Effect of constitutive *AIF* downregulation on oxidative stress mediated cell death of *D. discoideum*

4.1 Introduction

The apoptosis inducing factor (AIF) was discovered as a caspase independent mitochondrial inter-membrane space protein that translocates to the nucleus during cell death (Susin *et al*., 1999). In the nucleus, it binds to DNA, triggering its fragmentation and nuclear condensation (Susin *et al.,* 1999; Cande *et al*., 2002). The mitochondrio-nuclear translocation of AIF and large scale DNA fragmentation are the characteristic features of caspase independent cell death in mammalian cells (Cande *et al*., 2002). AIF is a ubiquitous Flavin Adenine Dinucleotide (FAD) comprising protein and displays low NADH oxidase activity (Susin *et al*., 1999). Remarkably, its NADH oxidase function was found to be independent of its apoptogenic function (Miramar *et al*., 2001). It was further shown that the human colon cancer cells with a mutation in the N-terminal of PRG3 (AIF homologue) lacking oxidoreductase activity, conserves its apoptotic ability (Ohiro *et al*., 2002). AIF is an essential protein as AIF knock out in mouse proves to be lethal at embryo stage (Porter and Urbano, 2006). Therefore, the role of AIF as an apoptosis executor or as an ROS scavenger is controversial. An earlier study indicates that reduced cellular *AIF* levels make the cells highly vulnerable to oxidative stress mediated cell death characterized by Mitochondrial Membrane Potential (MMP) loss, translocation of AIF to the nucleus, Phosphatidyl Serine (PS) exposure, etc. (Schulthess *et al*., 2009).

Arnoult *et al.* (2001) stated that AIF (DdAIF) is vital for cell death in *D. discoideum.* Our earlier reports also demonstrated that AIF is a downstream effector molecule in PARP-1 and staurosporine mediated caspase independent cell death in *D. discoideum* (Mir *et al*., 2012; Rajawat *et al*., 2014a). *D. discoideum*, being caspase independent organism, is useful to elucidate the mechanism of AIF in caspase independent paraptotic cell death. Nevertheless, the effect of *AIF* deficiency on cell death under physiological and oxidative stressed conditions is yet to be unraveled. Hence, the present study focuses on to examine the involvement of AIF in *D. discoideum* cell death. Our data confirms the importance of AIF in caspase independent cell death, strengthening AIF's role as an ROS regulator.

4.2 Results

Cell death can be stimulated by various chemical agents including hydrogen peroxide (Hasnain *et al*., 1999; Sah *et al*., 1999). Classically, AIF is known to be a pro-apoptotic factor. To understand the effect of *AIF* downregulation on cell fate during oxidative stress, various cell death parameters were studied in *D. discoideum*.

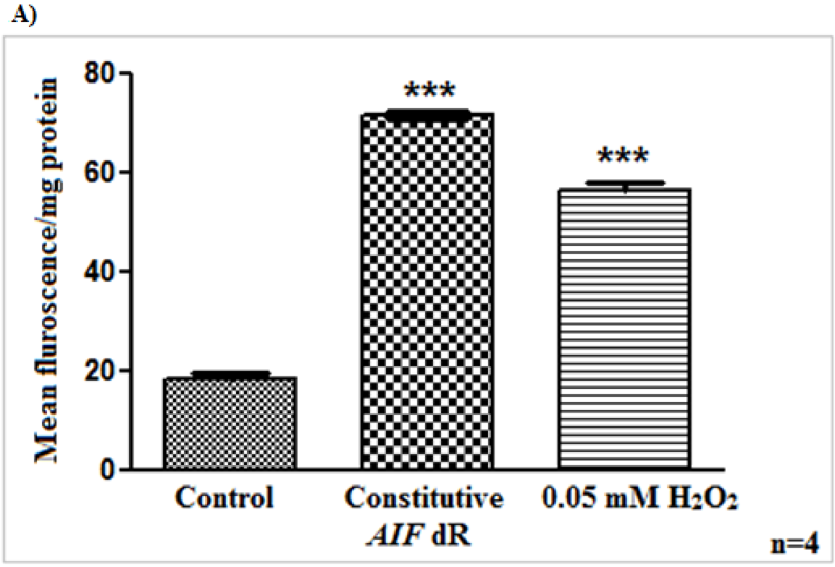
4.2.1 Assessment of relation between AIF and ROS

Cerebellar granule cells of Harlequin (Hq) mutant mouse characterized with oxidative trauma, indicate that AIF may be playing a pivotal role in ROS homeostasis (Klein *et al*., 2002). Hence, to study the same and also to comprehend the vacuolated phenotype of constitutive *AIF* dR cells, ROS levels were estimated. Higher ROS levels were found in constitutive *AIF* dR cells as compared to control cells (Fig. 4.1A).

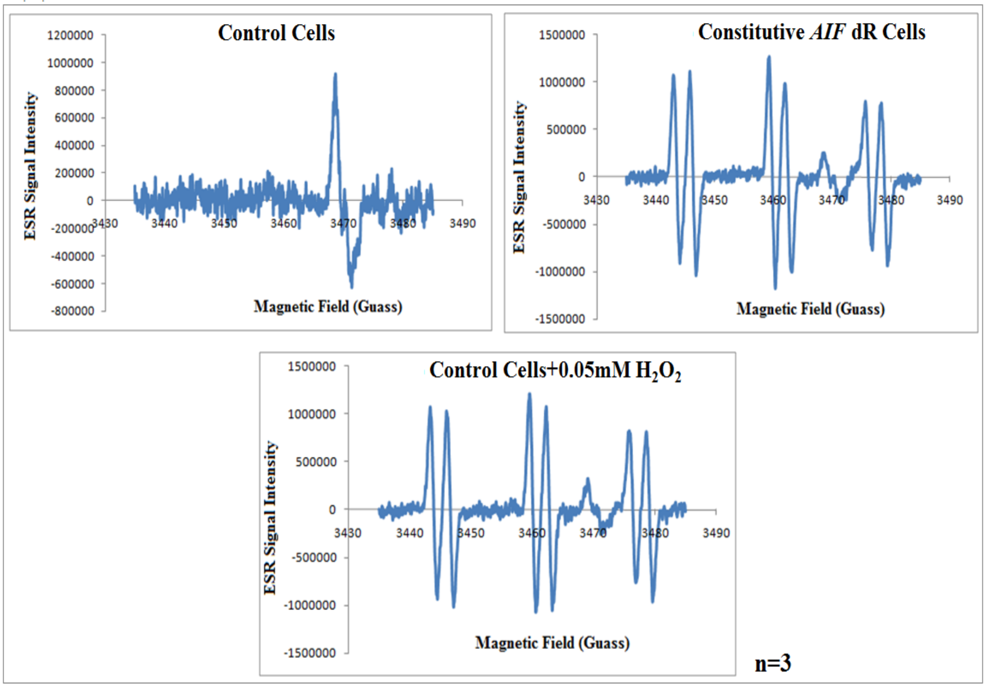
The presence of ROS was further monitored by EPR spectroscopy using POBN as a spin trap. Constitutive *AIF* dR cells exhibited triplets of POBN adducts which were absent in control cells, showing the presence of hydroxyl radicals ($OH^{\cdot}$) in constitutive *AIF* dR cells (Fig. 4.1B).

To support this data, we observed if supplementation of glutathione (GSH) could restore the growth and developmental morphogenesis of constitutive *AIF* dR cells by imitating the purpose of AIF because GSH is known to maintain the redox metabolism of the cell. Constitutive *AIF* dR cells in the presence of 10 mM GSH exhibited rescue in cellular growth (Fig. 4.1C) as compared to untreated constitutive *AIF* dR cells.

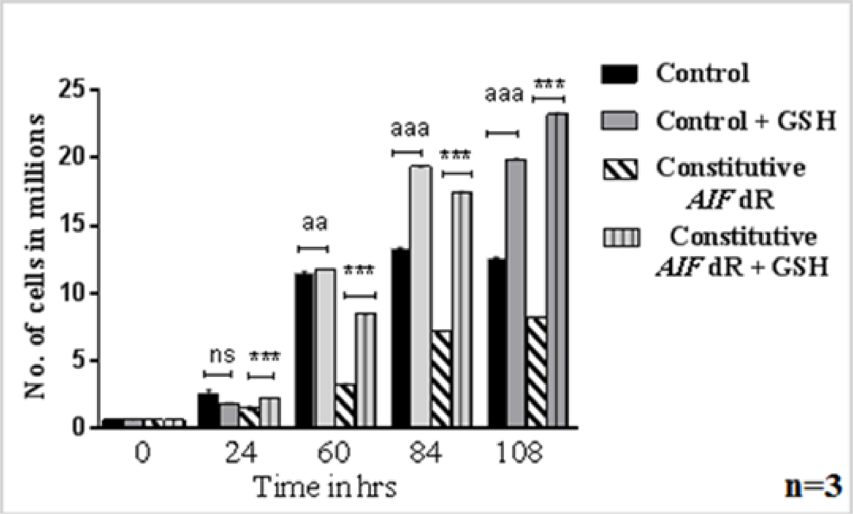
Regulated ROS levels were found to be indispensable during multicellular development in *D. discoideum* (Bloomfield and Pears, 2003). Hence, defects due to an increase in ROS levels in constitutive *AIF* dR cells could be restored by treating cells with GSH. Supplementation with GSH led to rescue in the multicellular developmental delay (Fig. 4.1D). Development was augmented by 12 hrs in GSH treated constitutive *AIF* dR cells as compared to untreated constitutive *AIF* dR cells, the former demonstrating mature fruiting bodies at 36 hrs (Fig. 4.1D) whereas untreated constitutive *AIF* dR cells showed the fruiting bodies formation at 48 hrs of treatment. Although control cells revealed fruiting body formation at 24 hrs with or without GSH, the cells treated with GSH contained abnormal cell structures that were not present in untreated control cells (Fig. 4.1D), suggesting that quenched ROS might be important for proper development (Covarrubias *et al.*, 2008). Our data reestablish that AIF could behave as a regulator of ROS.



B)


Control Cells
ESR Signal Intensity
1200000
1000000
800000
600000
400000
200000
0
-200000
-400000
-600000
-800000
3430
3440
3450
3460
3470
3480
3490
Magnetic Field (Guass)
Constitutive AIF dR Cells
1500000
1000000
500000
0
-500000
-1000000
-1500000
Control Cells+0.05mM H2O2
n=3

C)


No. of cells in millions
25
20
15
10
5
0
ns
***
aa
aaa
0
24
60
84
108
Time in hrs
Control
Control + GSH
Constitutive AIF dR
Constitutive AIF dR + GSH
n=3

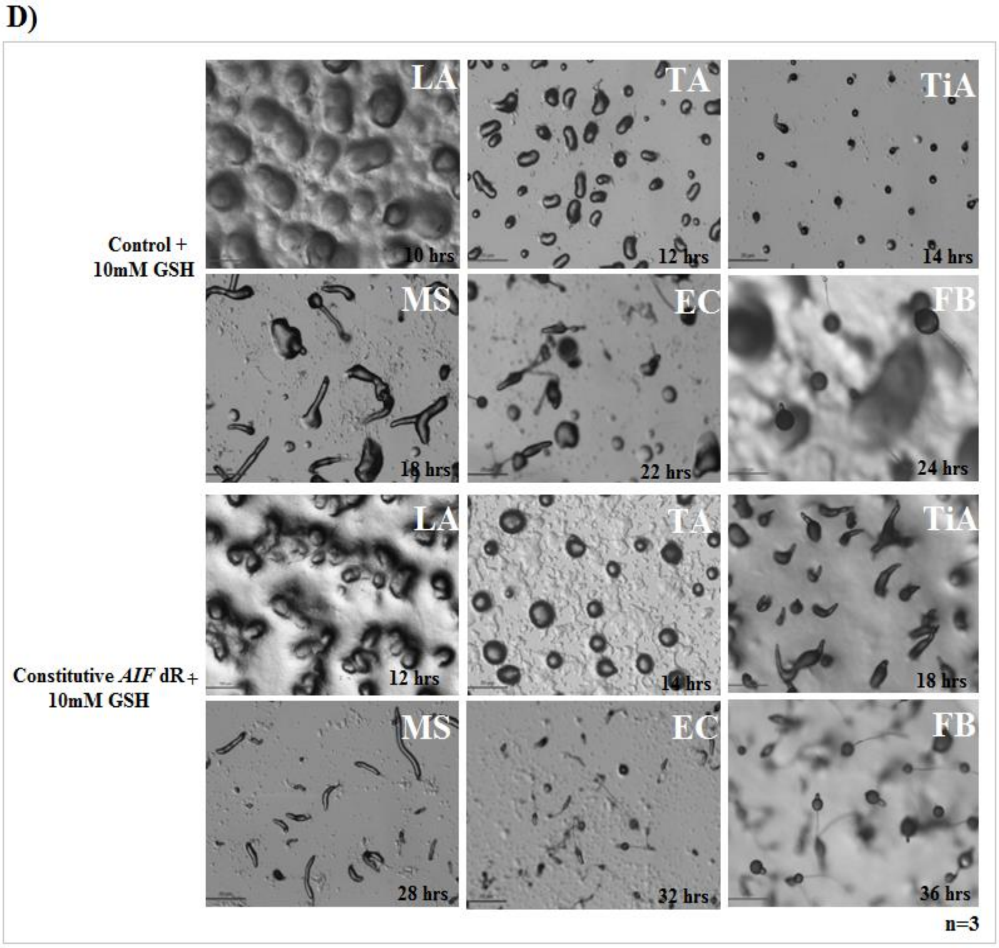
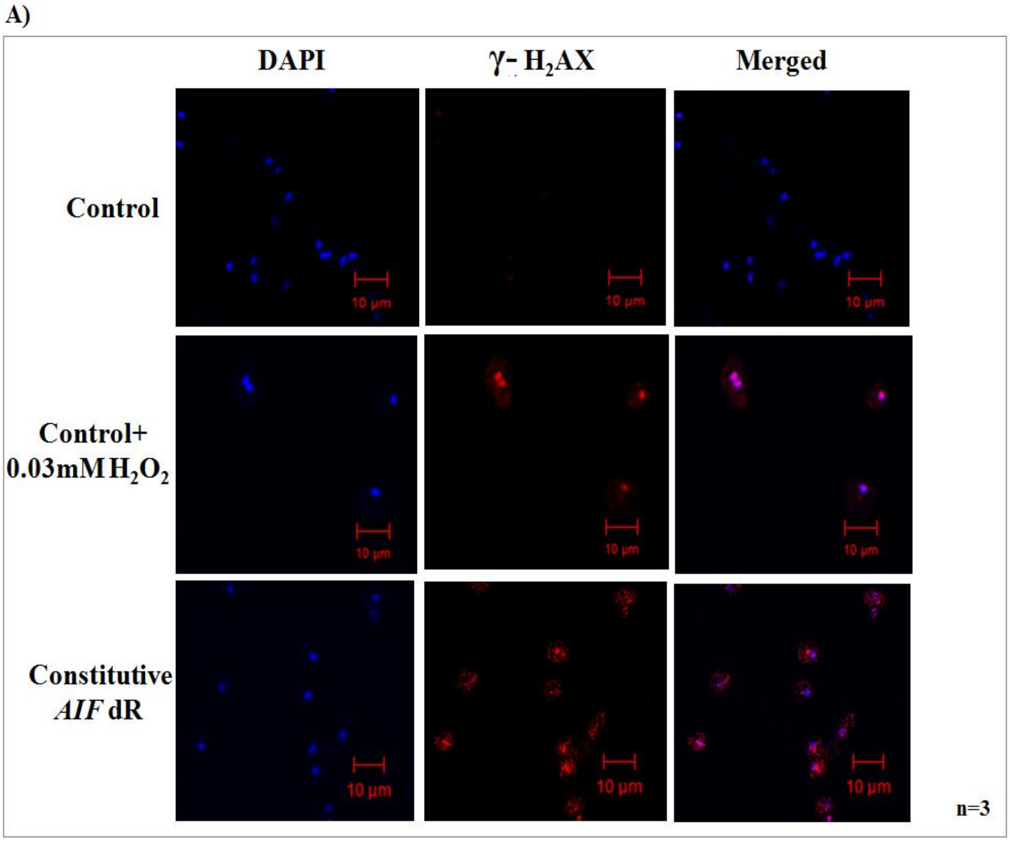


Fig. 4.1: Assessment of relation between AIF and ROS: A) Fluorimetric estimation of ROS using DCFDA dye. Significant elevation in ROS levels was observed in constitutive *AIF* dR cells compared to H_2O_2 treated control which was kept as a positive control. Data are a representation of mean ± S.E. values of four independent experiments. ***p<0.0001 compared to control. **B) EPR profile of *D. discoideum* cells**. The spin trapping spectra were achieved by three signal-averaged scans by EPR spectrometer. POBN adducts were detected in constitutive *AIF* dR cells but not in control cells. 0.05mM treated control cells were kept as a positive control. **C) Effect of GSH on the growth of constitutive *AIF* dR cells.** 10mM GSH partially restored the growth of constitutive *AIF* dR cells compared to untreated constitutive *AIF* dR cells. Data are representative of three independent experiments and represented as mean ± S.E. ns- non significant; ***p<0.0001 compared to untreated *AIF* dR cells. **D) Effect of GSH on the developmental morphogenesis of**

constitutive *AIF* dR cells. 10mM GSH rescued the development of constitutive *AIF* dR cells compared to untreated constitutive *AIF* dR cells. The developmental stages are named on images (LA- Loose Aggregate, TA- Tight Aggregate, TiA- Tipped Aggregate, MS- Migrating Slug, EC- Early Culminant, FB- Fruiting Body); Scale bar =10 µm, Magnification=4X.

4.2.2 Analysis of DNA damage and Protein Carbonyl (PC) content

ROS production leads to DNA damage; the most abundant being base modification and gamma-H2AX protein phosphorylation (Minami *et al*., 2005). Gamma-H2AX foci formation was observed in constitutive *AIF* dR cells as compared to control cells indicating DNA damage (Fig. 4.2A). PC is also one of the oxidative stress markers (Mekrungruangwong *et al*., 2012). Hence, PC content was monitored in constitutive *AIF* dR cells. PC content was significantly higher (*p*=0.0004) in constitutive *AIF* dR cells as compared to control cells (Fig. 4.2B).



B)

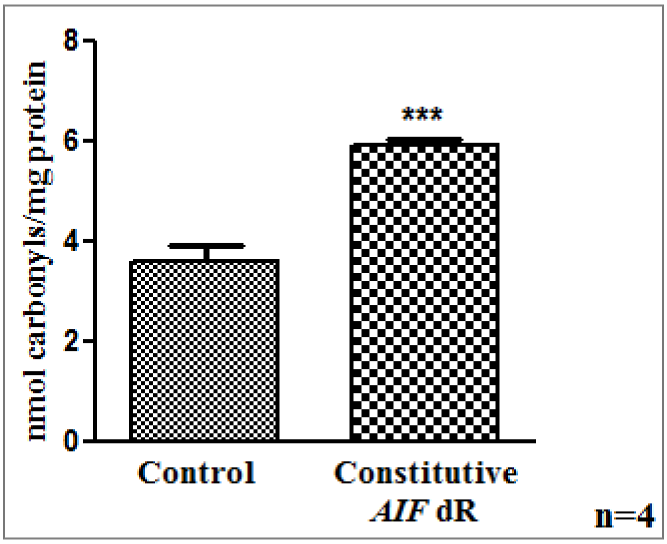


Fig. 4.2: A) DNA damage in constitutive *AIF* dR cells. Immunofluorescence staining for DNA damage was done using γ-H2AX Ab (red) and DAPI (blue). Constitutive *AIF* dR cells exhibited γ-H2AX foci formation (pink) indicating DNA damage compared to control cells. 0.03mM H_2O_2 treated cells were kept as a positive control. Scale bar =10 µm, Magnification: 60X. **B) Spectrophotometric determination of Protein Carbonyl (PC) content by DNPH assay.** The PC content was observed to be significantly higher in constitutive *AIF* dR cells compared to control cells. Data are representative of four independent experiments which represented as mean ± S.E. ***p<0.0001 compared to control.

4.2.3 Free cytosolic calcium [Ca^{2+}] levels

AIF downregulation and intensification in cellular ROS pool may affect cytosolic calcium homeostasis. Calcium estimation revealed enhanced free cytosolic calcium in constitutive *AIF* dR cells as compared to control cells (Fig. 4.3), signifying the role of AIF in sustaining calcium homeostasis via upholding the mitochondrial membrane potential and integrity of the mitochondrial membrane.

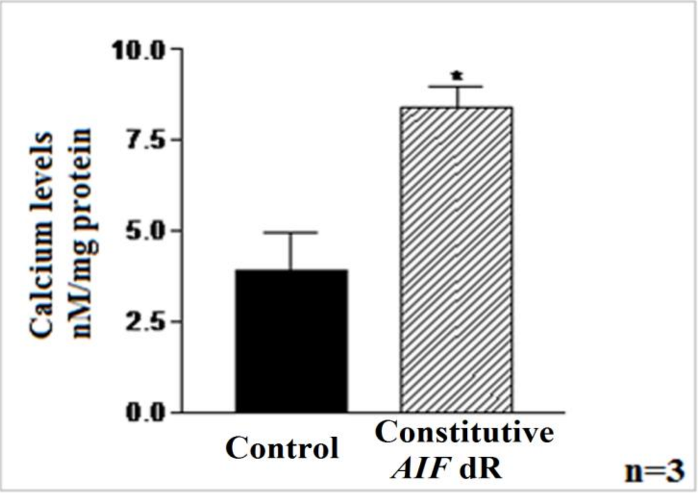


Fig. 4.3: Intracellular calcium levels in constitutive *AIF* dR cells: Fluorimetric measurement of intracellular calcium levels by Fura-2AM disclosed an increase in $[Ca^{2+}]_i$ levels in constitutive *AIF* dR cells compared to control cells. Data are represented as mean ± S.E. of three independent experiments. $^*p<0.01$ compared to control.

4.2.4 Mitochondrial membrane potential (MMP)

The loss of MMP is a hallmark feature of cell death. Studies on MMP alterations revealed that constitutive *AIF* dR cells unveiled reduced fluorescence i.e. lower $\Delta\psi m$, even without oxidant treatment as compared to control cells, specifying compromised MMP (Fig. 4.4A) which validates our earlier results of increased free calcium levels. Reduction in MMP was observed at 2 hrs post 0.03mM H_2O_2 treatment with significant MMP loss occurring at 3 hrs (Fig. 4.4A) in constitutive *AIF* dR cells. On the contrary, 0.03mM H_2O_2 treated control *D. discoideum* cells displayed MMP reduction at 3 hrs with significant reduction at 5 hrs.

Changes in MMP were confirmed by JC-1 staining also. Healthy control cells showed mitochondrial red JC-1 aggregates in FL2 channel while green JC-1 monomers in apoptotic cells are detectable in the FL1 channel. A decrease in red fluorescence i.e. ~95.54% and 95.79% in constitutive *AIF* dR cells is indicative of depolarization in the mitochondrial membrane at 3 hrs post 0.03mM H_2O_2 and 0.05mM H_2O_2 treatment respectively as compared to ~ 60% in control under both doses as shown in Fig. 4.4B. Constitutive *AIF* dR cells also showed a significant drop in red fluorescence in 95.42% cells compared to 1.67% control cells. These results were also supported by increased free cytosolic Ca^{2+} levels (Fig. 4.3).

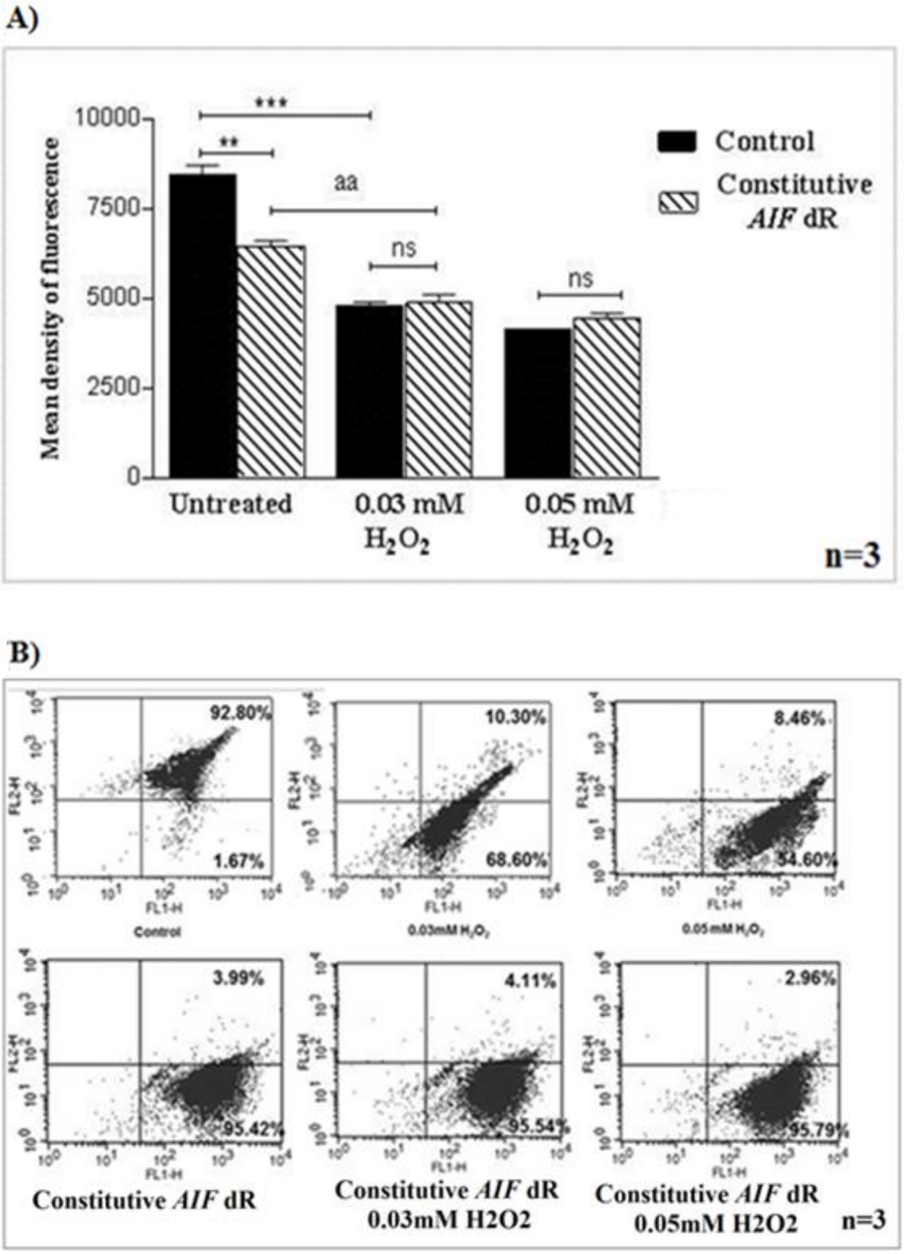


Fig. 4.4: Mitochondrial membrane potential changes induced by oxidative stress: A) Densitometric analysis of MMP changes post 3 hrs treatments of 0.03mM and 0.05mM H_2O_2. $^{***}p<0.001$ compared to oxidative stress; $^{**}p<0.01$ compared to control; $^{aa}p<0.01$ compared to oxidative stress. **B)** A dot plot of red fluorescence (FL2) versus green fluorescence (FL1) resolved in

control cells, constitutive *AIF* dR cells and 0.03mM and 0.05mM H_2O_2 treated (3 hrs) control and constitutive *AIF* dR cells. Constitutive *AIF* dR cells showed ~95% (FL1) cells with compromised mitochondrial membrane potential with and without oxidative stress as compared to 1.67% (FL1) without treatment and ~60% (FL1) in control cells under oxidative stress. Data are representative of three independent experiments.

4.2.5 AIF translocation under oxidative stress

Constitutive *AIF* downregulated cells exposed to 0.03mM and 0.05mM doses of H_2O_2 showed early translocation of AIF to the nucleus within 3 hrs (Fig. 4.5). 0.03mM H_2O_2 did not cause translocation of AIF whereas 0.05mM H_2O_2 caused translocation of AIF at 3 hrs in control cells, implying constitutive *AIF* dR cells are more prone to oxidative stress induced cell death.

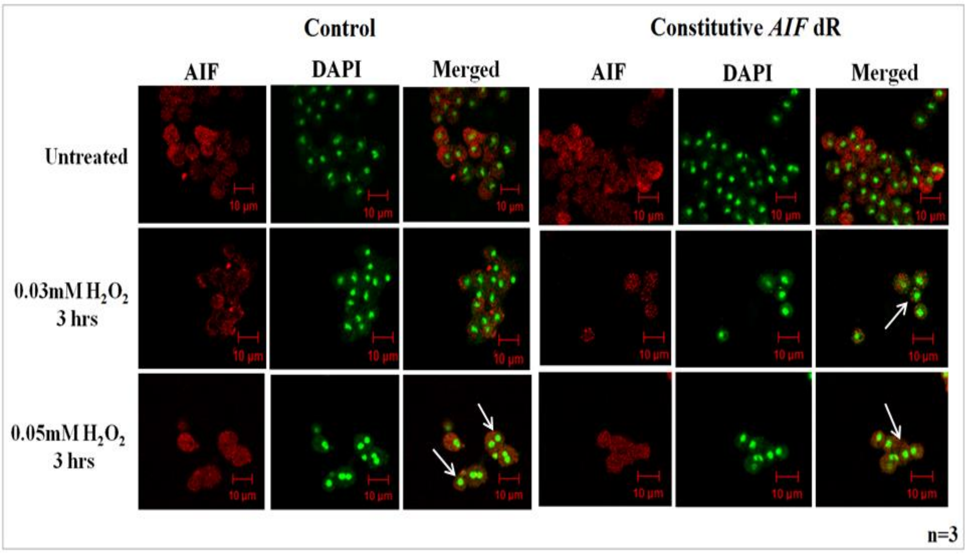


Fig. 4.5: Mitochondrio-nuclear translocation of AIF: In response to oxidative stress, constitutive *AIF* dR cells displayed AIF translocation from mitochondria to the nucleus as early as 3 hrs. Data are representative of three independent experiments. Red color: AIF, Green: DAPI (pseudo color), Fluorescent Green: Translocation of AIF to the nucleus. Scale bar =10 µm, Magnification: 60X.

4.2.6 Annexin V-FITC and PI staining under oxidative stress

Based on our earlier results, control *D. discoideum* cells subjected to 0.03mM H_2O_2 stress showed PS externalization (Annexin-V staining) at 5 hrs and PI staining at 12 hrs while 0.05mM H_2O_2 showed both Annexin-V staining and PI staining at 3 hrs (Rajawat *et al*., 2014a), suggesting *D. discoideum* exhibits paraptotic cell death at lower dose and necrotic cell death at higher dose of H_2O_2. Nonetheless, 0.03mM H_2O_2 caused Annexin-V as well as PI staining as early as 3 hrs in constitutive *AIF* dR cells, indicating that these cells exhibit necrotic cell death under 0.03mM H_2O_2 treatment as compared to paraptosis in control cells (Fig. 4.6). Interestingly, constitutive *AIF* dR cells showed PS externalization even under non-oxidant treatment (Fig. 4.6), further establishing compromised mitochondrial membrane structure.

All the above cell death factors exemplified *AIF* dR cells to demonstrate an oxidative stress sensitive phenotype. In conclusion, constitutive *AIF* dR cells are more vulnerable to cell death under oxidative stress (Fig. 4.7).

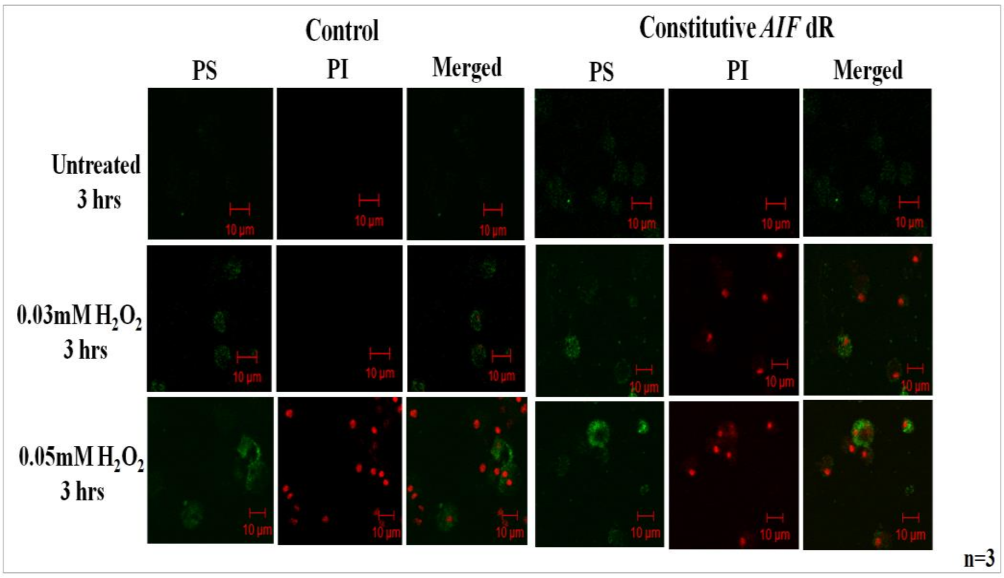


Fig. 4.6: Annexin V-PI dual staining of constitutive *AIF* dR cells under oxidative stress. Externalization of Phosphatidyl Serine (PS) was observed in constitutive *AIF* dR cells even in non-oxidant conditions. At 0.03mM and 0.05mM H_2O_2 doses, constitutive *AIF* dR cells exhibited Annexin V-PI dual positive cells as early as 3 hrs, while the same effect was found in control cells

with 0.05mM H_2O_2. Data are representative of three independent experiments. Scale bar =10 µm, Magnification: 60X.

4.3 Discussion

A mitochondrion is a potent source of ROS (Green and Reed, 1998). AIF, being a mitochondrial inner membrane flavoprotein, may have a role in maintaining the mitochondrial complex I assembly (Modjtahedi *et al*., 2006). *AIF* knockdown illustrates an increase in superoxide production due to diminished complex I activity leading to oxidative stress (Varecha *et al.*, 2012). Hq mice are associated with high oxidative stress, proposing AIF's function as an antioxidant (Lipton and Bossy-Wetzel, 2002; Krantic *et al*., 2007; Zhu *et al*., 2007). On the contrary, a few studies failed to support AIF's ROS scavenging activity (Pospisilik *et al*., 2007; Chinta *et al*., 2009). Increased ROS levels were found in constitutive *AIF* dR cells, confirming AIF as a ROS regulator (Fig. 4.1A, 4.1B). Alterations in *AIF* levels may lead to leakage of electrons (e^-) during electron transport, thus producing more ROS. Slower cell proliferation rate and multicellular development of constitutive *AIF* dR cells may be attributed to amplified ROS levels that were reversed upon exogenous treatment of GSH, a reducing agent signifying that it might be mimicking AIF in regulating ROS (Fig. 4.1C, Fig. 4.1D). The oxido-reductase domain [NAD(P)H and FAD binding domain] of AIF can nullify the ROS produced by mitochondrial complexes (mainly I and III), and depletion of the former, may deteriorate the function of these electron chain complexes, thereby enhancing the intracellular ROS pool which could result in oxidative DNA and protein damage in constitutive *AIF* dR cells (Fig. 4.2A, Fig. 4.2 B).

AIF is solely confined to mitochondria but apoptotic challenges cause its translocation from mitochondria to the nucleus with depolarization of mitochondrial membrane (Susin *et al*., 1999). Since mitochondrion is a reservoir of calcium, depolarization of MMP by higher ROS in constitutive *AIF* dR cells might also cause leakage of calcium pool from mitochondria to cytosol, thus amplifying cytosolic calcium (Fig. 4.3). *D. discoideum* cells in S and early G2 exhibit relatively high levels of cellular Ca^{2+} and displayed a prestalk tendency after starvation (Azhar *et al.*, 2001) which was observed in our study too. Constitutive *AIF* dR cells were arrested in S phase of cell cycle and exhibited enhanced Ca^{2+} levels and prestalk tendency during development (Fig. 3.6, Fig. 4.3). Oxidative stress due to the accumulation of excessive ROS stimulates cell death (Elmore, 2007). Furthermore, ~80% drop in *AIF* expression causes marked susceptibility to oxidative stress mediated cell death as observed in Hq mutant mice (Klein *et al.*, 2002). Under oxidative stress, *AIF* downregulated *D. discoideum* cells exhibited early loss of mitochondrial polarization compared to control cells (Fig. 4.4). AIF is also reported to be contributing in the maintenance of mitochondrial potential and morphology (Cheung *et al.*, 2006; Sevrioukova, 2011). In line with these reports, constitutive *AIF* dR cells presented compromised MMP even under normal conditions (Fig. 4.4), denoting AIF may be responsible for maintaining mitochondrial morphology. Mitochondrio-nuclear translocation of AIF and exposure of phosphatidylserine on the outer leaflet of the plasma membrane are the characteristics of caspase independent cell death (Cande *et al.*, 2002). Our data show early AIF translocation during oxidative stress induced cell death in constitutive *AIF* dR cells (Fig. 4.5). Annexin V and PI dual staining were noted positive as both Annexin- V, indicative of PS externalization and PI staining were observed in response to 0.03mM and 0.05mM H_2O_2 as early as 3 hrs. Annexin V staining was noticed even in normal physiological conditions in constitutive *AIF* dR cells (Fig. 4.6). Our results are consistent with the previous findings that *AIF* downregulation sensitizes the cells to oxidative stress induced cell death (Liu *et al.*, 2005; Schulthess *et al.*, 2009). The role of AIF in balancing the mitochondrial homeostasis would be discussed in the next chapter.

This work is published in the *BBA General Subject* journal entitled ‘Potential role of Apoptosis Inducing Factor in evolutionarily significant eukaryote, *Dictyostelium discoideum* survival’ (Kadam *et al.*, 2017).

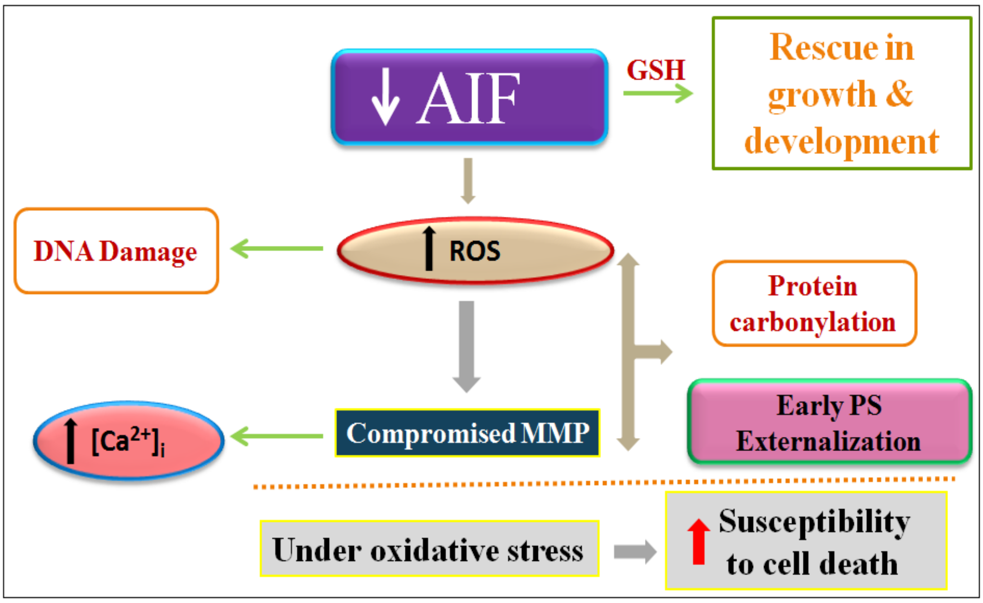


Fig. 4.7: Effect of *AIF* downregulation on cell death under oxidative stress in *D. discoideum*

4.4 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. Azhar M, Kennady PK, Pande G, Espiritu M, Holloman W, Brazill D, Gomer RH, Nanjundiah V (2001) Cell cycle phase, cellular Ca^{2+} and development in *Dictyostelium discoideum.* Int. J. Dev. Biol. 45: 405-414.
3. Bloomfield G, Pears C (2003) Superoxide signalling required for multicellular development of *Dictyostelium.* J Cell Sci 116:3387-3397.
4. Cande C, Cohen I, Daugas E, Ravagnan L, Larochette N, Zamzami N, Kroemer G (2002) Apoptosis-inducing factor (AIF): a novel caspase-independent death effector released from mitochondria. Biochimie 84:215-222.
5. Cheung EC, Joza N, Steenaart NA, McClellan KA, Neuspiel M, McNamara S, MacLaurin JG et al (2006) Dissociating the dual roles of

apoptosis-inducing factor in maintaining mitochondrial structure and apoptosis. EMBO J 25:4061-4073.

6. Chinta SJ, Rane A, Yadava N, Andersen JK, Nicholls DG, Polster BM (2009) Reactive oxygen species regulation by AIF and complex I-depleted brain mitochondria. Free Radic Biol Med 46:939-947.
7. Covarrubias L, Hernández-García D, Schnabel D, Salas-Vidal E, Castro-Obregón, S, (2008) Function of reactive oxygen species during animal development: Passive or active? Dev Biol 320:1-11.
8. Elmore S (2007) Apoptosis: A Review of Programmed Cell Death. Toxicol Pathol 35:495-516.
9. Green DR, Reed JC (1998) Mitochondria and apoptosis. Science 281: 1309-1312.
10. Hasnain SE, Taneja TK, Sah NK, Mohan M, Pathak N, Sahdev S, Athar, M, Begum R (1999) In vitro cultured Spodoptera frugiperda insect cells: model for oxidative stress-induced apoptosis. J Biosci 24:13-19.
11. Klein JA, Longo-Guess CM, Rossmann MP, Seburn KL, Hurd RE, Frankel WN, Bronson RT, Ackerman SL (2002) The harlequin mouse mutation down-regulates apoptosis-inducing factor. Nature 419:367-374.
12. Krantic S, Mechawar N, Reix S, Quirion R (2007) Apoptosis-inducing factor: a matter of neuron life and death. Prog Neurobiol 81:179-196.
13. Lipton SA, Bossy-Wetzel E (2002) Dueling activities of AIF in cell death versus survival: DNA binding and redox activity. Cell 111:147-150.
14. Liu PL, Chen YL, Chen YH, Lin SJ, Kou YR (2005) Wood smoke extract induces oxidative stress-mediated caspase-independent apoptosis in human lung endothelial cells: role of AIF and EndoG. Am J Physiol Lung Cell Mol Physiol 289:L739-L749.
15. Mekrungruangwong T, Seenak P, Luangaram S, Thongsri T, Kumphune S (2012) The serum protein carbonyl content level in relation to exercise stress test. Int J Health Allied Sci 1:200-203.
16. 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.

17. 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.
18. Miramar MD, Costantini P, Ravagnan L, Saraiva LM, Haouzi D, Brothers G, Penninger JM et al (2001) NADH oxidase activity of mitochondrial apoptosis-inducing factor. J Biol Chem 276:16391-16398.
19. Modjtahedi N, Giordanetto F, Madeo F, Kroemer G (2006) Apoptosis-inducing factor: vital and lethal. Trends Cell Biol 16:264-272.
20. Ohiro Y, Garkavtsev I, Kobayashi S, Sreekumar KR, Nantz R, Higashikubo BT, Duffy SL et al (2002) A novel p53-inducible apoptogenic gene, PRG3, encodes a homologue of the apoptosis-inducing factor (AIF). FEBS Lett 524:163-171.
21. Porter AG, Urbano AG (2006) Does apoptosis-inducing factor (AIF) have both life and death functions in cells? Bioessays 28:834-843.
22. Pospisilik JA, Knauf C, Joza N, Benit P, Orthofer M, Cani PD, Nakashima T et al (2007) Targeted deletion of AIF decreases mitochondrial oxidative phosphorylation and protects from obesity and diabetes. Cell 131:476-491.
23. 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.
24. Sah NK, Taneja TK, Pathak N, Begum R, Athar M, Hasnain SE (1999) The baculovirus anti-apoptotic p35 gene also functions via an oxidant dependent pathway. Proc Natl Acad Sci USA 96:4838-4843.
25. Schulthess FT, Katz S, Ardestani A, Kawahira H, Georgia S, Bosco D, Bhushan A, Maedler K (2009) Deletion of the mitochondrial flavoprotein apoptosis inducing factor (AIF) induces β-cell apoptosis and impairs β-cell mass. PLoS ONE 4:e4394.
26. Sevrioukova IF (2011) Apoptosis inducing factor: structure, function and redox regulation. Antioxid Redox Signal 14:2545-2579.

27. 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.
28. Varecha M, Paclova D, Prochazkova J, Matula P, Cmarko D, Kozubek M (2012) Knockdown of apoptosis-inducing factor disrupts function of respiratory complex I. Biocell 36:121-126.
29. Zhu C, Wang X, Huang Z, Qiu L, Xu F, Vahsen N, Nilsson M et al (2007) Apoptosis-inducing factor is a major contributor to neuronal loss induced by neonatal cerebral hypoxia-ischemia. Cell Death Differ 14:775-784.