Oxidative phosphorylation studies in constitutive *AIF* downregulated *D. discoideum* cells

5.1 Introduction

AIF is ubiquitously expressed mitochondrial protein which was initially recognized as a caspase independent cell death effector (Susin *et al*., 1999). AIF is a bi-functional protein with an oxidoreductase and apoptogenic function. Its apoptogenic function along with the underlying mechanism has been well documented (Wang *et al*., 2002; Cande *et al*., 2004). The FAD and NADH binding domains of AIF distinguish it from the pro-apoptotic proteins, imparting its dual role in cell death and cell survival. The strong structural and sequence homology to bacterial NADH oxidase, plant ascorbate and ferredoxin reductases denote that AIF has oxidoreductase activity (Senda *et al*., 2000). The information hinted towards the possibility that AIF can accomplish a non-apoptotic enzymatic function in the healthy cells. Interestingly, its inherent NADH oxidase activity which is independent of its apoptotic activity (Miramar *et al*., 2001) is linked to mitochondrial homeostasis maintenance (Churbanova and Sevrioukova, 2008). AIF mutation in humans has been associated with impaired Electron Transport Chain (ETC) function that results in mitochondrial encephalomyopathy. Arginine 201 (R201) residue of AIF is the part of the FAD binding pocket that confers conformational stability to the flavoprotein. An inherited tri-nucleotide deletion in exon 5 of human AIF caused the ablation of the Arg201 (R201deletion) which showed abnormal FAD binding to AIF and consequently impaired structural stability, redox activities and oxidative phosphorylation (OXPHOS) functioning (Ghezzi *et al*., 2010). The deletion or down-modulation of *AIF* causes dysfunction to the most essential energy generating machinery of the cell, mitochondrial respiratory chain and complex I was found to be most affected (van Empel *et al*., 2005; Brown *et al*., 2006; Cheung *et al*., 2006; Ishimura *et al*., 2008). Occasionally, a compromised complex III or IV activities could also be detected in cells or tissues lacking

AIF (Vahsen *et al*., 2004; Joza *et al*., 2005). AIF is projected to be involved in cellular bioenergetics, assisting in the stabilization and/ maintenance of assembly of the ETC (Vahsen *et al*., 2004). However, it is still not clear which ETC complex gets affected due to the loss of *AIF* and how AIF participates in mitochondrial metabolism, though it is largely being attributed to its NADH dependent oxidoreductase activity. Respiratory chain defects and the mtDNA quality are interconnected and it may vary depending upon the cell type and OXPHOS demand, suggesting that AIF can influence respiratory chain and mtDNA integrity. AIF, apart from stabilizing assembly of mitochondrial ETC complexes, it is implicated in mitochondrial DNA (mtDNA) maintenance (Ghezzi *et al*., 2010).

Recently, *D. discoideum* has been widely used to study mitochondrial biogenesis and diseases (Annesley and Fisher, 2009). Being caspase independent, it provides a better model to explore non-apoptotic functions of AIF without the interference of caspases. Hence, we aim to explore the conserved role of AIF in the maintenance of proper mitochondrial respiratory activity and mtDNA content during the unicellular and multicellular phases of *D. discoideum.*

The outcomes from the present study would be instrumental for the elucidation of AIF's potent non-apoptotic activity and its impact on cell survival.

5.2 Results

To elucidate the mitochondrial function of AIF, effect of *AIF* downregulation was studied on mitochondrial respiration and ETC assembly along with ATP production in *D. discoideum*.

5.2.1 Estimation of Oxygen Consumption Rate (OCR) in constitutive *AIF* dR cells

The life cycle of *D. discoideum* cells exists in both unicellular and multicellular phases. In the presence of nutrients, these cells multiply as unicellular amoebae (vegetative growth) while under nutrient deprivation, they

enter a developmental cycle to form mature fruiting bodies. In the present study, OCR was measured in both vegetative and starved constitutive *AIF* dR cells.

5.2.1.1 OCR studies during vegetative stage

The rate of oxygen consumption is a vital indicator of mitochondrial respiration. As AIF takes part in the maintenance of mitochondrial respiration, OCR was measured in vegetative as well as starving constitutive *AIF* dR cells. Reduced OCR at complex I, complex III and complex IV with increased OCR at complex II was found in constitutive *AIF* dR compared to control cells (Fig. 5.1A), indicating compromised mitochondrial respiration. Oxidation OCR ratio is accurate indicator of partial respiratory chain deficiencies. CI/CII OCR ratio confirms the deficiency in CI relative to succinate oxidation (Rustin *et al*., 1991). CI/CII OCR ratio revealed that constitutive *AIF* dR cells exhibited ~50% reduction in CI dependent substrate oxidation relative to CII dependent substrate oxidation compared to control cells (Fig. 5.1B).

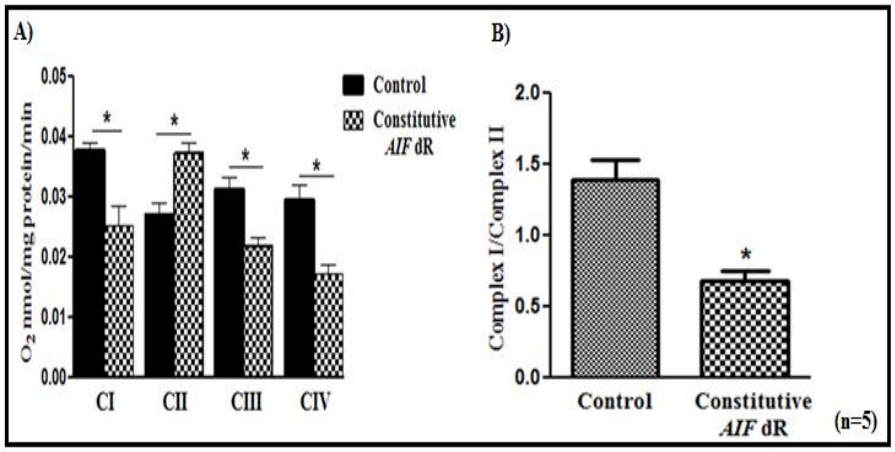


Fig. 5.1: Oxygen Consumption Rate (OCR) studies at vegetative stage of *D. discoideum*: A) Clark O_2 analysis showed reduced OCR at complex I (p=0.0243), complex III (p=0.0211) and complex IV (p=0.0127) activity in constitutive *AIF* dR cells compared to control cells. **B)** CI/CII OCR ratio in constitutive *AIF* dR cells showed reduced CI dependent substrate oxidation relative to CII dependent substrate oxidation as compared to control cells

(p=0.0102). Data are representation of SEM values of five independent experiments. *p<0.05 as compared to control.

5.2.1.2 OCR studies during developmental stages

Our previous results showed delayed development in constitutive *AIF* dR cells, signifying its role in multicellular development of *D. discoideum* (Kadam *et al*., 2017). Hence, *AIF* dR cells were subjected to development and OCR was measured at the major developmental stages of *D. discoideum*, i.e. aggregate, slug and fruiting body. Constitutively *AIF* dR cells showed significantly diminished OCR at complex I in the aggregate stage compared to control cells (Fig. 5.2A). Also, reduced OCR was observed at complexes I, III and IV at the slug stage (Fig. 5.2B) and at complex I and III activities at the fruiting body stage (Fig. 5.2C) of constitutive *AIF* dR compared to control cells. Taken together, these results indicate AIF's role towards regulating the assembly and/ activities of mitochondrial respiratory chain complexes and thereby OXPHOS maintenance during the developmental phase too in *D. discoideum*.

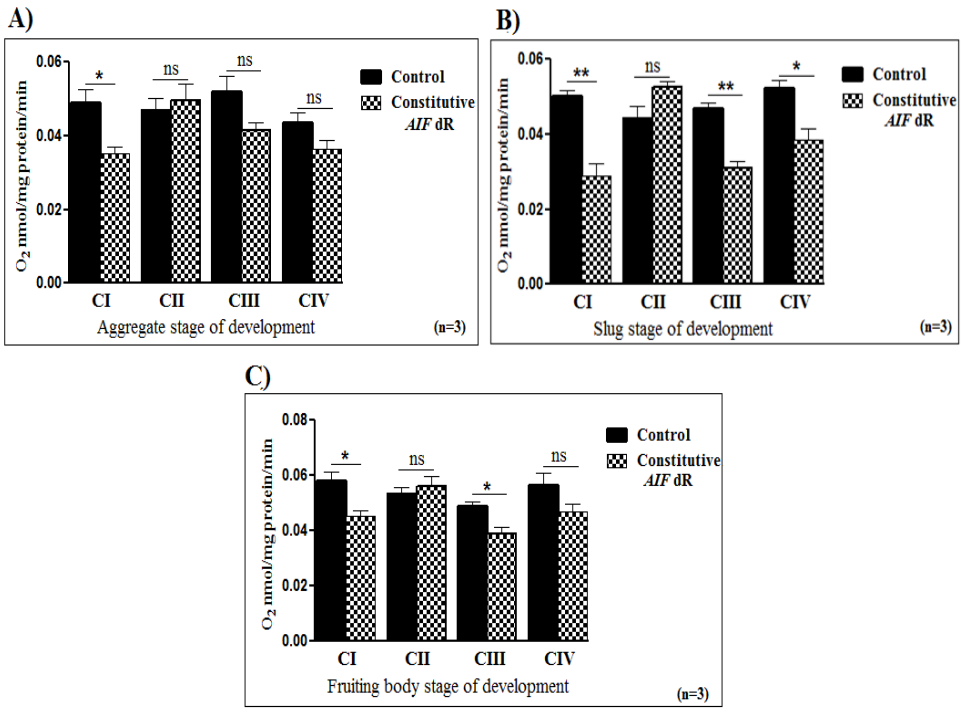


Fig. 5.2: Estimation of Oxygen Consumption Rate (OCR) studies at developmental stages in *D. discoideum*: A) Constitutive *AIF* dR cells exhibited significantly reduced activities of complex I (p=0.0248) compared to control cells during the aggregate stage. **B)** Constitutive *AIF* dR cells exhibited significantly reduced activities of complexes I (p=0.0038), III (p=0.0023) and IV (p= 0.0247) compared to control cells during the slug stage. **C)** Constitutive *AIF* dR cells showed significantly reduced activities at complexes I (p=0.0251) and III (p=0.0198) compared to control cells in the fruiting body. Data are representation of SEM values of three independent experiments. *p<0.05 and **p<0.01 as compared to control; ns= non-significant.

5.2.2 Investigating the effect of glutathione (GSH) on OCR

Previously it was shown that 10 mM GSH could restore the delay in *D. discoideum* growth and development regulating ROS levels in constitutive *AIF* dR cells (Kadam *et al*., 2017). To confirm the function of AIF as a ROS regulator, we monitored if exogenous treatment of GSH could restore activities of the ETC complexes in constitutive *AIF* dR cells as GSH is known to maintain the redox status of the cell. Constitutive *AIF* dR cells in the presence of 10 mM GSH showed restoration of OCR at complexes I and III of constitutive *AIF* dR as compared to untreated cells suggesting that oxido-reductase property of AIF might be implicated in balancing the ROS levels (Fig. 5.3).

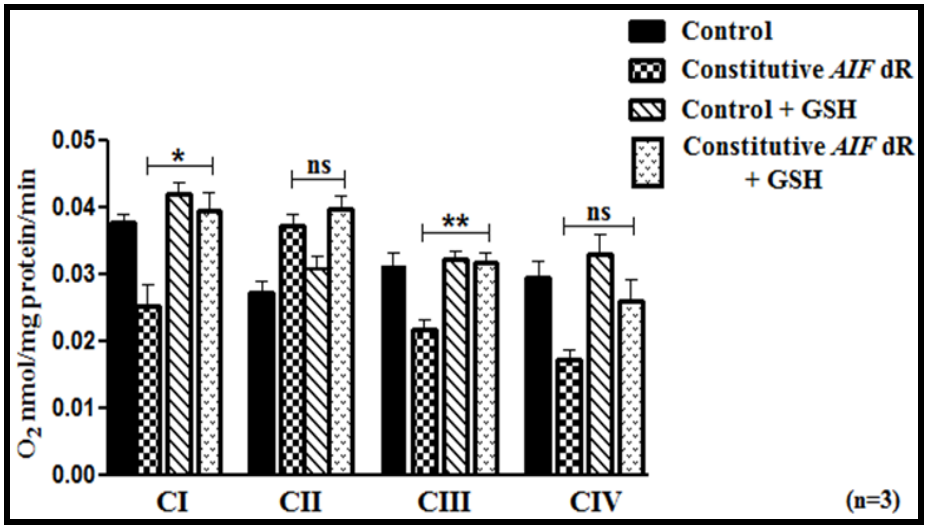


Fig. 5.3: OCR studies in constitutive *AIF* dR cells in the presence of 10mM GSH: 10 mM GSH could restore the oxygen consumption rate of constitutive *AIF* dR cells at complexes I (p=0.0272) and complex III (p=0.0079) compared to untreated constitutive *AIF* dR cells. Data are a representation of SEM values of three independent experiments. *p<0.05 and **p<0.01 as compared to control; ns= non-significant.

5.2.3 Total ATP and NAD^+ levels

Reduced AIF levels induced generation of more ROS due to impaired complex I function and thereby might lead to reduced ATP levels (Joza *et al*., 2008). Hence, high ROS in constitutive *AIF* dR cells may also be suggestive of impaired mitochondrial functioning, which could be established by estimating the ATP and NAD^+ levels. Constitutive *AIF* dR cells exhibited ~60% depletion in cellular ATP levels (Fig. 5.4A) and ~75% depletion in mitochondrial ATP levels (Fig. 5.4B). Also, ~30% reduction in NAD^+ levels was observed in constitutive *AIF* dR cells compared to control cells (Fig. 5.4C). These results indicate mitochondrial impairment and hence lower cellular energy supply owing to which proliferation rates were lower in constitutive *AIF* dR cells.

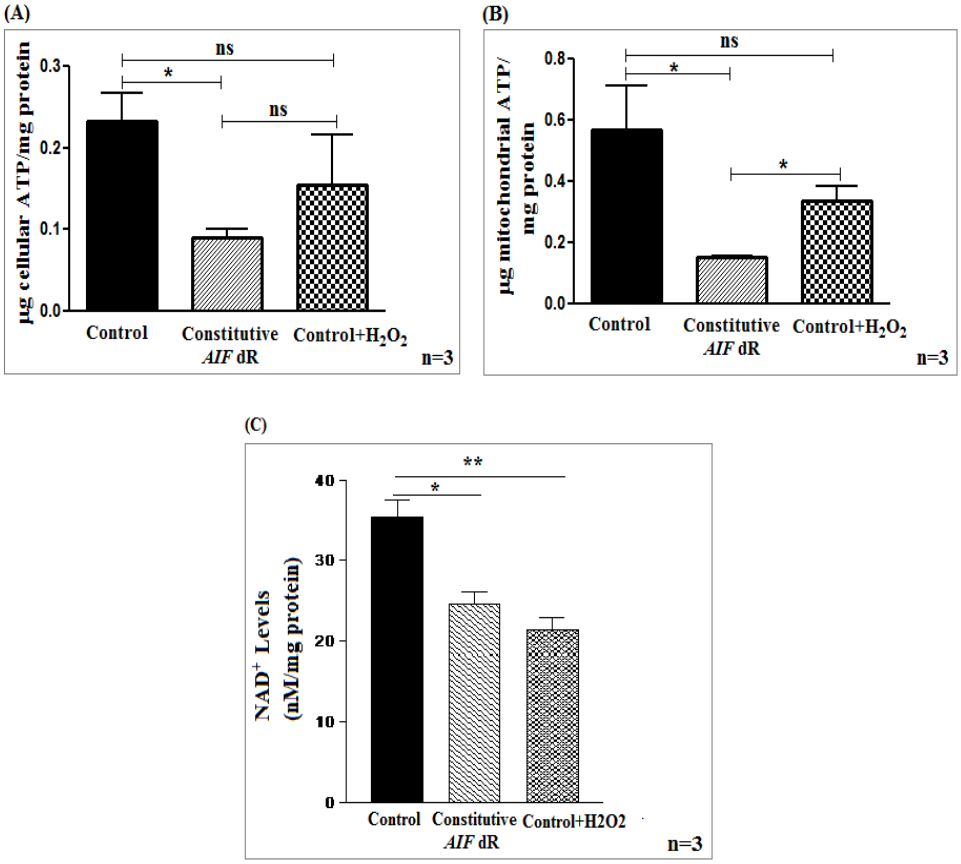
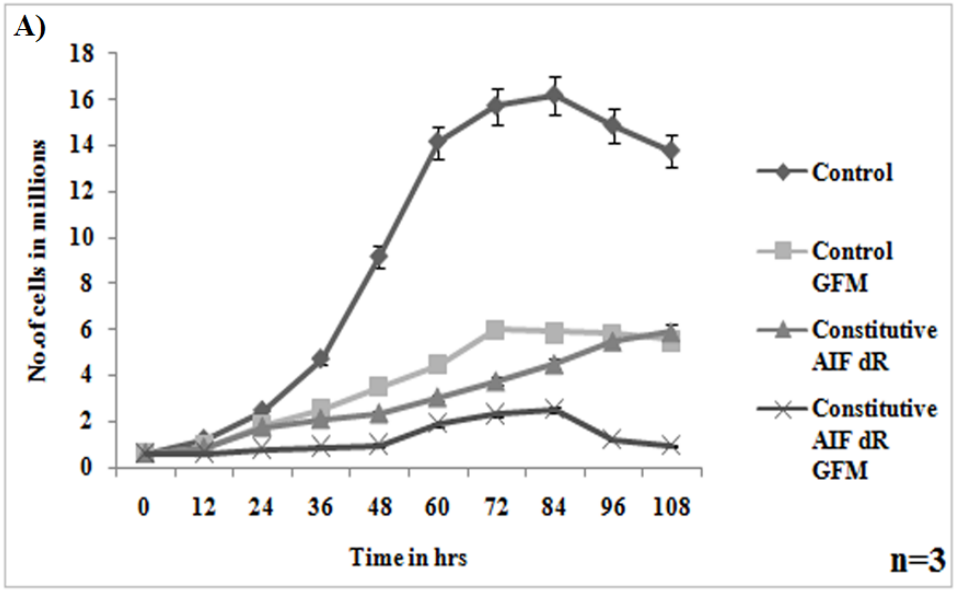


Fig. 5.4: Estimation of cellular & mitochondrial ATP and NAD^+ levels: A) and **B)** A significant drop in both cellular and mitochondrial ATP levels respectively were observed in constitutive *AIF* dR cells compared to control cells. 0.03mM H_2O_2 treated cells were used as a positive control. Data are a representation of SEM values of three independent experiments. **p*<0.05 compared to control. **C)** A significant reduction in NAD^+ levels was detected in constitutive *AIF* dR cells compared to control cells. 0.03mM H_2O_2 treated cells were kept as a positive control. Data are a representation of SEM values of three independent experiments. **p*<0.05 compared to control; ns=Non-significant.

5.2.4 Glucose dependency

As constitutive *AIF* dR cells have lessened energy production via oxidative phosphorylation, these cells might be dependent on glycolysis to compensate for the reduced mitochondrial ATP levels for survival. Hence, we monitored

the glucose dependency of these cells. This was demonstrated by the removal of glucose from the growth medium. Constitutive *AIF* dR cells failed to grow in glucose free medium (GFM) compared to control cells indicating that constitutive *AIF* dR cells may be more dependent on glucose for energy production where mitochondrial functioning is intact (Fig. 5.5A). Production of lactic acid is a downstream metabolic change showing more utilization of glycolysis for energy production. Quantitation of lactic acid showed a negligible difference in both control as well as constitutive *AIF* dR cells at 4^{th}, 6^{th} and 8^{th} day of cell growth (p=0.2927, p=0.1193 and p=0.244 respectively) (Fig. 5.5B) because another metabolic pathway might be operating for taking care of the excess lactic acid production.



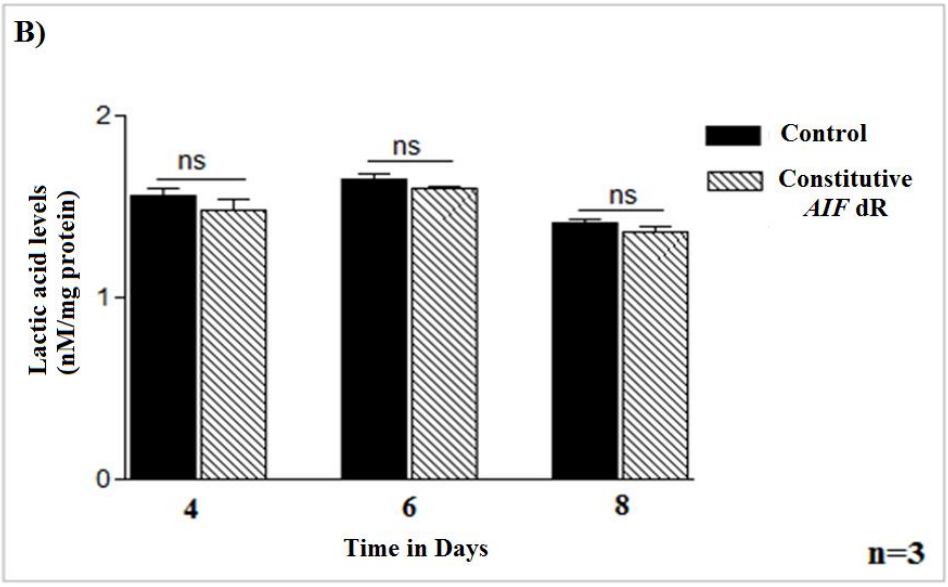
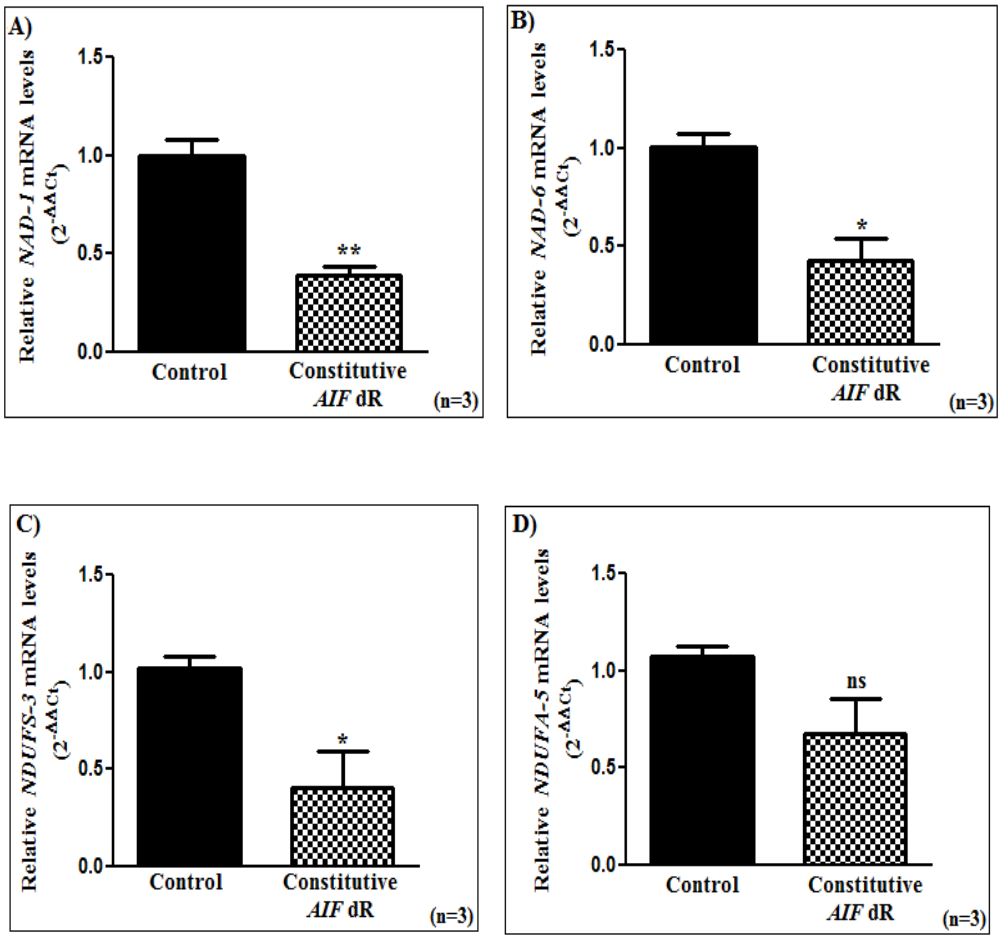


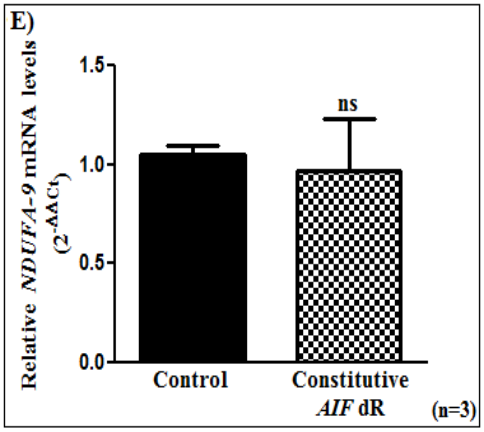
Fig. 5.5: Glucose dependency of constitutive *AIF* dR cells: A) Growth profile of *D. discoideum* in glucose free medium (GFM): Constitutive *AIF* dR GFM cells showed arrest in growth in the absence of glucose showing high glucose dependency whereas considerable growth was seen in control cells in GFM. Data is a representation of SEM values of three independent experiments. **B) Estimation of Lactic acid by HPLC in constitutive *AIF* dR cells:** No significant difference was found in lactic acid levels produced by control and constitutive *AIF* dR cells at 4^{th}, 6^{th} and 8^{th} day of cell growth. Data is a representation of SEM values of three independent experiments.

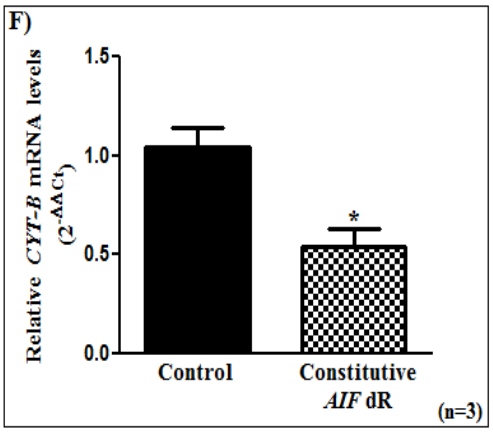
5.2.5 Analysis of the assembly of electron transport chain complexes

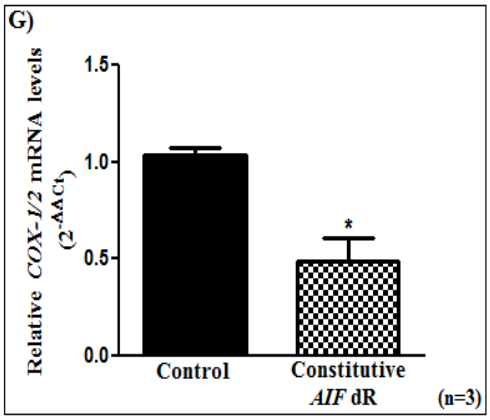
Due to loss of *AIF*, NAD1, NAD6, NDUFS3, NDUFA5 and NDUFA9 subunits of complex I, CYTB subunit of complex III, COX1/2 subunit of complex IV and ATP6 subunit of complex V were found to be affected in humans (Vahsen *et al*., 2004; Milasta *et al*., 2016). To study how AIF exerts its effect on the respiratory chain complexes of *Dictyostelium*, transcript levels of these subunits of the affected complexes were analyzed by Real Time PCR. Constitutive *AIF* dR cells exhibited decreased transcript levels of *NAD1* (Fig. 5.6A), *NAD6* (Fig. 5.6B), *NDUFS3* (Fig. 5.6C), *NDUFA5* (Fig. 5.6D), *NDUFA9* (Fig. 5.6E), *CYTB* (Fig. 5.6F), *COX1/2* (Fig. 5.6G) and *ATP6* (Fig.

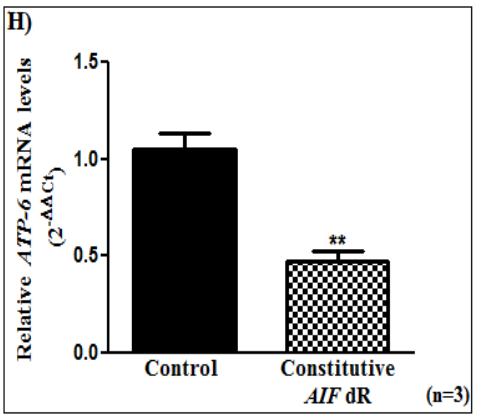
5.6H) subunits compared to control cells. An assembly of the ETC supercomplexes was observed by BN-PAGE. The abundance of complexes I, III and IV proteins was found to be reduced in constitutive *AIF* dR compared to control cells (Fig. 5.6I, 5.6J, 5.6K), suggesting assembly of ETC complexes is affected due to loss of AIF. Decreased ETC subunits' transcript levels and a defect in ETC assembly indicate that AIF may contribute to assembly, maintenance and/ stabilization of the mitochondrial ETC.

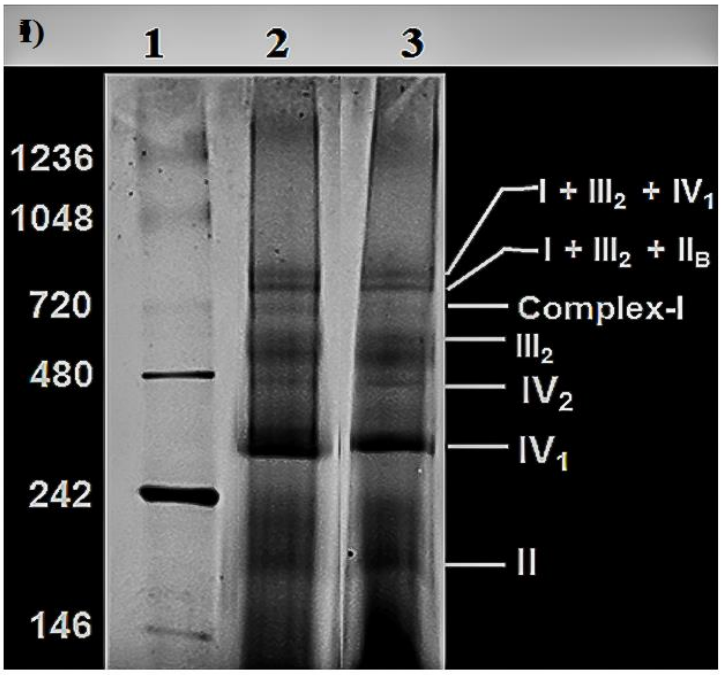



E)
Relative NDUFA-9 mRNA levels
(2-ΔΔCt)
1.5
1.0
0.5
0.0
ns
Control
Constitutive AIF dR
(n=3)


F)
Relative CYT-B mRNA levels
(2-ΔΔCt)
1.5
1.0
0.5
0.0
*
Control
Constitutive AIF dR
(n=3)


G)
Relative COX-I/2 mRNA levels
(2-ΔΔCt)
1.5
1.0
0.5
0.0
*
Control
Constitutive AIF dR
(n=3)


H)
Relative ATP-6 mRNA levels
(2-ΔΔCt)
1.5
1.0
0.5
0.0
**
Control
Constitutive AIF dR
(n=3)


I)
1
2
3
1236
1048
720
480
242
146
I + III2 + IV1
I + III2 + IIB
Complex-I
III2
IV2
IV1
II

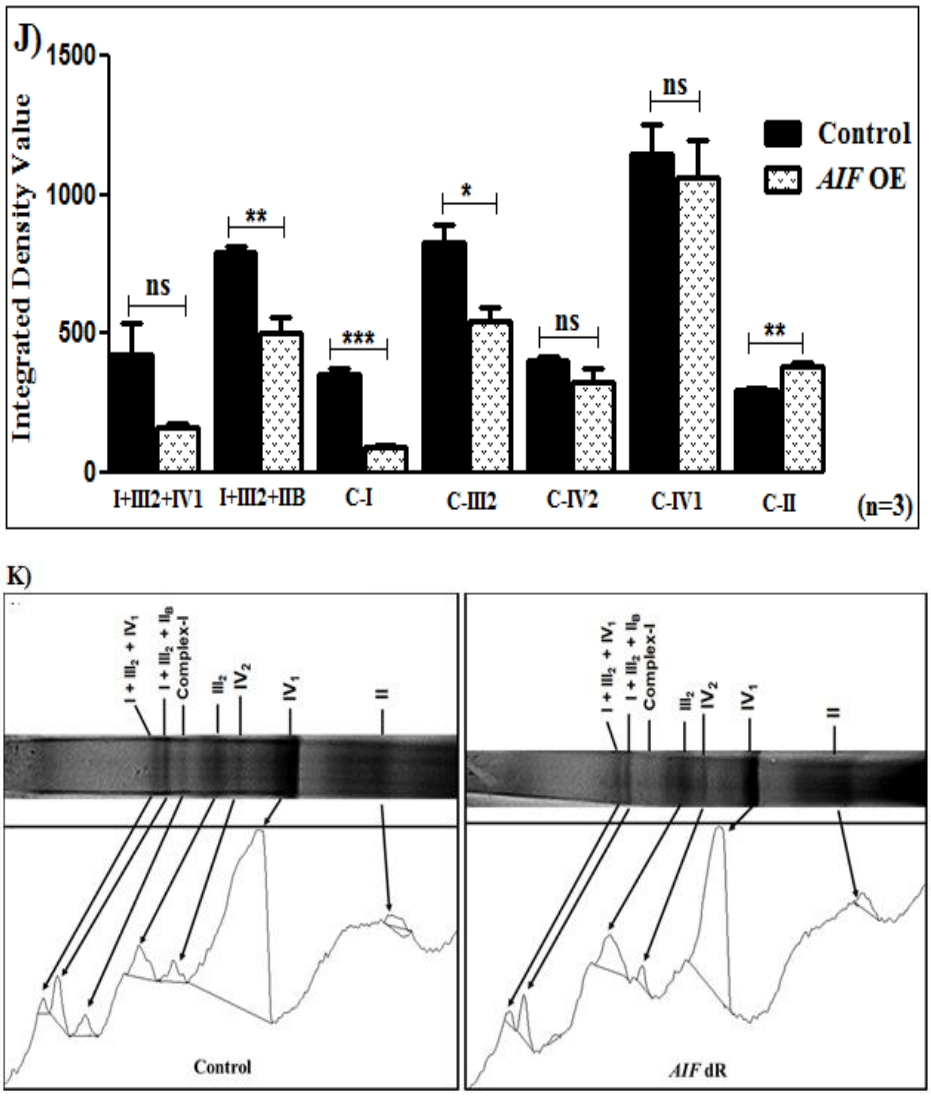


Fig. 5.6: Analysis of Electron transport chain complexes by Real Time PCR and BN-PAGE: A), B), C), D), E), F), G) and **H)** Real Time PCR analysis showed reduced relative transcript levels of *NAD1* (p=0.0024), *NAD6* (p=0.0105), *NDUFS3* (p= 0.0348), *NDUFA5* (p=0.1073), *NDUFA9* (p=0.7656), *CYTB* (p=0.0205), *COX1/2* (p=0.0125) and *ATP6* (p=0.0034) in constitutive *AIF* dR compared to control cells. Data are a representation of SEM values of three independent experiments. *p<0.05 and **p<0.01 as compared to control; ns= non-significant. **I)** Representative gel image of BN-PAGE revealed reduced protein abundance of complexes I, III and IV proteins in constitutive *AIF* dR cells. Lane 1: Native protein marker in kDa; Lane 2: control cells; Lane 3: constitutive *AIF* dR cells. **J)** Schematic representation of

densitometric analysis of BN-PAGE gel by imageJ software. Data are representation of the SEM values of three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 as compared to control; ns: non-significant. **K)** Schematic representation of densitometric analysis of BN-PAGE gel by imageJ software quantifying the area for intensity of a single gel lane.

5.2.6 Estimation of mtDNA content

As the transcript levels of mt DNA encoded subunits of the ETC complexes CI, CIII, CIV and CV were modulated in constitutive *AIF* dR cells, we strengthened this data by estimating mitochondrial DNA content in *AIF* dR cells under both vegetative and developmental stages. mtDNA content relative to nuclear DNA content was observed to be significantly reduced in constitutive *AIF* dR compared to both unicellular and multicellular stages of *D. discoideum* (Fig. 5.7A, Fig. 5.7B), implying AIF's role in mtDNA maintenance.

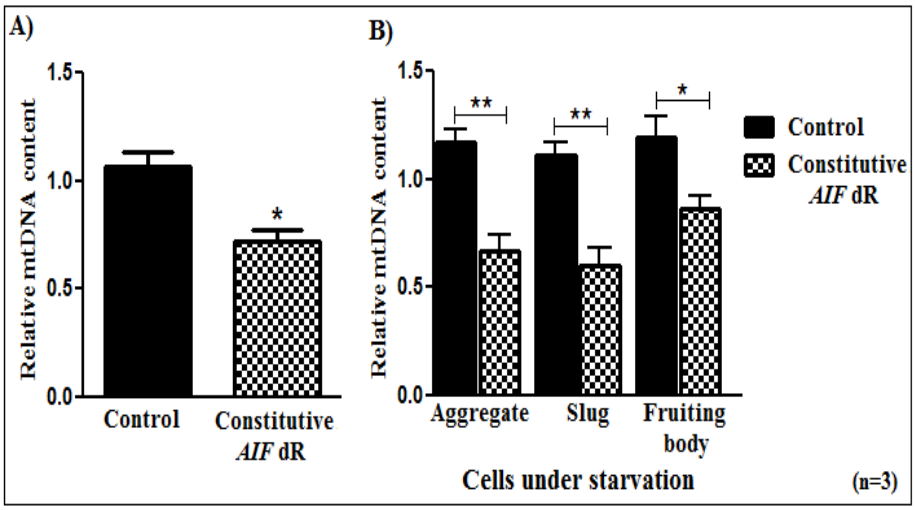


Fig. 5.7: Estimation of relative mtDNA content by Real Time PCR: A) A significant decrease in mtDNA content was found in vegetative constitutive *AIF* dR compared to control cells (p= 0.0140). **B)** Under the starvation condition, mtDNA content was found to be reduced significantly at the aggregate (p=0.0058), slug (p=0.0079) and fruiting body (p=0.0442) stages of development in constitutive *AIF* dR compared to control cells. Data are a

representation of SEM values of three independent experiments. *p<0.05 and **p<0.01 as compared to control; ns= non-significant.

5.3 Discussion

AIF is a mitochondrial protein pivotal for the maintenance of the OXPHOS and mtDNA system. Several *AIFM1* mutations have been recognized in patients with mitochondrial dysfunctions, further explaining the significance of AIF in pathophysiology (Rinaldi *et al*., 2012; Kettwig *et al*., 2015). Intending to make a new model of *AIF* deficiency, we study AIF mediated mitochondrial impairment in *D. discoideum*. Our data show that *AIF* downregulation affects mitochondrial homeostasis, reducing the respiration rate and ETC dysfunction. *In vitro* and *in vivo* studies showed an absence of *AIF* caused damage in the assembly and/or maintenance of the respiratory chain complex I (Pospisilik *et al*., 2007; Ghezzi *et al*., 2010). Moreover, deletion of *AIF* led to OXPHOS deficiency and a switch in cellular metabolism towards glycolysis in non-transformed pneumocytes and at early stages of tumor development (Rao *et al*., 2019). Interestingly, our study also reveals compromised OXPHOS function due to impairment not only in complex I but the entire ETC complex activities in both the vegetative and starved constitutive *AIF* dR cells (Fig. 5.1, Fig. 5.2). Reduced activities of complex I, III and IV along with their lower expression of the subunits at transcript and protein levels caused OXPHOS dysfunction (Fig. 5.6), explaining further the reduced total cellular and mitochondrial ATP levels (Fig. 5.4) and hence metabolic switch to enhanced glycolysis in constitutive *AIF* dR cells for energy need (Fig. 5.5). Reduced ATP levels could be the reason for a slower growth rate (Fig. 3.5) and delay in development (Fig. 3.8) as discussed in Chapter 3. Loss of AIF in heart and B cells also resulted in decrease in complex I along with increase in complex II activity (Milasta *et al*., 2016). Elevated complex II activity in constitutive *AIF* dR cells (Fig. 5.1A) can be a compensatory mechanism that links mitochondrial reserve respiratory capacity to cellular survival under compromised complex I and III

activities (Quinlan *et al*., 2012; Dhingra and Kirshenbaum, 2015). Moreover, complex II activity obligatorily links mitochondrial reserve respiratory capacity to cell survival in cardiac myocytes (Dhingra and Kirshenbaum, 2015) as AIF is neither an integral part of complex I of ETC or interacts with any ETC components (Vahsen *et al*., 2004; Sevrioukova, 2011). Oxidation OCR ratio are accurate indicators of partial respiratory chain deficiencies (Rustin *et al*., 1991). CI/CII OCR ratio in constitutive *AIF* dR cells confirmed the deficiency in CI relative to succinate oxidation (Fig. 5.1B). AIF is proposed to regulate mitochondrial biogenesis by interacting with proteins required for proper protein folding or recruitment of ETC subunits or stabilization of ETC assembly. With this hypothesis, Meyer group revealed AIF interacting partner which might mediate OXPHOS defect. NADH enhanced binding of AIF to oxido-reductase CHCHD4/MIA40 (Mitochondrial Inter-membrane Space Import and Assembly 40) in Hq mutant mice stabilizes ETC subunits assembly and thereby OXPHOS maintenance (Meyer *et al*., 2015). MIA40 is a crucial component of the IMS import and assembly machinery of complexes I & IV subunits. The loss of MIA40 protein was correlated with *AIF* deficiency. It acts downstream to AIF and is involved in the proper folding of ETC subunits, confirming optimal mitochondrial function at the selected ETC complexes (Meyer *et al*., 2015). AIF loss is also linked to an increase in ROS generation as a consequence of respiratory chain dysfunction (Klein *et al*., 2002; Urbano *et al*., 2005). NADH- and two FAD-binding domains of AIF may act as ROS regulators. They might be shielding the respiratory complexes from locally produced ROS (Sevrioukova, 2011) or modifying the electron flow through ETC complexes (Troulinaki *et al.*, 2018). This hypothesis is corroborated by our study wherein GSH supplementation partially restores the complex I and complex III activities in constitutive *AIF* dR cells, mimicking the AIF's function and strengthening the role of AIF as an oxidant regulator (Fig. 5.3). This is as per the report which suggested that antioxidant MitoQ restored impaired respiration in *AIF* silenced cells (Apostolova *et al*., 2006). AIF may modulate the electron flow through the

respiratory chain complexes, stimulating proton pumping across the mitochondrial membrane comparably to the yeast NADH-ubiquinone oxidoreductase (Ndi1) as discussed by Elguindy and Nakamaru-Ogiso, (2015) and Jafari *et al*., (2016). Together, these observations suggest that AIF behaves as a ROS regulator and its redox activity is necessary for optimum functioning of mitochondrial bioenergetics.

Mitochondrial pathologies are the result of mutations of nuclear and mitochondrial genes encoding for proteins essential for mitochondrial homeostasis or the OXPHOS (Schapira, 2012). Human patients carrying an arginine deletion (R201) in AIF led to severe mitochondrial encephalomyopathy with reduced respiratory CIII and CIV levels and depleted mtDNA content in muscle biopsies, suggesting AIF's involvement in mtDNA maintenance (Ghezzi *et al*., 2010). This was strengthened by reduced mtDNA copy number in EndoG knockout mice (McDermott-Roe *et al*., 2011), where direct interaction of AIF with the mitochondrial endonuclease EndoG during cell death is established (Wang *et al*., 2002). Troulinaki *et al*., (2018) showed that the worm AIF homolog, WAH-1 dependent regulation of OXPHOS is functionally associated with the mtDNA homeostasis. In corroboration with these reports, we also observed diminished mtDNA content in vegetative and starved constitutive *AIF* dR cells (Fig. 5.7), confirming AIF's regulatory role in mtDNA maintenance. A decrease in mtDNA pool could negatively affect the mitochondrial genes transcript as well as protein levels that eventually compromise the mitochondrial function (Barazzoni *et al.*, 2000). However, the exact underlying mechanism linking AIF and mtDNA needs to be investigated further.

In conclusion, these results establish the conserved role of AIF in the correct maintenance of a functional OXPHOS system and mtDNA content in *D. discoideum* (Fig. 5.8).

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*).

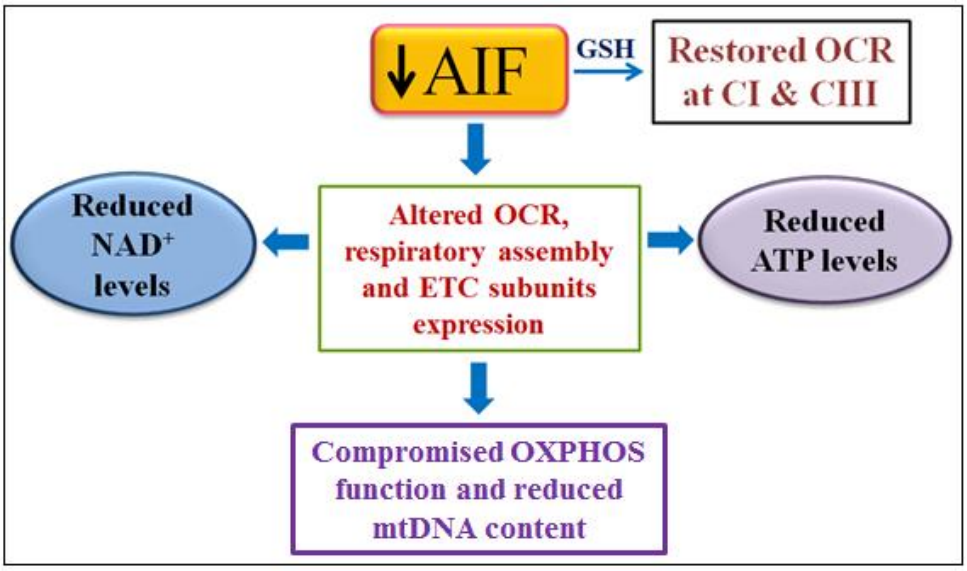


Fig. 5.8: Effect of *AIF* downregulation on mitochondrial activities

5.4 References

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