Chapter 2:
Materials and methods

2.1 CHEMICALS

Cumene H_2O_2, Benzamide, Trypan blue, NAD, Agarose, N-acetyl cysteine (Sigma), Propidium iodide(Sigma), Annexin V-FITC/PI dual staining kit (Invitrogen), 2',7' dichlorodihydrofluorescein diacetate (H2DCFDA-AM), 4',6-diamidino-2-phenylindole (DAPI) DiOC6 (3,3'-dihexyloxacarbocyanine iodide), Anti-mouse IgG (whole molecule) FITC conjugate, Anti-PAR mouse mAb (10H) (Calbiochem, Germany), Phospho-Histone H_2AX (S139) antibody (R&D systems), TRIZOL reagent (Invitrogen, USA). 3'5'-cyclic adenosine mono phosphate (SRL), Neutral Red (SRL), Light- CyclerH480 SYBR Green I Master Mix (Roche Diagnostics GmbH, Mannheim, Germany), Other reagents were of analytical grade from Merck and Himedia.

2.2 Materials and methods

2.2.1. *Dictyostelium discoideum* culturing

D. discoideum, Ax-2 strain (axenic derivative of Raper's wild type NC-4) cells were grown in HL5 medium, pH 6.5 with 150 rpm shaking at 22°C. All the experiments were carried out with mid-log phase *D. discoideum* cells at a cell density of 2-2.5 x 10^6 cells/ml with 95% viability (tested with Trypan blue exclusion). These cells were also maintained on nutrient agar with *Klebsiella aerogenes,* natural food and harvested using standard procedures (Watts and Ashworth, 1970; Kosta *et al*., 2001).

2.2.2Preparation of modified HL5 medium:

Proteose peptone (Oxoid)	-	14.3 gm
Yeast extract (Oxoid)	-	7.5 gm
Maltose	-	18 gm
Na_2HPO_4 $2H_2O$	-	0.616 gm
KH_2PO_4 $2H_2O$	-	0.486 gm

The above ingredients were added to 1000 ml of distilled water and pH was adjusted to 6.7 followed by autoclaving at 15 psi for 15 min.

2.2.3. Development of *D. discoideum* cells on non-nutrient agar (NNA)

For development, mid log phase cells were washed twice followed by spin at 300g/5min/4^0C. This pellet was further resuspended in 1X Sorenson's Buffer (SB) at a density of 1 x 10^8 cells/ml. The cell suspension was spotted on 2% non-nutrient agar and incubated at 22ºC to allow differentiation (Sussmann, 1987). Images were taken every 2 hrs initially till 12 hrs and then at 12 hrs interval. Development was synchronized by incubating the cells at 4^oC for 4-5 hrs and then transferring them to 22^oC for further development.

50 X Sorenson's buffer (SB): 2mM Na_2HPO_4 + 15mM KH_2PO_4, (pH 6.4)
It was sterilized by autoclaving at 15 psi for 15 min**.**

2.2.4 Revival of *Dictyostelium* Spores

The fruiting bodies (Spores) were picked up from the surface of NNA plates with a sterile Nichrome loop. The loop was not touched to the agar surface and all the necessary precautions were taken to avoid bacterial contamination. Few fruiting bodies were inoculated in a petri dish containing HL5 axenic medium and then incubated under shaking conditions at 22^oC. Confluency could be obtained after about a week.

2.2.5. Genomic DNA Isolation from *D. discoideum* (Pilcher *et al*., 2007)

10^7cells/ml were cultured and washed once with 1X SB, followed by one wash with ice cold [0.2 % NaCl (0.034M)]. 2% SDS was added and kept at 65°C for 15' incubation. One volume of TE buffer (pH 9.5) was added and then extracted with one volume of Phenol: Chloroform (1:1) treatment. The upper aqueous layer was taken after centrifugation at 9000g for 5'. The step was repeated with chloroform followed by spin at 9000g for 5' to get upper aqueous layer. 1/10th volume of sodium acetate and one volume of isopropanol was added into it and DNA was precipitated at -20°C for 30'. The DNA pellet was obtained by centrifuging at 9000g for 5' followed by a wash with 75% ethanol. DNA pellet was then dried and resuspended in NFW (nuclease free water).

2.2.6. Plasmid DNA isolation by alkaline lysis method (Sambrook and Russell, 2001)

A single colony was picked from transformed bacterial cells and inoculated in LB (Luria Broth) medium followed by overnight incubation at 37^0C with constant shaking. The grown culture was pelleted down and resuspended in ice cold alkaline solution I. Alkaline solution II (freshly prepared) was then added and mixed for a couple of time. After 5 min incubation, ice cold alkaline solution III was added and incubated in ice for 15 min followed by centrifugation. Equal volume of Phenol: Chloroform was then added to the supernatant followed by centrifugation. For precipitation of plasmid DNA, ice cold Isopropanol was added to the supernatant and kept for 30 min. at 4^0C. This mixture was again pelleted down and the pellet obtained was washed with 70% ethanol followed by drying at 37^0C. Plasmid DNA pellet was dissolved in TE buffer containing RNase. Integrity of the extracted plasmid DNA was checked by agarose gel electrophoresis.

2.2.7. Transformation of plasmid DNA in *E.coli* (Sambrook and Russell, 2001)

3 ml of LB was inoculated with *E.coli* DH5α and incubated at 37ºC till it reached OD ~ 0.4 - 0.6 (log phase). 3 ml of the log phase culture of *E.coli* was pelleted down at 5000g/5min. 1.8 ml 80 mM $MgCl_2$ + 20 mM $CaCl_2$ mixture was added to the culture and kept on ice for 20min. Culture was then pelleted and washed once with 1 ml 100 mM $CaCl_2$. 100 µl of 100 mM $CaCl_2$ was again added to the cells and incubated on ice for ~ 1 hr to prepare competent cells. Plasmid DNA was added to the competent cells followed by an incubation on ice for 30 min. Heat shock was given at 42ºC for 90 sec and immediately transferred to ice for 2-3 min. ~800 µl of sterile Luria broth (LB) was added and incubated at 37ºC for 45 min under shaking conditions. Finally cells were pelleted down and resuspended in 40 µl LB which was then spreaded on Luria agar plates containing Ampicillin (100mg/ml).

2.2.8. Generation of overexpression and knockout construct

2.2.8..1. PCR amplification

In order to get the full length *ADPRT1A* (3.04 kb) for overexpression construct and 3'& 5' homologous region (HR) for knockout construct of *ADPRT1A* using specific primers, PCR amplification was done using following PCR conditions:

PCR amplicon and size	**Denaturating**	**Annealing**	**Extension**	**No. of cycles**	**Modifications**
ADPRT1A overexpression 3046 bp	94°C - 3' Hot Start 94°C - 5' 94°C – 30 sec	Tagged: 63.5°C – 1' Untagged: 55°C – 1'	68°C - 3'30sec 68°C - 10'	35	Use Phusion enzyme only + 3.5 $MgCl_2$
ADPRT1A knockout 5'HR: 889 bp 3'HR: 875 bp	94°C - 3' Hot Start 94°C - 5' 94°C – 30 sec	5'HR: 58°C - 45 sec 3'HR: 53°C - 30 sec	68°C - 1'	35	Use Phusion enzyme only + (3.5 $MgCl_2$ - only for 3'HR)

Table1: PCR conditions for ADPRT1A overexpression and knockout amplicons

2.2.8.2. *ADPRT1A* overexpression construct

***PARP* (*ADPRT1A* Primers with Melting Temperature (Tm in °C)) specific**	**Enzyme**
Forward Primer: (44.7 °C) 5'-GT**GAGCTC**GCAACAAAAAATACATCTCCTTATG-3'	*Sac*I
Reverse Primer: (46.6°C) 5'- AGC**GGATCC**TTTTCCATTGTAAACTCTTAAG-3'	*Bam*HI

Table 2: Primers used for *ADPRT1A* overexpression construct

2.2.8.3. *ADPRT1A* knockout construct

***PARP* (*ADPRT1A*) specific Primers with Melting Temperature (Tm in ^{o}C)**	**Enzyme**
5'HR Forward Primer- (44.7^{o}C) 5'-ACG**GCGGCCGC**GCAACAAAAAATACATCTC-3'	*Not*I
5'HR Reverse Primer- (54.3^{o}C) 5'-AGC**TCTAGA**ATCTTGCTCTTGTTCCTCCTC-3'	*Xba*I
3'HR Forward Primer- (52.2^{o}C) 5'-ATC**AAGCTTA**CACAGCTAATCCAAGTCG-3'	*Hind*III
3'HR Reverse Primer- (54.6^{o}C) 5'-AGT**GGATCC**TACTATGGAATGGATGTGGTGC-3'	*Bam*HI

Table 3: Primers used for *ADPRT1A* knockout construct

2.2.9 Transformation of plasmid DNA in *D. discoideum* (Gaudet *et al*., 2007)

5 x10^{6} *D. discoideum* cells were pelleted down and washed twice with ice cold H50 buffer. Plasmid DNA (10-15µg) was added to *Dictyostelium* cells and incubated on ice for 5 min. The cell suspension was transferred to a cold 0.1 cm electroporation cuvette and electroporated in cold at 650 V, capacitance 25 µF pulses, twice for about 15 sec gap between 2 pulses using Gene Pulser Xcell^{TM} electroporator (BioRad). The cuvette was incubated on ice for 5 min and then cells were transferred to a 10 cm Petri dish containing sterile 10 ml HL5 for selection. Petri dish with cells was incubated in BOD at 22^{o}C for overnight. Antibiotics were added from the next day with medium change as follows:

Antibiotics	**1st day**	**2rd day**	**Day Foci is seen (approx. 7th day)**	**Alternate day**
Ampicillin	1 µg/ml	1 µg/ml	1 µg/ml	1 µg/ml
Geneticin (G418) for *ADPRT1A* overexpression clone	0 µg/ml	10 µg/ml	15 µg/ml	30 µg/ml till 100 µg/ml
Blasticidin for *ADPRT1A* knockout clone	0 µg/ml	2 µg/ml	5 µg/ml	10 µg/ml

Table 4: Antibiotic dose pattern after electroporation

2.2.10 RNA Isolation (Pilcher *et al*., 2007**)**

2 x 10^7 cells were harvested and washed with 1X SB buffer. These cells were then resuspended in Trizol reagent (Invitrogen, USA) followed by incubation for 5 min at room temperature (RT). 200 µl chloroform was added and stored at RT for 5 min followed by a spin for 10 min at 10,000 rpm. The upper phase was transferred to a fresh tube and 1 ml 100% ethanol was added. It was incubated for 20 min at -20 °C and centrifuged for 5 min at 13,000 rpm to get the RNA pellet. Pellet was further semidried and dissolved in 10 µl of nuclease free water. RNA integrity was verified by 1.5% agarose gel electrophoresis. RNA yield and purity was determined spectrophotometrically at 260/280 nm.

2.2.11. cDNA synthesis

cDNA synthesis was performed using 1.5µg of the total RNA by Thermo Scientific Verso™ DNA Synthesis Kit (Thermo Fisher Scientific, Inc.) as per the manufacturer's instructions. This cDNA was further used in a PCR to analyze the transcripts levels of respective genes with gene specific primers.

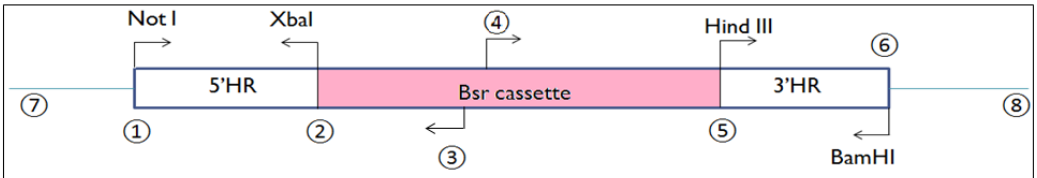
2.2.12. Confirmation of knockout strains

The knockout strains obtained were first checked by appropriate PCR amplifications and further confirmed by semi-quantitative and Real time PCR

Genomic DNA amplification

Genomic DNA was isolated from the knockout and random integrants. Wild type Ax2 and the linearized vector genomic DNA was taken as control. List of primers used for confirmation are mentioned below in the table. Their respective positions in the construct are shown in below in the table with their expected results in different strains and their product sizes.

(A)



(B)

Sr. no.	5' HR primer combi-nation	Product size (bp)	Expected result				Sr. no.	3' HR primer combi-nation	Product size (bp)	Expected result			
			PI	V	RI	AX2				PI	V	RI	AX2
1	1 & 2	889	+	+	+	+	5	5 & 6	872	+	+	+	+
2	1 &3	1333	+	+	+	-	6	4 & 6	1877	+	+	+	-
3	7 & 2	1516	+	-	+	-	7	8 & 5	1695	+	-	+	-
4	7 & 3	1920	+	-	-	-	8	8 & 4	2700	+	-	-	-

PI: Positional Integrant, V: Vector, RI: Random integrant, Ax2: Wild type

(C)

Primer No	Sequence (5'-3')
1	5'-ACGGCGGCCGCGCAACAAAAAATACATCTC -3'
2	5'-AGCTCTAGAATCTTGCTCTTGTTCCTCCTC -3'
3	5'- CCTATATACGCTTCAATATGTAC-3'
4	5'-GTACATATTGAAGCGTATATAGG-3'
5	5'-ATCAAGCTTACACAGCTAATCCAAGTCG
6	5'-AGTGGATCCTACTATGGAATGGATGTGGTGC -3'
7	5'-GGAAATATAATGGTACCT-3'
8	5' ACA ACA TTC ATT ACC AGA TTC 3'

Fig 2.1 Validation of ADPRT1A knockout strain. (A) strategy used for the validation of knockout strain **(B)** primer combinations used for the validation of various strains that include wild type Ax2, random integrants, vector as a positive control and *ADPRT1A*$^-$/Ax2with their respective product size **(C)** Primers used for the validation which are numbered according to their positions assigned in section (A) of this figure.

2.2.13. Functional characterization of *ADPRT1A* overexpression and knockout

ADPRT1A overexpression and knockout was confirmed by monitoring gene specific expression of *ADPRT1A* by Real time PCR with *RNLA* as an internal control using Light Cycler® 480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions and carried out in the LightCycler 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany).

2.2.14. 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.

2.2.15 Growth profile analysis (Kosta *et al*., 2001)

The growth profile of *Dictyostelium* cells was studied by inoculating mid log phase cells at a density of 0.6×10^6 cells/ml in HL5 medium at 22^0C under 150 rpm. Cell viability was checked after every 2 hrs initially till 12 hrs and after 12 hrs 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. Similar growth profile studies were also done in presence of Benzamide, a PARP inhibitor (1 mM) and NAC, an antioxidant (2.5 mM).

2.2.16 Cell cycle analysis (Chen *et al*., 2004)

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. The fixed cells were resuspended in staining solution (TritonX-100, DNase free RNase and Propidium Iodide) and incubated for 30 min followed by FACS analysis. Quantification was done by flow cytometry using FACS ARIA III (BD Biosciences) and data was analyzed with FACSDiva software.

2.2.17 Induction of oxidative stress (Rajawat *et al.,* 2014a)

In situ generation of H_2O_2 in *D. discoideum* cells was induced by exogenous addition of cumene H_2O_2 (Sigma), resulting in oxidative stress. Log phase cells at a density of ~2.5×10^6 cells/ml were exposed to paraptotic (0.03mM) and necrotic (0.05mM) doses of cumene H_2O_2 in HL-5 medium at 22°C in a sterile flask.

2.2.18 Sequence similarity, Domain and Phylogenetic analysis (Dereeper *et al.*, 2008)

The *ADPRT1A* protein was aligned by Clustal W. The aligned sequences were analyzed by the maximum likelihood method. The tree file 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 Clustal W.

2.2.19 Estimation of NAD^+ levels (Bernofsky and Swan, 1973)

Intracellular levels of NAD^+ were determined by enzymatic recycling method by Bernofsky *et al*., using alcohol dehydrogenase to reduce NAD^+ to NADH. Principle- Cellular NAD^+ is used to convert ethanol into acetaldehyde catalyzed by alcohol dehydrogenase. NADH produced in the first reaction is used to reduce MTT in to Formazan and NAD^+ is regenerated. In this way, cellular NAD^+ keeps on getting recycled and thereby enhances the yield of Formazan, a chromophore which gives absorbance at 570 nm. NAD^+ was extracted with 1 ml of 0.5 M perchloric acid and then neutralized with 1 N KOH. NAD^+ levels were estimated at 570 nm and protein concentration was estimated by Lowry method (Lowry *et al*., 1951).

2.2.20 ROS estimation (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 H_2DCFDA 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. $2x10^6$ cells were harvested and washed with 1X SB twice. DCFDA (50 nM) was added to the cells and was incubated for 15 min at 22^0C 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 1X SB. Excitation (λex) and emission (λem) wavelengths used for fluorimetric studies were 480 and 525 nm respectively.

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

Phospho-Histone H_2AX (S139) antibody at a concentration of 2 µg/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 hrs in primary antibody. After incubation, the cells were washed 2-3 times with blocking solution and further incubated for 1 hr 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).

2.2.22. PARP activation (Cole and Perez-Polo, 2002)

PARP was assayed by indirect immunofluorescence using anti-PAR mouse mAb (10H) (Calbiochem) at a concentration of 0.5µg/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.

2.2.23. Evaluation of mitochondrial membrane potential (MMP) (Koning *et al.*, 1993)

Potential sensitive dye $DiOC_6$ (3, 3'-dihexyloxacarbocyanine iodide) (Sigma) was used to evaluate changes in mitochondrial membrane potential. ~2.0 x 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 quantitated by flow cytometry using a FACS ARIA (BD Biosciences). Data were analyzed with FACSDiva software.

2.2.24. Assessment of cell death by AnnexinV-FITC/PI dual staining (Miller, 2004)

To differentiate between apoptotic and necrotic cell death, dual staining with Annexin V-FITC/PI was performed using apoptosis detection kit (Molecular Probes). ~2.0 x 10^6 cells were pelleted and washed twice with 1X SB. These 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 22^0C. Fluorescence was monitored under 63X by Zeiss confocal laser scan fluorescence-inverted microscope (LSM 710; Carl Zeiss).

2.2.25 Effect of Oxidative stress on *ADPRT1A* knockout cells

The growth profile of *ADPRT1A* knockout cells in presence of 0.03 mM H_2O_2 was studied by inoculating mid log phase cells at the density of 2×10^6 cells/ml in HL5 medium. Cell viability was checked after every after every 12 hrs interval thereafter till 120 hrs. 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).

2.2.26. Neutral red staining (Dormann *et al.*, 1996)

For development, mid log phase cells were washed twice and resuspended in 1X SB at a density of 1 x 10^8 cells/ml. The cells were labelled with the vital dye, neutral red, by incubating them in 0.06% neutral red (in SB) for 1 minute, followed by a final

wash in distilled water. Slugs were obtained by placing drops of cells (1 x 10^8 cells/ml) on 1% NNA plates .The plates were then incubated in the dark for 24-48hrs hours at 22°C and observed for slugs under 4X objective in a stereo microscope (Nikon,SMZ-1000, Japan)

2.2.27 cAMP estimation

Log phase cells were resuspended at a density 1×10^8 cells/ml and subjected to development for indicated time points on 2% non- nutrient agar plates, followed by harvesting by centrifugation at 1500 g/5 min/4°C. Cells were then collected, and intracellular cAMP was estimated using an ELISA kit method according to the manufacturer's instructions (Calbiochem, Gibbstown, NJ, USA). Extracellular levels were checked by same kit as described in Mir *et al*., 2015. Protein normalization was done by estimating protein levels using Folin Lowry method.

2.2.28. Chemotaxis assay and cAMP pulsing

Chemotaxis assay towards cAMP was performed as described (Mir *et al.,* 2015) with a few modifications. Axenically grown cells in shaking culture were harvested in the log phase, washed in Sorenson's buffer (SB -2 mM Na_2HPO_4, 15 mM KH_2PO_4, pH 6.4) and were starved at a density of $2x10^7$ cells/ml in SB for 6 hrs. Then, starved cells were washed twice in SB and resuspended in SB to a final density of 1 x 10^7/ml. Three droplets of starved cells, 2 µl each, were placed on 2% Non-nutrient agar (NNA) plate in the petri dish. Chemotaxis towards cAMP was tested by placing 1µM of cAMP in a well at 3 mm distance from the cell-filled droplet. The distribution of cells within their droplet was observed after 5 hrs from placing on the plate and images were captured using stereo microscope (Nikon, SMZ 1000, Japan). Relative number and distance moved by cells was an indication of their chemotactic activity.

To study the effect of cAMP pulsing on cells, they were harvested from the exponential growth phase and washed twice in SB. Washed cells were resuspended at a density of 2 x 10^7 cells/ml in SB and then stimulated with 50 nM cAMP pulses every 6 min for 6 hrs. The pulsed cells were washed and then used for the chemotaxis assay or developed on NNA plates.

2.2.29. Gene expression analysis by Real Time PCR (qPCR)

RNA extraction and cDNA synthesis: Total RNA from developing *Dictyostelium* cells plated on NNA were harvested every 2 hrs until 8 hrs, isolated and purified using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. RNA integrity was verified by 1.5% agarose gel electrophoresis/ethidium bromide staining and O.D. 260/280 absorbance ratio 1.95. RNA was treated with DNase I (Ambion inc. Texas, USA) before cDNA synthesis to avoid DNA contamination. 1.5µg of total RNA was used to prepare cDNA. cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions in the CG Palmcycler (Genetix, India).

The expression of specific transcripts was measured by qPCR using gene specific primers (Eurofins, Bangalore, India) as shown in Table 1. qPCR was performed in duplicates in 10µl volume using Light- CyclerH480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions and carried out in the LightCyclerH480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany). The thermal cycling conditions included an initial activation step at 95^{o}C for 10 min. followed by 45 cycles of denaturation, annealing and extension. The fluorescence data collection was performed during the extension step. At the end of the amplification phase a melting curve analysis was carried out on the product formed. The value of Cp was determined by the first cycle number at which fluorescence was greater than the set threshold value. Results of qPCR were analyzed using the comparative CT method (Livak and Schmittgen, 2001) with the amplification of *RNLA* (mitochondrial large rRNA) as a control. Relative gene expression was plotted and analyzed by nonparametric unpaired t-test using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003).

Sr. No.	Gene	Primers [Forward primer (FP) & Reverse primer (RP)]	Size (bp)
1.	*REGA*	FP: ATAAAGTGCGGTGATATTTC RP: AAGAACTTTGCCATACTTTGG	225
2.	*PDSA*	FP: ACT AAA GAT TGT AGG GAT GC RP: AACTGCCCATGATGGATAG	216
3.	*ADPRT1A*	FP: TCTTTCAGAGTTTGCTGAGCG RP: TGGTTCAATTTCTTGAGAGGTTG	216
4.	*ADPRT2*	FP: TTGCTATTGTATGTCACTTTCTGC RP: TTGGATCTGGTTCAACAGTACC	182
5.	*ADPRT1B*	FP: TGGTCAAGAGGTTAAAAGATATCG RP: TGAATCTGAAATTGATAAAGCTG	190
6.	*EcmA*	FP: AGTTAATGCGGAAACTGAAACC RP: ATTGGGGTATGAACACAACC	210
7.	*EcmB*	FP: ACCGGTTGTGTCCACACTC RP: TGATTGGAGTGTGACAACAACC	166
8.	*D19*	FP: TGTCCGTTACATTCCTGATGC RP: AACTGTTGGTGTAACTGTTGG	205
9.	*CYC B*	FP: ACACATCAAACAACTGGAATGGC RP: TCCATTGGCATTTGGTAAAACTCC	180

Table 5: Primers used for gene expression analysis

2.2.30. Data analysis and statistics

Flow cytometry and colorimetric assay experiments were repeated at least three times. Data were analysed 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.

2.3 References:

1. Kosta, A., Laporte, C., Lam, D., Tresse, E., Luciani, M. F. & Golstein, P. (2001). How to assess and study cell death in *Dictyostelium discoideum.* Methods Cell Biol. 346, 535-550.
2. Watts, D. J. & Ashworth, J. M. (1970). Growth of myxamoebae of the cellular slime mould *Dictyostelium discoideum* in axenic culture. Biochem. J. 119, 171-174.
3. Sussman, M. (1987). Cultivation and synchronous morphogenesis of *Dictyostelium* under controlled experimental conditions. Methods Cell Biol. 28, 9-29.
4. Pilcher, K. E., Fey, P., Gaudet, P., Kowal, A. S., & Chisholm, R. L. (2007). A reliable general purpose method for extracting genomic DNA from Dictyostelium cells. *Nature protocols*, *2*(6), 1325-1328.
5. Pilcher K.E., Gaudet P., Fey P., Kowal A.S. and Chisholm R.L. A general purpose method for extracting RNA from *Dictyostelium* cells. (2007) *Nature Protocols* 2, 1329 – 1332.
6. Sambrook, J., Russell, D. W., & Russell, D. (2001). Plasmids and their usefulness in molecular cloning. *Molecular cloning*, *1*(1), 31-35.
7. Gaudet, P., Pilcher, K. E., Fey, P., & Chisholm, R. L. (2007). Transformation of Dictyostelium discoideum with plasmid DNA. *Nature protocols*, *2*(6), 1317-13
8. Chen, G., Shaulsky, G. & Kuspa, A. (2004). Tissue-specific G1-phase cell-cycle arrest prior to terminal differentiation in *Dictyostelium.* Development 131, 2619-2630.
9. Rajawat, J., Mir, H., Alex, T., Bakshi, S., Begum, R., 2014a. Involvement of poly(ADP-ribose) polymerase in paraptotic cell death of *D. discoideum.* Apoptosis 19, 90-101.
10. Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J. F., Guindon, S., Lefort, V., Lescot, M., Claverie, J. M. & Gascuel,

O. (2008). Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Res. 1, 36.

11. Bernofsky, C. & Swan, M. (1973). An improved cycling assay for nicotinamide adenine dinucleotide. Anal Biochem. 53, 452-458.
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.
13. Degli Esposti, M. (2002). Measuring mitochondrial reactive oxygen species. Methods 26, 335-340.
14. Minami,T., Adachi, M., Kawamura, R., Zhang, Y., Shinomura, Y. & Imai, K. (2005) Sulindac enhances the proteasome inhibitor bortezomib-mediated oxidative stress and anticancer activity. Clin. Cancer Res. 11, 5248-5256.
15. Mir, H., Alex, T., Rajawat, J., Kadam, A., Begum, R., 2015. Response of *D. discoideum* to UV-C and involvement of poly(ADP-ribose) polymerase. Cell Prolif. 48, 363-74.
16. Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method.*methods*, *25*(4), 402-408.
17. Cole, K. K. & Perez-Polo, J. R. (2002). Poly (ADP-ribose) polymerase inhibition prevents both apoptotic-like delayed neuronal death and necrosis after H_2O_2 injury. J. Neurochem. 82, 19-29.
18. Koning, A. J., Lum, P. Y., Williams, J. M. & Wright, R. (1993). $DiOC_6$ staining reveals organelle structure and dynamics in living yeast cells. Cell Motil. Cytoskeleton, 25, 111-128.
19. Miller, E. (2004). Apoptosis measurement by annexin v staining. Methods Mol. Med. 88, 191-202.
20. Dormann, D., Siegert, F., & Weijer, C. J. (1996). Analysis of cell movement during the culmination phase of Dictyostelium development. *Development*, *122*(3), 761-76.