using semi-quantitative RT-PCR.

Results: We report that the *D. discoideum* cells displayed PARP activation within 2 min of UV-C irradiation and there was increase in NO levels in a dose-dependent manner. UV-C-irradiated cells had impaired growth, delayed or blocked development and delayed germination compared to control cells. In our previous studies we have shown that inhibition of PARP recovered oxidative stress-induced changes in *D. discoideum*; however, intriguingly PARP inhibition did not correct all defects as effectively in UV-C-irradiated cells. This possibly was due to interplay with increased NO signalling.

Conclusions: Our results signify that UV-C and oxidative stress affected growth and development in *D. discoideum* by different mechanisms; these studies could provide major clues to complex mechanisms of growth and development in higher organisms.

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Abbreviations

Ax-2: axenic 2

FITC: fluorescein isothiocyanate

HA: hydroxylamine

iNOS: inducible nitric oxide synthase

NO: nitric oxide

PAR: poly-ADP ribose

PARP: poly(ADP-ribose) polymerase

PBA: phosphate-buffered agar

SB: Sorenson's buffer

G0, G1, G2, M, S: phases of the cell cycle

L-NIO: iNOS inhibitor

Introduction

Dictyostelium discoideum is a unicellular organism that feeds on bacteria and divides (under favourable environmental conditions) approximately every 4 h. It divides every 8–12 h in axenic media. During starvation, cells become chemotactically sensitive to cAMP pulses and initiate their developmental program to ultimately form a multicellular fruiting body, consisting of spores within a sorocarp, and a stalk. Spores germinate to form unicellular amoebae under favourable conditions. *Dictyostelium discoideum,* being a eukaryote that stands at the transition point of unicellularity and multicellularity, is an exceptional model system to study various signal transduction pathways (1) that can be extrapolated to mammalian systems. The unicellular stage is known to be highly resistant to DNA-damaging agents and oxidative stress (2,3).

Ionising radiation and chemical mutagens are direct DNA-damaging agents. Ultraviolet (UV) radiation, of a broad band of energy extending from 200 to 400 nm, is one of the frequently used types of radiation in biological studies. UV wavelengths of the solar electromagnetic spectrum can be subdivided into three regions: UV-C (200–280 nm), UV-B (280–315 nm) and UV-A (315–400 nm). Of these, UV-C is the most harmful although its rays do not reach the surface earth, due to the protective ozone layer. All UV-C and approximately 90% UV-B radiation is absorbed by ozone, water vapour, oxygen and carbon dioxide [WHO] as sunlight passes through the atmosphere. However, consequent to ozone depletion, UV-C could become a great challenge to living organisms in the future. Oxidative stress induced by ionizing radiation and alkylating agent accounts for DNA damage induced by these agents. UV-C can cause formation of thymine glycol (4) but the main lesions are caused by cyclobutane pyrimidine dimers (CPDs) and pyrimidine-(6-4)-pyrimidone photoproducts (5). In double-stranded DNA, these lesions lead to generation of SSBs (single-strand breaks) when they are repaired by NER (nucleotide excision repair) (6). They also lead to distortion in the DNA double helical structure which is sufficient to activate the nuclear enzyme, poly(ADP-ribose) polymerase (PARP) (7). PARP uses NAD^+ as donor of poly(ADP-ribose) and catalyses poly(ADP ribosylation) (PARylation), of itself and of a variety of other proteins. Numerous substrates list PARP and link it to a wide range of physiological processes. Primarily, it is involved in chromatin remodelling, DNA repair and maintenance of genomic integrity (8–13). PARP contributes to cellular homoeostasis under conditions of basal DNA damage, wherein it is involved in cell cycle arrest (14). During conditions of moderate/severe cell stress, PARP over-activation leads to cell death resulting in various pathological conditions (15). PARP inhibition during moderate/severe cellular stress is beneficial (16) and we have reported long-term consequences of PARP inhibition and down-regulation (17) in oxidative stressed *D. discoideum* cells (18,19). Also, staurosporine-induced cell death has been studied in *D. discoideum* (20). In the present study, the role of PARP in UV-C-induced growth and developmental changes in *D. discoideum* has been addressed by inhibiting its activity with benzamide, and also long-term effects of PARP inhibition for two successive generations and interplay with nitric oxide (NO) have been explored.

Materials and methods

Cell death after UV-C stress

Dictyostelium discoideum cells (Ax-2 strain) were grown in suspension in HL5 medium (21), with shaking at 3 g, at 22 °C. All experiments were carried out with *D. discoideum* cells at mid log phase expansion, cell density 2.5×10^6 cells/ml. Cells were washed in 1× SB (1× Sorenson's buffer from 50× SB, 2 mM Na_2HPO_4, 15 mM KH_2PO_4, pH 6.4) by centrifugation, at 300 ***g*** for 5 min, then exposed to different doses of UV-C (254 nm) (10.4 J/m^2, 13 J/m^2, 65 J/m^2 and 130 J/m^2). Cells were then resuspended in HL5 after pelleting, and incubated at 22 °C, with shaking at 150 rpm, for 24 h.

Growth curve studies

For growth curve studies after UV-C irradiation, 1% inoculations (50 μl from ~2.5×10^6 cells/ml into 5 ml) were performed in HL5 medium, and cell viability was assayed using the trypan blue exclusion technique, every 24 h until death (22).

Cell cycle analysis

The cell cycle was analysed by flow cytometry using propidium iodide. Mid-log-phase cells were fixed by drop wise addition of 70% ethanol, and incubated at 4 °C overnight. Fixed cells were resuspended in staining solution (TritonX-100, DNase-free RNase and propidium iodide) and incubated for 30 min, followed by FACS analysis (23). Quantification was performed using flow cytometry, with FACS ARIA (BD Biosciences, San Jose, CA, USA). Data were analysed with FACSDiva software.

Developmental studies

2.5×10^6 cells were harvested and processed as described above for UV-C treatment (10.4 J/m^2, 13 J/m^2, 65 J/m^2 and 130 J/m^2), then were resuspended in 100 μl 1× SB and spread on non-nutrient agar plates (2% agar in 1× SB) kept at 22 °C, and different stages of development were studied.

Chemotactic assay

Chemotactic studies were performed according to the method of Wallace and Fraizier (24). Exponentially growing cells were washed free of medium with 1× SB and starved for 5–6 h after treatment. Of the cell suspension, 5 μl were placed on 2% agar surfaces at a distance of 2 mm from wells containing 1 μM cAMP. Movement of cells towards wells was observed using phase contrast microscopy (Nikon TE-2000S, Tokyo, Japan) and was photographed. Relative number and distance moved by cells was an indication of their chemotactic activity.

cAMP pre-treatment. Exogenous (1 μM) cAMP was added 2.5 h prior to UV-C treatment to *D. discoideum* cells in 1× SB. Plates were then kept at 22 °C to observe different stages of development, or cells were harvested to study developmental gene expression, at various time points.

cAMP estimation in UV-C-irradiated cells

5×10^6 cells were harvested by centrifugation at 300 ***g***/5 min/4 °C and subjected to UV-C irradiation and they were resuspended in Sorenson's buffer for 6 h. Cells were then collected, and extracellular cAMP was estimated in the buffer, using an ELISA kit method according to the manufacturer's instructions (Calbiochem, Gibbstown, NJ, USA). A total of 200 μM L-NIO (iNOS inhibitor) pre-treated cells were also collected, and extracellular cAMP was estimated.

Nitric oxide estimation

NO generation was estimated according to the method of Green *et al.* (25). 5×10^6 cells were suspended in 1 ml 1× SB and incubated at 22 °C for 20 min, to allow for accumulation of NO. 1 ml Griess reagent was added and mixed in well. Incubation was again carried out at 22 °C for 15–30 min. Absorbance was measured at 546 nm.

PARP activation under UV-C stress

Cells treated with different doses of UV-C were processed for PARP assay (19). Data were analysed using Image Proplus software to calculate mean intensity of fluorescence from different fields and ~50 cells were examined for each dose. PARP was inhibited by 12 h pre-treatment with 1 mM benzamide (Sigma Aldrich, St Louis, MO, USA), prior to UV-C irradiation.

Expression analysis of developmentally regulated genes, by RT-PCR

RNA extraction and cDNA synthesis. Dictyostelium discoideum cells were exposed to UV-C stress as mentioned above. After specific pre-treatment, cells were pelleted and washed in 1× SB before finally being resuspended in 1× SB. Total RNA was isolated from cells at two time points (as specified in figure 4) using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). RNA integrity was verified by 1.5% agarose gel electrophoresis and 260/280 absorbance ratio >1.95. RNA was treated with DNase I (Ambion Inc., Austin, TX, USA) before cDNA synthesis, to avoid DNA contamination. Reactions were performed by reverse transcriptase using RevertAid First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions, in MJ Research Thermal Cycler (Model PTC-200, Watertown, MA, USA).

Second strand synthesis. First-strand cDNA was used as a template with gene-specific primers to synthesize second strand DNA by conventional PCR. Expression kinetics of DYRK family protein kinase (*yakA*), cAMP receptor-1 (*car1*), adenylyl cyclase (*aca*),cell adhesion molecule–contact site A protein (*csA*), component of the counting factor complex–countin A (*ctnA*) and countin B (*ctnB*), calcium-dependent cell adhesion molecule-1 (*gp24*), cAMP phosphodiesterase (*regA*), a heat shock protein of Hsp90 family (*hspD*) and discoidin (*dsn*) genes were analysed. *rnlA* was used as internal control (Table 1). Reactions were performed according to the manufacturer's instructions (Fermentas, Burlington, ON, Canada). DNA fragments were amplified for 24 cycles and signal intensities were analysed on 2% agarose gel stained with ethidium bromide. Densitometric analysis was performed using AlphaImager software, and mean density for respective genes with respect to *rnlA*, was plotted.

Fate of spores formed under UV-C stress. Spores formed from cells irradiated with 10.4 J/m² UV-C in the presence and absence of benzamide (Sigma Aldrich)

Table 1. Primer sequences used for gene expression analysis.

Gene	Primer sequence
*yak*A Forward	5′-GCTTAGAGGACTTTCAACCAATTT-3′
*yak*A Reverse	5′-GATTTTTCATAACAAGCAGATCCA-3′
*cAR*1 Forward	5′-TGTGGACTTTATGTCTTGCAATTAG-3′
*cAR*1 Reverse	5′-CCAATACTGCTGAAATTGCCC-3′
aca Forward	5′-GTGATACTGCCAATACCGCC-3′
aca Reverse	5′-ACCCAAGAGAGTTCCAGATAATGG-3′
*cs*A Forward	5′-ATAGTGCACATTCAGCTCC-3′
*cs*A Reverse	5′-AAGAACTTTGCCATACTTTGG-3′
*ctn*A Forward	5′-ATGAATAAATTATTTTCATTAATTTTAG CTTTATTCCTTGTCAACTCCGC-3′
*ctn*A Reverse	5′-TTAAAATAAAGCAAAACCTGAACCTGA ACCAGAGGCGGCACC-3′
*ctn*B Forward	5′-GTGGTGCCGTTTGTTCATTACTCCC-3′
*ctn*B Reverse	5′-CCAGTTGGGTCAGTTACCATAACAGCAAC-3′
*gp*24 Forward	5′-CCAGGAGCTTTTCAATGGGCAGTTGATG-3′
*gp*24 Reverse	5′-GTGTAACAGTCATATTCTTTGGGAATGTCTC-3′
dsn Forward	5′-CCACCCATTAACCTGGAATG-3′
dsn Reverse	5′-TGGTGGCATCAGTACAATCG-3′
*hsp*D Forward	5′-ACATTCCAAGCTGAAATTAATCAGC-3′
*hsp*D Reverse	5′-GTGTAAGAGTTTTGGCAGTCTTATC-3′
*reg*A Forward	5′-AATTGTTGGGGATACTGAATCAGC-3′
*reg*A Reverse	5′-ATAAAGTGCGGTGATATTTC-3′
*rnl*A Forward	5′-TTACATTTATTAGACCCGAAACCAAGCG-3′
*rnl*A Reverse	5′-TTCCCTTTAGACCTATGGACCTTAGCG-3′

were picked from different areas, with the aid of sterilized nichrome loops, and added to 5 ml HL5 medium. Flasks were kept shaking at 150 rpm/22 °C. After germination, cells were counted using a haemocytometer, every 12 h, for growth curve experiments.

Assessment of DNA damage in cells germinated from spores (26). Histones become phosphorylated during oxidative stress. Hence, presence of phosphorylated histone indicates oxidative stress-induced DNA damage. Anti-H2AX at 0.5 μg/ml, and anti-mouse IgG (whole molecule)–TRITC conjugate (Sigma, St. Louis, MO, USA), 1:200, were used to study presence of oxidative stress. Cells were pelleted and washed once in phosphate-buffered saline (PBS) pH 7.4, fixed in 70% chilled methanol for 10 min at −20 °C, then washed in blocking solution (1.5% BSA with 0.05% Tween 20 in PBS) and incubated for 1 h in primary antibody. After incubation, cells were washed 2–3 times in blocking solution and further incubated for 1 hr with TRITC-labelled secondary antibody. Then, they were washed 2–3 times in PBS and fluorescence was observed under 60× magnification. Data were analysed using Image Proplus software to calculate mean density of fluorescence and graphs were plotted using Graphpad prism software.

Results

Cell death and PARP activation after UV-C irradiation

Cell death was monitored after different doses of UV-C irradiation. UV-C dose (10.4 J/m^2 and 13 J/m^2) led to less than 10% cell death (Fig. 1a). UV-C is a potent DNA damage inducer and is well reported to activate PARP (7,15). PARP was assayed at various time points post UV-C stress. 10.4 J/m^2 and 13 J/m^2 UV-C-treated cells had highest PARP activity at 2 min (Fig. 1b), which then declined, and reached basal levels after 5 min. Benzamide pre-treated cells had reduced activation of PARP (Fig. 1b).

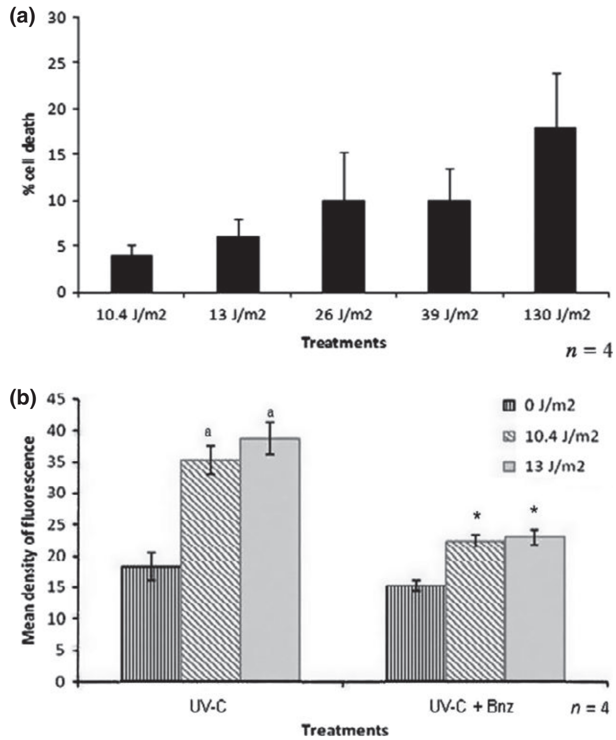

Figure 1. Cell death and PARP activation in UV-C-irradiated cells. (a) UV-C induced dose-dependent cell death as monitored by the trypan blue exclusion method. Results are mean ± SE of four independent experiments. (b) Peak PARP activity induced by UV-C irradiation was intercepted by benzamide. Benzamide inhibited PARP activity at 2 min post 10.4 J/m^2 and 13 J/m^2 irradiation respectively. Data (mean ± SE) are from four independent experiments. *$P < 0.05$ compared to control; $^{a}P < 0.05$ compared to respective dose of UV-C irradiation, without benzamide pre-treatment.

Dictyostelium discoideum growth and development under UV-C stress

Growth analysis revealed dose-dependent increase in lag phase with UV-C dose. As can be seen from Fig. 2a, with increase in UV-C dose, there was increase in lag phase and consequently late entry into the log phase. Also, the stationary phase was achieved at lower density compared to control cells. Pre-treatment of cells with benzamide reseulted in partial rescue in UV-C-induced changes in growth (Fig. 2a). Lag phase growth of UV-C-stressed cells can be clearly explained by cell cycle analysis studies, wherein UV-C-stressed cells were seen to be arrested in G0/G1 (~77%) as opposed to untreated control cells (~68%), at 48 h growth (Fig. 2b). Also, marked reduction is seen in percentage of cells in S phase after UV-C irradiation at 10 J/m^2 (13.6%) and 13 J/m^2 (12.1%) compared to untreated control cells (22.1%) (Fig. 2b), explaining the slower growth in stressed cells.

Effects of UV-C on development also reflected dose dependency as seen in growth (Fig. 3); however, it was more drastic. Cells exposed to 13 J/m^2 (Fig. 3) and higher doses (65 J/m^2 and 130 J/m^2) (data not shown) UV-C failed to undergo complete development. 10.4 J/m^2 UV-C-treated cells displayed development that was delayed compared to control cells. Delay in development induced by 10.4 J/m^2 was rescued when cells were pretreated with benzamide (Fig. 3). Interestingly after 12 h, benzamide-pretreated cells exposed to higher doses (65 J/m^2) of UV developed streaming structures and incompetence to form aggregates, compared to untreated control and UV-C-exposed cells.

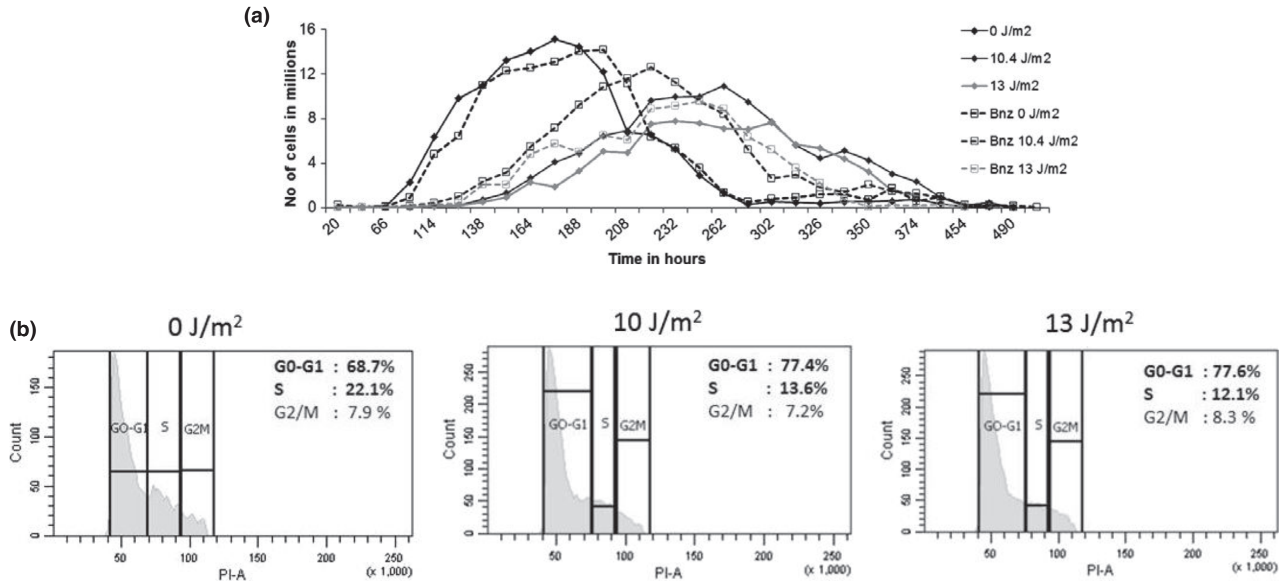

Figure 2. Effect of PARP inhibition on UV-C induced growth changes and fate of spores in *Dictyostelium discoideum*. (a) Effect of PARP inhibition on UV-C induced growth changes Benzamide rescued UV-C-induced changes as shown in the growth curve. The stationary phase was achieved at higher cell density in benzamide pre-treated cells compared to respective control. Results are mean of four independent experiments. (b) Cell cycle analysis of UV-C-irradiated cells by propidium iodide staining. The 10.4 J/m^2 and 13 J/m^2 UV-C-treated cells had G0/G1 arrest, i.e. ~77% cells were in G0/G1 phase in contrast to ~68% untreated control cells. This figure is a representative picture of three independent experiments performed.

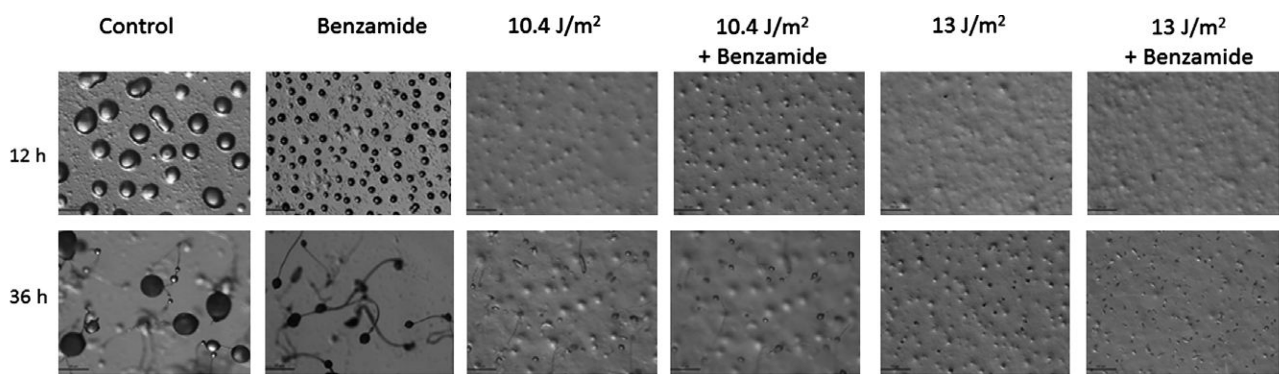

Figure 3. Development of *Dictyostelium discoideum* cells under UV-C stress. *Dictyostelium discoideum* cells after UV-C treatment were allowed to develop on nutrient-free agar medium and were observed at various time intervals. Benzamide pre-treatment restored the delay induced by 10.4 J/m^2 but did not lead the 13 J/m^2 UV-C-treated cells to complete development, though the cells entered it. Photographs taken with 4× objective. Scale = 100 μm.

Also, developing fruiting bodies formed after 10.4 J/m^2 UV-C stress were smaller and ~40% greater in number. Cells subjected to UV-C stress formed less compact aggregates (Fig. 3). This observation led us to assess expression profile of development-related genes such as *ctnA*, *ctnB*, *dsn1*, *hspD*, *csA* and *gp24*. These are associated with regulation of aggregate size (27–29). Expression profiles of *yakA*, *car1*, *aca* and *regA* were also studied as these genes are important for growth to differentiation transition and cAMP-mediated signalling, respectively (1,30). As shown in Fig. 4a and 4b, UV-C affected levels of *ctnA*, *yakA*, *car1*, *aca, csA* and *regA* mRNA. Benzamide pre-treatment prevented UV-C-induced changes in expression of *ctnA* and *regA*; however, *yakA*, *car1*, *aca* and *csA* expression levels could not be restored. Altered expression levels of *yakA, car1, aca, csA* and *regA* during UV-C stress indicated that the finely tuned cAMP signalling network of development was affected, which was re-established by *yakA, car1* and *aca* restoration in presence of exogenous cAMP. To probe effects on cAMP signalling, chemotactic assay and cAMP estimations were undertaken. Control amoebae moved toward 1 μM cAMP wells, while UV-C-irradiated cells failed to sense cAMP in cups (Fig. 5a). These results justify effects of exogenous cAMP on altered expression profiles of developmentally regulated genes (Fig. 4a, b) and on developmental arrest (Fig. 5b). Also cAMP levels were found reduced in UV-C-irradiated cells (Fig. 6). Spores developed after 10.4 J/m^2 UV-C irradiation were delayed by around 27 h in revival (111 h $\pm$ 6.0) compared to control cells (81 h $\pm$ 6.658) (Fig. 7a). These spores also had longer lag phases compared to controls. However, benzamide pre-treatment had no effect on revival of spores formed after 10.4 J/m^2 UV-C exposures as shown in (Fig. 7a). Also this second generation of UV-C-exposed cells did not differ from that of control cells with respect to damage in DNA (Fig. 7b); ensuring that partial inhibition of PARP did not interfere with basal repair of the cells.

UV-C-induced developmental changes restored by iNOS inhibition

UV-C radiation, unlike oxidative stress, leads to increased NO generation which may further interfere with *D. discoideum* development. Hence, we monitored effects of inducible nitric oxide synthase inhibitor (L-NIO) on UV-C-induced developmental changes. UV-C dose of 13 J/m^2 caused arrested development. However, 200 μM L-NIO (iNOS inhibitor) pre-treated cells exhibited partial rescue in developmental delay due to UV-C treatment (Fig. 8a). These results were further confirmed by monitoring NO production under UV-C stress (Fig. 8b). Results clearly suggest that *D. discoideum* cells exposed to UV-C irradiation had dose-dependent increase in production of nitric oxide, and NO levels decreased significantly ($P < 0.05$) on benzamide pre-treatment. Also inhibition of iNOS partially restored cAMP levels (Fig. 6). Thus, increased NO generation affected signalling in UV-C-exposed cells, thereby impeding their development.

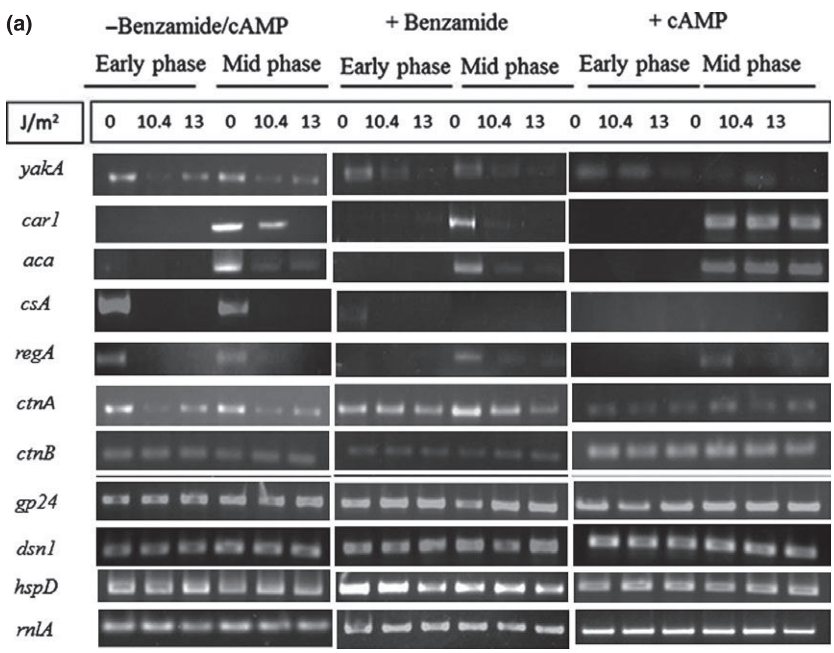

Figure 4. (a) Irradiation with UV-C affected mRNA expression of various genes involved in *Dictyostelium discoideum* development. Expression of *yakA*, coding for cell cycle arrest protein; cAMP receptor – *cAR1*, *aca* coding for adenyl cyclase A, countin, coding for a protein involved in cell counting mechanism, *csA*, a cell adhesion molecule and *regA*, a phosphodiesterase were found to be reduced in UV-C-treated cells compared to internal control *rnlA* (mitochondrial rRNA IG7). Benzamide pre-treatment restored expression levels of countin, however, it did not affect *yakA* and *cAR1* mRNA levels. Levels of countin (another protein of cell counting mechanism) mRNA were found increased in benzamide pre-treated cells. Exogenous cAMP restored *cAR1* without affecting *yakA* and *csA*. Expression of *dsn,* a gene coding for a protein which helps cells to adhere to the substratum, *hspD*, a heat shock protein and *gp24* (intercellular contacts and aggregation) were not affected. mRNA levels of *yakA*, *cAR1, aca*, *csA* and *regA* were assayed at 0 and 9 h, while the rest of the genes were assayed at 6 and 10 h of development induction. (b) Densitometric analysis of various genes involved in *D. discoideum* development. $^{***,aaa,+++,\$\$\$,\#\#\#,\&\&\&}P < 0.001$ compared to respective controls; $^{**,aa,++,\$\$,\#\#,\&\&}P < 0.05$ compared to respective controls and $^{*,a,+,\$,\#,\&}p < 0.05$ compared to respective controls. For benzamide pre-treated and 13 J/m^2 UV-C-treated cells, $^{bbb}P < 0.001$, $^{bb}P < 0.01$ compared to 13 J/m^2 UV-C-treated cells (-benzamide/-cAMP). For cAMP pre-treated and 13 J/m^2 UV-C-treated cells, $^{qqq}P < 0.001$, $^{qq}P < 0.01$ compared to 13 J/m^2 UV-C-treated cells (benzamide/cAMP).

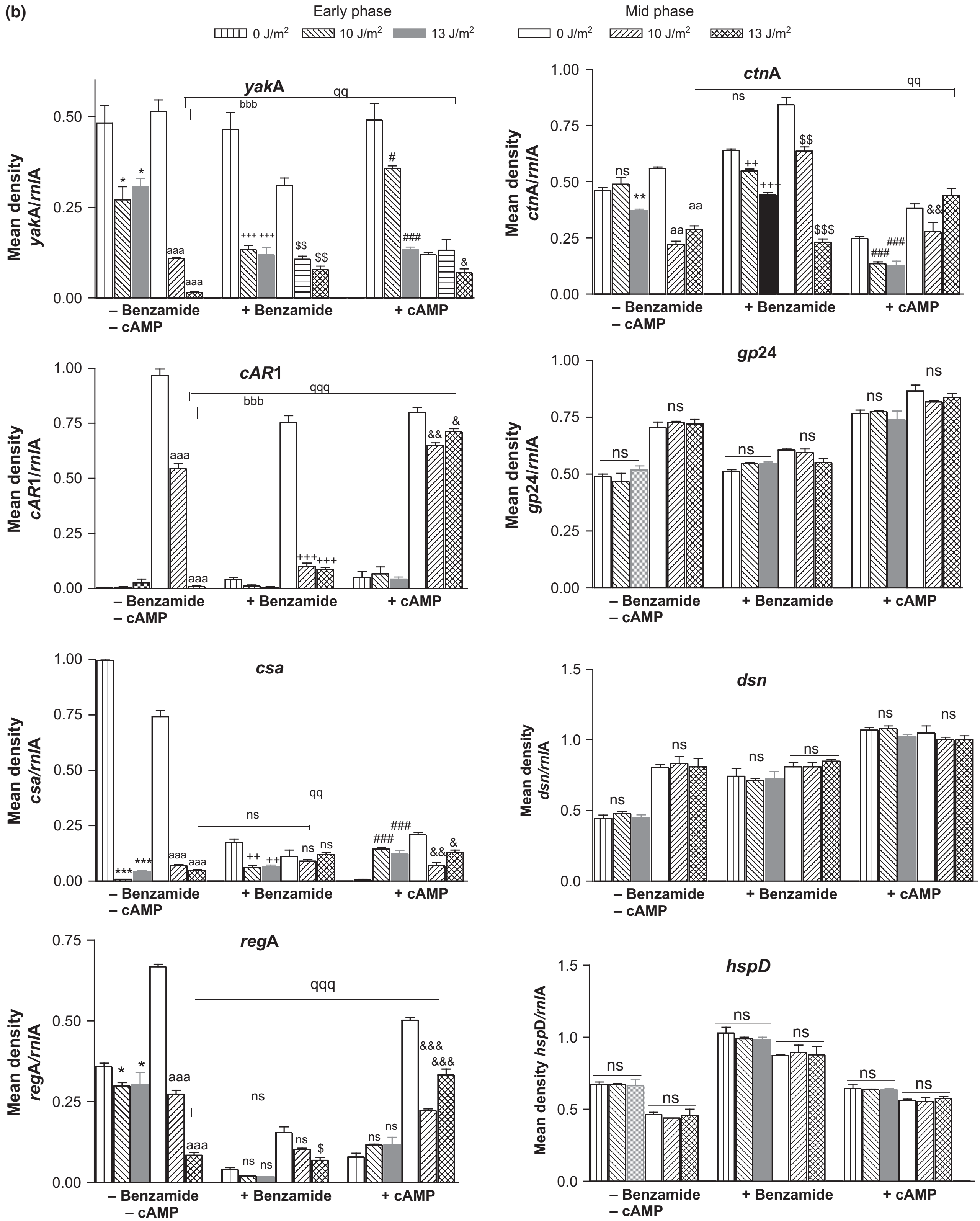

Figure 4. (Continued).

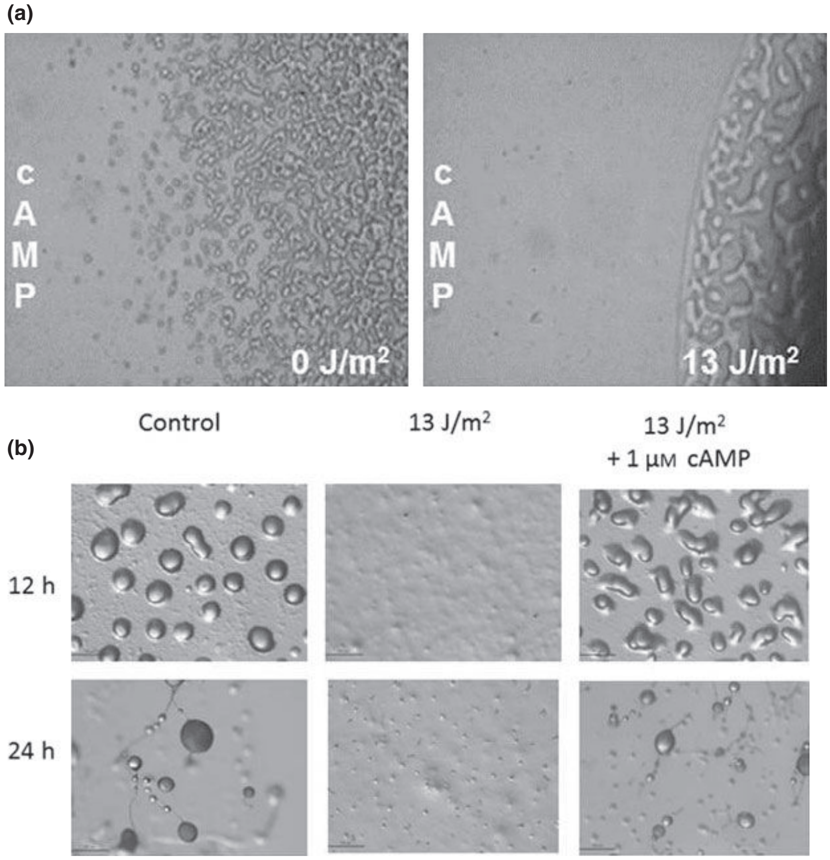

Figure 5. Effect of UV-C irradiation on chemotaxis in *Dictyostelium discoideum.* (a) UV-C-exposed *D. discoideum* cells failed to move towards cAMP. Wells were formed on PBA plates using a cup borer and were filled with 100 μl of 1 μM cAMP; cells were placed at a distance of 2 mm from wells. Photographs were captured 6 h after plating the cells at 4× magnification. Results are representative of three independent experiments. (b) Exogenous cAMP resumed development of UV-C-exposed *D. discoideum* cells. Exposure to 13 J/m^2 UV-C blocked initiation of development; however supplementation with cAMP (1 μM) restored development. Photographs were captured at 4× magnification. Results representative of three independent experiments. Scale = 100 μm

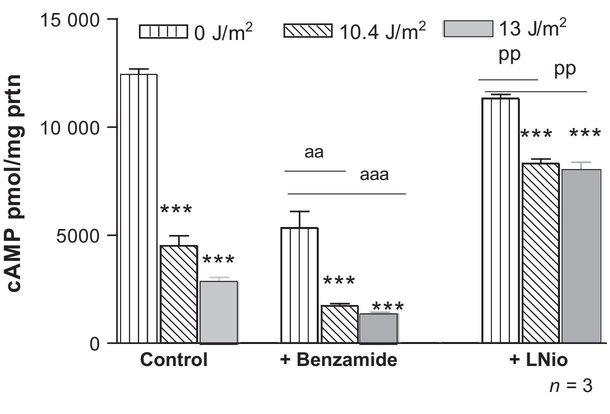

Figure 6. cAMP levels in UV-C irradiated *Dictyostelium discoideum* were reduced in NO-dependent manner. UV-C irradiated *D. discoideum* cells had reduced cAMP levels. Cells pre-treated with iNOS inhibitor, L-NIO, retained levels of cAMP even after UV-C exposure. Results are mean ± SE of three independent experiments. $^{*}P < 0.05$, $^{***}P < 0.001$ compared to control (0 J/m^2), $^{a}P < 0.05$ and $^{aa}P < 0.01$ compared to benzamide pre-treated cells and $^{PP}P < 0.01$ compared to L-NIO pre-treated cells.

Discussion

PARP characteristics render it an ideal candidate for participation in cell responses to UV stress (31). UV-C induces direct DNA lesions such as CPDs (e.g. T-T, T-C or C-C) or 6,4-photoproducts. These lesions distort DNA structure. DNA strand breaks generated during excision repair of such lesions activate PARP (32–35). Using elegant techniques that allow visualization of events occurring in UV-C-irradiated zone, Vodenicharov *et al.,* (36) demonstrated that UV-C causes immediate PARP activation within the first 15 s to 5 min. Our results corroborate these reports. UV-C treatment lead to both dose-dependent cell death and PARP activation within 2 min (Fig. 1). Cells exposed to higher doses of UV-C (130 J/m^2) had a longer lag phase (~100 h) than at milder doses, which could be due to seized cell cycle (Fig. 2b). This is in accordance with the finding that UV-C lead to cell cycle arrest after activation of the repair machinery (37). PARP inhibition allowed cells exposed to 10.4 J/m^2 UV-C to enter the log phase earlier than UV-C-only exposed cells. These also attained higher cell density compared to10.4 J/m^2 UV-C-only irradiated cells, suggesting that PARP played a role in cell cycle arrest and DNA repair.

Dictyostelium discoideum, during its unicellular vegetative phase, exhibits higher resistance to oxidative stress (3) and other DNA-damaging agents (2,20). Very high doses of hydroxylamine (4 mM) leading to ~90%

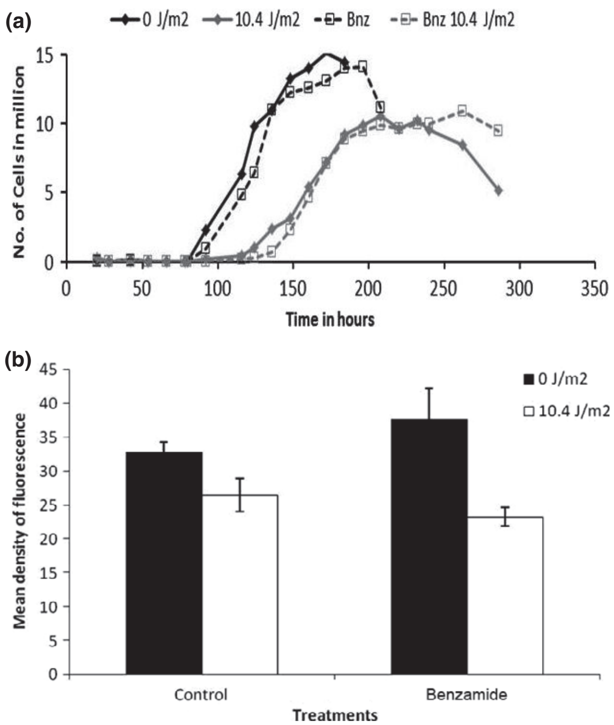

Figure 7. (a) Effect of PARP inhibition on fate of spores developed under UV-C stress. Spores of control cells revived within 81 h whereas spores formed after 10.4 J/m^2 UV-C stress exhibited ~27 h of delay in spore revival, which could not be rescued by benzamide pre-treatment. Data are mean of four independent experiments. (b) DNA damage monitored in second-generation *Dictyostelium discoideum* cells. No significant damage was observed in second-generation cells.

cell death, is required to block *D. discoideum* development (17,18). Surprisingly, the effect of UV-C is more drastic on *D. discoideum* development. Doses of UV-C equivalent to 13 J/m^2 (~6% cell death) or higher, completely arrested *D. discoideum* development at the loose aggregate stage, whereas 10.4 J/m^2 UV-C-irradiated cells completed development albeit delayed (Fig. 3). Benzamide pre-treatment of 13 J/m^2 UV-C-exposed cells had streaming structures indicating a definite role of PARP in development. This is supported by work previously published (19) from our laboratory, where we showed that both constitutive and slug stage-specific down-regulation of PARP lead to blocked or arrested development respectively. Protective effects of PARP inhibition were not seen at doses higher than 13 J/m^2, as cells died (38).

Azzam *et al.* (39) demonstrated cell type-specific effect of UV-C irradiation on expression of connexin 43 native isoform, in human fibroblasts and AG1522 cells. Experiments performed with UV-C irradiation in human skin fibroblasts (37) showed that UV-C reduced transcription of certain proteins involved in adhesion and motility. Hence, expression of certain genes crucial to various aspects of *Dictyostelium* development were assessed. Our results indicate that heat shock protein D, a cytosolic protein was required for early developmental stages. Discoidin-1, a marker of growth to differentiation transition that aids differentiating cells adhere to the substratum and gp24, required for filopodia formation and

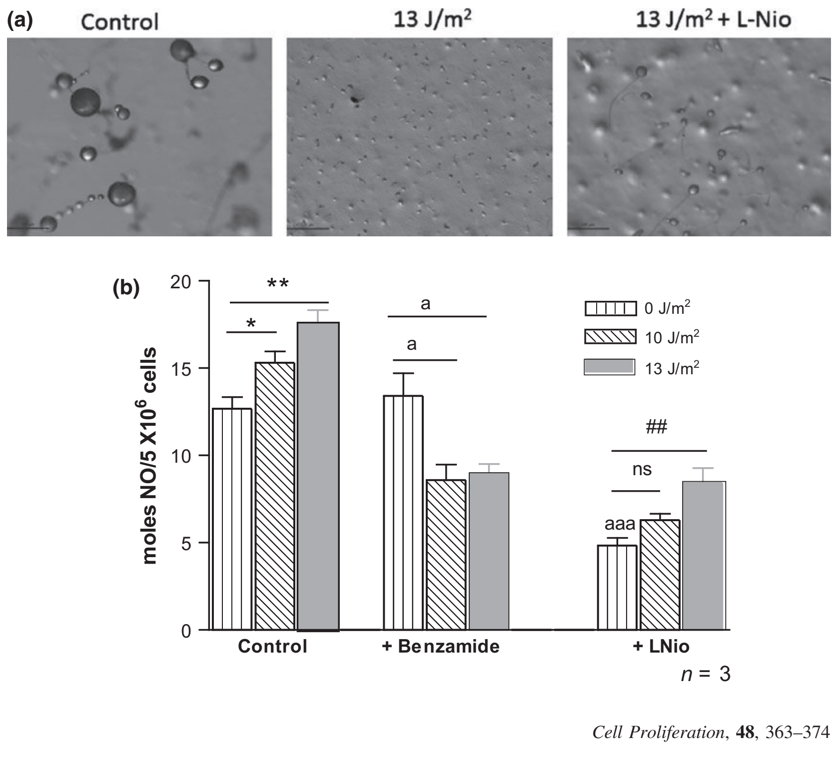

Figure 8. Effect of UV-C on development of *Dictyostelium discoideum* under iNOS inhibition and nitric oxide generation. (a) Development of UV-C-irradiated cells pre-treated with iNOS inhibitor. L-NIO-treated cells developed like control untreated *D. discoideum* cells within 24 h. Photographs were captured at 4× magnification 24 h after plating the cells. Results are representative of three independent experiments. Scale = 100 μm. (b) Nitric oxide generation increased in UV-C-treated *D. discoideum* cells. NO generation was estimated 30 min after UV-C treatment by the Griess method and was found to increase with increasing doses of UV-C. Results are the mean ± SE of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to untreated control cells, $^{a}P < 0.05$ compared to benzamide pre-treated cells and $^{###}P < 0.001$ compared to L-NIO pre-treated cells.

aggregation, was not affected in UV-C-treated cells. Nevertheless, expression of *csA* and *ctnA* was markedly reduced without great alteration in *ctnB* mRNA levels (Fig. 4a, b). *csA* is involved in Ca^{2+} independent cell–cell adhesion during aggregation, and countin and countin 2 along with at least three other polypeptides form a > 450 kDa counting factor complex, involved in maintaining aggregate size. *ctn* null cells had increased group size (40) similar to larger aggregate size seen in UV-C-treated cells (Fig. 3), unable to progress to fruiting bodies. Such cells have been reported to have reduced cAMP-induced cAMP pulse, and decrease in cAMP-stimulated Akt/PKB membrane translocation and kinase activity, which in turn lower cell motility (40–42). This is substantiated by reduced expression of *cAR1* and *aca* (Fig. 4a, b) and lower cAMP levels (Fig. 6), quintessential for cAMP signalling. In addition to this, reduced *regA* expression underlay compromised motility of UV-C-irradiated cells (Fig. 5a), as the protein is a phosphodiesterase that regulates PKA activity *via* reducing cAMP levels during chemotactic aggregation. cAMP addition was capable of restoring UV-C-induced changes in *car1* and *aca* expression (Fig. 4a, b). This explains that some UV-C-induced developmental defects were *via* modulating cAMP signalling to alter expression of genes associated with growth to differentiation transition, chemotaxis and aggregate size regulation (43–45).

Interfering PARP activity partly rescued development of UV-C-irradiated cells. It restored countin levels to normal but failed to affect expression of *car1, aca, yakA,* crucial for regulating cell cycle exit and growth to differentiation transition, and *csA*. Also, expression of *gp24*, a cell adhesion protein, increased in the presence of benzamide. PARP activity has previously been associated with expression of adhesion proteins (46). As increased adhesion proteins are known to hinder chemotaxis (47), the effect of benzamide on the gp(s) may mask its effect on development *via* restoration of countin levels.

On the other hand, UV irradiation induces NO generation in keratinocytes; this serves as a signal for melanogenesis (48). NO functions as a signalling molecule for *D. discoideum* cells also. NO-treated cells transiently activate their adenylyl cyclase and produce pulses of cAMP when stimulated with exogenously applied cAMP (49). However, physiological or environmental conditions that enhance external NO levels delay initiation of cAMP pulses, which are essential for cell differentiation (50). Estimation of NO (Fig. 8b) during development with L-NIO, iNOS (inducible nitric oxide synthase) inhibitor, showed NO generation *via* iNOS in *D. discoideum* after UV-C irradiation. Also, this NO production (Fig. 8b) was dependent on PARP. Activated PARP-1 up-regulates iNOS expression (51,52) and further iNOS byproducts may modulate PARP-1 enzymatic activity by nitration (53) making PARP activity and NO production interdependent. In *D. discoideum* cells, UV-C irradiation affects expression of certain developmentally important genes (*yakA*, *aca, car1*) and hence affects cAMP pulses *via* PARP activation and NO production. This has also been hinted at by increase in levels of cAMP with iNOS inhibition after UV-C exposure. An alternative sequence of events in which UV-C induces NO production prior to PARP activation is also possible. Further experiments need to be performed to pinpoint the affected pathways. Nevertheless, our results suggest interplay between PARP and NO with respect to regulation of gene expression during developmental defects induced by UV-C. Delayed revival of spores could be attributed to down-regulation of certain genes involved in spore revival, by UV-C. PARP may not be involved in increasing dormancy of spores induced by UV-C, as benzamide pre-treatment did not show any significant change in the revival of spores compared to UV-C-only treated cells. Cells germinated from spores did not show any significant damage (Fig. 7b) signifying that reduced activity of PARP (Fig. 1b) may be sufficient to repair DNA damage induced by 10.4 J/m^2 UV-C.

Interestingly, effects of PARP inhibition on UV-C-induced changes in *D. discoideum* growth and development also differed from our oxidative stress response results (17,18). Spores formed under oxidative stress exhibited delayed revival compared to benzamide-pre-treated cells suggesting that PARP inhibition during oxidative stress not only resumed delayed development but also retained normal spore revival (17). However, the present study suggests that PARP inhibition and UV-C treatment did not have any effect on spore development of second-generation cells. This, in addition to the observation that lower doses of UV-C caused developmental defects in *D. discoideum* cells, a few of which were unaffected by PARP inhibition, raises a question concerning therapeutic significance of PARP inhibition in various DNA damage-related diseases. Thus, the concept of PARP inhibition being beneficial in various DNA associated diseases should be considered cautiously.

On a different note, *D. discoideum* despite being a lower eukaryote shows differential effects of oxidative and UV-C stress on development and spore germination. Hence, this organism has the complex signalling machinery to deal with different stresses such as oxidative stress and UV-C, in diverse ways. This fact emphasizes the importance of signalling pathway studies in *D.*

discoideum, which is simple and easy to handle compared to mammalian cell lines. Proteases involved in *D. discoideum* cell death downstream of PARP have been identified in oxidative stress-induced cell death (54). Further work needs to be performed to explore downstream targets of PARP during *D. discoideum* development for understanding the role of this multifunctional enzyme, in developmental cell death.

Acknowledgements

Infrastructure facilities provided by The Maharaja Sayajirao University and FACS facility provided by DBT-MSUB ILSPARE are gratefully acknowledged. RB thanks the Department of Biotechnology, New Delhi (BT/PR4383/BRB/10/1014/2011), Department of Science and Technology, New Delhi (SR/SO/BB-03/2010) and Council of Scientific and Industrial Research (38 (1383)/14/EMR-II) for research support; JR thanks the Council of Scientific and Industrial Research (New Delhi) for awarding SRF and TA thanks UGC- SAP DRS for fellowship.

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Involvement of poly(ADP-ribose) polymerase in paraptotic cell death of D. discoideum

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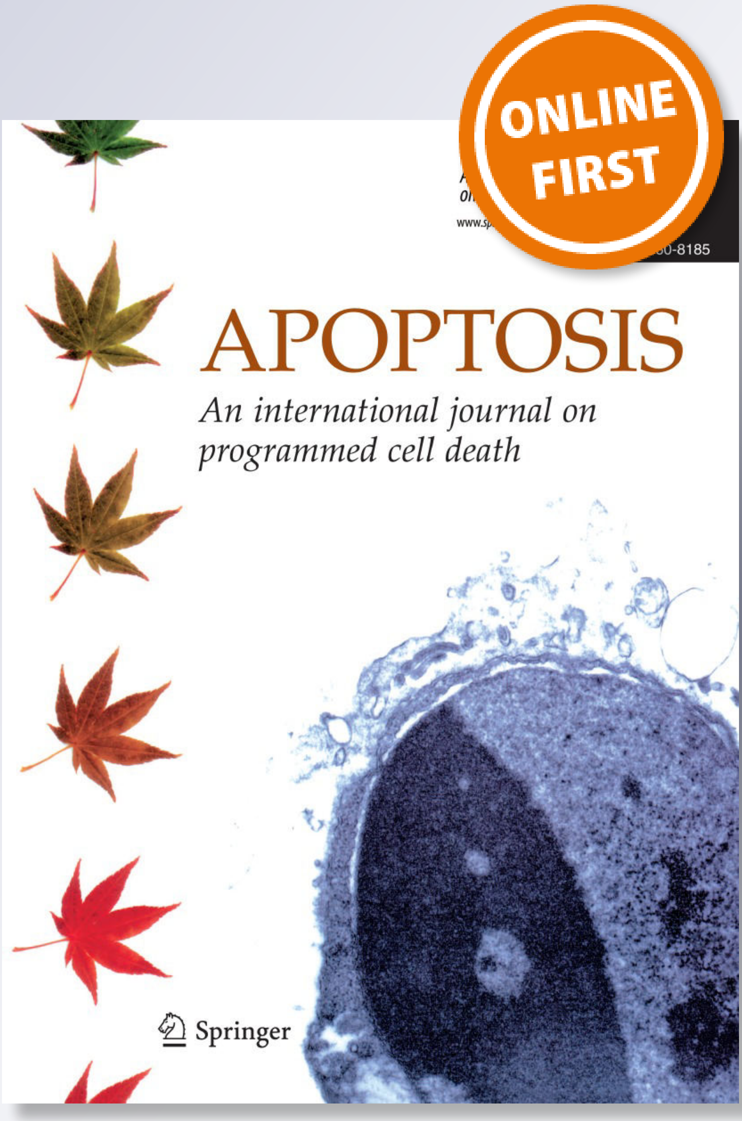
Apoptosis
An International Journal on Programmed Cell Death

ISSN 1360-8185

Apoptosis
DOI 10.1007/s10495-013-0920-9



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Apoptosis
DOI 10.1007/s10495-013-0920-9

ORIGINAL PAPER

Involvement of poly(ADP-ribose) polymerase in paraptotic cell death of *D. discoideum*

Jyotika Rajawat · Hina Mir · Tina Alex · Sonal Bakshi · Rasheedunnisa Begum

Abstract Paraptosis is mediated by several proteins, poly(ADP-ribose) polymerase being one of them. *D. discoideum* lacks caspases thus providing a better system to dissect out the role of PARP in paraptosis. The cell death phenotype in unicellular eukaryote, *D. discoideum* is similar to the programmed cell death phenotype of multicellular animals. However, the events downstream to the death signal of PCD in *D. discoideum* are yet to be understood. Our results emphasize that oxidative stress in *D. discoideum* lacking caspases leads to PARP activation, mitochondrial membrane potential changes, followed by the release of apoptosis inducing factor from mitochondria. AIF causes large scale DNA fragmentation, a hallmark feature of paraptosis. The role of PARP in paraptosis is reiterated via PARP inhibition by benzamide, PARG inhibition by gallotannin and PARP down-regulation, which delays paraptosis. PARP, PARG and AIF interplay is quintessential in paraptosis of *D. discoideum*. This is the first report to establish the involvement of PARP in the absence of caspase activity in *D. discoideum* which could be of evolutionary significance and gives a lead to understand the caspase independent paraptotic mechanism in higher organisms.

Keywords Poly(ADP-ribose) polymerase · Paraptosis · MMP · Apoptosis inducing factor · PARP antisense · Poly(ADP-ribose) glycohydrolase

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Abbreviations

PCD	Programmed cell death
PARP	Poly(ADP-ribose) polymerase
PARG	Poly(ADP-ribose) glycohydrolase
AIF	Apoptosis inducing factor
MMP	Mitochondrial membrane potential
ROS	Reactive oxygen species
H_2O_2	Cumene H_2O_2
PS	Phosphatidylserine
PI	Propidium iodide

Introduction

Programmed cell death or apoptosis which is mediated by caspases is essential for normal development and homeostasis in multicellular organisms. Cell death can occur in a programmed manner independent of caspases, and paraptosis is one such type [1, 2]. Paraptosis is mediated among others by poly(ADP-ribose) polymerase (PARP) activation via DNA damage [3, 4]. Cell death can be induced by a number of agents including reactive oxygen species (ROS) [5–7]. ROS in addition to other apoptotic stimuli activate PARP [8] which PARylates several target proteins [9]. Poly(ADP-ribose) (PAR) attached to acceptor proteins are rapidly degraded by poly(ADP-ribose) glycohydrolase (PARG). Downstream to PARP activation, a cascade of events occurs wherein; mitochondrial protein such as apoptosis inducing factor (AIF) [10] has been implicated in the execution of paraptosis. However, the role of PARP in paraptosis and the cascade of events are yet to be fully understood.

Unlike yeast *D. discoideum,* a unicellular eukaryote has nine potential PARP genes [11]. Absence of caspases [12] makes this organism a good model to study paraptosis and

Published online: 16 October 2013

the probable role of PARP in this type of cell death. Studying PARP and cell death mechanisms in *D. discoideum* would throw light on the evolutionary aspects of programmed cell death [13, 14]. Developmental cell death in *D. discoideum* exhibits a few but not all the features of apoptotic cell death reported in multicellular organisms [15]. Also, cell death in *D. discoideum* is shown to be caspase-independent [16]. We have earlier reported high resistance of the unicellular stage of *D. discoideum* to oxidative stress [17], activation of PARP during oxidative stress [4] and involvement of PARP in *D. discoideum* development [18]. The current study attempts to dissect out the role of PARP during oxidative stress induced paraptosis in *D. discoideum* using PARP inhibitor-benzamide, down-regulation of PARP by antisense and its indirect inhibition by PARG inhibitor-gallotannin. The study focusses on the cascade of events following PARP activation, a key player in caspase-independent cell death.

Materials and methods

D. discoideum culturing conditions

Dictyostelium discoideum cells (Ax-2 strain) were grown in suspension in HL5 medium and also maintained on nutrient agar with *Klebsiella aerogenes* and harvested using standard procedures [19]. All the experiments were carried out with the *D. discoideum* cells at mid-log phase at a cell density of 2×10^6 cells/ml with >95 % viability (tested with Trypan blue exclusion).

PARP down-regulation by antisense

Antisense of 500 bp was designed for the catalytic domain sequence of *PARP* which is conserved in all PARP family members and was PCR amplified by using forward (5′-*Kpn*I AAAACGGGTTCCTCACTTTG 3′) and reverse (5′-*Bam*HI CGGCGATTAGAATTCTTCGT 3′) primers. The confirmed amplicon was cloned in *D. discoideum* constitutive expression vector and the clone, pTX-*PARP* thus obtained was used to generate *D. discoideum* transformants with constitutive down-regulation of *PARP*. Further experiments were carried out with these transformed *D. discoideum* cells.

PARP and PARG inhibition

Dictyostelium discoideum culture with a count of 2×10^6 cells/ml was incubated with different doses (1.0, 2.0, 3.0, 4.0 mM) of benzamide (Sigma, India) [20] and (5, 15, 25, 50 µM) gallotannin (Sigma, India) (PARG inhibitor) for 12 h. For further experiments 1 mM benzamide and 15 µM gallotannin were used as they showed 2 % cell death only (data not shown). Cells were pre-incubated with benzamide or gallotannin and then treated with cumene H_2O_2 (Sigma, India).

Induction of oxidative stress and assessment of cell death by Annexin V-FITC/PI dual staining

Oxidative stress was induced in *D. discoideum* cells by exogenous addition of cumene H_2O_2. 2.5×10^6 cells/ml were exposed to different doses of cumene H_2O_2 (H_2O_2) (0.03, 0.05 mM) in HL-5 medium at 22 °C in a sterile flask. Cell viability was assayed using Trypan blue after 12 h of stress [21]. To differentiate between apoptotic and necrotic cell death, dual staining with Annexin V-FITC/PI was performed using apoptosis detection kit (Molecular Probes). Dose and time dependent studies were done to standardize the paraptotic and necrotic doses. Cells were analyzed qualitatively by Zeiss confocal laser scan fluorescence-inverted microscope (LSM 710; Carl Zeiss) and quantitated by flow cytometry using a FACS ARIA (BD Biosciences). Data were analysed with FACSDiva software.

Measurement of PARP activity

PARP was assayed by indirect immunofluorescence [22] using anti-PAR mouse mAb (10H) (Calbiochem) at a concentration of 0.5 µg/ml and anti-mouse IgG (whole molecule) FITC conjugate (Sigma) at a dilution of 1:200. Cells were observed by fluorescence microscope (Nikon TE-2000S, Tokyo, Japan) and photographed. Mean density of fluorescence was plotted for quantification.

Estimation of NAD^+ and ATP levels

Intracellular levels of NAD^+ were determined by enzymatic recycling method [23] employing alcohol dehydrogenase to reduce NAD^+ to NADH. NAD^+ levels were estimated by taking the absorbance at 570 nm and protein concentration was estimated by Lowry method [24]. ATP was determined by HPLC in aliquots extracted with alkali [25].

Evaluation of mitochondrial membrane potential (MMP)

Potential sensitive dye $DiOC_6$ (3,3′-dihexyloxacarbocyanine iodide) (Sigma) at a concentration of 5 nM was used to evaluate changes in MMP [26]. Time dependent study was done using H_2O_2. Cells were observed by fluorescence microscope (Nikon TE-2000S, Tokyo, Japan) and photographed. Results were expressed as the proportion of cells exhibiting low mitochondrial membrane potential estimated by the reduced $DiOC_6$ uptake. Mean density of fluorescence was plotted for quantification.

MMP was also measured by flow cytometry, by incubating *D. discoideum* cells (1×10^6/ml) with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carbocyanine iodide (JC-1) (Molecular Probes) [27] and quantitated by flow cytometry using a FACS ARIA (BD Biosciences). Data were analysed with FACSDiva software.

Monitoring apoptosis inducing factor (AIF) release

AIF release from mitochondria to cytosol and translocation to nucleus was monitored by immunofluorescence [28]. Rabbit anti-AIF polyclonal antibodies against amino acids 151–180 of human AIF (Cayman Chemical), 1:1,000 dilution and anti-rabbit IgG (whole molecule) TRITC conjugate (Sigma), 1:400 dilution were used. Nuclear counterstaining with DAPI (1 μg/ml) for 5 min was performed and observed the fluorescence. Cell specimens were observed with a Zeiss Confocal Laser scan fluorescence-inverted microscope (LSM 710; Carl Zeiss).

Monitoring DNA fragmentation

Cells were exposed to 0.03 mM H_2O_2 for 6 h and DNA isolation was done from the cells by the conventional method and was run on 0.8 % agarose gel. TUNEL assay (Terminal deoxynucleotidyl transferase dUTP nick end labeling) [29] was performed as per the manufacturer's instructions (Sigma).

Data analysis and statistics

Flow cytometry and colorimetric assay experiments were repeated at least three times. Data were analyzed according to mean fluorescence intensity or optical density and plotted on dot plots or on graphs. Statistical analysis was performed by *t* test for experiments with single comparisons.

Results

Induction of cell death in *D. discoideum* by exogenous addition of H_2O_2

The susceptibility of *D. discoideum* cells to death was examined by treating them with H_2O_2. In response to increasing doses of H_2O_2 from 0.03 to 0.1 mM, the percentage of cells undergoing death significantly increased from 15 to 90 % after 12 h treatment regime, as examined

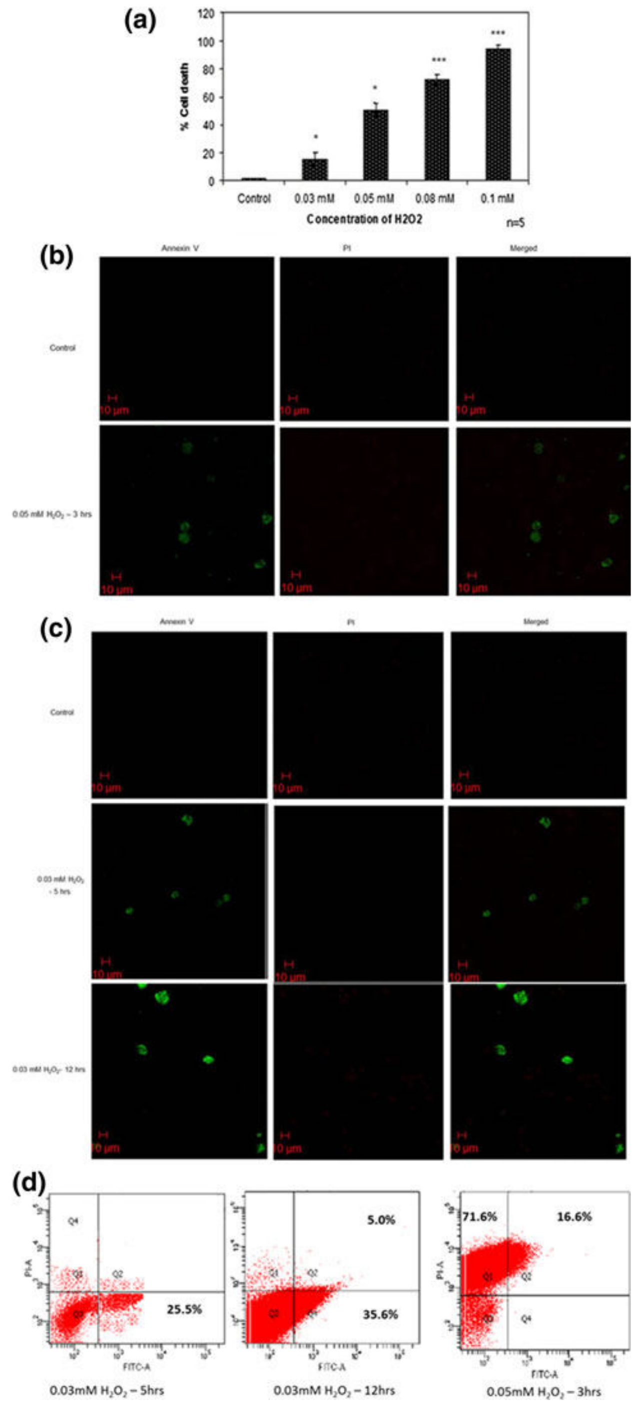

Fig. 1 H_2O_2 induces dose dependent cell death. **a** Monitored by trypan blue exclusion method. Dose dependent increase in cell death was observed with H_2O_2. $***p < 0.001$, $**p < 0.01$, $*p < 0.05$ compared to control. **b** Annexin V-PI dual staining of 0.05 mM H_2O_2 stressed *D. discoideum* cells. 0.05 mM H_2O_2 treated cells showed Annexin V-PI dual positive cells as early as 3 h. **c** Annexin V-PI dual staining of 0.03 mM H_2O_2 stressed *D. discoideum* cells. Annexin V staining due to PS externalization was seen at 5 h while PI staining at 12 h with 0.03 mM H_2O_2. Data are representative of three independent experiments. Photographs were taken with ×60 objective. **d** Annexin V-PI staining for *D. discoideum* cells subjected to 0.03 and 0.05 mM H_2O_2 quantitated by flow cytometry

by Trypan blue exclusion (Fig. 1a). The LD_{50} was found to be 0.05 mM. However, based on our Annexin V-PI dual staining results, 0.05 mM H_2O_2 was found to be necrotic as cells exhibited PI and Annexin V staining at 3 h (Fig. 1b, d). In contrast, 0.03 mM H_2O_2 was found to be paraptotic, as cells showed Annexin V staining at 5 h and during this period, the majority of the cells were negative for PI, implicating the early stage of paraptosis. With increased exposure time (12 h), in addition to Annexin V staining, a few cells exhibited PI staining consistent with a more advanced stage of paraptosis (Fig. 1c, d).

Quantitation of Annexin V-PI dual staining by FACS showed 0.03 mM H_2O_2 treated cells exhibited 25.5 % Annexin V staining at 5 h with negligible PI staining, while at 12 h around 5 % cells showed Annexin V-PI dual staining. On the contrary, at 0.05 mM H_2O_2 dose ~17 % cells showed Annexin V-PI dual staining within 3 h (Fig. 1d).

Functional characterization of PARP antisense

pTX-*PARP* was transformed into *D. discoideum* cells and *PARP* down-regulation was confirmed by monitoring its expression by semi quantitative Reverse Transcriptase PCR and it was found that *PARP* transcript was reduced by 60 % (Fig. 2a, b). PARP activity in *PARP* down-regulated cells was also found to be 50 % lower than basal level (Fig. 2c, d).

PARP inhibition delays oxidative stress induced cell death

The ability of PARP to modulate oxidative stress induced cell death was then determined. The mean viability of H_2O_2 treated cells was partially intercepted by benzamide and PARP down-regulation as observed with Trypan blue staining (Fig. 3a). 0.03 mM H_2O_2 treated cells exhibited Annexin V staining at 5 h however, PI staining was seen only at 12 h. Nevertheless, at 0.03 mM H_2O_2 dose, PARP down-regulated as well as benzamide treated *D. discoideum* cells showed Annexin V staining at 12 h without PI staining suggesting membrane integrity was still maintained (Fig. 3b). This confirms the involvement of PARP in oxidative stress mediated paraptosis in *D. discoideum*. However, at 0.05 mM H_2O_2 dose, Annexin V and PI staining were seen as early as 3 h (Fig. 1b). Consistent with the inhibition studies cell death was also partially intercepted by PARG inhibition, PARP inhibition and down-regulation (Fig. 3b).

PARP activation induced by oxidative stress

PARP activation is seen in response to DNA damage resulting in the formation of poly(ADP-ribose) polymers (PAR). PARP activity was assayed at various time points after oxidant treatment by indirect immunofluorescence method using PAR antibody. Peak PARP activity was seen at

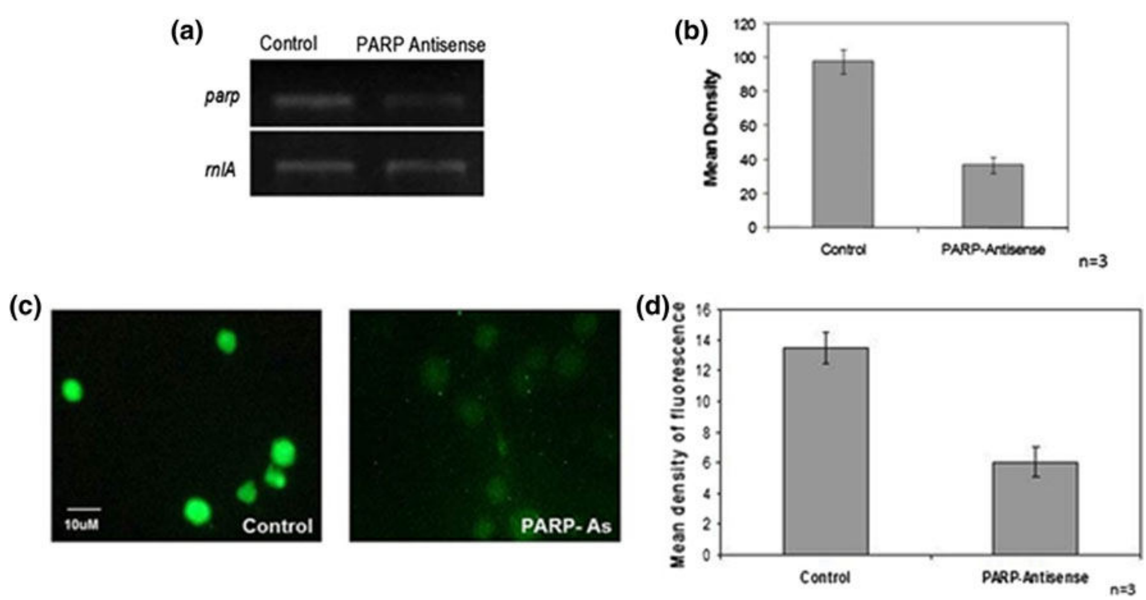

Fig. 2 Functional characterization of *PARP* antisense (As). **a** RT-PCR of *PARP* down-regulated *D. discoideum.* Gene specific expression of *PARP* exhibits ~60 % reduction while no change was observed with internal control *rnl*A. **b** Densitometric analysis of *PARP* expression at the transcript level. **c** PARP activation monitored in PARP down-regulated cells. **d** Densitometric analysis of PARP activation

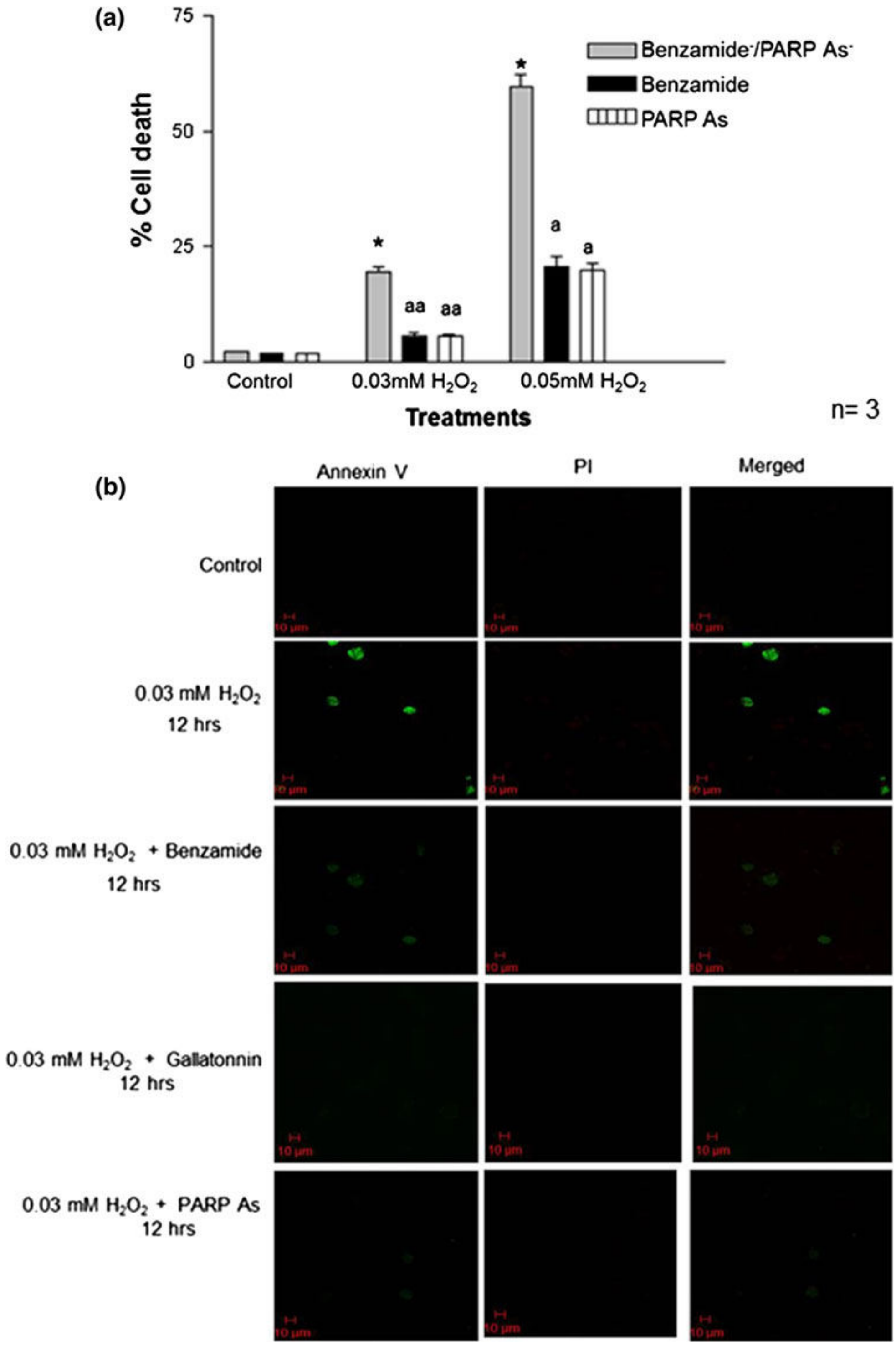

Fig. 3 Effect of PARP inhibition on H_2O_2 induced cell death. **a** PARP inhibition partially prevented the cell death induced by H_2O_2 as monitored by Trypan blue. $^{*}p < 0.05$ compared to control; $^{aa}p < 0.01$, compared to 0.03 mM H_2O_2; $^{a}p < 0.01$ compared to 0.05 mM H_2O_2. **b** Monitoring cell death by Annexin V-PI dual staining. At 12 h, cells showed only Annexin V staining and no PI staining with PARP down-regulation, PARP inhibition and PARG inhibition

10 min post 0.03 mM H_2O_2 treatment ($p < 0.01$) (Fig. 4a). Results indicated no significant difference in PARP activity at 15 min. High PAR levels were maintained for a few minutes and basal level was regained after 10 min of H_2O_2 (Fig. 4a). Peak PARP activity at 10 min was significantly inhibited by 1 mM benzamide (Fig. 4b, c). In addition,

PARP antisense partially prevented the increase in PARP activity due to oxidative stress ($p < 0.01$) (Fig. 4b, c).

PARP activation leads to adenine and pyridine nucleotides depletion

PARP activation consumes cellular NAD^+ and ATP pools and depletion of adenine nucleotides offers one possible signal responsible for the downstream events in the paraptotic pathway. Thus PARP activation was also confirmed by monitoring cellular NAD^+ and ATP levels and the results are shown in Fig. 5a, b. NAD^+ and ATP contents were reduced to 60 and 45 % of control values respectively within 1 h of 0.03 mM H_2O_2 treatment (Fig. 5a, b). These results support the early activation of PARP.

Our results on PARP inhibition with benzamide showed significant rescue in the depletion of cellular NAD^+ and ATP levels. 0.03 mM H_2O_2 stressed cells with benzamide pretreatment could intercept the depletion in NAD^+ and ATP levels by 30 and 35 % respectively, indicating that PARP activation caused reduction in energy levels. PARG inhibition also partially restores the NAD^+ levels (Fig. 5a).

Mitochondrial membrane potential (MMP) changes under oxidative stress

Lower H_2O_2 doses led to an increase in MMP at an early stage. Significant reduction in MMP was observed at 3 h post 0.03 mM H_2O_2 ($p < 0.01$). In accordance with nucleotide depletion results benzamide pretreatment also delayed MMP changes in cells exposed to 0.03 mM H_2O_2 for 7 h (Fig. 6a,

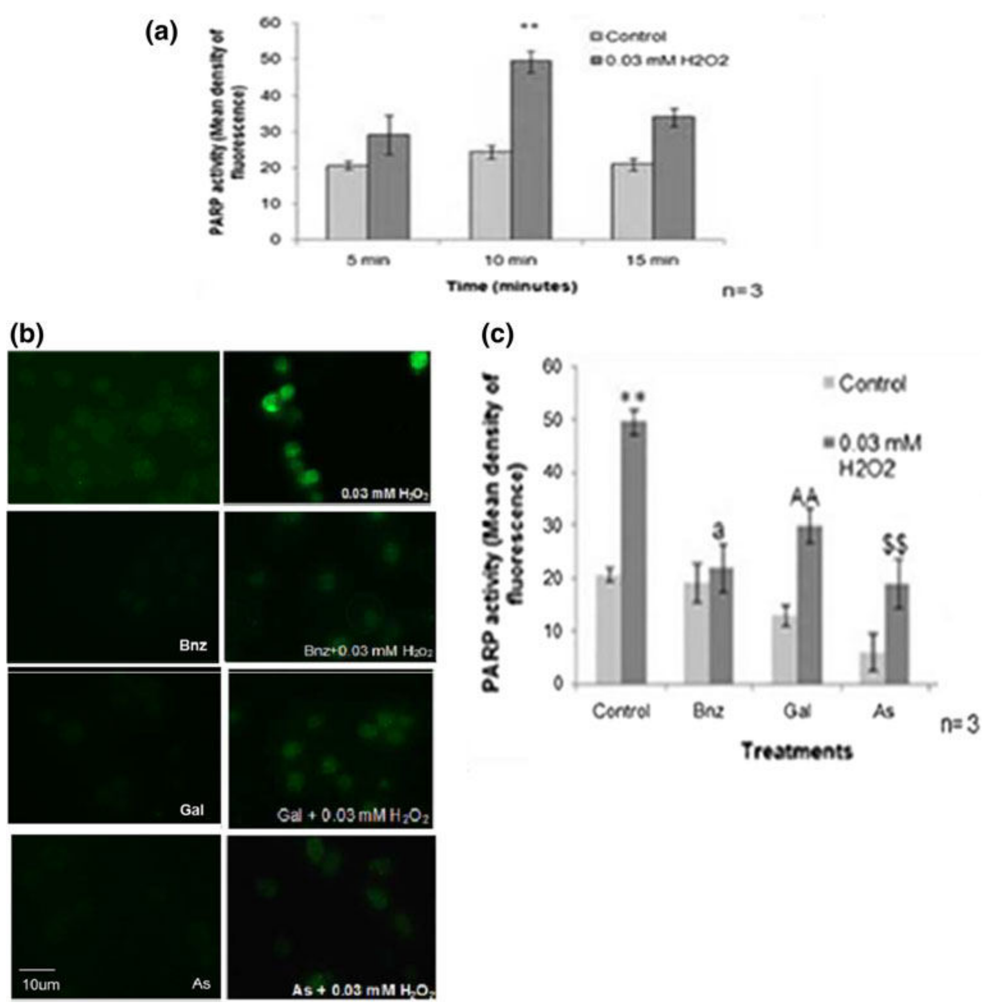

Fig. 4 Peak PARP activity induced by H_2O_2 at 10 min was intercepted by PARP inhibition. **a** Peak PARP activity was observed at 10 min post 0.03 mM cumene H_2O_2 stress. **b** Benzamide, gallotannin and PARP down-regulation prevented peak PARP activation at 10 min during oxidative stress induced paraptosis. **c** Densitometric analysis of PARP activation in the presence of benzamide, gallotannin and PARP down-regulation. $^{**}p < 0.01$ compared to control; $^{a}p < 0.05$ compared to 0.03 mM H_2O_2; $^{AA,\$\$}p < 0.01$ compared to 0.03 mM H_2O_2 stress

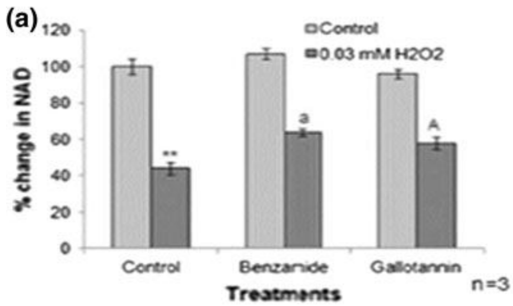

Fig. 5 Oxidative stress depletes NAD^+ and ATP content of *D. discoideum* cells in a PARP dependent manner. **a** NAD^+ estimation post 0.03 mM cumene H_2O_2 stress in presence of benzamide and gallotannin.**$p < 0.01$ compared to control; $^{a,A}p < 0.05$ compared to H_2O_2 stress. **b** ATP estimation in presence of benzamide post 0.03 mM cumene H_2O_2 stress. *$p < 0.05$ compared to control; $^{a}p < 0.05$ compared to H_2O_2 stress

b). MMP changes induced by oxidative stress were also found to be partially intercepted by PARP down-regulation ($p < 0.05$) (Fig. 6b). The magnitude of this effect mediated by PARP antisense was comparable with the PARP inhibitor, benzamide and by PARG inhibition (Fig. 6b).

To confirm the change in MMP, JC-1 staining was performed. Mitochondria containing red JC-1 aggregates in healthy cells and are detectable in FL2 channel, and green JC-1 monomers in apoptotic cells are detectable in FITC channel (FL1). JC-1 staining also shows reduction in red fluorescence in almost 72 % cells indicating mitochondrial membrane depolarization at 3 h post 0.03 mM H_2O_2 treatment which is partially rescued by PARP down-regulation (59.34 %), pretreatment with benzamide (62 %) and gallotannin (65.32 %) as shown in Fig. 6c.

AIF translocation is downstream to PARP activation

As AIF has been identified as a mediator of PARP induced cell death, we have monitored the release of AIF during oxidative stress induced cell death. AIF release under 0.03 mM H_2O_2 dose was seen at 5 h and its translocation to nucleus was observed by 6 h (Fig. 7). These changes in the localization of AIF coincide with the MMP changes. PARP inhibition intercepted AIF release at 0.03 mM H_2O_2 stress. AIF translocation was also partially prevented by gallotannin and PARP down-regulation (Fig. 7).

Oxidative stress induced apoptosis leads to large scale DNA fragmentation

Oligonucleosomal DNA fragmentation is the hallmark of apoptosis while paraptosis exhibits large scale DNA fragments that cannot be resolved on agarose gel. However DNA fragmentation can be detected by TUNEL assay. *D. discoideum* cells exposed to 0.03 mM H_2O_2 showed absence of oligonucleosomal DNA ladder (Fig. 8a). A significant increase in TUNEL positive cells was seen (Fig. 8b, c) at 6 h post 0.03 mM H_2O_2 stress suggesting that under oxidative stress *D. discoideum* cells undergo large scale DNA fragmentation.

Discussion

Apoptosis is mediated by caspases which exist as inactive precursors, or procaspases, and are activated by either extracellular or intracellular death signals. Caspase activation is regulated by members of the Bcl-2 family. However, the mediators of non-apoptotic cell death programs are much less characterized. Earlier report demonstrates non-apoptotic caspase independent cell death pathway operates via alternative caspase-9 activity (1). Notably, in *Dictyostelium*, there are no caspase-family members, except one paracaspase gene which was previously shown not to be involved in autophagic and necrotic cell death [16]. The types of cell death reported in *Dictyostelium* are autophagy, necrosis and developmental cell death [30–32]. Also, in silico investigation showed that the genome of *Dictyostelium* harbored no detectable member of the Bcl-2 family and no single BH3 domain-bearing molecule. Lam et al. [33] also showed that cell death in *Dictyostelium* can occur without caspases and Bcl-2 members. Our earlier report showed oxidant induced PARP mediated cell death in *D. discoideum* [4]. The present study is the first detailed report that enlightens paraptosis induced by oxidative stress in *D. discoideum* without the involvement of procaspase-9. It demonstrates that cell death is mediated by PARP as it occurs without caspase activation and it could be intercepted by the PARP inhibitor, benzamide. Caspase inhibitors are found to be ineffective in blocking the cell death (data not shown). Also, our present study delineates the ensuing cascade of events following PARP activation finally culminating in cell death.

Oxidative stress is a known inducer of cell death and severity of the stress determines whether cells undergo

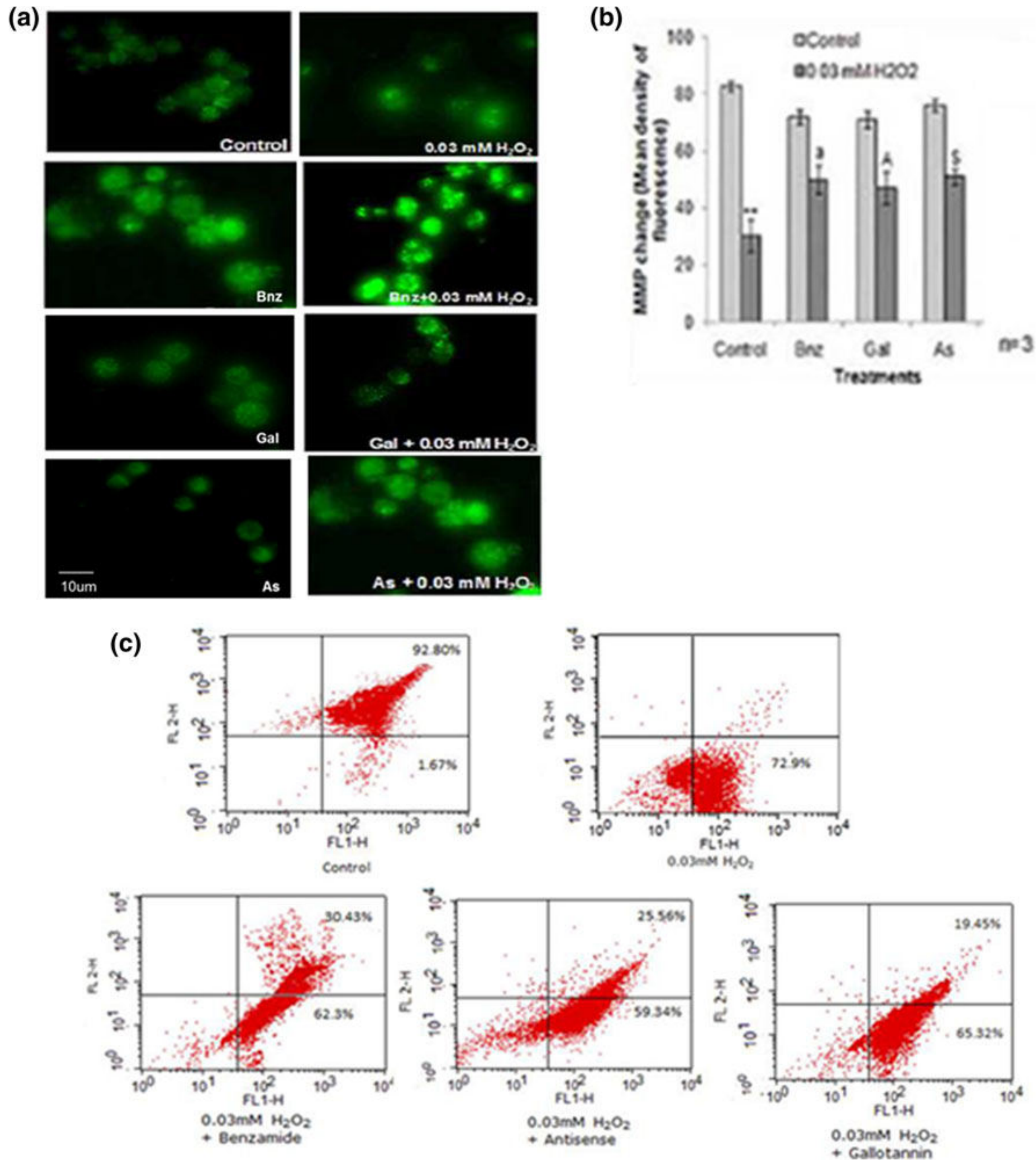

Fig. 6 Mitochondrial membrane potential changes induced by oxidative stress at 3 h. **a** Benzamide, gallotannin and PARP down-regulation partially restored the changes in MMP. **b** Densitometric analysis of the MMP changes in presence of benzamide, gallotannin and PARP down-regulation. $^{**}p < 0.01$ compared to control; $^{a,A,\$}p < 0.05$ compared to H_2O_2 stress. **c** A dot plot of *red fluorescence* (FL2) versus *green fluorescence* (FL1) resolved in live cells with intact mitochondrial membrane potential and from 0.03 mM H_2O_2 treated cells (in presence of benzamide, gallotannin and PARP down-regulation) with loss of mitochondrial membrane potential. Mitochondria containing *red* JC-1 aggregates in healthy cells and are detectable in FL2 channel, and *green* JC-1 monomers in apoptotic cells are detectable in FITC channel (FL1)

necrosis or apoptosis. Annexin V and PI dual staining results suggest that 0.05 mM H_2O_2 dose is necrotic (Fig. 1b, d). Nevertheless, 0.03 mM H_2O_2 stress yielded paraptotic cell death (Fig. 1c, d). Thus, H_2O_2 at different concentrations can induce different types of cell death in *D. discoideum*. Similar results were also obtained with another oxidant, hydroxylamine (data not shown). These results are in parallel with a murine macrophage like tumor cell line which exhibits apoptosis and necrosis when exposed to lower and higher doses of H_2O_2 respectively

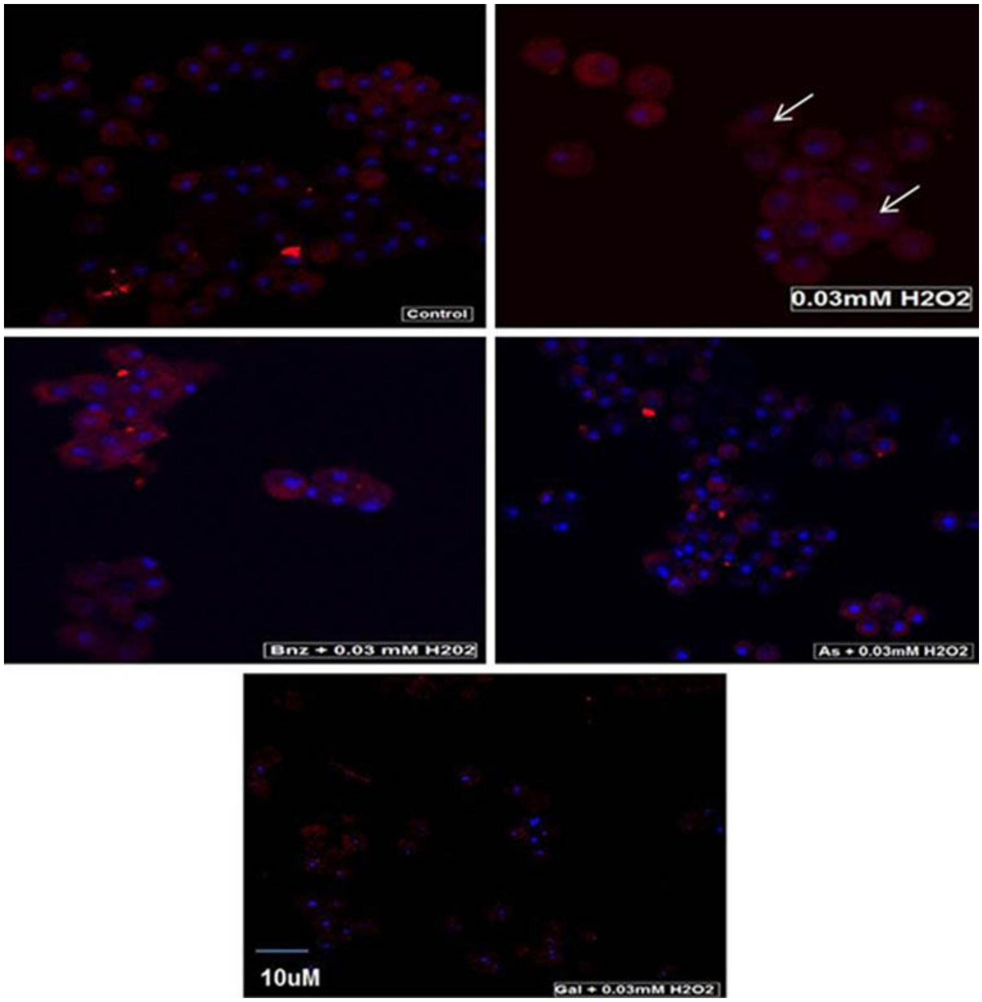

Fig. 7 Mitochondrio-nuclear translocation of AIF 5 h post oxidative stress. Confocal microscopy images where nucleus of control *D. discoideum* cells is *blue* and translocation of AIF (*red* coloured) to nucleus during oxidative stress turns the nuclear stain into *pink* (Color figure online)

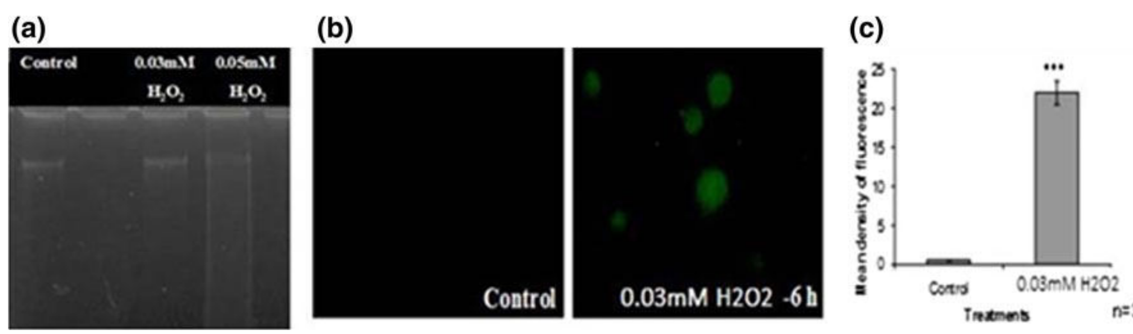

Fig. 8 DNA fragmentation was monitored under oxidative stress. **a** By agarose gel electrophoresis. **b**, **c** Using TUNEL assay and its densitometric analysis ***$p < 0.001$ compared to control

[34]. Cell death induced by phagocyte derived oxygen radicals as well as exogenous hydrogen peroxide was efficiently prevented by PARP inhibitors [35].

PARP-1 knockout can prevent energy depletion and hence increased viability of the cells exposed to oxidative stress [36]. In higher organisms PARP-1 and PARP-2 double knockouts are lethal due to loss of DNA repair function [37]. In view to these reports and to rule out the non specific effects of benzamide we have made an attempt to down-regulate PARP by antisense to dissect out its role in *D. discoideum* cell death. Peak PARP activity was observed at 10 min of 0.03 mM H_2O_2 stress (Fig. 4a). It has also been demonstrated that PARP is activated under oxidative stress in HeLa cells within 10 min [38]. PARP inhibition by antisense confers survival advantage to oxidative stress exposed endothelial cells by maintaining cellular energy levels and activating pro-survival pathway [39]. Benzamide pretreated and PARP down-regulated cells subjected to H_2O_2 showed reduction in the peak PARP activity (Fig. 4b).

PARP is known to be activated rapidly in response to different DNA damaging agents and the type of cell death a cell exhibits depends on the extent of PARP activation in the nucleus [36]. Oxidant imbalance in the cell leads to potential DNA damage. Within minutes following oxidative stress H2AX phosphorylation was observed in *D. discoideum* cells (data not shown). To elucidate the role of PARP in paraptosis PARP inhibition studies were attempted by using benzamide. In the presence of benzamide, paraptosis was delayed by 7 h (Fig. 3b). PARP down-regulation results were comparable to PARP inhibition studies by benzamide where Annexin V staining which was seen at 5 h with paraptotic dose of H_2O_2 was delayed by 12 h and PI staining was not seen at 12 h suggesting a delay in paraptotic cell death (Fig. 3b). PARP inhibition prevented cell death by 50 % while 60 % rescue was observed with PARP down-regulation.

As a consequence of PARP activation, cellular NAD^+ and ATP levels were reduced to 60 % and 45 % of control *D. discoideum* cells within 1 h of oxidative stress (Fig. 5a, b). This reduction in the pyridine and adenine nucleotides is a supportive evidence for PARP activity as NAD^+ serves as the substrate for PARP, and ATP is required for NAD^+ replenishment [40] as opposed to our earlier report on staurosporine induced cell death in *D. discoideum* wherein PARP activation was not seen and there was no concomitant change in cellular NAD^+ and ATP levels as compared to control cells [41]. Also, NAD^+ supplementation partially intercepted oxidative stress induced cell death in *D. discoideum* cells (data not shown). Cytoprotection by PARP inhibition is due to the prevention of NAD^+ and ATP depletion caused by PARP overactivation [42]. Ying et al. [43] reported that PARG inhibition is protective in function during oxidative stress. Also, our results showed that pretreatment of *D. discoideum* cells with benzamide and PARG inhibitor gallotannin prevented PARP activation and rescued the NAD^+ and ATP levels by $\sim$30 and 35 % respectively at paraptotic dose (Fig. 5a, b), thus maintaining the energy levels.

Loss of MMP was observed downstream to PARP activation during *D. discoideum* paraptosis. Benzamide pretreatment delayed the change in MMP (Fig. 6a–c) which correlates with the delay seen in PS exposure. Similar results were obtained with PARP down-regulation (Fig. 6a–c).

AIF is known to be an important executioner of caspase independent cell death. ROS mediated PARP activation is necessary for the AIF release from mitochondria [44]. PAR polymer itself or PARylated proteins or NAD^+ depletion could lead to mitochondrial permeability transition, linking PARP activation to AIF translocation [45]. Our findings support this hypothesis as PARP inhibition partially prevented the release of AIF from mitochondria (Fig. 7) and also delayed DNA fragmentation (Fig. 8). We have proposed a pathway which shows that paraptosis is partially rescued by PARP inhibition (Fig. 9).

PARG inhibition prevents cell death by slowing the turnover of PAR and thus preventing NAD^+ depletion. Reduced PARP activity might also contribute to cell death prevention in presence of PARG inhibitor. Thus, paraptosis induced by oxidative stress can be intercepted by PARG inhibition, suggesting that PARP-mediated cell death requires the concomitant action of PARG. Our findings support the hypothesis suggested by Ying et al. [43].

Interestingly, paraptosis also can be seen in mammalian cells, but paraptosis lacks many of the molecular and biochemical characteristics of apoptosis. Paraptosis and apoptosis represent two separate programs of cell death that are induced via distinct molecular pathways, but may be induced simultaneously by a single insult or agent. One feature distinguishing paraptotic cell death from apoptotic cell death is that paraptosis is not affected by broad caspase inhibitor (data not shown). In particular, apoptosis is inhibited by caspase inhibitors whereas paraptosis is resistant to such inhibitors. Involvement of procaspase-9 in

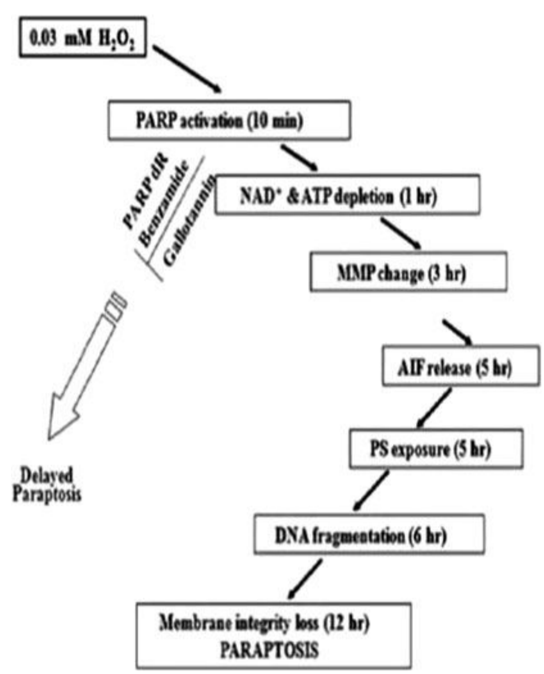

Fig. 9 Proposed pathway for oxidative stress induced paraptosis

paraptosis is independent of its proteolytic activity in the apoptotic pathway as evidenced by the resistance of paraptosis to Apaf-1. However, *D. discoideum* paraptosis may not be dependent even on procaspase 9 function as this organism lacks caspases [12]. Thus our results point out that paraptotic pathway could be further divided into procaspase-9 dependent and procaspase-9 independent types.

A striking similarity has been observed at the morphological level between paraptosis, type 3 (cytoplasmic) cell death, and the neuronal cell death observed in some neurodegeneration models, which suggests that paraptosis may be a physiologically relevant process. It will be important to characterize the molecular mechanisms underlying paraptosis to identify specific inhibitors for such non-apoptotic programmed cell death. These results support the concept that multiple, distinct cell death programs may be employed in a cell depending upon the nature of stress and its severity. So far there are no reports which throw light on the protein/s involved in mediating *D. discoideum* paraptotic cell death.

Dictyostelium discoideum, a protist that emerged in evolution after plants and from an ancestor common to fungi and animals shows developmental cell death which is of paraptotic type. We have established the role of PARP in *D. discoideum* during oxidative stress induced paraptotic cell death. Our PARP and PARG inhibition studies by benzamide and gallotannin respectively, and our PARP antisense results furthermore suggest that PARP down-regulation has delayed the oxidative stress induced paraptosis. Thus we have demonstrated that *D. discoideum* is an excellent model to dissect out and study the molecular mechanisms of PARP mediated paraptosis in complete absence of caspases.

This is the first report where the involvement of PARP and the downstream events during oxidative stress induced paraptosis in *D. discoideum,* an ancient eukaryote, are established. PARP and AIF are the major players governing *D. discoideum* cell death kinetics during paraptosis induced by oxidative stress. PARG inhibition is also protective in function during oxidative stress induced cell death. The elucidation of the mechanisms by which PARP-1 activation signals translocation of PARG would be of paramount importance. Therefore, PARP, PARG and AIF could be potential targets for therapy of neurological disorders. This model could also be exploited to screen the compounds/drugs that can intercept or activate paraptotic type of cell death during different disease conditions involving PARP.

Acknowledgments RB thanks the Department of Biotechnology, New Delhi (BT/PR9496/BRB/10/562/2007) and Department of Science and Technology, New Delhi (SR/SO/BB-03/2010) for research support. JR thanks Council Scientific Research, New Delhi for awarding JRF/SRF. We thank DBT-MSUB-ILSPARE program for providing the FACS and Confocal facilities. We also thank Dr. Shweta Saran for generous gift of vectors. Our sincere thanks are to Ms. Bandhana Katoch from M.S. University of Baroda, Vadodara; Prof. V. Nanjundiah and Dr. Ritwick Sarwarkar from Indian Institute of Science, Bangalore for their help in standardization of the fluorescence techniques.

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 DOI 10.1099/mic.0.076620-0

Proteases involved during oxidative stress-induced poly(ADP-ribose) polymerase-mediated cell death in *Dictyostelium discoideum*

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Received 4 January 2014
Accepted 6 April 2014

Apoptosis involves a cascade of caspase activation leading to the ordered dismantling of critical cell components. However, little is known about the dismantling process in non-apoptotic cell death where caspases are not involved. *Dictyostelium discoideum* is a good model system to study caspase-independent cell death where experimental accessibility of non-apoptotic cell death is easier and molecular redundancy is reduced compared with other animal models. Poly(ADP-ribose) polymerase (PARP) is one of the key players in cell death. We have previously reported the role of PARP in development and the oxidative stress-induced cell death of *D. discoideum. D. discoideum* possesses nine PARP genes and does not have a caspase gene, and thus it provides a better model system to dissect the role of PARP in caspase-independent cell death. The current study shows that non-apoptotic cell death in *D. discoideum* occurs in a programmed fashion where proteases cause mitochondrial membrane potential changes followed by plasma membrane rupture and early loss of plasma membrane integrity. Furthermore, the results suggest that calpains and cathepsin D, which are instrumental in dismantling the cell, act downstream of PARP. Thus, PARP, apoptosis inducing factor, calpains and cathepsin D are the key players in *D. discoideum* caspase-independent cell death, acting in a sequential manner.

INTRODUCTION

Cells contain a specific death-governing network associated with different effector and/or dismantling mechanisms. Apoptosis consists of events occurring via a cascade of caspase activation leading to ordered dismantling of critical cell components and pathways. At the end of apoptosis, the cell is fragmented into apoptotic bodies that undergo phagocytosis by neighbouring cells (Katoch *et al.*, 2002). Thus, these morphological changes are the manifestation of the cell undergoing systematic dismantling and further packaging itself into membrane-bound vesicles to be taken up by neighbouring cells. Because cellular contents are not released, apoptosis occurs without inflammation. However, little is known about the dismantling process in non-apoptotic cell death where caspases are not involved. A major task, for the less well-known non-apoptotic types of cell death, will be the identification and study of cell death types, which reflect dismantling events of effector mechanisms. *Dictyostelium discoideum* is a good model system to study dismantling events where it shows poly(ADP-ribose) polymerase (PARP)-mediated caspase-independent cell death (Rajawat *et al.*, 2014). The evolutionary aspects of programmed cell death would be illuminated by studying cell death mechanisms in *D. discoideum* (Mir *et al.*, 2007; Kawal *et al.*, 2011).

Oxidative stress has been shown to be associated with cell death in most systems (Hasnain *et al.*, 1999; Sah *et al.*, 1999; Mohan *et al.*, 2003). We have reported the high resistance of the unicellular stage of *D. discoideum* to oxidative stress (Katoch & Begum, 2003). *D. discoideum* devoid of caspases exhibits non-apoptotic cell death mediated by several proteins or factors, PARP being one of them (Rajawat *et al.*, 2014). We have established the role of PARP during development (Rajawat *et al.*, 2007, 2011) and staurosporine- (Mir *et al.*, 2012) and oxidative stress-induced cell death (Rajawat *et al.*, 2014) along with the downstream effects. Our results suggest that PARP and apoptosis inducing factor (AIF) may be the key players in regulating caspase-independent cell death. In the current study we have explored the biochemical events occurring during oxidative stress-induced cell death in *D. discoideum*.

Abbreviations: AIF, apoptosis inducing factor; ALLN, N-Acetyl-L-leucyl-L-leucyl-L-norleucinal; AMC, 7-amino-4-methyl-coumarin; $DiOC_6$, 3,3′-dihexyloxacarbocyanine iodide; DPH, 1,6-diphenyl-1,3,5-hexatriene; HA, hydroxylamine; MMP, mitochondrial membrane potential; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; PIC, protease inhibitor cocktail; PS, phosphatidylserine; zVAD-fmk, N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone.

In mammalian systems, probable candidates involved in cell dismantling could be lysosomal proteases or cytosolic protease, namely calpain. Partial destabilization of lysosomal membrane leads to release of cathepsin D in the cytosol. Cathepsin D triggers Bax activation, which induces the release of AIF into the cytosol (Bidère *et al.*, 2003). Cathepsin D also induces generalized proteolysis leading to caspase-independent cell death. Calpains are believed to participate in intracellular signal processing via limited proteolysis of their targets. Calpains have been shown to act downstream of caspase activation and contribute to the degradation phase of campthotecin-induced apoptosis in HL-60 cells (Wood *et al.*, 1998; Wood & Newcomb, 1999). Sanvicens *et al.* (2004) have shown that both caspases and calpains contribute to oxidative stress-induced apoptosis in retinal photoreceptor cells. Thus, to explore the role of proteases in *D. discoideum* dismantling, we initiated our study with protease inhibitor cocktail (PIC) to inhibit most of the proteases and then used specific inhibitors for calpain and cathepsin D.

METHODS

***D. discoideum* culture conditions.** *D. discoideum* strain Ax-2, which is an axenic derivative of Raper's wild-type NC-4, was used. *D. discoideum* was grown under different culture conditions. Growing cells (unicellular) were maintained in a liquid suspension (HL5 medium). *D. discoideum* cells were grown in HL5 medium, pH 6.5, with shaking (150 r.p.m.) at 22 °C (Watts & Ashworth, 1970). Exponential phase cells at a density of ~2.5×10^6 cells ml^{-1} were used for experiments.

Induction of oxidative stress. Oxidative stress was induced in *D. discoideum* cells by *in situ* generation of H_2O_2 upon addition of hydroxylamine (HA; Sigma), a catalase inhibitor (Kono & Fridovich, 1983), or by exogenous addition of cumene hydroperoxide (Sigma). Exponential phase cells at a density of ~2.5×10^6 cells ml^{-1} were exposed to different doses of HA (0, 1, 2.5 mM) and paraptotic (0.03 mM) and necrotic (0.05 mM) doses of cumene H_2O_2 as described by Rajawat *et al.* (2014) in HL-5 medium at 22 °C in a sterile flask.

Assessment of cell death by Annexin V-FITC/propidium iodide (PI) dual staining. To differentiate between apoptotic and necrotic cell death, dual staining with Annexin V-FITC/PI (Miller, 2004) was performed using an apoptosis detection kit (Molecular Probes). Then, ~2.0×10^6 cells were pelleted and washed twice with 1× Sorenson's buffer (SB). *D. discoideum* cells were then suspended in binding buffer provided in the kit and incubated with Annexin V for 10 min and then with PI for 5 min in the dark at 22 °C. Fluorescence was monitored at ×63 using a Zeiss confocal laser scanning fluorescence-inverted microscope (LSM 710; Carl Zeiss) and quantified by flow cytometry using a FACS ARIA cytometer (BD Biosciences). Data were analysed with FACSDiva software. A dose- and time-dependent study was done to standardize the paraptotic and necrotic doses of HA for further experiments.

Evaluation of mitochondrial membrane potential (MMP). The potential sensitive dye 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$; Sigma) was used to evaluate changes in MMP (Koning *et al.*, 1993). To observe the change in MMP, a time-dependent study was done using paraptotic and necrotic doses of HA as standardized by Annexin V–PI dual staining. Approximately 2.0×10^6 cells were pelleted and washed twice with 1× SB. Cells were stained with $DiOC_6$ (400 nM) for 15 min in the dark and then washed once with 1× SB and monitored for fluorescence using a Nikon Eclipse TE2000S fluorescence microscope.

MMP was also measured by flow cytometry, by incubating *D. discoideum* cells (1×10^6 ml^{-1}) with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carbocyanine iodide (JC-1; Molecular Probes) (Cossarizza & Salvioli, 2001), and quantified by flow cytometry using a FACS ARIA cytometer. Data were analysed with CellQuest software.

Monitoring AIF release by immunofluorescence. Release of AIF from mitochondria to cytosol and its translocation to nucleus were monitored by detecting immunofluorescence (Bidère *et al.*, 2003) at different time intervals. *D. discoideum* cells were pelleted and washed once with PBS (pH 7.4), fixed in 70 % chilled methanol for 10 min at −20 °C and then washed with blocking solution (1.5 % BSA with 0.05 % Tween 20 in PBS) followed by incubation for 1 h in primary antibody using rabbit anti-AIF polyclonal antibodies raised against amino acids 151–180 of human AIF (Cayman Chemical) at 1 : 1000 dilution and then anti-rabbit IgG (whole molecule) TRITC conjugate (Sigma) at 1 : 400 dilution. Nuclear counterstaining with DAPI (1 μg ml^{-1}) for 5 min was performed after the removal of excess secondary antibody and observed for fluorescence.

Detection of caspase activity. The substrate DEVD-AMC is an oligopeptide that is covalently linked with the fluorophore 7-amino-4-methylcoumarin (AMC). DEVD is the cleavage site for caspases (3 and 7). Cleavage takes place at the C terminus of the last aspartate residue, thus liberating the fluorophore AMC, which can be estimated and/or visualized under the fluorescence microscope (AMC λ_{ex}=380 nm; λ_{em}=420 nm).

Approximately 2.0×10^6 cells were harvested and washed with PBS. Cells were resuspended in 1 ml PBS and an aliquot of 100 μl was taken from it and 10 μl of DEVD-AMC substrate (1 mg ml^{-1}) was added. After incubation for 1 h, cells were observed under the Nikon Eclipse TE2000S fluorescence microscope. AMC liberated from the fluorogenic substrate was measured at 380 nm using a UV filter (Olie *et al.*, 1998). Caspase activity as a function of AMC fluorescence was monitored at different time intervals. The entire procedure was carried out using a caspase-3 assay kit (Sigma) as per the manufacturer's instructions. The effect of caspases on cell death was also studied by using a broad caspase inhibitor (zVAD-fmk, Sigma).

Characterization of vesicles formed during paraptotic cell death. Isolation of vesicles was performed from *D. discoideum* culture after 16 h of 1 mM HA stress as mentioned by Gautam & Sharma (2002a). The culture was centrifuged at 1000 ***g*** for 4 min at 4 °C; the supernatant was collected and centrifuged at 21 000 ***g*** for 45 min at 4 °C. The pellet obtained was washed once with 1× SB and used for further analysis. Formation of these vesicles was monitored at different time intervals. Isolated vesicles were stained separately with a fluorescent membrane probe, 1,6-diphenyl-1,3,5-hexatriene (DPH), at a concentration of 1 μM, DAPI and Annexin V–PI and then observed using a confocal laser scanning fluorescence-inverted microscope (LSM 710; Carl Zeiss) and fluorimeter (F7000; Hitachi).

Vacuolization in paraptotic cell death. Cultured exponential-phase cells exposed to 0.03 mM H_2O_2 were rinsed in 0.1 M phosphate buffer, and fixed in 2.5 % glutaraldehyde and 2.0 % paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4 °C for 6–8 h. The pellet was then stored in paraformaldehyde and 0.1 M phosphate buffer (1 : 1) and processed for microtomy. Sections were obtained with a Reichert Ultracut E ultramicrotome, stained and examined with a Morgagni 268D transmission electron microscope.

Investigation of proteases involved in cell death. Cells were pre-incubated with PIC (Sigma) followed by oxidative stress, and MMP change was monitored. Similarly, MMP change, phosphatidylserine

(PS) exposure and vesicle formation were followed in oxidative stress-treated cells pre-incubated for 12 h with 7.5 μM pepstatin A, a cathepsin D inhibitor, and 10 μM N-acetyl-L-leucyl-L-leucyl-L-norleucinal (ALLN), a calpain inhibitor.

Calpain activity. Calpain activity present in total cell lysates of 2.0×10^6 cells of control and oxidative stressed *D. discoideum* was determined by cleavage of the fluorescent substrate *N*-succinyl-LLVY-AMC (Calbiochem). Calcium-dependent fluorescence was measured after 30 min incubation at 37 °C in buffer containing 63 mM imidazole-HCl, pH 7.3, 10 mM *β*-mercaptoethanol and 5 mM $CaCl_2$ (Moubarak *et al.*, 2007). Fluorescence was recorded in a fluorimeter. Data were normalized per milligram of protein, as estimated by the Lowry method (Lowry *et al.*, 1951).

RESULTS

Induction of cell death in *D. discoideum* cells by oxidative stress

Experiments were designed to establish the ability of *D. discoideum* to undertake cell death as a function of oxidative stress. This was achieved by intracellular build up of H_2O_2 by HA treatment. Cells were treated with different concentrations of HA (0, 1, 2.5, 3, 4 mM), a known inhibitor of catalase, leading to intracellular accumulation of reactive oxygen species, and cell death was measured by the trypan blue exclusion method (Fig. 1a). The LD_{50}, i.e. the concentration of HA inducing 50 % cell death at 12 h post-treatment, was 2.5 mM.

Staining with Annexin V-FITC in conjunction with vital dyes such as PI allows us to distinguish apoptotic cells (Annexin V-positive, PI-negative) from necrotic cells (Annexin V-positive, PI-positive). Thus, based on Annexin V–PI dual staining results, 2.5 mM HA was necrotic as cells exhibited both Annexin V staining due to PS exposure and PI staining at 3 h. In contrast, 1 mM HA was paraptotic as Annexin V staining due to PS exposure was exhibited by cells after 5 h while PI was observed at 12 h of oxidant treatment (Fig. 1b, c). Paraptotic and necrotic doses of cumene H_2O_2 were also used for further experiments, as described previously (Rajawat *et al.*, 2014).

Oxidative stress-induced cell death in *D. discoideum* is caspase-independent

Oxidative stress was generated with HA or cumene H_2O_2. Caspase-3 activity was monitored during oxidative stress-induced cell death. No significant change in caspase activity was seen in basal fluorescence in control and 1 mM HA-treated cells at 1 and 6 h post-stress. A caspase-3-specific inhibitor (DEVD-CHO) could not inhibit the observed caspase activity (Fig. 2a), suggesting absence of caspase activation during oxidative stress-induced cell death in *D. discoideum*. HA at 2.5 mM also exhibited non-significant caspase activity (Fig. 2a).

To demonstrate that the cytotoxicity effect is not due to a caspase-dependent pathway, cell death study was carried out with a broad caspase inhibitor (zVAD-fmk). Our previous reports show that MMP changes following post-oxidative stress. zVAD-fmk had no effect on MMP changes (Fig. 2b) induced by oxidative stress and also on plasma membrane integrity as monitored by PI staining (Fig. 2c). In the presence of 10 μM zVAD-fmk, the cytotoxicity induced by oxidative stress was not inhibited, indicating that *D. discoideum* cells take up a caspase-independent pathway. Further increasing the concentration of zVAD-fmk up to 25 μM did not change the results.

Characterization of vesicles formed during non-apoptotic cell death

To examine the fate of dying paraptotic cells, paraptotic vesicle formation was assessed. We could observe the formation of vesicles at a later stage after loss of plasma membrane integrity. Our results suggest that the vesicles formed by paraptotic doses of HA and cumene H_2O_2 were membranous in nature (Fig. 3a, b) and contain DNA (Fig. 3c). PS staining could not be seen, suggesting that vesicles did not exhibit PS exposure (data not shown). Interestingly such vesicles were not observed with 2.5 mM HA/0.05 mM cumene H_2O_2 stress, which is the necrotic dose.

Vacuolization in paraptotic cell death

Under the electron microscope, vegetatively growing *D. discoideum* cells showed a highly developed cytoplasmic system of small vacuoles. After 16–22 h of oxidative stress-induced paraptotic cell death (1 mM HA/0.03 mM cumene H_2O_2), vacuoles were more abundant in stressed cells than in control cells. After 12 h in the presence of 1 mM HA/0.03 mM cumene H_2O_2, the cell borders were altered. The most prominent difference was the presence of large cytoplasmic vacuoles, either appearing empty or containing residual material (Fig. 3d).

Proteases involved in *D. discoideum* caspase-independent cell death

Effect of PIC on caspase-independent cell death. PIC had no effect at 3 h while partial rescue in MMP changes was observed at 5 h (Fig. 4a). Protease inhibition results showed that MMP changes were unaffected, suggesting that lysosomal proteases are not involved in the early phase of paraptotic cell death. However, during the late phase of paraptotic cell death lysosomes seem to be involved in dismantling events, as caspases are absent in *D. discoideum*.

Calpain activity during caspase-independent cell death

To explore a possible role of calpain in oxidative stress-induced cell death we examined the effect of HA and H_2O_2 on calpain activity. The kinetics of calpain activity was studied using the fluorescent calpain peptide substrate *N*-succinyl-leu-leu-val-tyr-7-amino-4-methylcoumarin. Our results suggest an increase in calpain activity by 3 h during 1 mM HA and 0.03 mM cumene H_2O_2 treatments (Fig. 4b).

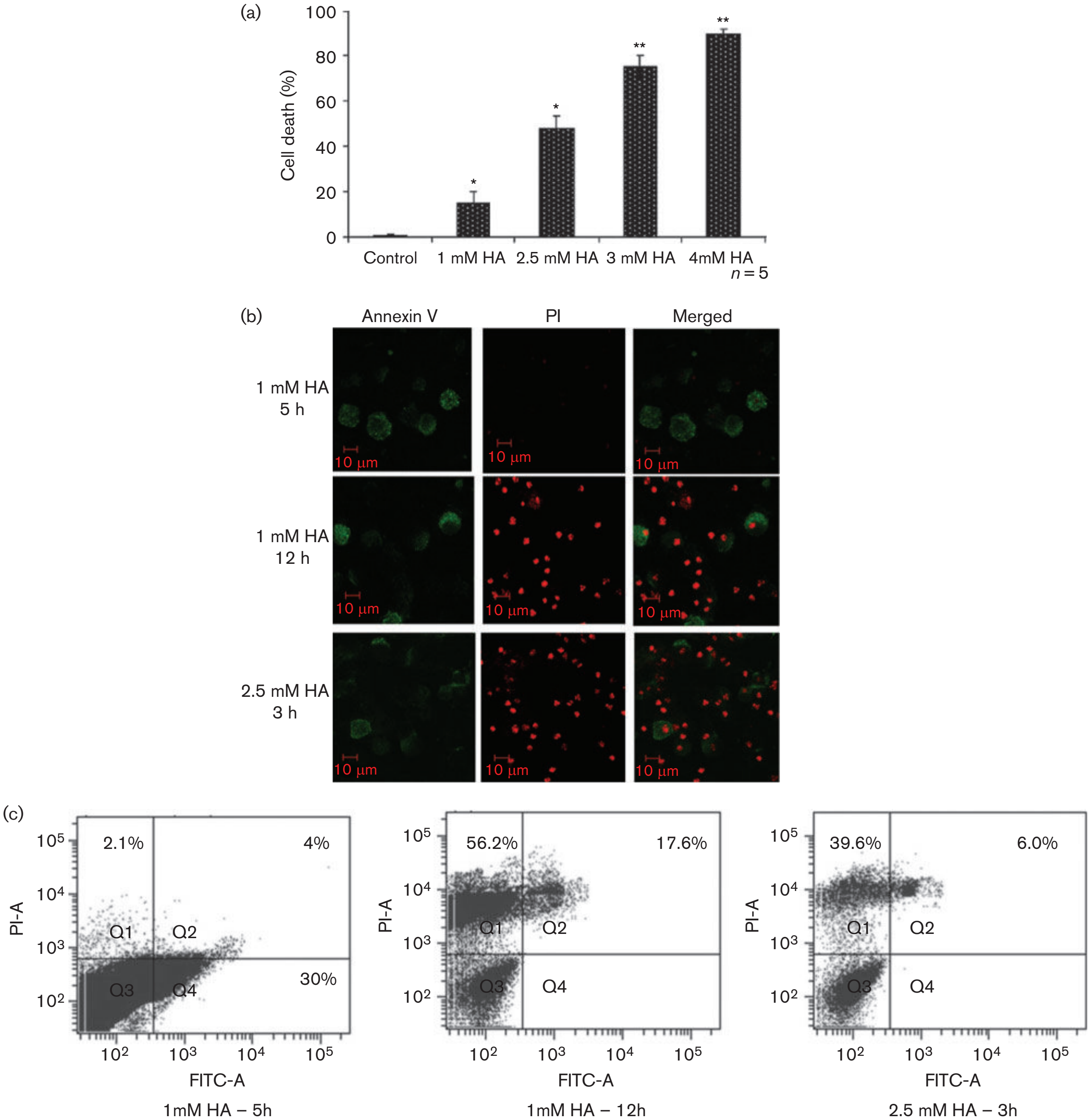

Fig. 1. HA induces dose-dependent cell death. (a) Dose-dependent increase in cell death was observed with HA, as monitored by trypan blue exclusion. Results are the mean ± SEM of five independent experiments. $**P<0.01$, $*P<0.05$ compared with control. (b) 2.5 mM HA-treated cells showed Annexin V–PI dual positive cells as early as 3 h. Annexin V–PI dual staining of 1 mM HA-stressed *D. discoideum* cells. Annexin V staining due to PS externalization was seen at 5 h and PI staining at 12 h with 1 mM HA. Data are representative of three independent experiments. Photographs were taken using a ×60 objective. (c) Annexin V–PI staining for *D. discoideum* cells subjected to 1 mM HA and 2.5 mM HA quantified by flow cytometry.

Effect of calpain and cathepsin D inhibition on MMP changes during oxidative stress-induced cell death

To further identify the specific protease(s) involved during oxidative stress-induced cell death we used ALLN, a calpain inhibitor, and pepstatin A, a cathepsin D inhibitor, and monitored their effects on MMP changes and PS–PI staining. To study the effect of calpain and cathepsin D inhibition during oxidative stress-induced cell death, cells were pre-incubated with 10 μM calpain inhibitor (ALLN)

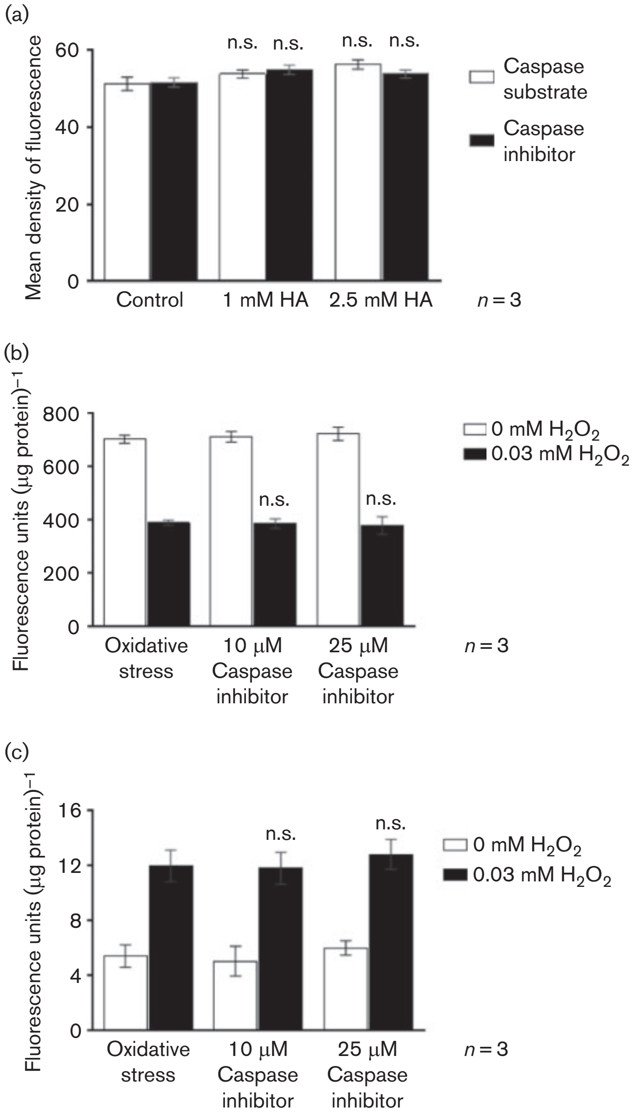

Fig. 2. Caspase-independent cell death in *D. discoideum.* (a) Caspase activity during paraptotic cell death. *D. discoideum* cells were treated with HA stress and caspase activity was assayed at 6 h in the form of DEVD-AMC cleavage and also in the presence of a caspase-3 inhibitor. Caspase activity was non-significant (n.s.). (b) MMP changes in the presence of a broad caspase inhibitor during paraptosis. Data (mean ± SE) are from three independent experiments. No significant (n.s.) effect was observed. (c) Propidium iodide staining in the presence of a broad caspase inhibitor during paraptosis. Data (mean ± SE) are from three independent experiments.

and 7.5 μM pepstatin A before inducing oxidative stress. No significant effect was observed on MMP with pepstatin alone (Fig. 5a). Nevertheless, calpain inhibitor (ALLN) alone could partially retrieve the changes in MMP induced during stress with 1 mM HA and 0.03 mM H_2O_2. Further combinatorial effects of both inhibitors on cell death were also monitored. The resultant MMP changes as monitored using $DiOC_6$ are shown in Fig. 5(b, c). As can be seen, calpain inhibitor and pepstatin A pretreatment together gave significant rescue in MMP changes as compared with pepstatin A or ALLN alone on 1 mM HA- and 0.03 mM H_2O_2-induced MMP changes. To confirm the change in MMP, JC-1 staining was performed. Mitochondria containing red JC-1 aggregates in healthy cells are detectable in the FL2 channel, and green JC-1 monomers in apoptotic cells are detectable in the FITC channel (FL1). JC-1 staining also showed a reduction in red fluorescence in ~64 % of cells, indicating mitochondrial membrane depolarization at 3 h post-1 mM HA treatment, which showed marked rescue by the calpain inhibitor ALLN and pepstatin A pretreatment: an ~37 % increase in red fluorescence as opposed to 5.6 % with pepstatin A or 12.56 % with ALLN (Fig. 5d). As pepstatin alone could not rescue MMP changes, cathepsin D probably does not have a major role in inducing cell death.

As shown in Fig. 6, cells pre-incubated with calpain inhibitor (ALLN) and cathepsin D inhibitor (pepstatin A) subjected to 1 mM HA stress exhibited only PS exposure at 12 h but no PI staining, indicating that plasma membrane integrity remains intact upon treatment with calpain and cathepsin inhibitors during oxidative stress.

Effect of calpain inhibition on AIF translocation

AIF translocation to nucleus was observed at 6 h of 1 mM HA treatment (cells exhibited pink fluorescence). Pretreatment of ALLN and pepstatin A partially prevented the translocation of AIF to nucleus in 1 mM HA-stressed *D. discoideum* cells (Fig. 7).

Vesicle formation in the presence of calpain and cathepsin D inhibitors

The vesicles were formed by 16 h with a paraptotic dose of oxidative stress. As can be seen from the fluorescence images, the vesicles are membranous in nature and contain DNA as indicated by staining with DPH and DAPI, respectively. Cells pretreated with cathepsin D and calpain inhibitors, then exposed to 1 mM HA and 0.03 mM H_2O_2 stress showed no vesicle formation while pre-incubation with calpain inhibitor alone followed by treatment with 1 mM HA and 0.03 mM H_2O_2 showed vesicle formation (Fig. 8) although less than under oxidative stress alone.

Proteases involved during oxidative stress-induced necrotic cell death

Calpain activity during necrosis. A significant increase in calpain activity was observed in cells subjected to 2.5 mM HA and 0.05 mM H_2O_2 treatments as compared with control at 3 h (Fig. 9a).

Effect of PIC on necrosis

Partial rescue in MMP was seen at 2 h with PIC during necrotic cell death, confirming that lysosomal proteases are involved in MMP changes (Fig. 9b).

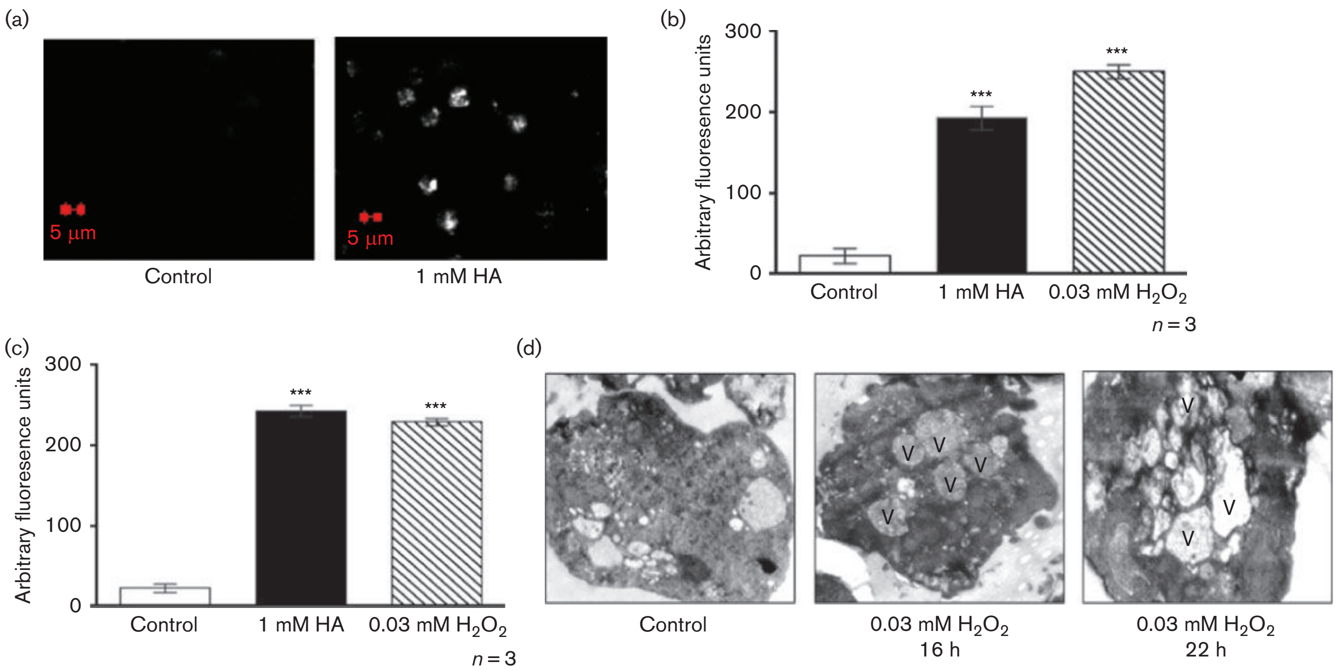

Fig. 3. Vesicle formation and vacuolization during oxidative stress-induced paraptosis. (a) Characterization of paraptotic vesicles formed during oxidative stress using the membrane probe DPH by confocal microscopy. Data are representative of at least three independent experiments. Photographs were taken using a ×63 objective. (b) Fluorimetric analysis of paraptotic vesicles using DPH staining. ***$P<0.001$ compared with control. Error bars, ±SEM. (c) Characterization of paraptotic vesicles formed during oxidative stress using the DNA binding dye DAPI. Data are representative of at least three independent experiments. ***$P<0.001$ compared with control. Error bars, ±SEM. (d) Electron microscopy of 0.03 mM H_2O_2-stressed *D. discoideum* Ax-2 cells at 16 and 22 h. Cells exhibit an irregular shape with a highly developed vacuolar system (V) under oxidative stress.

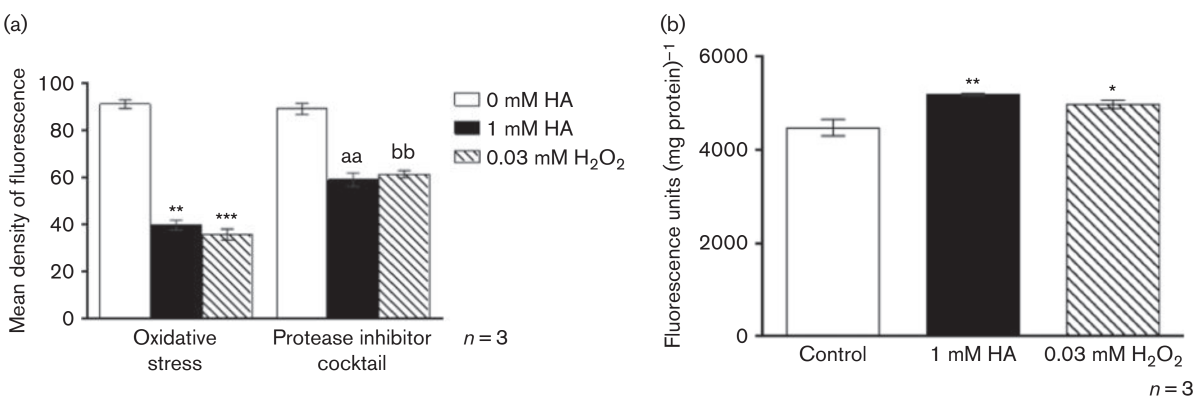

Fig. 4. Effect of PIC on MMP changes and calpain activity during paraptotic cell death. (a) PIC exhibited partial restoration of MMP changes at 5 h. Results are the mean of three independent experiments±SEM. ***$P<0.001$, **$P<0.01$ compared with control; ^{aa}, $^{bb}P<0.01$ compared with the respective treatments. (b) Calpain activity measured by using its substrate succinyl-AMC during paraptosis at 3 h post-oxidative stress. **$P<0.001$, *$P<0.01$ compared with control.

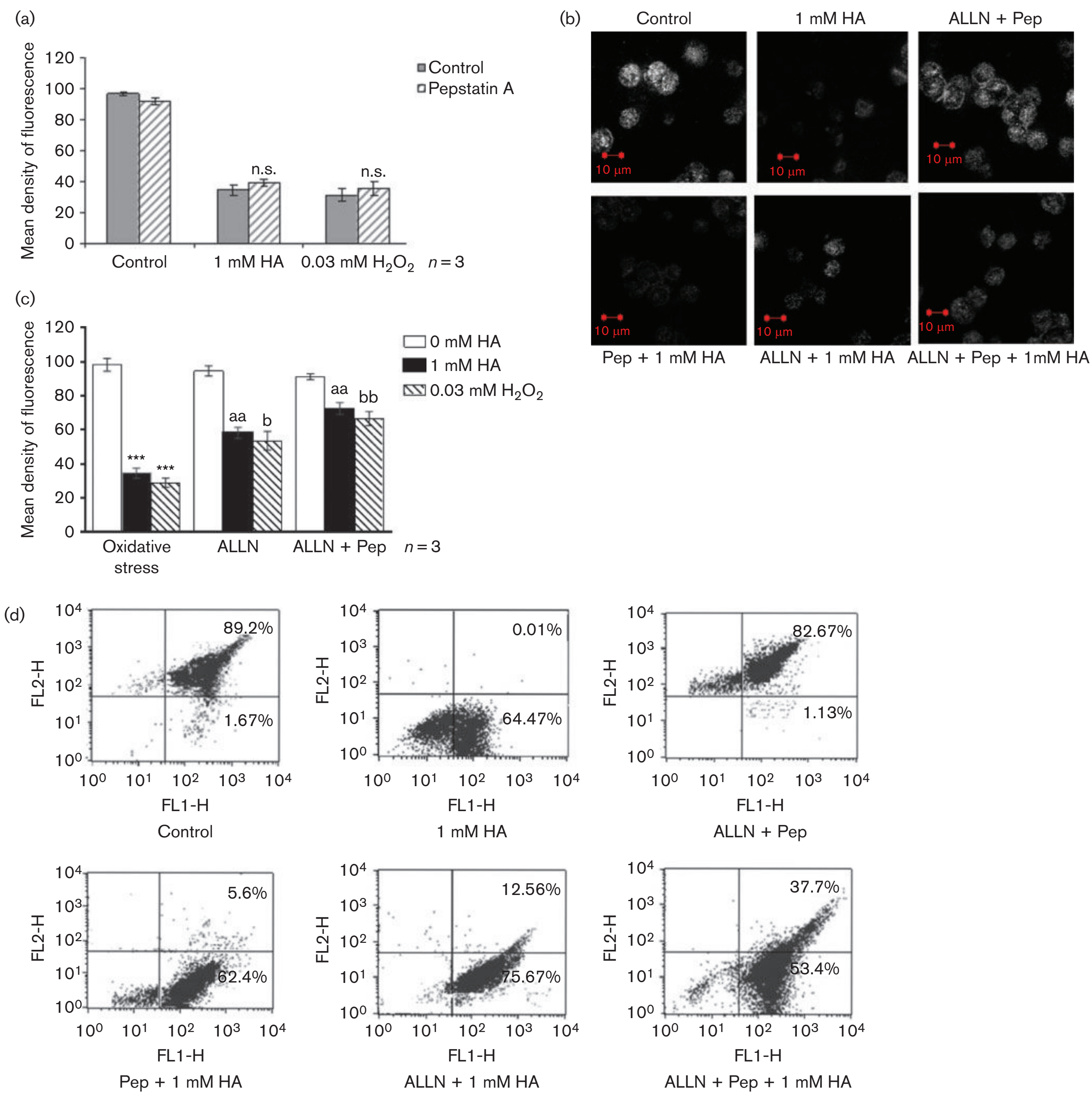

Fig. 5. MMP changes during paraptosis in *D. discoideum*. (a) Effect of cathepsin D inhibition on MMP changes during oxidative stress. No significant changes were observed with cathepsin D inhibitor, pepstatin A. Results are the mean of three independent experiments ± SEM. (b) Fluorescence images of MMP changes monitored using $DiOC_6$ dye. Data are representative of three independent experiments. Photographs were taken using a ×60 objective. (c) Densitometric analysis of fluorescence data. Calpain inhibition partially intercepts the MMP changes at both 1 mM HA and 0.03 mM H_2O_2. $^{***}P<0.001$ compared with untreated control; $^{aa,\,bb}P<0.01$ compared with the respective treated controls; $^{b}P<0.05$ compared with H_2O_2 treatment. Error bars, ± SEM. (d) Dot plot of red fluorescence (FL2) versus green fluorescence (FL1) resolved in live cells with intact MMP and from 1 mM HA-treated cells (in the presence of ALLN, pepstatin and ALLN+pepstatin) with loss of MMP. Mitochondria containing red JC-1 aggregates in healthy cells are detectable in the FL2 channel, and green JC-1 monomers in apoptotic cells are detectable in the FITC channel (FL1).

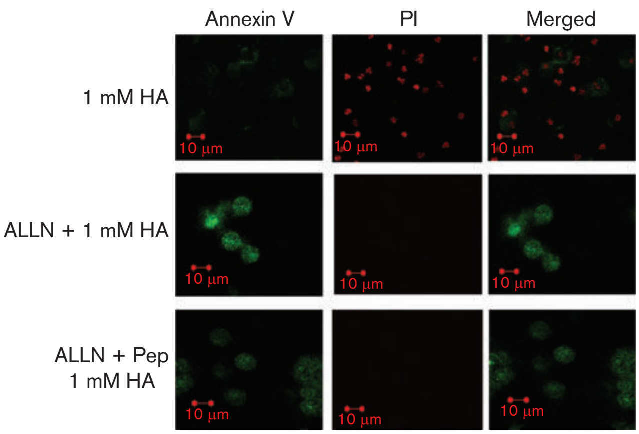

Fig. 6. Effect of calpain and cathepsin D inhibition on plasma membrane integrity as monitored by PS–PI dual staining. Both ALLN and pepstatin A delayed the PS exposure during paraptosis. Photographs were taken using a ×60 objective.

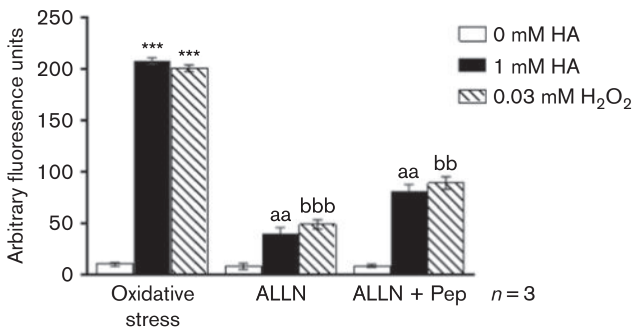

Fig. 8. Paraptotic vesicles stained with membrane probe DPH. Vesicles collected after 12 h of HA and H_2O_2 treatments were incubated with DPH and fluorescence was observed by fluorimetry. $^{***}P<0.001$ compared with control; $^{aa,\ bb}P<0.01$ compared with the respective treatments; $^{bbb}P<0.001$ compared with H_2O_2 treatment. Error bars, ±SEM.

Effect of calpain and cathepsin D inhibition on MMP changes during oxidative stress-induced necrotic cell death

No effect was observed on 2.5 mM HA- and 0.05 mM H_2O_2-induced MMP changes by ALLN, a calpain inhibitor, alone. Calpain and cathepsin D inhibition collectively prevented the MMP changes during necrosis (Fig. 9c).

DISCUSSION

Caspase activation is the hallmark feature of apoptotic cell death seen in all multicellular eukaryotes (Saraste & Pulkki, 2000). Interestingly, caspase activation has also been reported during prokaryotic cell death (Gautam & Sharma, 2002a, b) and also during oxidative stress-induced cell death in yeast (Madeo *et al.*, 2002). We have characterized the oxidative stress-mediated cell death in *D. discoideum*, which was found to be PARP- and AIF-mediated but caspase-independent. Reports suggest that developmental cell death is caspase-independent (Roisin-Bouffay *et al.*, 2004). However, Olie *et al.* (1998) reported that extracts from vegetative Ax-2 cells showed labelling with z-EK(bio)D-aomk, which labels activated caspases and caspase inhibitor, blocked morphogenesis and not cell death. Nevertheless, caspase activation could not be seen in *D. discoideum* under oxidative stress (Fig. 2a), strengthening caspase independence during oxidative stress-induced cell death in *D. discoideum* unlike the yeast system which showed caspase activation during oxidative stress-induced cell death (Madeo *et al.*, 2002). The absence of caspases led us to characterize caspase-independent cell dismantling mechanisms. The sequence of events between oxidative insult and cell dismantling remain unclear. Along with caspase independence, vacuolization continues to be a morphological change in non-apoptotic PARP-mediated death called ‘paraptosis’ (Sperandio *et al.*, 2000). Vacuolization observed under oxidative stress indicates paraptotic cell death (Fig. 3).

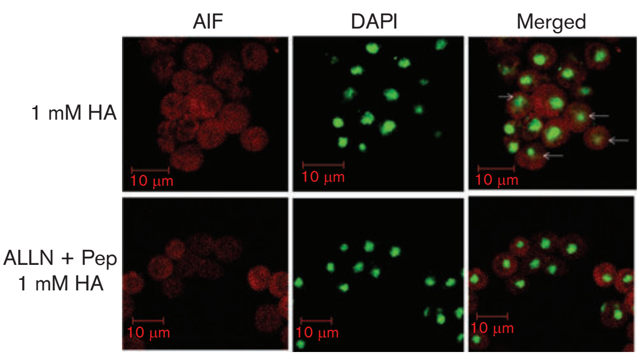

Fig. 7. Effect of calpain inhibition on AIF translocation. Intensity of green fluorescence was monitored after 6 h of oxidative stress-induced paraptotic cell death. Red colour: AIF; fluorescent green: DAPI (pseudo colour); green: AIF translocated to nucleus (indicated by arrow). Photographs were taken using a ×60 objective.

To explore the possibility of lysosomal involvement during oxidative stress-induced cell death in *D. discoideum*, PIC was used to monitor MMP changes, which exhibited partial rescue, suggesting that proteases could be acting upstream of MMP changes (Fig. 4a). Cathepsin D and calpain inhibition studies were performed to further elucidate the involvement of specific proteases in dismantling during paraptosis. Cathepsin D, being a lysosomal protease known to be active at cytosolic pH (Zong & Thompson, 2006), could serve as the protease in dismantling the cell (Turk & Stoka, 2007) during oxidative stress-induced cell death. In addition, calpain, a cytosolic protease known to affect MMP (Polster & Fiskum, 2004), and further downstream events during caspase-independent cell death could be involved. Hence

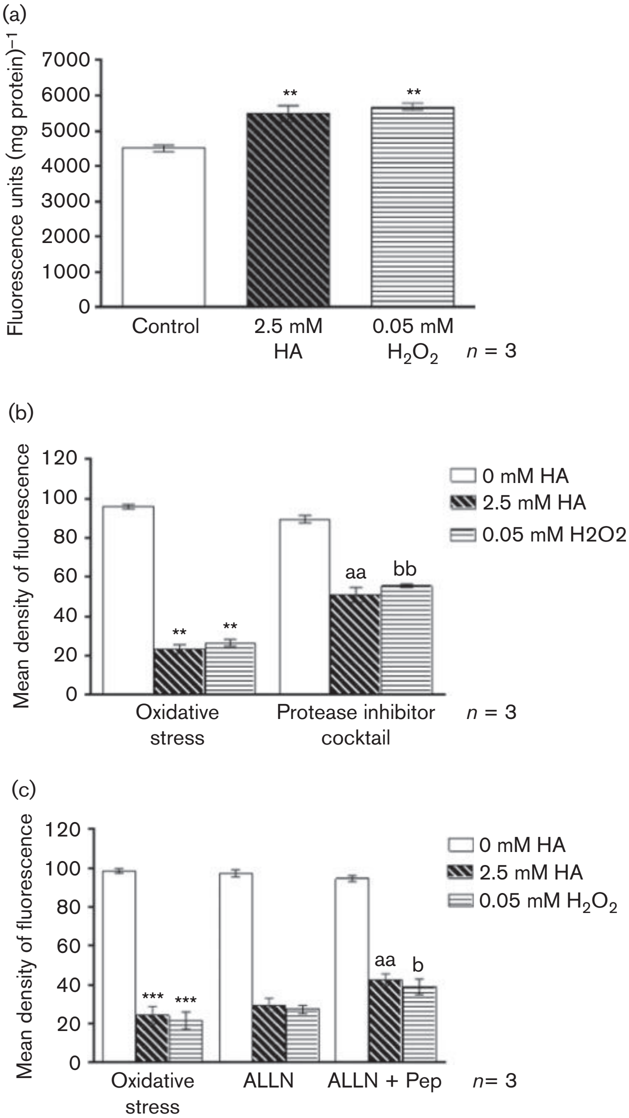

Fig. 9. Programmed necrosis exhibited at higher oxidative stress in *D. discoideum*. (a) Calpain activity measured during necrosis. $^{**}P<0.01$ compared with control. (b) Effect of PIC on MMP changes during necrosis. Results are the mean of three independent experiments. $^{**}P<0.01$ compared with control; $^{aa,\ bb}P<0.01$ compared with the respective treatments. (c) Effect of calpain and cathepsin D inhibition on MMP changes during necrosis. MMP changes during necrosis are partially rescued by both calpain and cathepsin D inhibition. $^{***}P<0.001$ compared with control; $^{aa}P<0.01$ compared with the respective treatments; $^{b}P<0.05$ compared with H_2O_2 treatment. Error bars, ±SEM.

experiments were done using a cathepsin D inhibitor (pepstatin A) and calpain inhibitor (ALLN).

Calpain inhibition studies showed a partial rescue in caspase-independent cell death while cathepsin D inhibition alone could not delay death of the cell. When pre-incubated with the calpain inhibitor ALLN, cells showed rescue in MMP changes at 3 h (Fig. 5b, c), while pepstatin A did not show any effect on MMP (Fig. 5a). During necrosis MMP change was partially rescued by PIC (Fig. 9b) and by both calpain and cathepsin D inhibition at 3 h (Fig. 9c). Inhibition of these two proteases also prevented the loss of plasma membrane integrity (Fig. 6). It has been reported that calpain inhibitor is able to block translocation of AIF and further cell death both *in vivo* and *in vitro* (Sanges *et al.*, 2006). AIF translocation to the nucleus was also partially rescued upon calpain inhibition (Fig. 7). Thus, our results imply that calpains function upstream while lysosomal proteases function downstream of mitochondrial changes during oxidative stress-induced cell death.

Cells pre-incubated with both cathepsin D and calpain inhibitors showed complete inhibition of vesicle formation (Fig. 8), suggesting that cathepsin D and calpains facilitate cell dismantling during oxidative stress-induced cell death. Calpain activation as well as its blockade during PARP-mediated cell death was confirmed by using a fluorescent substrate for calpain in the total cell lysate (Figs 4b and 9a), and our results suggest that calpain regulates AIF release during oxidative stress-induced PARP-mediated cell death in *D. discoideum*. Hence, protection observed with protease inhibitors could be attributed mainly to calpains as caspases are absent in *D. discoideum* (Olie *et al.*, 1998). Our data suggest that oxidative stress promotes calpain activity in *D. discoideum*. We have also shown that proteases are involved in causing MMP changes and downstream events including cell dismantling in the absence of caspases. Our studies suggest that proteases, particularly calpains and cathepsin D, could be the main players involved in the downstream events during oxidative stress-induced cell death as their inhibition prevented dismantling of the cell and thus delayed cell death in *D. discoideum*.

Mitochondrial uncoupling leads to plasma membrane rupture in necrotic cell death induced by DIF in *D. discoideum*, whereas exogenous glucose delays it non-glycolytically (Laporte *et al.*, 2007). Our study suggests that necrosis occurs in a programmed fashion with MMP changes manifested by proteases followed by plasma membrane rupture. Proteolysis during oxidative stress-induced necrotic cell death involves calpains and lysosomal proteases. While the pathways for apoptosis and necrosis are distinct, they nonetheless overlap and cross talk *in vivo* (Hasnain *et al.*, 2003). It would be of interest to explore if such cross talk exists.

Thus, from the above results we suggest an alternative cell death programme that may be regulated by PARP, AIF, calpains and cathepsin D as key players, where PARP may activate calpains by bringing a change in calcium homeostasis, as shown by Moubarak *et al.* (2007). Activated calpains would cause the release of AIF from mitochondria and cathepsin D from lysosomes. In this context we have revealed two proteases that could be orchestrating cell dismantling. This pathway opens the possibility to further characterize the mechanism involved in cell dismantling during caspase-independent cell death. Moreover, *D. discoideum* can thus be used as a model to study the molecular mechanisms involved in non-apoptotic and programmed necrosis, which can then be substantiated in mammalian cells.

ACKNOWLEDGEMENTS

R. B. thanks the Department of Biotechnology, New Delhi (BT/PR9496/BRB/10/562/2007) and Department of Science and Technology, New Delhi (SR/SO/BB-03/2010) for research support. J. R. thanks the Council of Scientific and Industrial Research, New Delhi, for JRF/SRF awards. We thank the DBT-MSUB-ILSPARE program and Dr Shweta Saran, JNU, for providing the FACS and confocal microscopy facilities. We acknowledge SAIF-AIIMS for use of their electron microscopy facility.

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Edited by: N. L. Glass