2.1 CHEMICALS

Trypan blue (Himedia, India), Cumene hydroperoxide (Sigma, USA), HEPES (Sigma, USA), Malate (Sigma, USA), Sodium Pyruvate (Sigma, USA), Ascorbate (Sigma, USA), Succinate (Sigma, USA), α-glycerophosphate (Sigma, USA), NAD^+ (SRL, India), 5′-ATP-Na_2 (SRL, India), Agarose (Sigma, USA), Digitonin (Himedia, India), Coomassie Brilliant Blue G-250 (Himedia, India), 6-aminohexanoic acid (SRL, India), Imidazole (Sigma, USA), Geneticin (G418) (SRL, India), Mannitol (Sigma, USA), Sucrose (Sigma, USA), EGTA (Himedia, India), Glutaraldehyde (Sigma, USA), POBN [(α-(4-Pyridyl N-oxide)-N-tert-butylnitrone) (Sigma, USA), Dimethyl Sulphoxide (DMSO) (Sigma, USA), Tricine buffer (SRL, India), Osmium tetroxide 2% EM grade (Sigma, USA), Propidium Iodide (PI) (Sigma, USA), Triton X-100 (Sigma, USA), DNAse-free RNAse (Ambion inc. Texas, USA), 2',7' dichlorodihydrofluorescein diacetate (H_2DCFDA-AM), $DiOC_6$ (3,3'-dihexyloxacarbocyanine iodide) (Sigma, USA), 4',6-diamidino-2-phenylindole (DAPI) (Sigma, USA), Fura-2 AM (Molecular probe, USA), TRIzol reagent (Invitrogen, USA), Bovine Serum Albumin (BSA) (SRL, India), Tween 20 (Himedia, India), Light- CyclerH480 SYBR Green I Master Mix (Roche Diagnostics GmbH, Mannheim, Germany), Annexin V-FITC/PI dual staining kit (Invitrogen, USA), Pluronic F-127 (Molecular probe, USA), Anti-rabbit IgG (whole molecule) TRITC conjugate (Sigma, USA), Anti-mouse IgG (whole molecule) FITC conjugate (Sigma, USA), Rabbit anti-AIF polyclonal Ab (Cayman chemical, USA), Phospho-Histone H_2AX (S139) antibody (R&D systems, Minneapolis, MN) were used in this study. Other reagents used in this study were of analytical grade from Merck Millipore and Himedia.

2.2 Materials and methods

2.2.1 *Dictyostelium discoideum* culture

D. discoideum, Ax-2 strain, axenic derivative of Raper's wild type NC-4 cells were grown in HL-5 medium, pH 6.5 with 150 rpm shaking at 22°C. All the

experiments were performed with mid-log phase cells at a density of 2-2.5 x 10^6 cells/ml with 95% viability which was assessed by Trypan blue exclusion. These cells were also maintained on a solid substratum containing Non-Nutrient Agar (NNA) with *Klebsiella aerogenes* and harvested using standard procedures (Watts and Ashworth, 1970; Kosta *et al*., 2001).

2.2.2 Composition of modified HL-5 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 constituents were added to 1000 ml of distilled water and pH was adjusted to 6.5 followed by autoclaving at 15 psi for 15 min.

2.2.3 Development of *D. discoideum* cells

For multicellular development, mid log phase cells were washed twice followed by spin at 300g/5min/4^0C. The cell pellet was further resuspended in 1X Sorenson's Buffer (SB) at a density of ~1 x 10^8 cells/ml and spotted on 2% NNA plate followed by incubation at 22ºC to allow differentiation (Sussmann, 1987). Images were taken every 2 hrs initially till 12 hrs and then at an interval of 12 hrs by using a stereo-microscope ((Nikon SMZ 1000, Japan). Development was synchronized by incubating the starved cells at 4ºC for 4-5 hrs and then transferring them to 22ºC for further development. The cells from different developmental stages were collected for respiration studies, RNA isolation and gene expression analysis.

50X Sorenson's buffer (SB):

Na_2HPO_4 - 2 gm

KH_2PO_4 - 0.29 gm

The above constituents were added to 1000 ml of distilled water and pH was adjusted to 6.4 followed by autoclaving at 15 psi for 15 min.

2.2.4 Revival of *D. discoideum* spores

The fruiting bodies (spores) were picked up from the surface of NNA plates with a sterile nichrome wire loop without touching to the agar surface. All the necessary precautions were taken to avoid bacterial contamination. A few fruiting bodies were inoculated in a petri plate containing sterile HL-5 axenic medium and kept for incubation under shaking conditions at 22ºC. Cell confluency was obtained after ~ a week.

2.2.5 Genomic DNA Isolation from *D. discoideum*

10^7cells/ml were grown and washed once with 1X SB, followed by one wash with ice cold buffer [0.2 % NaCl (0.034M)]. 2% SDS was added and kept at 65°C for 15 min incubation. 1 volume of TE buffer (pH 9.5) was added and then extracted with 1 volume of phenol:chloroform (1:1) treatment. The upper aqueous layer was separated after spinning/centrifuging at 9,000g for 5 min. The step was repeated with chloroform followed by centrifugation at 9,000g for 5 min to get upper aqueous layer. 1/10th volume of sodium acetate and 1 volume of isopropanol was added into it for DNA precipitation at -20°C for 30 min. The DNA pellet was obtained by spinning/centrifuging it at 9,000g for 5 min followed by a wash with 75% ethanol. DNA pellet was then dried and resuspended in NFW (Nuclease Free Water) (Pilcher *et al*., 2007).

2.2.6 Plasmid DNA isolation by alkaline lysis method

Plasmid DNA was isolated by alkaline lysis method (Sambrook and Russell, 2001). A single colony was taken from transformed bacterial cells and inoculated in LB (Luria Broth) medium followed by overnight incubation at 37^0C under constant shaking condition. The culture was centrifuged and resuspended in ice cold alkaline solution I. Alkaline solution II (freshly prepared) was then added and mixed for a couple of time. Ice cold alkaline solution III was further added and incubated it in ice for 15 min followed by centrifugation at 15000rpm/15min/4^0C. Equal volume of phenol:chloroform was added to the supernatant followed by centrifugation at

10000rpm/10min/4^{0}C. To precipitate plasmid DNA, ice cold isopropanol was added to the supernatant and incubated for 30 min at 4^{0}C. This mixture was again centrifuged at 15000rpm/15min/4^{0}C to obtain the pellet which was then washed with 70% ethanol and dried at 37^{0}C. Plasmid DNA pellet was dissolved in 1X TE buffer containing RNase (10mg/ml; Thermo Fischer Scientific, USA). The integrity of plasmid DNA was assessed by agarose gel electrophoresis.

2.2.7 Transformation of plasmid DNA into *E. coli*

Competent cells were prepared by inoculating *E. coli* DH5α cells in ~3 ml of LB and incubated at 37ºC until the OD_{600} reached 0.6 (logarithmic phase). The grown culture was pelleted down at 5000g/5min and 1.8 ml of 80 mM $MgCl_2$ + 20 mM $CaCl_2$ were added to it followed by incubation on ice for 20 min. Culture was then centrifuged and washed once with 1 ml of 100 mM $CaCl_2$. 100 µl of 100 mM $CaCl_2$ was again added to the cells and incubated on ice for 1 hr. 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 shifted to ice for 2-3 min. ~800 µl of sterile LB was added and incubated at 37ºC for 45 min with shaking. Finally, cells were centrifuged and resuspended in 40 µl LB which was then spread on Ampicillin (100mg/ml) containing Luria agar plates (Sambrook and Russell, 2001).

2.2.8 Generation of *AIF* antisense constructs (constitutive and prestalk specific)

2.2.8.1 PCR amplification

In order to get *AIF* constitutive and prestalk specific antisense constructs, PCR amplification was done using following conditions.

PCR amplicon size	Denaturation	Annealing	Extension	No. of

				cycles
AIF constitutive antisense amplicon (353 bp)	94°C - 10’ 94°C - 30 sec	61°C - 20 sec	72°C - 25 sec 72°C - 10’	39
AIF prestalk specific antisense amplicon (520 bp)	94°C - 10’ 94°C - 30 sec	60.5°C - 25 sec	72°C - 25 sec 72°C - 10’	39

Table 1: PCR conditions for *AIF* amplification for *AIF* antisense constructs

2.2.8.2 *AIF* constitutive and prestalk specific antisense constructs

Constitutive downregulation of *AIF* was under actin promoter whereas prestalk specific downregulation of *AIF* was under EcmB (inducible) promoter which is expressed only during the slug stage of *D. discoideum* development. For constitutive *AIF* downregulation, PCR amplification was done using 5’ region of *AIF* (353 bp) and the amplicon was directionally cloned into constitutive *D. discoideum* expression vector (pTX-GFP) by replacing GFP using *Xba*I and *Kpn*I enzymes. For prestalk specific *AIF* downregulation, PCR amplification was done using 5’ region of *AIF* (520 bp) and the amplicon was directionally cloned into prestalk specific vector (pEcmB-Gal) using *Xho*I and *Cla*I enzymes. *AIF* antisense clones (pTX-*AIF* and pEcmB-*AIF*) were selected under Geneticin (100µg/ml) and confirmed by PCR amplification and restriction enzyme digestion.

***AIF* specific Primers**	**Enzyme**
***AIF* constitutive antisense construct** Forward Primer	*Xba*I

5'-ACG**GCGGCCGC**GCAACAAAAAATACATCTC-3'	
***AIF* constitutive antisense construct** Reverse Primer 5'-AGC**TCTAGA**ATCTTGCTCTTGTTCCTCCTC-3'	*Bam*HI
***AIF* prestalk specific antisense construct** Forward Primer 5'-ATC**AAGCTTA**CACAGCTAATCCAAGTCG-3'	*Xho*I
***AIF* prestalk specific antisense construct** Reverse Primer 5'-AGT**GGATCC**TACTATGGAATGGATGTGGTGC-3'	*Cla*I

Table 2: Primers used for *AIF* antisense constructs

2.2.9 Generation of *AIF* constitutive overexpression constructs

2.2.9.1 PCR amplification

In order to get full length *AIFA* (*AIF*) PCR amplification from the genomic DNA of *D. discoideum* using *AIF* specific primers was performed using following conditions.

PCR amplicon size	Denaturing	Annealing	Extension	No. of cycles
AIF constitutive overexpression amplicon (1.8kbp); **PCR master mix modifications:** 3.5mM $MgCl_2$ + 1% DMSO + 80% Glycerol	95°C - 5' 95°C - 45 sec	61.2°C - 1'	72°C - 3' 72°C - 10'	39

Table 3: PCR conditions for *AIF* amplification for *AIF* overexpression constructs

2.2.9.2 *AIF* constitutive overexpression constructs

PCR amplification (1.8kbp) was done using *AIF* specific primers and cloned into act15-EYFP vector under actin promoter. Purified PCR product digested with *SacI* and *BamHI* was ligated into act15-EYFP. The positive clones, *AIF*-EYFP OE (*AIF* OE) and EYFP vector control were then transformed into *D. discoideum* cells by electroporation. EYFP-vector control cells were selected at 100 µg/mL G418. For selection of *AIF* OE, we went up to 30 µg/mL G418. However, these cells could not withstand 30µg/mL concentration. Hence, we reduced G418 to 10 µg/mL. All the experiments were performed with 10 µg/mL G418. Positive clone is confirmed by PCR amplification and restriction digestion.

***AIF* specific Primers**	**Enzyme**
***AIF* constitutive overexpression construct** Forward Primer 5'- TTT**GAGCTC**CAACATCCCCAACCC -3'	*Sac*I
***AIF* constitutive overexpression construct** Reverse Primer 5'- AGC**GGATCC**GGCTTGTGTATGAATAATTAC-3'	*BamH*I

Table 4: Primers used for *AIF* overexpression construct

2.2.10 Transformation of plasmid DNA into *D. discoideum*

Transformation of plasmid DNA into *D. discoideum* was performed by electroporation method (Gaudet *et al*., 2007). ~5x10^6 *D. discoideum* cells were centrifuged and washed twice with ice cold H-50 buffer (20 mM HEPES+50 mM KCl+10 mM NaCl+1 mM $MgSO_4$+5 mM $NaHCO_3$+1 mM NaH_2PO_4, pH-7.0). Plasmid DNA (10-15 µg) was added to *Dictyostelium* cell pellet in 100-200 µl H-50 buffer 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, Hercules, CA, USA). The cuvette was kept on ice for 5 min and then cells were shifted to a 10 cm Petri plate containing sterile 10 ml HL-5 for selection. Petri plate with cells was incubated in BOD at 22^{o}C for overnight. Then 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**
Geneticin for *AIF* constitutive and prestalk specific antisense clones	0 μg/ml	10 μg/ml	15 μg/ml	30 μg/ml till 100 μg/ml
Geneticin for *AIF* constitutive overexpression clone	0 μg/ml	10 μg/ml	15 μg/ml	For *AIF* OE cells: 30 μg/ml for 7-10 days. Later, 30 μg/ml was reduced to 10 μg/ml. For EYFP-vector control cells, selection was done till 100 μg/ml

Table 5: Antibiotic dose pattern after electroporation

2.2.11 RNA Isolation and cDNA synthesis

~2.5 x 10^7 cells were harvested and washed with 1X SB buffer. These cells were then resuspended in TRIzol reagent (Invitrogen, USA) and incubated for 5 min at room temperature (RT). 200 μl chloroform was added and kept it at RT for 5 min followed by centrifugation at 10000rpm/10min/4^{0}C. The upper aqueous layer was transferred to a fresh tube and 1 ml 100% ethanol was added followed by incubation for 20 min at -20°C and then centrifugation at 12000rpm/5min/4^{0}C to obtain the RNA pellet. RNA pellet was further semidried and dissolved in 10-20 μl of Nuclease Free Water (NFW). RNA integrity was assessed by 1.5% agarose gel electrophoresis. RNA yield and

purity were analyzed spectrophotometrically at 260/280 nm (Pilcher *et al*., 2007). RNA was treated with DNase I (Ambion inc. Texas, USA) for 15-30 min at RT before cDNA synthesis to avoid DNA contamination. 1.5µg of total RNA was used to prepare cDNA. cDNA synthesis was performed using 1.5µg of the total RNA by Thermo Scientific Verso^{TM} DNA Synthesis Kit (Thermo Fisher Scientific, Inc. USA) as per the manufacturer's instructions. This cDNA was further used to analyze the transcript levels of respective genes with gene specific primers by Real Time PCR.

2.2.12 Functional characterization of *AIF* downregulation

AIF downregulation was confirmed by monitoring the expression of *AIF* with gene specific primers by Real Time PCR 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). *RNLA* was used as an internal control.

2.2.13 Cell growth profile

To study growth profile of constitutive *AIF* dR cells, mid log phase cells at a density of ~0.6×10^6 cells/ml were inoculated in HL-5 medium. Cells were collected after every 2 hrs till 12 hrs and thereafter at 12 hrs interval till 144 hrs for the cell count. The cell suspension was mixed with trypan blue solution [0.4% (w/v) in phosphate buffer] in the ratio of 2:1. After ~2 min of incubation, the cell count was taken using hemocytometer (Kosta *et al*., 2001). To analyze the effect of antioxidant on cell growth, the cell growth profile was assayed in the presence of 10mM glutathione (GSH).

2.2.14 Cell cycle analysis

Cell cycle was assessed by Flow cytometry using PI (Sigma, USA) (Chen *et al*., 2004). Mid log phase cells were fixed with a drop wise addition of 70% ethanol and incubated at 4°C overnight. These cells were then resuspended in

500 µl of staining solution [0. 1 % (v/v) Triton X-100+ 2 mg DNAse-free RNAse+ 500 µg/ml PI in Phosphate Buffered Saline (PBS)] with incubation 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.

2.2.15 Induction of oxidative stress

For induction of oxidative stress, 0.03mM H_2O_2 (cumene H_2O_2) (paraptotic dose) and 0.05mM H_2O_2 (necrotic dose) were used (Rajawat *et al.*, 2014a). Mid-log phase cells at a density of ~2.5×10^6 cells/ml were exposed to these doses in HL-5 medium at 22°C in a sterile flask and then used for the respective cell death experiments.

2.2.16 Estimation of NAD^+ and ATP levels

Intracellular levels of NAD^+ were determined by enzymatic recycling method using alcohol dehydrogenase to reduce NAD^+ to NADH (Bernofsky and Swan, 1973). Cellular NAD^+ is taken up to convert ethanol into acetaldehyde which is catalyzed by alcohol dehydrogenase. NADH produced in the first reaction is used to reduce MTT into Formazan and NAD^+ is regenerated. In this way, cellular NAD^+ keeps on getting recycled and thereby increases the yield of Formazan, a chromophore giving absorbance at 570 nm. NAD^+ was extracted with 1 ml of 0.5 M perchloric acid (Pubchem, USA) and then neutralized with 1 N KOH (SRL, India). Protein concentration was estimated by Lowry method (Lowry *et al*., 1951).

Total cellular and mitochondrial ATP levels were estimated by HPLC in aliquots extracted with alkali (Leoncini *et al*., 1987). Mitochondria were isolated from log phase vegetative *D. discoideum* cells (Nagayama *et al*., 2008).

2.2.17 Estimation of intracellular ROS generation

The production of ROS was measured using DCFDA (2'-7'dichlorofluorescin diacetate) dye (1µg/ml) (Esposti, 2002). Oxidation of H_2DCFDA by ROS

converts the molecule to 2',7' dichlorodihydrofluorescein (DCF), which is highly fluorescent. Upon stimulation, the resultant ROS generation causes an increase in fluorescence over time. ~2.0×10^6 *D. discoideum* cells were washed twice with 1X SB followed by addition of membrane permeable dye, DCFDA and incubated for 15 min at 22^0C under constant shaking condition. Fluorescence was measured by Fluorimeter (F7000, Hitachi, Japan) and λ_{ex} and λ_{em} used for these studies were 480 nm and 525 nm respectively.

2.2.18 Electron Paramagnetic Resonance (EPR) spectroscopy

ROS formation was also detected using an EPR-based spin trapping system using POBN and DMSO (Wadhawan *et al*., 2010). ~5.0×10^7 *D. discoideum* cells were washed with 1X SB and then mixed with POBN (200mM) and DMSO (250mM) and subjected to an X band-EPR spectrometer (EMX series, Bruker, Germany) for ROS formation analysis. The spin trapping spectra were obtained by three signal-averaged scans at ambient temperature (25 ± 2^0C). The instrument settings were as follows: power, 15.94mW; receiver gain, 1×10^5; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; sweep width, 50 G; and sweep time, 40 ms.

2.2.19 Estimation of Protein Carbonyl (PC) content

Protein carbonyl content was estimated by spectrophotometric DNPH (2,4-dinitrophenylhydrazine) assay (Mekrungruangwong *et al*., 2012). ~2.0×10^6 cells were washed once with 1X SB followed by its extraction in a final concentration of 10% (w/v) Trichloroacetic acid (TCA) (Pubchem, USA). The precipitates were then treated with 0.2% DNPH (Pubchem, USA) and incubated at RT for 1 hr. The cell pellets were washed 2-3 times with ethanol:ethyl acetate (1:1) mixture and then finally dissolved in 6M guanidine hydrochloride (Himedia, India). The absorbance was measured at 370 nm and the PC was estimated.

2.2.20 Fluorimetric estimation of intracellular calcium $[Ca^{2+}]_i$ levels

Intracellular calcium levels were measured using Fura-2AM dye (Nakamura *et al*., 1996). ~2.5×10^6 cells were washed with 1X SB and loaded with 5µM Fura-2AM (Molecular probe, USA) and 0.1% Pluronic F-127 (Molecular probe, USA) for 30 min at 22^0C followed by washing with 1X SB. The fluorescence intensities were recorded at 340/380 nm excitation and 510 nm emission by Fluorimeter (F7000, Hitachi, Japan). The ratio of peak amplitude at 340 nm and 380 nm was used to evaluate the calcium levels using the formula, $[Ca^{2+}]_i = K_d\,[(R-R_{min})/(R_{max}-R)]\beta$.

2.2.21 DNA damage assay

DNA damage was analyzed using anti-human phospho-histone γ-H2AX (R&D Systems, Minneapolis, MN, USA; 2µg/ml; rabbit polyclonal) (Roh *et al*., 2008). Cells were washed once with PBS (pH 7.4) and fixed in 70% chilled methanol for 10 min at -20°C followed by washing with blocking solution (1.5% BSA with 0.05% Tween 20 in PBS). Cells were then incubated with anti-γ-H2AX for 7-8 hrs followed by 1 hr incubation with secondary anti-rabbit IgG (whole molecule) TRITC conjugate (Sigma, St. Louis, MO, USA; 1:400 dilutions). The nucleus was counterstained with DAPI (1µg/ml) for 5 min and fluorescence was analyzed by using confocal laser scan fluorescence-inverted microscope (LSM 710; Carl Zeiss, Jena, Germany).

2.2.22 Analysis of Mitochondrial Membrane Potential (MMP)

$DiOC_6$ (3, 3'-dihexyloxacarbocyanine iodide) (Sigma, USA) was used to evaluate changes in mitochondrial membrane potential (Koning *et al*., 1993). ~2.0×10^6 cells were washed twice with 1X SB and stained with $DiOC_6$ (400 nM) for 15 min in dark. 1X SB wash was given to the cells and fluorescence was monitored under 63X by Zeiss confocal laser scan fluorescence-inverted microscope (LSM 710; Carl Zeiss, Jena, Germany). MMP was also measured by flow cytometry, by incubating *D. discoideum* cells (~$1\text{-}2\times10^6$/ml) with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol carbocyanine iodide (JC-1) (Molecular Probe, USA) (Smiley *et al*., 1991) and quantitated by flow

cytometry using FACS ARIA (BD Biosciences, Franklin Lakes, New Jersey). Data were analyzed with FACSDiva software.

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

Apoptotic and necrotic cell death are differentiated by dual staining with Annexin V-FITC/PI apoptosis detection kit (Molecular Probes, USA) (Miller, 2004). ~2.0 x 10^6 cells were washed twice with 1X SB and resuspended in binding buffer provided in the kit. Cells were treated 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, Jena, Germany).

2.2.24 Monitoring AIF release

AIF translocation from mitochondria to the nucleus was monitored by immunofluorescence (Bidere *et al*., 2003). ~2.0 x 10^6 cells were washed with 1X SB and then treated with primary Rabbit anti-AIF polyclonal antibody (1:1000 dilution) (Cayman chemical, USA) against amino acids 151-180 of human AIF and secondary anti-rabbit IgG (whole molecule) TRITC conjugate (1:400 dilution) (Sigma, USA) were used. Nuclear counterstaining with DAPI (4', 6 diamidino-2-phenylindole) (1µg/ml) was performed for 5 min and was analyzed for fluorescence. Cell specimens were observed with confocal laser scan fluorescence-inverted microscope (LSM 710; Carl Zeiss, Jena, Germany).

2.2.25 Glucose dependency

To study the dependency of glucose, mid log phase *D. discoideum* cells (~1×10^6) were washed once with 1X SB and allowed to grow in glucose free HL-5 medium (GFM) and cell viability was monitored by trypan blue exclusion method (Kosta *et al*., 2001).

2.2.26 Estimation of lactic acid

Lactic acid was estimated in culture medium by HPLC at 4^{th}, 6^{th} and 8^{th} day of cell growth. Log phase vegetative cells (~3.5×10^6) were collected and washed

with 1X SB. To measure the production of lactic acid, cells were subjected to freeze lysis and cell lysate was obtained by centrifugation at 10000rpm/15min/RT (Jang *et al*., 2009). This suspension was used to analyze lactic acid levels by HPLC (Remme and Woll, 2006).

2.2.27 Transmission Electron Microscopy (TEM)

TEM was performed to analyse the morphometric mitochondrial and cellular alterations in *D. discoideum*. Log phase cells were fixed with 2% glutaraldehyde and 2% osmium tetroxide in PBS, pH 7.2 at 4^0 C for 1 hr and processed as described by Rajawat *et al*., (2014b) and Kosta *et al*., (2008). Thin sections were obtained with a Reichert Ultracut E ultramicrotome, stained and examined using TEM (Morgagni 268D, FEI electron optics company Philips, Hillsboro, Oregon, USA). Mitochondrial surface area was calculated using ImageJ software.

2.2.28 Estimation of Oxygen Consumption Rate (OCR)

OCR was measured in saponin (5mg/ml) (Himedia, India) permeabilized cells (~12 X 10^6 cells/ml) using Oxytherm Clark-type oxygen electrode (Hansatech Instruments, Norfolk, UK) containing respiration buffer (80 mM KCl+ 0.1% BSA+ 50 mM HEPES+ 2 mM $MgCl_2$+ 2.5 mM KH_2PO_4; pH 7.2) (Finner and Newell, 1987). Log phase cells were washed with 1X SB and treated with saponin for 15 min followed by three washes of isolation buffer (10 mM HEPES+ 200 mM D-Mannitol+ 70 mM Sucrose+ 1 mM EGTA; pH7.5). These cells were then resuspended in respiration buffer and used for OCR studies. Respiratory chain complexes I-IV activities were recorded using 100 mM Sodium Pyruvate & 800 mM Malate (complex I), 1M Succinate (complex II), 10 mM α-glycerophosphate (complex III), and 200 mM Ascorbate (complex IV) (Li and Graham, 2012) and the protein concentration was estimated by Lowry method (Lowry *et al*., 1951). Mitochondrial outer membrane integrity of saponin-treated cells was assessed by impermeability to exogenous cytochrome c (Sigma, USA), which was consistently greater than

95%. OCR was determined by measuring the amount of oxygen (nmol) consumed, divided by the time elapsed (min) and amount of protein.

2.2.29 Isolation of mitochondria from *D. discoideum* cells

Log phase (~12 x 10^6cells/ml) of *D. discoideum* cells were washed in mitochondrial isolation buffer (10 mM HEPES+ 200 mM D-Mannitol+ 70 mM Sucrose+ 1 mM EGTA; pH7.5) and subjected to approximately 80 strokes with 24G syringe. Cells were then pelleted down at 2000g/15min/4°C and cell debris was removed. Supernatant was collected followed by centrifugation at 8000rpm/15min/4°C to get mitochondria pellet which was then washed with mitochondrial isolation buffer and used for BN-PAGE (Nagayama *et al.,* 2008).

2.2.30 Blue Native-PAGE (BN-PAGE)

Mitochondrial respiratory chain complexes assembly was analyzed by BN-PAGE (Jha *et al*., 2016). Mitochondria were isolated from vegetative *D. discoideum* cells (Liza *et al.,* 2010) and mitochondrial pellet was resuspended in solubilization buffer (50 mM NaCl+ 50 mM Imidazole/HCl+ 2 mM 6-aminohexanoic acid+ 1 mM EDTA; pH 7) (Nagayama *et al*., 2008). The amount of protein was quantified by Lowry method (Lowry *et al*., 1951). Mitochondrial pellet was then solubilized using Digitonin (5%) and the samples were centrifuged at 12000rpm/20min/4°C. Coomassie blue-G250 dye (5% in 750 mM 6-aminohexanoic acid) was added to the supernatant to set a detergent/Coomassie-dye ratio of 4 (g/g). This supernatant was then subjected to 1.0 mm sample wells of BN-PAGE using 3-12% acrylamide gradient gels and run at 250V for 2-3 hrs at RT.

2.2.31 Gene expression analysis by Real Time PCR

Total RNA was isolated from *D. discoideum* cells and gene transcript analysis was performed using gene specific primers (Eurofins, Bangalore, India) using LightCycler® 480 SYBR Green I Master (Roche Diagnostics GmbH,

Mannheim, Germany) in the LightCycler® 480 Real Time PCR (Roche Diagnostics GmbH, Mannheim, Germany). The thermal cycling conditions included an initial activation step at 95°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 the fluorescence was greater than the set threshold value. Results were analyzed using the comparative CT method with the amplification of *RNLA* (mitochondrial large rRNA) as an internal control. Fold change in the transcript levels ($2^{-\Delta\Delta Ct}$) was shown graphically (Livak and Schmittgen, 2001). Relative gene expression was plotted and analyzed by nonparametric unpaired t-test using GraphPad PRISM®6, GraphPad software Inc., USA. Table 6 shows primers used for gene expression analysis.

Gene	Primer
AIF/ AIFA	Forward Primer (FP): 5'-CCAATCCTCATCAAAAGGAAG-3' Reverse Primer (RP): 5'-AGCTGCTGTTCCACCACC-3'
AIFB	FP: 5'-TGTAATCAATGAGTCTGGAC-3' RP: 5'-AAAGACTAAGTATTGGACCTG-3'
AIFC	FP: 5'-TGGAGGTAGCCAAGTTG-3' RP: 5'-TTCTGGTTCAACTATTGCTC-3'
AIFD	FP: 5'-TTCAGGTAGTATTGTAGCAC-3' RP: 5'-TTGAGCCAGAGTTTATCA C-3'
NAD1	FP: 5'-AAGAGGACCAAATGTAGTAGGG-3' RP: 5'-GCTGCTGATCGTAATCCTC-3'
NAD6	FP: 5'-GAGTCGTAGGTCTTCTGG-3' RP: 5'-CATTGTTCGTATATCTGTCG-3'
NDUFS3	FP: 5'-ACGGATTATGGATTTGTAGG- 3'

	RP: 5'-TCT CCC CAT ACT GCT CC-3'
CYTB	FP: 5'-GGAATTTATGAAGCAGGCG-3' RP: 5'-GCTAAATCGACATGTGCCG-3'
COX1/2	FP: 5'-AGCAGAGAAAGAAGGAAAC-3' RP: 5'-TTGACCGTCTCCATCTAAC-3'
ATP6	FP: 5'-GGCAACCATAATAGGTGG-3' RP: 5'-AACCCCAAATTGTTATTCC-3'
NDUFA5	FP: 5'-GTTGAACCAAATGCAAGAC-3' RP: 5'-TCATGGACTAAATCGACAAC- 3'
NDUFA9	FP: 5'-CTCGTAATTTCTCACTCGACG-3' RP:5'-CACCGATTGCTT TTGAACGTG-3'
CLUA	FP: 5'-TGTTACAGCATCAACTCAAGG -3' RP: 5'-ACTGGTAATACGCCTGGAAG-3'
DYMA	FP: 5'-CCAATTGCTGATGATGGATCAC-3' RP: 5'-GCTGGTGTCACTGCAACG-3'
DYMB	FP: 5'-GGTCAGAGATTACTCTACCAC-3' RP: 5'-GTCCAAATTCTCCCCATTCC-3
FSZA	FP: 5'-ACAAGAGGATTAGGAGCAGGAGC-3' RP: 5'-CAACTCTATCAAACCTTGTTCTGCCA-3'
FSZB	FP: 5'-TGGTGAAGCATCTGGTGAGGATAG-3' RP: 5'-ACGAAAACTTGAACATCAGGATCAGC-3'
GAPDH	FP: 5'-TCAACTGATGCCCCAATGTA-3' RP: 5'-CGTGAACGGTGGTCATTAAA-3'
COX1/2	FP: 5'-ACAAACTAAATGCGGGAACG-3' RP: 5'-TTAAATTTACGCCCCCACAG-3'
RNLA	FP: 5'-TTACATTTATTAGACCCGAAACCAAGC-3' RP: 5'-TTCCCTTTAGACCTATGGACCTTAGCG-3'

Table 6: Primers used for gene expression analysis

2.2.32 Estimation of mtDNA content (mtDNA/nDNA)

Total cellular DNA was isolated from *D. discoideum* and mtDNA content was estimated by Real Time PCR using the SYBR Green PCR Master Mix. mtDNA content was calculated by taking the ratio of mtDNA to the nuclear DNA (nDNA). The relative mtDNA content was calculated as the difference in the numbers of threshold cycles (Ct) between the nuclear-specific glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene and the mitochondrial-specific cytochrome c oxidase subunit 1/2 (*COX1/2*) gene (ΔCt), in which the amount of mtDNA calculated per cell ($2^{-\Delta Ct}$) represents a relative measure of mtDNA content (Kim *et al*., 2007). Table 7 shows primer sequences used for the estimation of mtDNA content.

Gene	Primer
GAPDH	FP: 5'-TCAACTGATGCCCCAATGTA-3' RP: 5'-CGTGAACGGTGGTCATTAAA-3'
COX1/2	FP: 5'-ACAAACTAAATGCGGGAACG-3' RP: 5'-TTAAATTTACGCCCCCACAG-3'

Table 7: Primers used for estimation of mtDNA content

2.2.33 Data analysis and statistics

Data are presented as a mean and Standard Error of the Mean (SEM) or Standard deviation (SD) as applicable. Statistical analysis was performed using GraphPad PRISM®6, GraphPad software Inc., USA. Student's unpaired t-test assessed statistical significance for experiments with the single comparison. 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.

2.3 References

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