Effect of constitutive *AIF* downregulation on mitochondrial fusion-fission mechanism of *D. discoideum*

6.1 Introduction

Mitochondria play central role as an energy producer and a station for cell death signaling (McBride *et al*., 2006). The dynamic network of mitochondria constantly remodels itself through the contrasting processes of mitochondrial fusion and fission, which critically maintains mitochondrial morphology and function (Detmer and Chan, 2007).

Apoptosis Inducing Factor (AIF) was first revealed as a pro-apoptotic protein but progressively it became clear that AIF also has a pro-survival role inside the mitochondria (Vahsen *et al.,* 2004). It is required to regulate proper mitochondrial metabolism. Later, *AIF* deficient models were found to be associated with fragmented mitochondria with defective cristae, suggesting an additional role of AIF in regulating mitochondrial structure too. Neuronal mitochondria from forebrain of specific *AIF* null (tel. AIFΔ) mice were dilated and fragmented with aberrant cristae structure (Cheung *et al*., 2006). Further, Harlequin (Hq) mutant mice showed the reduced levels of cerebellar Mitofusin 1 (*MFN1*), suggesting that alterations of mitochondrial fusion led to cerebellar degeneration. A few reports demonstrate the link between AIF and mitochondrial fusion-fission processes (Chung *et al*., 2011; Cheung *et al*., 2006). Moreover, mitochondrial fission process upon *AIF* loss is yet to be explored. Hence, we investigated the effect of down-modulation of *AIF* on both the mitochondrial fusion and fission processes by analyzing the transcript levels of mitochondrial fusion and fission genes and mitochondrial structure in *D. discoideum*.

Though mitochondrial fusion-fission have been implicated in lower eukaryotes such as *D. discoideum*, the process and effect is not yet well studied (Schimmel *et al*., 2012). Dynamin related proteins such as Dynamin A (DYMA), Dynamin B (DYMB) and mitochondrial division proteins viz FSZA and FSZB are involved in mitochondrial fission whereas Clustered A (CLUA)

is involved in mitochondrial fusion in *D. discoideum* (Rai *et al*., 2011; Schimmel *et al*., 2012; Rai *et al*., 2013; DictyBase).

Our results specify the altered mitochondrial fusion and fission in *AIF* dR cells. These findings can be useful for further mechanistic studies on AIF-related oxidative stress, cell death and mitochondrial fusion-fission processes.

6.2 Results

As shown in Chapter 5, *AIF* downregulation affected mitochondrial respiratory activities along with ATP production and mtDNA content. Thus, to study AIF's function in regulating the mitochondrial structure, gene expression analysis of the fusion-fission mechanism was carried out by Real Time PCR in unicellular and multicellular stages of *D. discoideum.*

6.2.1 Analysis of the transcript levels of mitochondrial fusion-fission genes in vegetative cells

Loss of *AIF* resulted in compromised mitochondrial function in constitutive *AIF* dR cells while the knockdown of *AIF* led to fragmented mitochondria with aberrant cristae structure, indicating the role of AIF in maintaining mitochondrial structure (Milasta *et al*., 2016). Since the mitochondrial structure is maintained by the balanced processes of fusion and fission (Chan, 2006), we studied the effect of reduced *AIF* on the mitochondrial structure by analysing the transcript levels of mitochondrial fusion-fission genes (*CLUA, DYMA, DYMB, FSZA,* and *FSZB*). Constitutive *AIF* dR cells exhibited reduced *CLUA* and elevated *DYMA, DYMB, FSZA* and *FSZB* transcript levels (Fig. 6.1). This altered fusion-fission balance in *AIF* dR cells uncovered the additional function of AIF in controlling mitochondrial morphology.

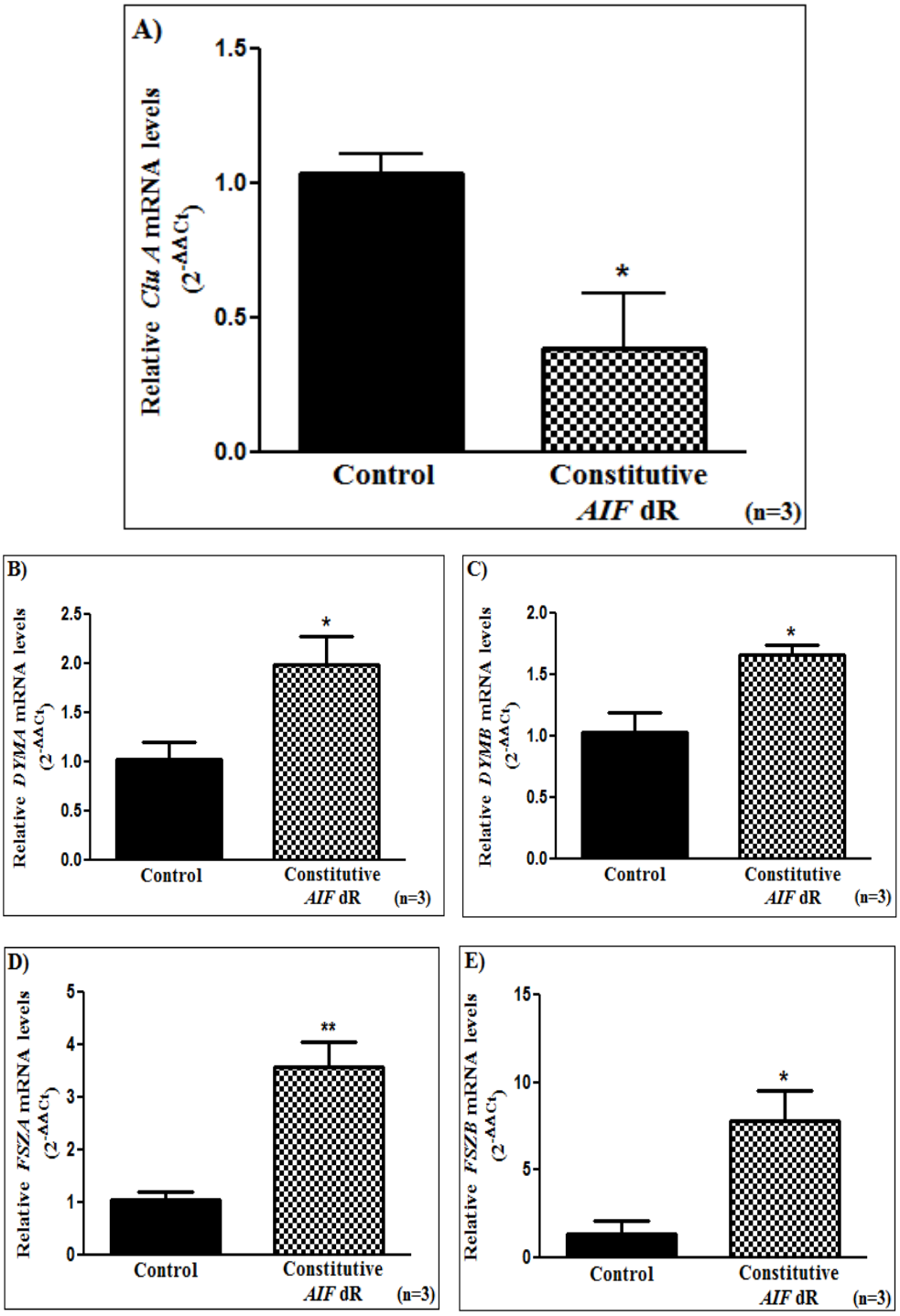
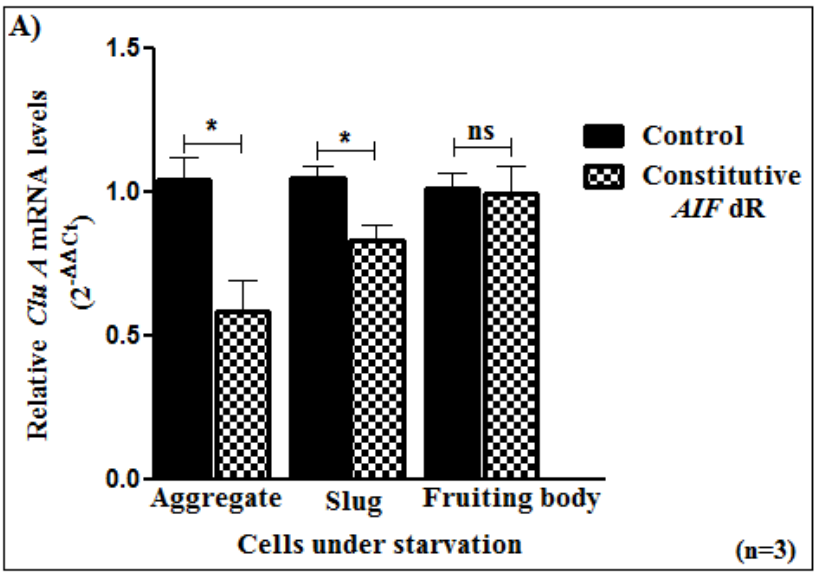


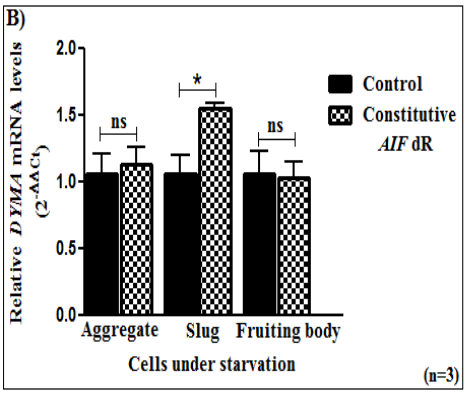
Fig. 6.1: Analysis of transcript levels of mitochondrial fusion-fission genes: A) Real Time PCR analysis showed significantly lower relative *CLUA* mRNA transcript levels in constitutive *AIF* dR cells as compared to control cells (p=0.0386). **B), C), D)** and **E)** Relative *DYMA* (p=0.0453), *DYMB* (p=0.0276), *FSZA* (p=0.0067) and *FSZB* (p=0.0277) transcript levels were

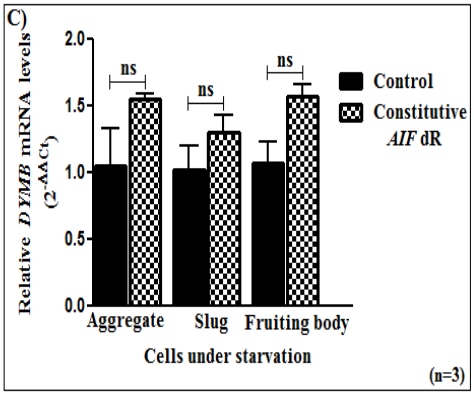
found to be significantly higher in constitutive *AIF* dR cells as compared to control cells. Data are a representation of SEM values of three independent experiments. *p<0.05 and **p<0.01 as compared to control; ns= non-significant.

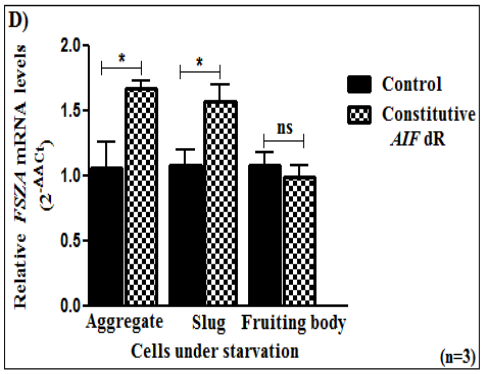
6.2.2 Analysis of transcript levels of mitochondrial fusion-fission genes in starving cells

As starved *AIF* dR cells exhibited impaired mitochondrial respiration, we were also interested to explore its effect on fusion-fission under starvation. Hence, the transcript levels of mitochondrial fusion-fission genes were analysed at each of the developmental stages (aggregate, slug and fruiting bodies). *CLUA* transcript levels were observed to be significantly reduced at aggregate and slug stages (Fig. 6.2A) while *DYMA* and *FSZB* transcript levels were found to be significantly increased at the slug stage of constitutive *AIF* dR compared to control cells (Fig. 6.2B, Fig. 6.2C). *FSZA* transcript levels were observed to be significantly elevated at aggregate and slug stages (Fig. 6.2D) whereas no significant difference was found in *DYMB* transcript levels of constitutive *AIF* dR compared to control cells (Fig. 6.2E). The altered fusion-fission process was demonstrated during developmental stages also due to the absence of AIF in *D. discoideum.* Overall, our study suggested the importance of AIF in controlling the mitochondrial structure under both vegetative and development stages.









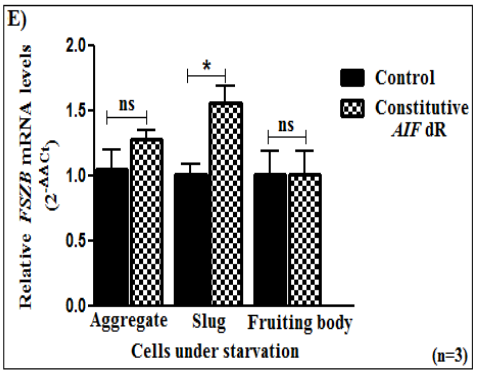


Fig. 6.2: Analysis of transcript levels of mitochondrial fusion-fission genes under starvation: A) Real Time PCR analysis showed significantly reduced relative *CLUA* transcript levels at the aggregate (p=0.0246) and slug (p=0.0291) stages of development in constitutive *AIF* dR as compared to control cells. **B)** Real Time PCR analysis showed significantly reduced

relative *DYMA* transcript levels at the slug stage (p=0.0284) of development in constitutive *AIF* dR as compared to control cells. **C)** No significant difference was found in *DYMB* transcript levels of constitutive *AIF* dR cells under starvation. **D)** Significantly higher relative *FSZA* transcript levels were observed at the aggregate (p=0.0407) and slug (p=0.0471) stages of development in constitutive *AIF* dR as compared to control cells. **E)** Constitutive *AIF* dR cells exhibited significantly elevated *FSZB* transcript levels at the slug stage (p=0.0228) of development compared to control cells. Data are a representation of SEM values of three independent experiments. *p<0.05 as compared to control; ns= non-significant.

6.2.3 Mitochondrial morphology

Loss of respiratory capacity and altered mitochondrial fusion-fission mechanism might contribute to modulation of the mitochondrial structure due to *AIF* deficiency (Cheung *et al*., 2006). In line with this report, perturbations in mitochondrial morphology were visualized by TEM. Mitochondria of constitutive *AIF* dR cells were defective with aberrant cristae and dilations. Interestingly, constitutive *AIF* dR mitochondria exhibited intra-mitochondrial 'holes' likely due to dilation of cristae which were absent in control cells (Fig. 6.3A). However, no significant difference was observed in the mitochondrial average surface area in constitutive *AIF* dR compared to control cells (Fig. 6.3B). Further, constitutive *AIF* dR exhibited greater number of mitochondria compared to control cells, corroborating increased mitochondrial fission and reduced mitochondrial fusion process (Fig. 6.1, Fig. 6.2). These results highlight the prime role of AIF in maintaining mitochondrial structure and morphology.

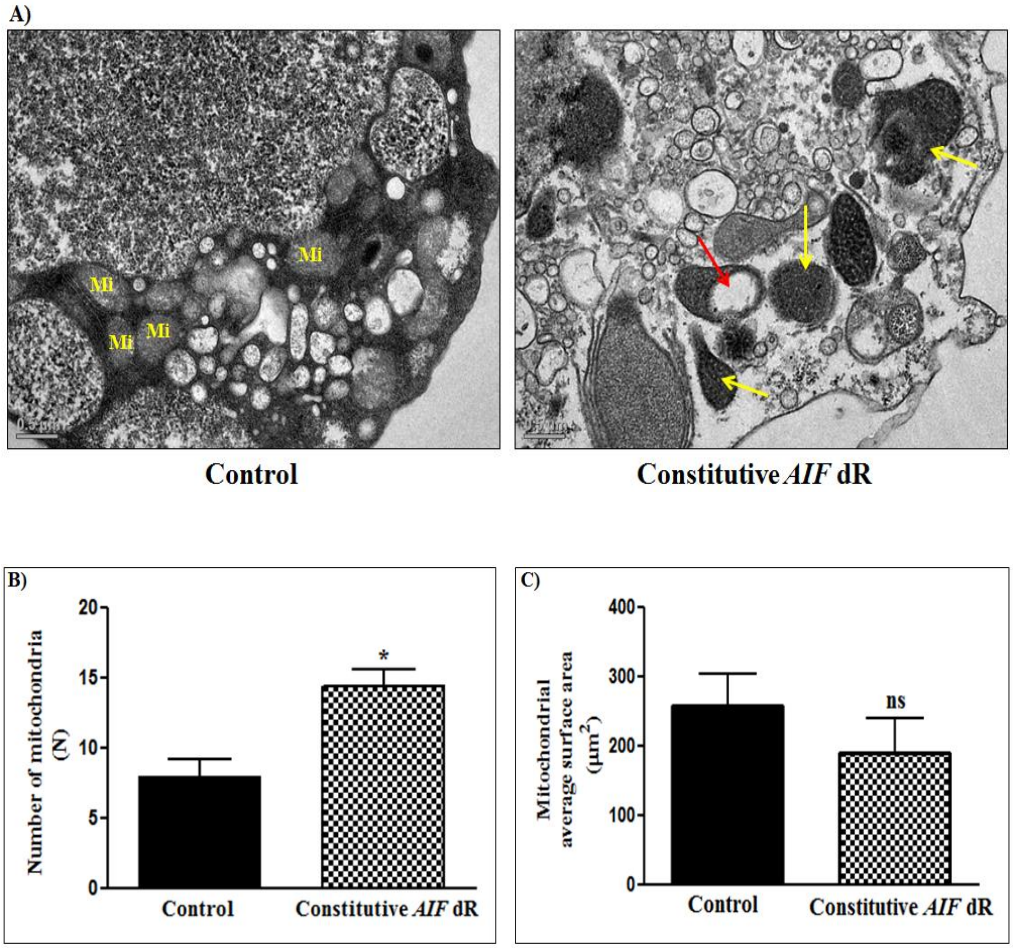


Fig. 6.3: Mitochondrial morphometric analysis by TEM: A) Representative electron micrographs showed compact Mitochondria (Mi) in control cells while defective (tubular and fragmented) mitochondria with a 'hole' in constitutive *AIF* dR cells. Red arrow indicates intra-mitochondrial 'hole' and orange arrow indicates defective mitochondria. Bar: 0.5µm. **B)** Histogram showed a significant increase in the number of mitochondria in constitutive *AIF* dR compared to control cells (p=0.0191). **C)** The mitochondrial average surface area showed a non-significant difference in constitutive *AIF* dR cells as compared to control cells. *p<0.05 as compared to control; ns= non-significant.

6.3 Discussion

Based on our previous results, AIF is found to be an essential protein for the maintenance of mitochondrial metabolism controlling ETC assembly and OXPHOS system. Mitochondrial metabolism is tightly linked to the organelle structure and morphology (McBride *et al.,* 2006). Deletion of arginine residue (R201) of AIF caused fragmentation in mitochondrial network in fibroblasts (Ghezzi *et al*., 2010). Moreover, knockdown of *AIF* caused mitochondrial fragmentation with abnormal cristae structure, suggesting AIF's auxiliary role in regulating mitochondrial structure possibly through its fusion-fission processes (Milasta *et al*., 2016). Thus, we monitored the effect of *AIF* down-regulation on the mitochondrial fusion-fission mechanism in *D. discoideum. CLUA*, a mitochondrial fusion gene, was found to be reduced significantly in constitutive *AIF* dR cells compared to control cells (Fig. 6.1A). However, mitochondrial fission genes *DYMA, DYMB, FSZA* and *FSZB*, were found to be increased significantly in constitutive *AIF* dR cells compared to control cells (Fig. 6.1B, 6.2C, 6.3D, 6.4E). Additionally, the transcript levels of mitochondrial fusion-fission genes were also analyzed at different developmental stages (aggregate, slug and fruiting bodies). *CLUA* transcript levels were observed to be significantly reduced in constitutive *AIF* dR cells compared to control cells during aggregate as well as slug stages (Fig. 6.2A). *DYMA* and *FSZB* transcript levels were significantly increased at slug stage of constitutive *AIF* dR cells compared to control cells (Fig. 6.2B, E). *FSZA* transcript levels were observed to be significantly elevated in constitutive *AIF* dR cells compared to control cells during aggregate and slug stages (Fig. 6.2D) whereas no significant difference was found in *DYMB* transcript levels of constitutive *AIF* dR cells compared to control cells (Fig. 6.2E). Overall, constitutive *AIF* dR cells exhibited increased mitochondrial fission and decreased mitochondrial fusion which was further validated by the perturbations in the mitochondrial morphology observed by TEM. Mitochondria of constitutive *AIF* dR cells were defective with dilations (mitochondrial hole) and aberrant cristae formation (Fig. 6.3A). Interestingly,

constitutive *AIF* dR cells exhibited greater number of mitochondria compared to control cells (Fig. 6.3B), substantiating increased mitochondrial fission and reduced mitochondrial fusion process.

In context to mitochondrial health, AIF might be interacting with mitochondrial fusion-fission genes to maintain the mitochondrial structure. The immune-precipitation experiment showed the physical interaction between AIF and OPA1, mitochondrial fusion protein and both the proteins are associated with subunits of the respiratory complexes (NDUFA9 subunit of complex I, the FP subunit of complex II, the core II subunits of complex III, the subunit I of complex IV) which is important for the maintenance of OXPHOS and mitochondrial morphology (Zanna *et al*., 2008). Altered levels of mitochondrial fusion-fission genes were also caused depletion in mtDNA number and thereby mitochondrial dysfunction. A decrease in mitochondrial fusion by deletion of *MFN1* and *MFN2* led to mtDNA depletion followed by mitochondrial dysfunction (Chen *et al*., 2010). In line with this observation, diminished levels of *CLUA* fusion gene could be the reason for reduced mtDNA content in *AIF* dR cells as demonstrated in Chapter 5 (Fig. 5.7).

Summing up the findings, the energy state of the cell is closely linked to the mitochondrial network organization, highlighting the role of AIF in both mitochondrial function and structure. The present results highlight the prime role of AIF in maintaining mitochondrial structure and morphology (Fig. 6.4).

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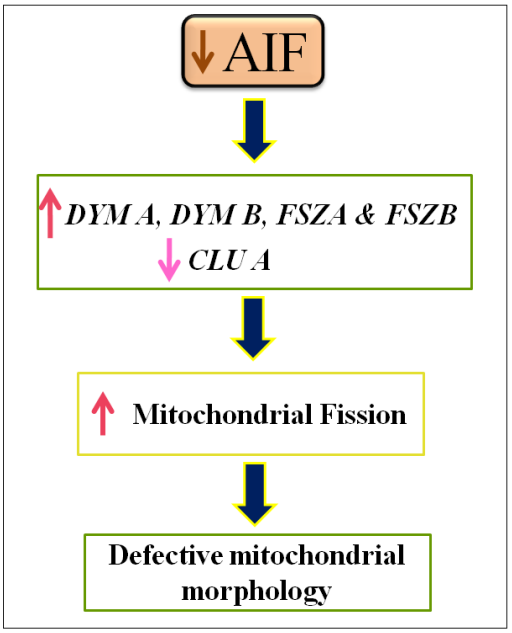

Fig. 6.4: Effect of *AIF* downregulation on mitochondrial fusion-fission mechanism

6.4 References

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