Effect of *AIF* overexpression on mitochondrial parameters of *D. discoideum*

7.1 Introduction

AIF is a double-edged sword, having pro-death and pro-survival functions. In a healthy cell, AIF is confined to the mitochondrial inter-membrane space. Upon the induction of apoptosis, it translocates from mitochondria to the nucleus. Here, it takes part in apoptosis-associated chromatinolysis (Susin *et al.*, 1999). AIF's apoptotic function points an important role in several cell death scenarios like ischemia-reperfusion, a condition relevant to brain damage and myocardial infarction, or cavitation of embryoid bodies (Zhu *et al.*, 2003).

Downregulation or overexpression studies of *AIF* states its complex function during cell survival or cell death. Despite of this complexity, various evidences suggest that AIF can be a prognostic marker of disease progression in certain tumors and elevated *AIF* levels sensitizes cells for oxidative stress mediated cell death (Bano and Prehn, 2018; Zhang and Csaky, 2006). High expression of *AIF* proved to be predictive of a longer overall survival in B-cell lymphoma patients (Troutaud *et al.*, 2010). Similarly, *AIF* overexpression was seen in chronic myeloid leukemia patients and was associated with reduced responsiveness to tyrosine kinase inhibitors (Quintas-Cardama *et al*., 2012). Overexpression of *AIF* also induced cell death by increasing ROS levels, loss of mitochondrial membrane potential (MMP) and the release of cytochrome c, while inhibition of its expression or activity delayed or abrogated apoptosis upon microinjection of recombinant AIF into the cytoplasm (Susin *et al.*, 1999) or overexpression of AIFΔ1-100 (Loeffler *et al.*, 2001). Overexpression of *AIF1* in HeLa cells also inhibited cell growth and led to caspase-3 and caspase-9 activation (Lv *et al.*, 2006). Moreover, overexpression of *N*-terminal *AIF1* in the gastric cancer cell line, MKN45 caused upregulation of *PSMC2, PSMD13, TXNRD1* and *NDUSF1*, and cell death (Yang *et al.*, 2014).

AIF (DdAIF) is vital for cell death in *D. discoideum* (Arnoult *et al*., 2001). Our earlier reports also demonstrated that AIF is a downstream effector in PARP-1 and staurosporine mediated caspase-independent cell death in *D. discoideum* (Mir *et al.*, 2012; Rajawat *et al.*, 2014a; 2014b). Our *AIF* dR studies indicate AIF's role as the ROS regulator and mitochondrial functions. The present study aims to study the effect of *AIF* overexpression on total cellular ROS levels, mitochondrial fission-fusion mechanism and mtDNA content in *D. discoideum*.

7.2 Results

In order to study the mitochondrial role of AIF, we cloned full length *AIF* with an aim to generate *AIF* overexpressing *D. discoideum* cells to monitor its effect on total cellular ROS levels, mitochondrial fission-fusion profile and mtDNA amount.

7.2.1 Cloning of *AIF* (*AIFA*) in EYFP vector

For generation of *AIF* overexpression construct, EYFP vector was used with *actin15* promoter. Full length *AIF* (1.8kbp) was PCR amplified from the genomic DNA of *D. discoideum* (Fig. 7.1) and cloned into act15-EYFP vector using *AIF* specific primers (Table 4). *Dictyostelium* genome being AT rich is difficult to amplify and hence modifications in PCR master mix (Table 3) were made to amplify full length *ADPRT1A.* Purified PCR product digested with *SacI* and *BamHI* was ligated into act15-EYFP. This ligation mixture was transformed into *E. coli DH5α* and the transformants were selected on Luria Bertani Agar containing ampicillin (100µg/ml). Randomly selected colonies were screened for the presence of recombinant plasmid EYFP-*AIF* colonies.

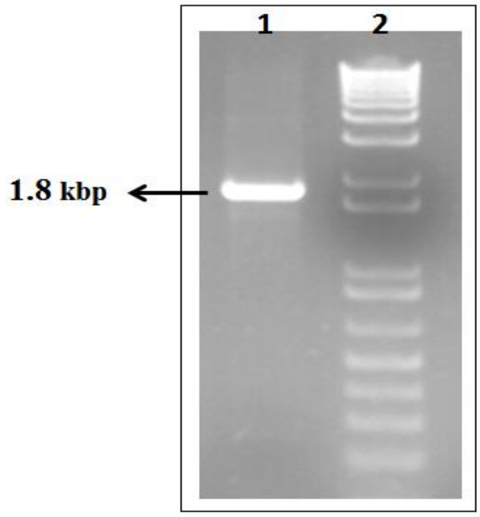


Fig. 7.1: PCR amplification of full length *AIF*: Lane 1: *AIF* amplicon***;*** Lane 2: 1kbp DNA ladder.

7.2.2 Confirmation of *AIF* overexpression construct in EYFP using Restriction digestion and PCR

The positive resultant EYFP-*AIF* overexpression clone was confirmed by PCR (Fig. 7.2) and restriction digestion (Fig. 7.3). The resultant positive clone (EYFP-*AIF)* was re-digested with *BamH*I and *Sac*I giving a release of 1.8 kbp. Presence of insert would give a single band of a positive resultant clone of ~9.3 kbp upon *BamHI* digestion, confirming EYFP-*AIF* construct whereas *BamHI* digested uncut EYFP would give a single band of ~7.5 kbp (Fig. 7.3). The confirmed clone containing full length *AIF* was used for transformation of *D. discoideum* cells to generate *AIF* overexpressing cells.

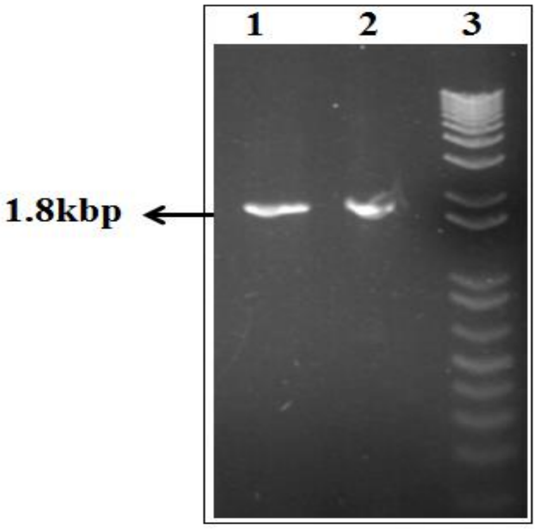


Fig. 7.2: EYFP-*AIF* clone confirmation by PCR: Lane 1: AIF amplicon from genomic DNA (positive control), Lane 2: AIF amplicon from EYFP-*AIF* clone; Lane 3: 1 kbp DNA ladder.

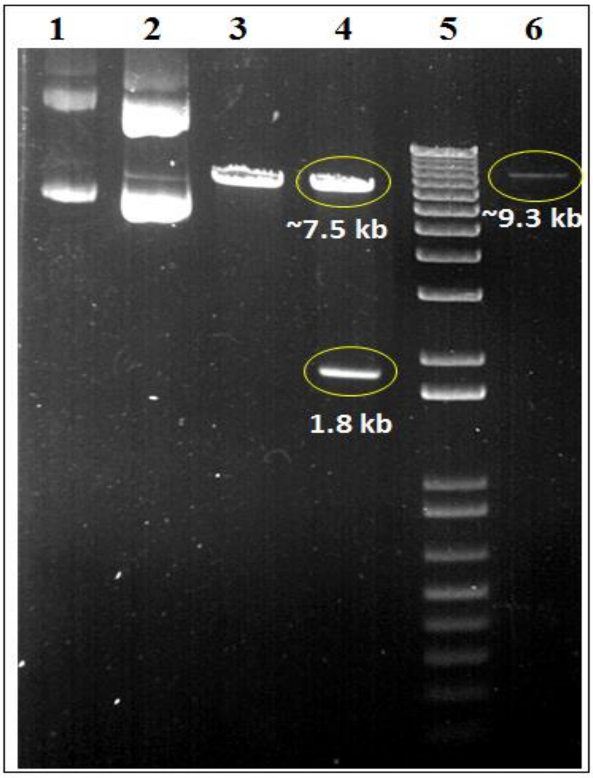


Fig. 7.3: EYFP-*AIF* clone confirmation by RE digestion: Lane 1: Uncut EYFP- *AIF* Lane 2: Uncut EYFP; Lane 3: EYFP digested with *BamH*I; Lane

4: EYFP-*AIF* digested with *BamH*I and *SacI*; Lane 5: 1 kbp DNA ladder; Lane 6: EYFP-*AIF* digested with *BamH*I.

7.2.3 Functional characterization of *AIF* overexpression

AIF overexpression was confirmed by monitoring gene specific expression of *AIF* using Real Time PCR. *AIF* OE cells showed ~36% higher expression of *AIF* transcript compared to control and vector control cells (EYFP-vector control) (Fig. 7.4).

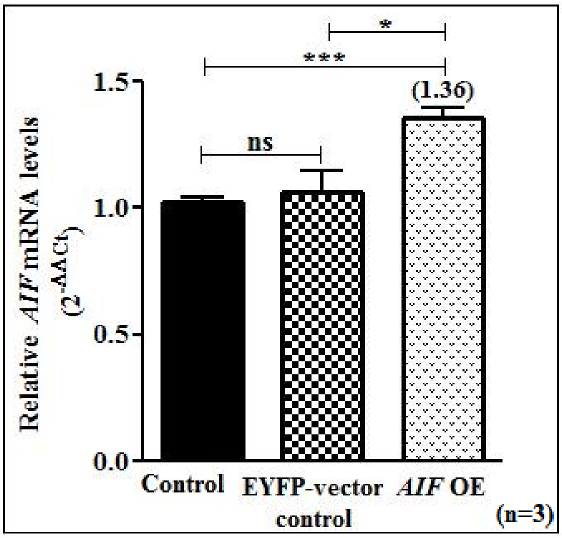


Fig. 7.4: Functional characterization of *AIF* overexpression by Real Time PCR: *AIF* OE cells exhibited significantly increased *AIF* transcript levels compared to control (p<0.0001) and EYFP-vector control (p=0.0114) cells. Data are representation of SEM values of three independent experiments. *p<0.0001 as compared to control and *p<0.05 as compared to EYFP-vector control; ns= non-significant.

7.2.4 Cellular ROS levels in *AIF* OE cells

As AIF is mainly involved in inducing cell death, higher *AIF* expression may lead to increase in ROS levels. Hence, ROS levels were estimated. A significant increase in ROS levels were observed in *AIF* OE cells as compared to control cells (Fig. 7.5), suggesting *AIF* OE cells exhibited high oxidative stress that might make the cells sensitive to oxidative stress mediated cell death.

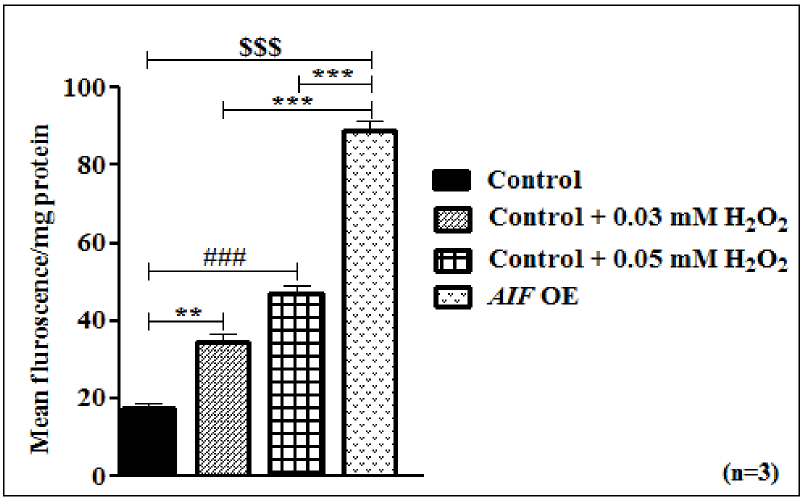


Fig. 7.5: Fluorimetric analysis of ROS levels using DCFDA dye: A significant rise in ROS levels was observed in *AIF* OE cells compared to control and 0.03mM and 0.05mM H_2O_2 treated control cells which were kept as positive controls. Data are representation of SEM values of three independent experiments. $^{\$}p<0.0001$ as compared to control; *$p<0.0001$ as compared to 0.03mM and 0.05mM H_2O_2 treated controls.

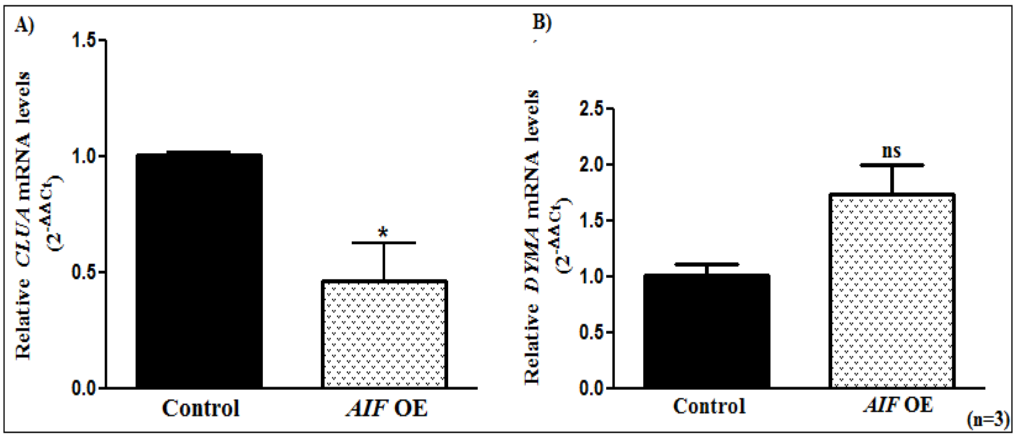
7.2.5 Analysis of transcript levels of mitochondrial fusion-fission genes in *AIF* OE cells

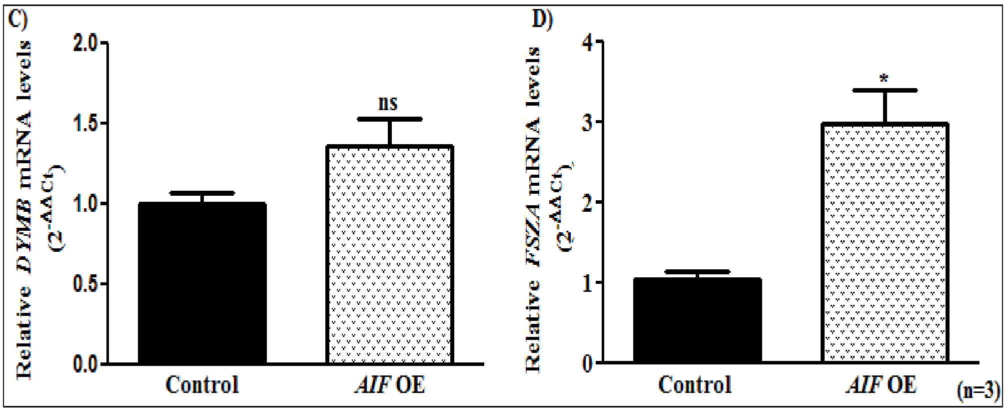
To examine the functional state of mitochondria in *AIF* OE cells, we further monitored the effect of *AIF* overexpression on mitochondrial fusion-fission mechanism and mtDNA content. Interestingly, mitochondrial fusion-fission mechanism and mtDNA content were found to be affected upon *AIF* overexpression.

Significantly reduced *CLUA* mRNA transcript levels were seen in *AIF* OE cells, while *FSZA* and *FSZB* transcript levels were found to be significantly elevated in *AIF* OE cells compared to control cells (Fig. 7.6A, 7.6D, 7.6E). No significant difference was found in *DYMA* and *DYMB* expression levels of *AIF* OE cells compared to control cells (Fig. 7.6B, 7.6C).

Excessive oxidative stress can damage and affect mtDNA content (Van Houten *et al.*, 2016). Hence, we analyzed mtDNA content upon *AIF* overexpression. Significant reduction in mtDNA content was observed in *AIF* OE cells compared to control cells (Fig. 7.6F).

Overall, AIF overexpression affected mitochondrial fission-fusion process and mtDNA amount due to high oxidative stress, suggesting that fine tuning of *AIF* levels are crucial in maintaining the mitochondrial homeostasis.





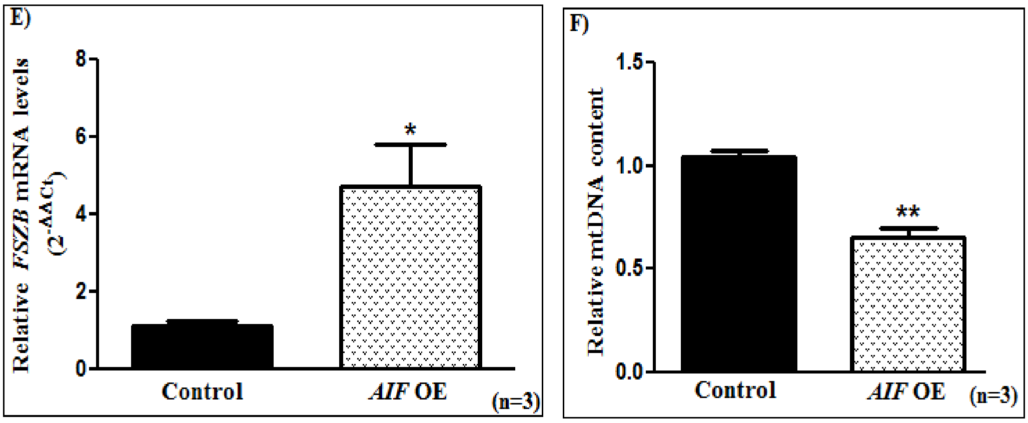


Fig. 7.6: Analysis of transcript levels of mitochondrial fusion-fission genes and mtDNA content by Real Time PCR: A) Significantly decreased *CLUA* transcript levels were seen in *AIF* OE cells as compared to control cells (p=0.0293). **B)** and **C)** No significant difference was found in *DYMA* (p=0.0570) and *DYMB* (p=0.1170) expression of *AIF* OE cells as compared to control cells. **D)** and **E)** Significantly higher *FSZA* (p=0.0407) and *FSZB* (p=0.0471) transcript levels were found in *AIF* OE cells as compared to control cells. **F) Estimation of relative mtDNA content:** Significant diminution in mtDNA content was found in *AIF* OE cells compared to control cells (p= 0.0014). Data are representation of SEM values of three independent experiments. *p<0.05 as compared to control; ns= non-significant.

7.3 Discussion

AIF is a nuclear-encoded mitochondrial inter-membrane space protein having several vital functions. It has been known for its translocation from mitochondria to the nucleus during apoptosis (Cao *et al.*, 2003; Susin *et al.*, 1999). It is also engaged in cell survival; its mechanism in the latter is not clearly understood. Excessive ROS levels elevate the oxidative stress which further stimulates cell death (Elmore, 2007). In *S. cerevisiae, AIF1P* overexpression resulted cell death due to high oxidative stress in the cell (Wissing *et al.*, 2004). Our results also showed that ~36% *AIF* overexpression (Fig. 7.4) caused high cellular ROS levels in *D. discoideum* cells (Fig. 7.5). Oxidative stress and the mtDNA quality are interconnected and it may diverge depending upon the cell type (Malena *et al*., 2016). We observed decreased mtDNA content in constitutive *AIF* overexpressed (OE) cells (Fig. 7.6F), confirming *AIF* overexpression affects mtDNA maintenance due to enhanced ROS levels. The decrease in mtDNA pool could affect the mitochondrial function (Van Houten *et al*., 2016; Barazzoni *et al.*, 2000). Several reports on *in vitro* phenotypes associated with overexpression of *AIF* have discovered the significance of AIF in the maintenance of mitochondrial energy metabolism by regulating the oxidative phosphorylation (Coughlan *et al*., 2016). In accordance with this report, we further monitored the effects *AIF* overexpression on the mitochondrial fission-fusion mechanism. *CLUA*, a mitochondrial fusion gene, transcript levels were found to be reduced significantly in *AIF* OE cells compared to control cells (Fig. 7.6A) whereas mitochondrial fission genes, *FSZA* and *FSZB* transcript levels were found to be increased significantly in constitutive *AIF* OE cells compared to control cells (Fig. 7.6D, Fig. 7.6E). No significant difference was observed in *DYMA* and *DYMB* mRNA transcript levels (Fig. 7.6b, Fig. 7.6C). Imbalance in mitochondrial fission-fusion genes expression levels depletes mtDNA amount and hence could cause mitochondrial dysfunction (Chen *et al*., 2010).

Summing up the results, our study suggests that the fine tuning of *AIF* level is essential in maintaining the mitochondrial homeostasis (Fig. 7.7).

This work has been accepted in the *BBA Bioenergetics* journal entitled ‘Apoptosis Inducing Factor: Cellular protective function in *Dictyostelium discoideum*’. Kadam *et al*., (2020) [https://doi.org/10.1016/j.bbabio.2020.148158] (*In Press*).

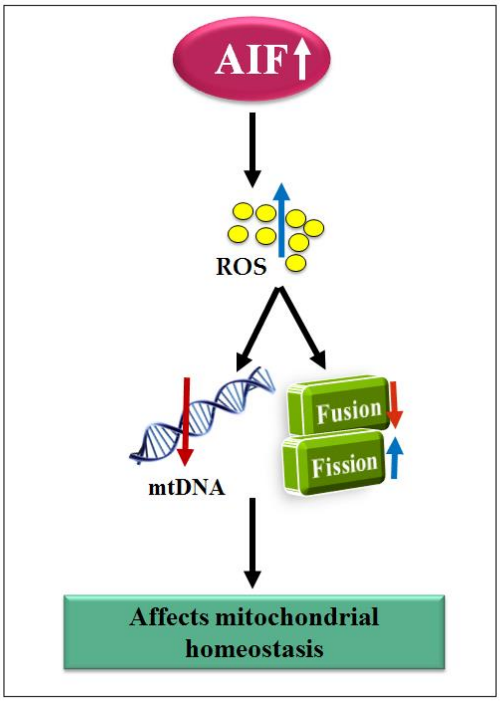


Fig. 7.7: Effect of *AIF* overexpression on cellular ROS levels, mitochondrial fusion-fission mechanism and mtDNA content

7.4 References

1. Arnoult D, Tatischeff I, Estaquier J, Girard M, Sureau F, Tissier JP, Grodet A et al (2001) On the evolutionary conservation of the cell death pathway: mitochondrial release of an apoptosis-inducing factor during *Dictyostelium discoideum* cell death. Mol Biol Cell 12:3016-3030.
2. Bano D, Prehn JHM (2018) Apoptosis Inducing Factor (AIF) in physiology and disease: The tale of repented natural born killer. EBioMedicine 30:29-37.
3. Barazzoni R, Short KR, Nair KS (2000) Effects of aging on mitochondrial DNA copy number and cytochrome c oxidase gene expression in rat skeletal muscle, liver, and heart. J Biol Chem 275:3343-3347.
4. Cao G, Clark RSB, Pei W, Yin W, Zhang F, Sun FY, Graham SH, Chen J (2003) Translocation of Apoptosis-Inducing Factor in Vulnerable Neurons After Transient Cerebral Ischemia and in Neuronal Cultures

After Oxygen-Glucose Deprivation. J Cerebral Blood Flow Metab 23:1137-1150.

5. Chen H, Vermulst M, Wang YE, Chomyn A, Prolla TA, McCaffery JM, Chan DC (2010) Mitochondrial fusion is required for mtDNA stability in skeletal muscle and tolerance of mtDNA mutations. Cell 141:280-289.
6. Coughlan MT, Higgins GC, Nguyen TV, Penfold SA, Bonke VT, Tan Sm et al (2016) Deficiency in Apoptosis-Inducing Factor Recapitulates Chronic Kidney Disease via Aberrant Mitochondrial Homeostasis. Diabetes 65:1085-1098.
7. Elmore S (2007) Apoptosis: a review of programmed cell death. Toxicol Pathol 35:495-516.
8. Loeffler M, Daugas E, Susin SA, Zamzami N, Metivier D, Nieminen A-L, Brothers G et al (2001) Dominant cell death induction by extramitochondrially targeted apoptosis-inducing factor. FASEB J 15:758-767.
9. Lv B, Shi T, Wang X, Song Q, Zhang Y, Shen Y, Ma D, Lou Y (2006) Overexpression of the novel human gene, nuclear apoptosis-inducing factor 1, induces apoptosis. Int J Biochem Cell Biol 38:671-683.
10. Malena A, Pantic B, Borgia D, Sgarbi G, Solaini G, Holt IJ et al (2016) Mitochondrial quality control: Cell-type-dependent responses to pathological mutant mitochondrial DNA. Autophagy 12:11 2098-2112.
11. Mir H, Rajawat J, Begum R. (2012) Staurosporine induced cell death in *D. Discoideum* is independent of PARP. Ind J Exp Biol 50:80-86.
12. Quintas-Cardama A, Qiu YH, Post SM, Zhang Y, Creighton CJ, Cortes J, et al (2012) Reverse phase protein array profiling reveals distinct proteomic signatures associated with chronic myeloid leukemia progression and with chronic phase in the CD34-positive compartment. Cancer 118:5283-5292.
13. Rajawat J, Alex T, Mir H, Kadam A, Begum R (2014b) Proteases involved during oxidative stress induced poly(ADP-ribose)

polymerase mediated cell death in *D. discoideum*. Microbiol 160:1101-1111.

14. 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.
15. Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J et al (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397:441-446.
16. Troutaud D, Petit B, Bellanger C, Marin B, Gourin-Chaury MP, Petit D, et al (2010) Prognostic significance of BAD and AIF apoptotic pathways in diffuse large B-cell lymphoma Clin. Lymphoma Myeloma Leuk 10:118-124.
17. Van Houten B, Hunter SE, Meyer JN (2016) Mitochondrial DNA damage induced autophagy, cell death, and disease. Front Biosci (Landmark Ed) 21:42-54.
18. Wissing S, Ludovico P, Herker E, Büttner S, Engelhardt SM, Decker T, Link A, et al. (2004) An AIF orthologue regulates apoptosis in yeast. J Cell Biol 166:969-974.
19. Yang M, Zhong J, Zhao M, Wang J, Gu Y, Yuan X, Sang J, Huang C (2014) Overexpression of Nuclear Apoptosis-Inducing Factor 1 Altered the Proteomic Profile of Human Gastric Cancer Cell MKN45 and Induced Cell Cycle Arrest at G1/S Phase. PLOS ONE 9(6): e100216.
20. Zhang C, Csaky KG (2006) Over Expression Apoptosis Inducing Factor (AIF) sensitizes Human Retinal Pigment Epithelium (HRPE) to oxidant induced cell death. Investig Ophthalmol Vis Sci 47:1380.
21. Zhu C, Qiu L, Wang X, Hallin U, Cande C, Kroemer G, Hagberg H, Blomgren K (2003) Involvement of apoptosis-inducing factor in neuronal death after hypoxia-ischemia in the neonatal rat brain. J Neurochem 86:306-317.