Effect of constitutive and prestalk specific downregulation of Apoptosis Inducing Factor (*AIF*) on growth and development of *D. discoideum*

3.1 Introduction

The cellular growth and development absolutely depend on energy metabolism, which is encountered predominantly through mitochondrial oxidative phosphorylation (OXPHOS). Deficiencies in OXPHOS and mitochondrial energy metabolism are associated with a variety of human disorders, including myopathies, diabetes mellitus, neurodegenerative disease etc (Chomyn and Attardi, 2003). AIF is one of the mitochondrial proteins which is implicated in mitochondrial metabolism. In various cellular contexts including yeast, *Caenorhabditis elegans* and mammals, it has been traditionally known as a cell death-promoting protein that translocates from mitochondria to the nucleus after pro-apoptotic stimuli and mediates chromatin condensation followed by large-scale DNA fragmentation (Joza *et al*., 2001). In addition to its cell death effector role, it is required for cell survival regulating ROS levels (Klein and Ackerman, 2003). Germline AIF ablation is embryonically lethal leading to cavitation defects in the embryoid bodies (Joza *et al*., 2001). Subsequent studies showed that the loss of *AIF* during embryogenesis retards embryonic growth and ultimately results in death during midgestation. In addition, mice lacking muscle AIF is characterized by severely dilated cardiomyopathy and skeletal muscle atrophy that was found to be associated with a severe deficiency in respiratory chain complex I (Joza *et al*., 2005). AIF is hence a requisite for efficient assembly of complex I of the Electron Transport Chain (ETC) and thus maintains OXPHOS (Vahsen *et al*., 2004; Hangen *et al*., 2015). Further, the significance of AIF in T cell development was earlier examined using Harlequin (Hq) mice. The authors concluded that the decrease in peripheral T cells is due to defective thymocyte development, affected by elevated ROS (Banerjee *et al*., 2012). In contrast, Milasta group observed that the absence of AIF in T cells affects the number of peripheral T cells and their ability to undergo proliferation, while not affecting their thymic development. However, B cells with *AIF* deletion develops and works normally (Milasta *et al*., 2016). Based on these studies, AIF's role in inducing cell death versus cell survival remains controversial and hence its role during development and differentiation is yet to be fully understood.

AIF in *D. discoideum*

Like mammalian AIF, *D. discoideum* AIF (DdAIF or AIFA or AIF) of 532 aa residues (~60kDa) encompasses an MLS (1-26 aa) (Claros and Vincens, 1996), a putative NLS (400-406 aa) and FAD (98-440) (Boulikas, 1993). It also contains a putative helix-turn-helix DNA binding motif (Dodd and Egan, 1990) at its C-terminal end, indicating that DdAIF may bind to DNA. Its oxidoreductase domain is phylogenetically conserved. All the amino acids are thought to interact with the FAD and NAD as in human AIF (Lorenzo *et al*., 1999). It shares >30% identity and 60% similarity with human AIF (Claros and Vincens, 1996). It has been shown to localize to the mitochondria in living cells, and to translocate to the nucleus during cell death (Arnoult *et al*., 2001). Other than AIF/AIFA, there are three more isoforms of AIF in *D. discoideum* i.e. AIFB, AIFC and AIFD. AIFA or AIF (henceforth AIF will be mentioned instead of AIFA) is mainly present in mitochondria which are responsible for caspase independent apoptosis while other isoforms' functions are not yet studied. *D. discoideum* is the simplest studied eukaryote that exhibits a transition of unicellularity to multicellularity in its life cycle (Raper, 1984). Thus, it provides a suitable platform to study the functions of AIF in cell growth as well as in multicellular development. Thus, given the existing reports, the present study aims to pin-point the role of AIF in *D. discoideum* growth and development.

3.2 Results

In order to unravel the role of AIF in growth and multicellular development, we generated *AIF* downregulated *D. discoideum* cells by antisense strategy. Constitutive downregulation of *AIF* was under *actin15* promoter whereas prestalk specific downregulation of *AIF* was under *EcmB* (inducible) promoter. The rationale behind prestalk specific *AIF* antisense was to understand the role AIF in developmental cell death of *D. discoideum* as it is expressed only during the slug stage of development.

3.2.1 Cloning of *AIF* antisense in constitutive (pTX-GFP) and prestalk specific (pEcmB-Gal) vectors

For the generation of *AIF* constitutive and prestalk specific antisense constructs, pTX-GFP vector with *actin15* promoter and pEcmB-Gal vector with *EcmB* (inducible) promoter were used. 5' region of *AIF* amplicon (353 bp) was PCR amplified using gene specific primers (Fig. 3.1A) and directionally cloned into constitutive *D. discoideum* expression vector (pTX-GFP) by replacing GFP using *Xba*I and *Kpn*I enzymes. 5' region of *AIF* amplicon (520 bp) was PCR amplified using gene specific primers (Fig. 3.1B) and directionally cloned into prestalk specific *D. discoideum* expression vector (pEcmB-Gal) by replacing Gal using *Xho*I and *Cla*I enzymes. These ligated vectors were transformed into *E. coli DH5α* and the transformants were selected on Luria Bertani Agar containing ampicillin (100µg/ml).

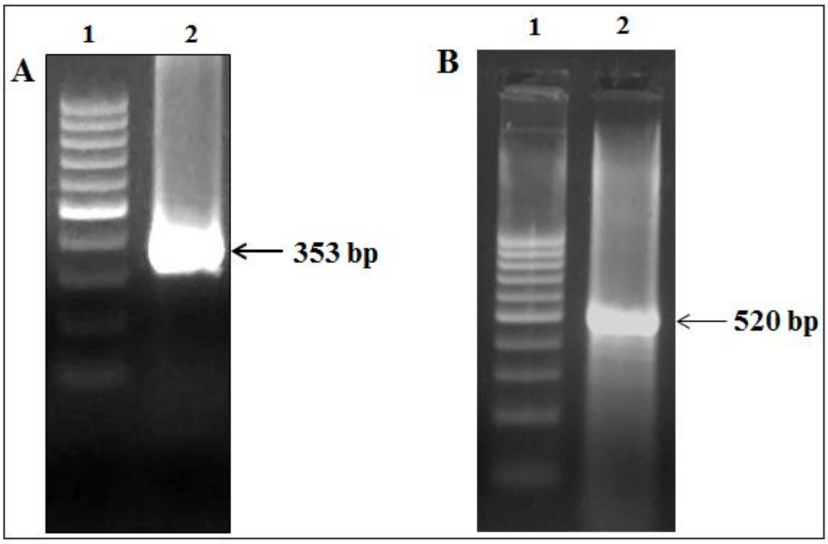

Fig. 3.1: A) PCR amplification of 5' region of *AIF* for constitutive antisense construct- Lane 1: 100 bp DNA ladder, Lane 2: *AIF* Amplicon. **B) PCR amplification of 5' region of *AIF* for prestalk specific antisense construct**- Lane 1: 100 bp DNA ladder, Lane 2: *AIF* Amplicon.

3.2.2 Confirmation of *AIF* antisense constructs using restriction digestion

To confirm both the *AIF* antisense constructs, randomly selected colonies were screened for the presence of recombinant plasmids pTX-*AIF* colony giving a release of 353 bp and 11.1 kb on restriction digestion using *BamH*I & *XbaI* whereas pEcmB-*AIF* gave release of 520 bp and 6.2 kb on restriction digestion using *Xho*I and *Cla*I (Fig. 3.2) Presence of insert of 353 bp would give a single linear band of ~11.4 kb on *BamH*I digestion (Fig. 3.2A). The confirmed clones were then used for transformation of *D. discoideum* cells to generate constitutive and prestalk specific *AIF* downregulated cells.

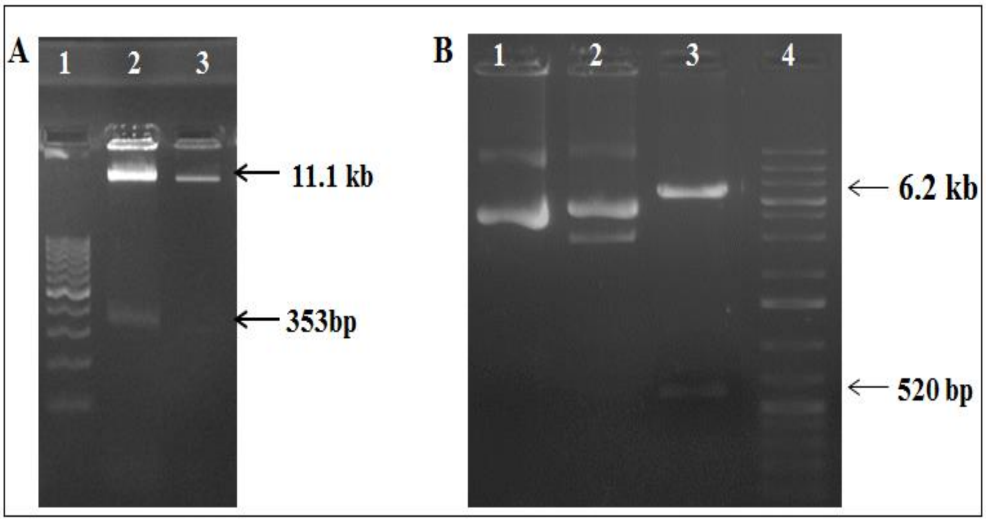

Fig. 3.2: A) Clone confirmation of pTX-*AIF* by RE digestion- Lane 1: 100 bp DNA ladder, Lane 2: Digestion of ***pTX-AIF*** with *BamH*I and *XbaI*, Lane 3: Linearization of clone by *BamH*1. **B) Clone confirmation of pEcmB-*AIF* by RE digestion-** Lane 1: Undigested EcmB-*GAL* vector, Lane 2: Undigested EcmB-*AIF*, Lane 3: Digestion of pEcmB-*AIF* with *ClaI* and *XhoI*, Lane 4: 1 kb DNA ladder.

3.2.3 Transformation of pTX-*AIF* and pEcmB-*AIF* antisense construct in *D. discoideum*

Transformation of *D. discoideum* cells was performed by electroporation method (Gaudet *et al*., 2007). Cells were transformed with pTX-*AIF* and pEcmB-*AIF* using Gene Pulser Xcell^{TM} electroporator (BioRad) at 650Volts, capacitance 25µF, 2 pulses with 12 sec gap between the 2 pulses. Geneticin (G418) was used as a selection marker (100µg/ml). *AIF* downregulated (*AIF* dR) cells were further stored as a spore glycerol stock after developing into fruiting bodies on NNA plate with 100µg/ml G418. Transformation of pTX-GFP and pEcmB-Gal vectors in *D. discoideum* was also carried out similarly and these transformed cells were used as the respective vector controls (pTX-control & pEcmB-control) for our further studies.

3.2.4 Functional characterization of *AIF* antisense

Since, *AIF* knockout is lethal and there are no known inhibitors of AIF, downregulation of *AIF* was carried out by using antisense strategy to establish constitutive and prestalk specific *AIF* antisense models. The rationale behind prestalk specific *AIF* antisense was to understand the role AIF in developmental cell death of *Dictyostelium*. *AIF* downregulation was confirmed by monitoring gene specific expression of *AIF* using Real Time PCR. *AIF* transcript levels displayed ~70% reduction in constitutive (p=0.0149) and ~73% reduction in pre-stalk specific *AIF* dR cells (p=0.0109) compared to control and the respective vector control cells (pTX-control and pEcmB-control) (Fig. 3.3).

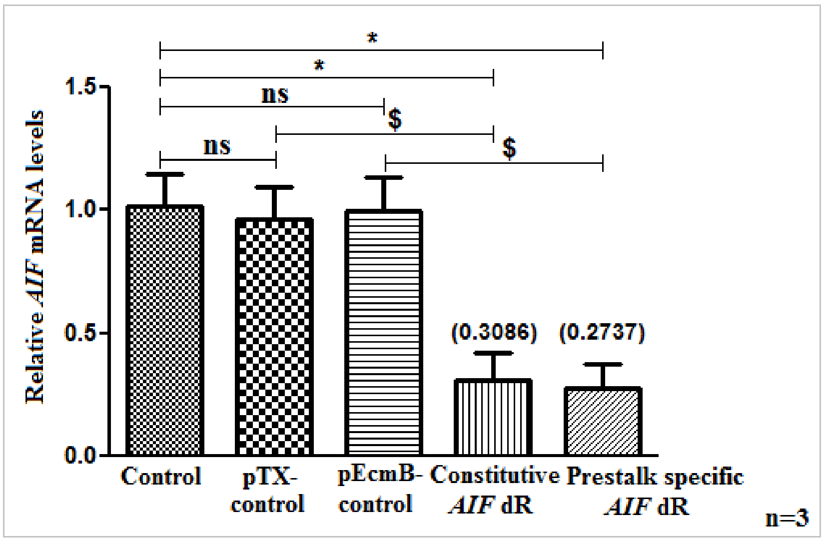

Fig. 3.3: Functional characterization of *AIF* antisense: Real Time PCR analysis showed ~70% & ~73% reduction in *AIF* transcript levels in

constitutive and prestalk specific *AIF* dR cells respectively. Data are representative of three independent experiments which represented as mean ± S.E. ns= non-significant; **p*<0.05 compared to control; $^{\$}p$<0.05 compared to respective vector controls (pTX-control & pEcmB-control).

3.2.5 Relative expression of AIFB, AIFC and AIFD

Other than AIF (AIFA), there are three more isoforms of AIF in *D. discoideum* i.e. AIFB, AIFC and AIFD. We further assessed the transcript levels of *AIFB*, *AIFC* and *AIFD* in constitutive *AIF* downregulated (dR) cells to ensure that only *AIF*/*AIFA* was downregulated. No significant difference was observed in the transcript levels of *AIFB, AIFC* and *AIFD* in constitutive *AIF* dR cells compared to control and vector control cells (Fig. 3.4), suggesting AIFB, AIFC and AIF D could not compensate the function of AIFA.

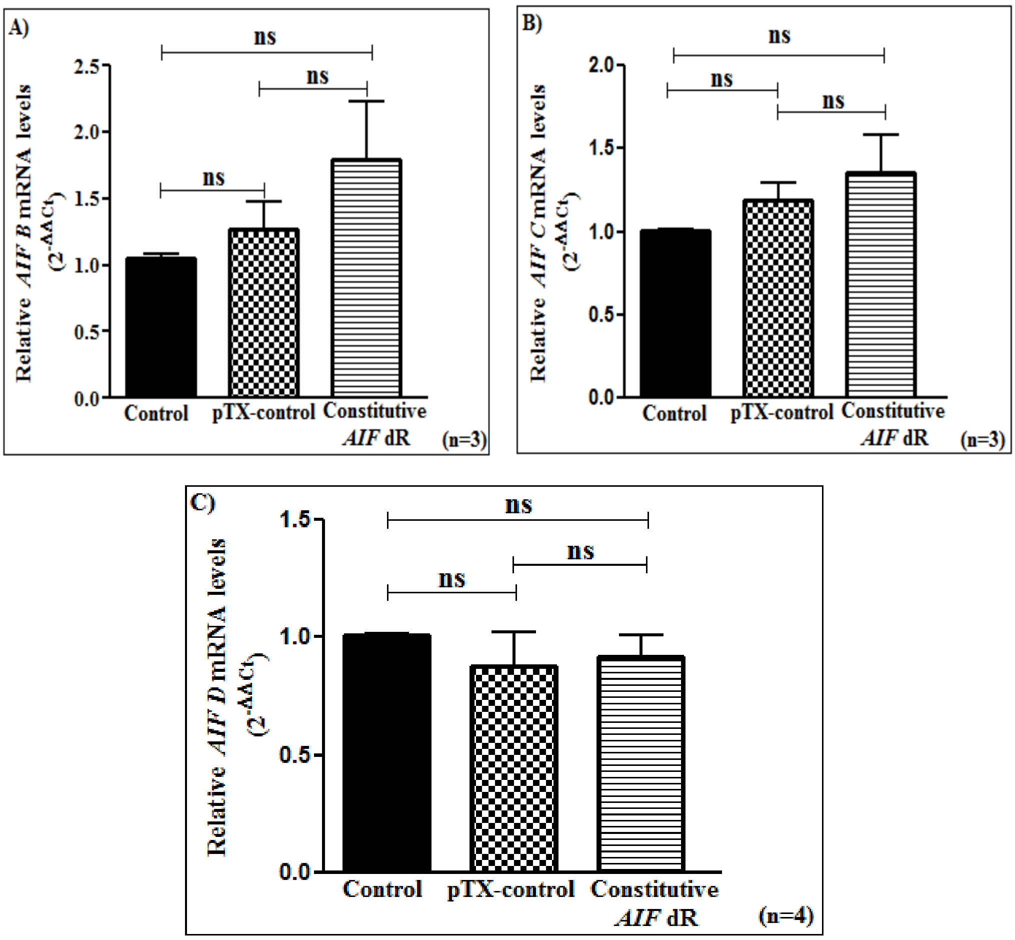

Fig. 3.4: Analysis of *AIFB, AIFC* and *AIFD* transcript levels in constitutive *AIF* dR cells by Real Time PCR: A) Relative transcript levels of *AIFB* in constitutive *AIF* dR cells compared to vector control and control cells. Data are representation of SEM values of three independent experiments. **B)** Relative transcript levels of *AIFC* in constitutive *AIF* dR cells compared to vector control and control cells. Data are representation of SEM values of three independent experiments. **C)** Relative transcript levels of *AIFD* in constitutive *AIF* dR cells compared to vector control and control cells. Data are representation of SEM values of four independent experiments. *RNLA* used as an internal control. pTX-control used as a vector control. ns= non-significant.

3.2.6 Cell growth profile

Constitutive *AIF* dR cells exhibited a slower growth rate in logarithmic phase as compared to control cells (p=0.0016) as well as pTX-control cells (p=0.0184) (Fig. 3.5). However, prestalk specific *AIF* dR cells displayed no significant change in growth as compared to control cells (p=0.9524) (Fig. 3.5). The doubling time of constitutive *AIF* dR cells and prestalk specific *AIF* dR cells were 25.71±2.2 hrs and 14.37±0.75 hrs respectively whereas control cells, pTX-control and pEcmB-control cells exhibited doubling time of 14.24±0.77 hrs, 16.42±2.22 hrs and 14.95±0.80 respectively.

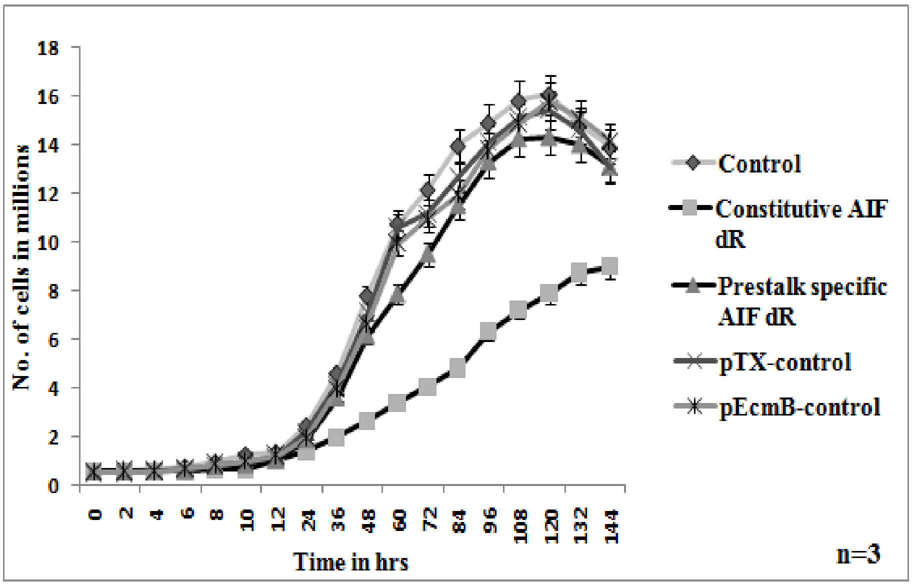

Fig. 3.5: Effect of *AIF* downregulation on *D. discoideum* growth: Constitutive *AIF* dR cells exhibited a slower growth rate whereas growth in prestalk specific *AIF* dR cells was comparable to control cells. pTX-control and pEcmB-control were used as respective vector controls. Data are representative of three independent experiments which represented as mean ± S.E. ns= non-significant. **p<0.01 compared to control and pTX-control.

3.2.7 Analysis of cell cycle

Cell cycle analysis revealed significant decrease in the number of constitutive *AIF* dR cells in G0-G1 phase with a simultaneous increase in the number of cells in S phase at 48 hrs of growth compared to control cells (Fig. 3.6) upon constitutive *AIF* downregulation. This change in the cell cycle profile as compared to control justifies the slower proliferation rate of constitutive *AIF* dR cells.

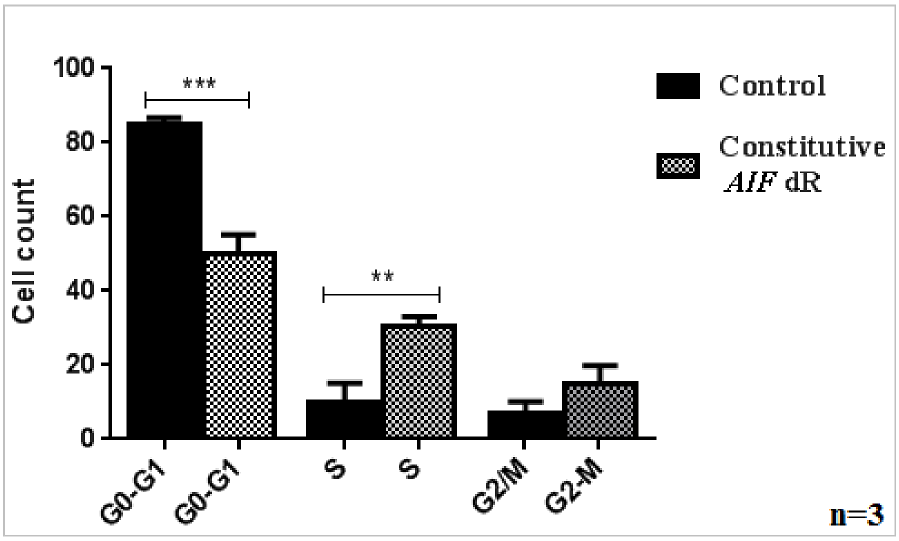

Fig. 3.6: Cell cycle analysis by FACS in constitutive *AIF* dR cells: Significant increase in the percentage of cells in S phase and decrease in the percentage of cells in G0-G1 phase was seen in constitutive *AIF* dR cells at 48 hrs. Data are representative of three independent experiments and represented as mean ± S.E. **p<0.01 compared to control, ***p<0.001 compared to control.

3.2.8 Cell Morphology

In addition, constitutive *AIF* dR cells showed a stressed phenotype. This was confirmed by studying the cellular morphology of constitutive *AIF* dR cells under a Transmission Electron Microscope (TEM). TEM analysis revealed vacuole like structures (shown by arrows) in constitutive *AIF* dR cells as compared to control which may be indicative of intrinsic stress in constitutive *AIF* dR cells (Fig. 3.7).

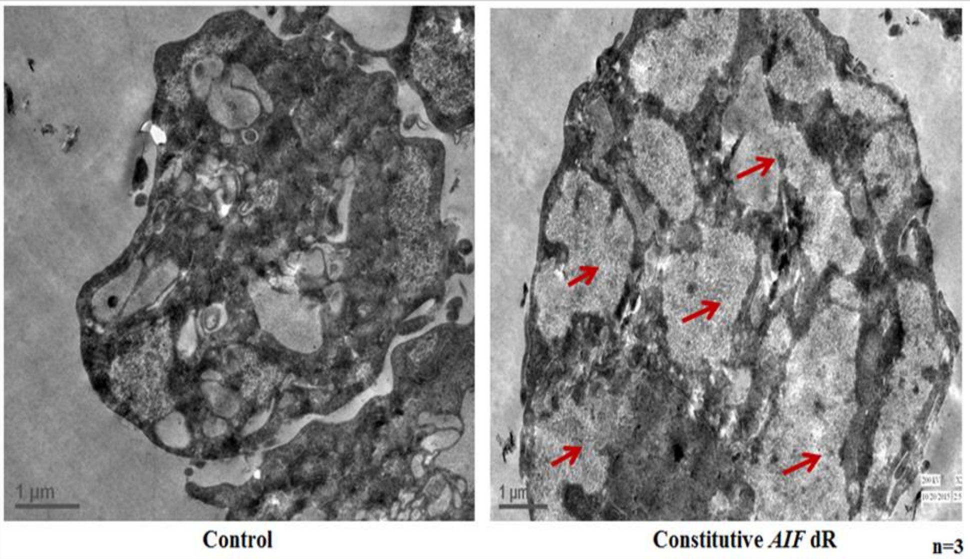

Fig. 3.7: TEM analysis of constitutive *AIF* dR cells: Vacuole like structures (shown by arrows) were observed in constitutive *AIF* dR cells compared to control cells.

3.2.9 Effect of *AIF* downregulation on development

AIF downregulation led to significant effects in the unicellular stage of *D. discoideum*. Hence, we further intended to study its effect on the developmental phase of the slime mold. Under the starvation conditions, *D. discoideum* cells aggregate and differentiate into multicellular structures like the loose aggregate, tight aggregate, the migrating slug and finally culminates into a fruiting body with spore case at the tip consisting of viable cells and a stalk composed of dead cells (Whittingham and Raper, 1960). Constitutive and prestalk specific *AIF* downregulation led to delayed morphogenesis as compared to control cells (Fig. 3.8). Constitutive *AIF* downregulation displayed a delay in initial stages of development i.e. tipped aggregate to migrating slug transition. Tipped aggregate and migrating slug were seen at 22 hrs and 36 hrs in constitutive *AIF* dR cells; while in control cells these stages were observed at 14 hrs and 18 hrs respectively. Mature fruiting bodies were formed only at 48 hrs in constitutive *AIF* dR cells which were smaller as compared to fruiting bodies of control cells at 24 hrs. Nevertheless, prestalk specific *AIF* dR cells exhibited delayed development during migrating slug (36-38 hrs) and fruiting body formation (48 hrs) (Fig. 3.8).

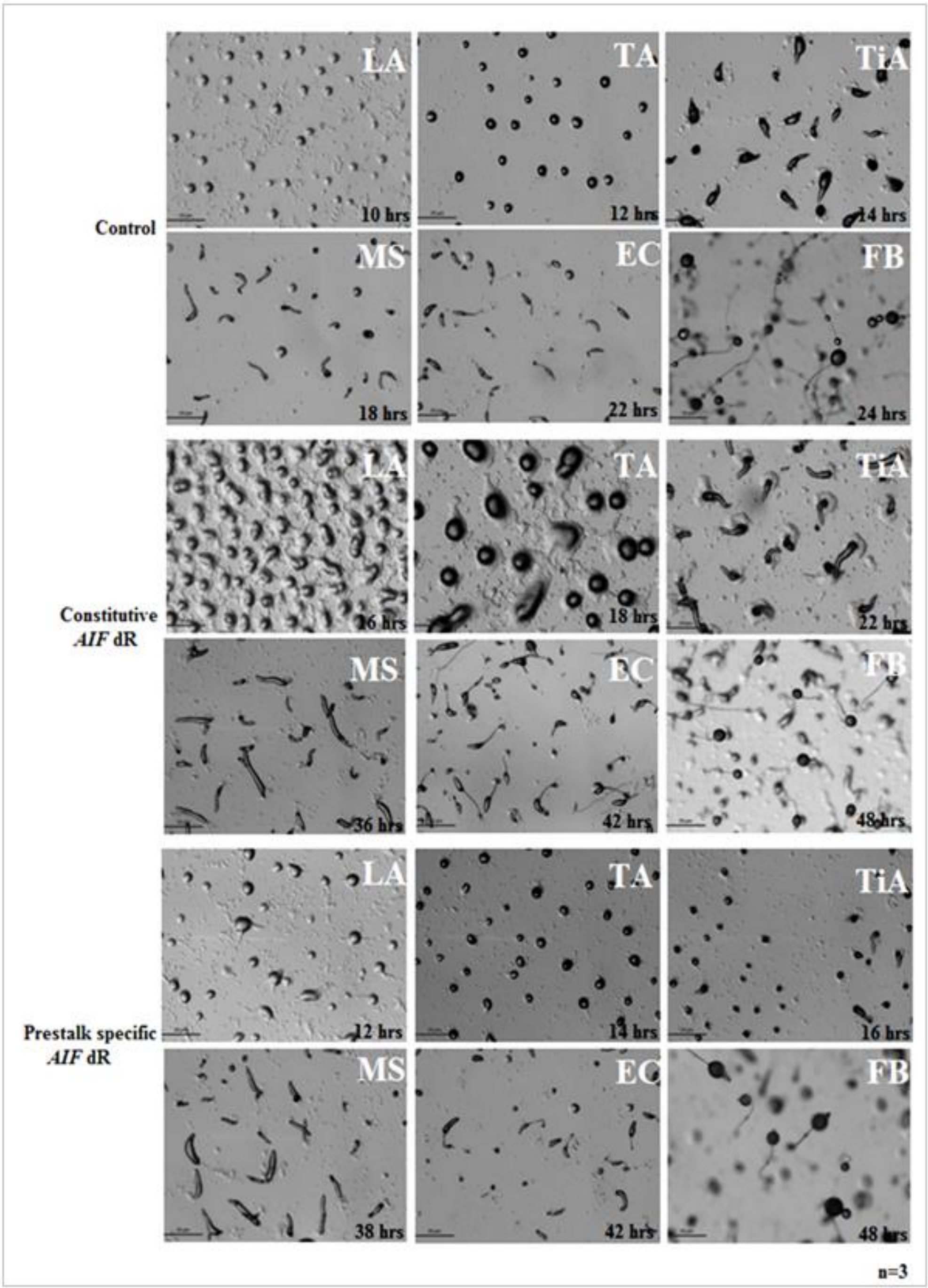

Fig. 3.8: Effect of *AIF* downregulation on *D. discoideum* development: Cells were starved and development was monitored till 48 hrs. Delayed development was observed in both, constitutive and pre-stalk specific *AIF* dR cells by 24 hrs and the formation of fruiting bodies were observed by 48 hrs. The 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=10µm, Magnification=4X). Data are representative of three independent experiments.

3.2.10 Effect of *AIF* downregulation on cell differentiation

Delay in development in *AIF* dR cells was substantiated by analyzing the transcript levels of differentiation prespore and prestalk markers. Real Time PCR analysis showed an increase in *EcmB* (prespore marker) (p=0.0441) and decrease in *D19* (prestalk marker) (p=0.0094) transcript levels in constitutive *AIF* dR cells compared to control cells (Fig. 3.9) and hence delay in developmental morphogenesis of *AIF* dR cells. Thus, these results indicate a role of AIF in cellular differentiation which needs to be further investigated.

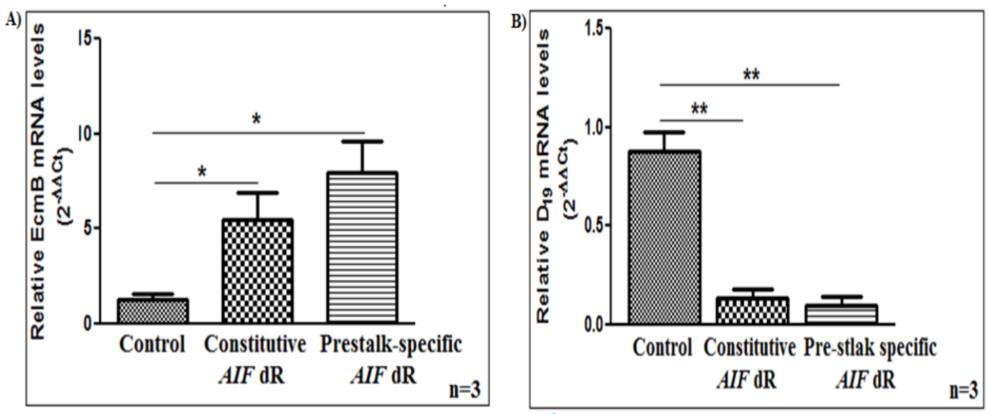

Fig. 3.9: Real time analysis of prespore-prestalk markers in constitutive *AIF* dR slugs: A) *ECMB* transcript levels were found to be increased significantly while **B)** *D19* transcripts transcript levels were found to be decreased significantly in both *AIF* dR cells compared to control. Data are a representation of as mean ± S.E values of three independent experiments. $^{*}p<0.05$ as compared to control; $^{**}p<0.01$ compared to control.

In conclusion, the above results illustrate a definite role of *AIF* in *D. discoideum* growth and multicellularity (Fig. 3.10).

3.3 Discussion

Although AIF is engaged in cell death and survival, its mechanism in the latter is not clearly understood. Hence, analyzing the function of AIF in cell survival seems quite promising. An understanding of how AIF functions during growth and development of *D. discoideum* would aid us to pinpoint its survival role which remains ambiguous.

Various reports on *in vivo* phenotypes associated with loss of *AIF* have discovered the significance of AIF in the maintenance of mitochondrial energy metabolism which is supposed to contribute for cell growth and development. Fruit flies with *AIF* deficiency led to growth arrest and exhibited imperfect complex I function and compromised ATP levels (Joza *et al*., 2008). *AIF* knockdown in *Caenorhabditis elegans* also showed a slower growth rate (Wang *et al*., 2002). In line with these reports, our findings suggest that diminution in *AIF* transcript levels (Fig. 3.3) resulted in to reduced cellular growth (Fig. 3.5) and delay in developmental morphogenesis (Fig. 3.8), accentuating its essential role in *D. discoideum* cell growth and development. Previous studies have shown that loss of AIF causes a reduction in the growth of embryonic stem cells (Vahsen *et al*., 2004) and abortive embryogenesis in mice (Brown *et al*., 2006; Porter and Urbano, 2006). The slower growth rate in constitutive *AIF* dR cells was explained by cell cycle analysis which revealed an enhanced number of cells in S phase of constitutive *AIF* dR cells (Fig. 3.6), suggesting *AIF* deficiency also impacts cell cycle. Klein group observed that post-mitotic retinal neurons and cerebellar granule cells of Hq mouse re-entered the cell cycle and subsequently underwent cell death (Klein *et al*., 2002). Moreover, *AIF* knockout in the cerebellum of the mouse displayed an increased number of Purkinje cells in S phase, which could not undergo mitosis during neurogenesis (Ishimura *et al*., 2008). Oxidative stress is known to be associated with abnormal cell cycle functions. Under normal conditions, the cell cycle proceeds without disturbances. However, if cycling cells are damaged by oxidative stress, cell growth would be arrested (Shackelford *et al*., 2000). Given the function of AIF in mitochondrial respiration as a ROS scavenger, absence of AIF could result in an accumulation of ROS, which would then dysregulate cell cycle, cell death and development (Schulthess *et al*., 2009). This is supported by the study wherein β-cells from AIF deficient mice and human islets from AIF-siRNA-treated exhibited sensitivity to oxidative stress induced cell death (Maechler *et al.,* 1999). The *D. discoideum* developmental cycle shows cell differentiation into prespore and prestalk cells (Jeremyn *et al*., 1989). Delayed development in *AIF* dR cells could be due to altered prespore-prestalk differentiation markers (EcmB and *D19*), implying the AIF's function in cellular differentiation too (Fig. 3.9). A proposed mechanism for slower growth rate and delayed development in *AIF* dR cells could be affected mitochondrial activities such as respiration, ATP production, ROS generation, cell death as AIF is suggested to maintain the mitochondrial functions. Thus, it would be interesting to further study how AIF impacts *D. discoideum* mitochondrial activities which would be addressed in the next chapters.

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).

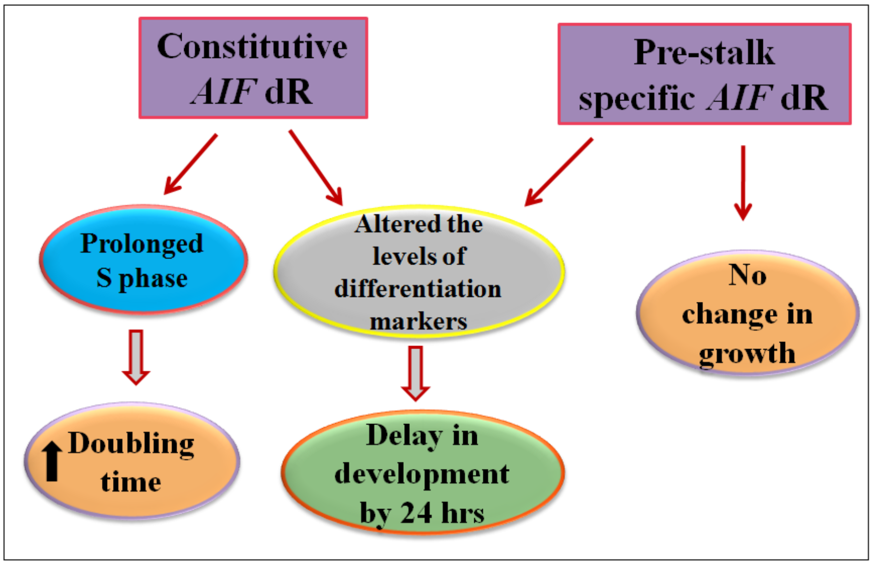

Fig. 3.10: Role of AIF in cellular growth and multicellular development of *D. discoideum*.

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