encompass regions necessary and sufficient for the vital mitochondrial and lethal nuclear activities of AIF, respectively.

Human AIF is coded by nuclear genome. The N-terminal MLS (Mitochondrial localization signal) of the precursor protein of 67 kDa is cleaved by a peptidase in the inter-membrane space. This trimmed protein then non-covalently incorporates FAD during refolding in the mitochondrial inter-membrane space. The recent members of this family of proteins: AMID and AIFL lack N-terminal localization sequence however, these proteins also localize in the mitochondria, suggesting the existence of an internal MLS (Fig. 8.2). AMID differs from AIF in one more aspect; it uses 6-hydroxy FAD as an oxidoreductase cofactor rather than FAD. Phylogenetic analysis suggests that AIF, AMID and AIFL were present in eukaryotic organisms before the emergence of metazoans.

Presence of an oxidoreductase domain and the localization in mitochondria assign significance to this protein in the functioning of electron transport chain. On the other hand the cell-death pathway initiated by PARP activation appears to be mediated by AIF. Probably AIF functions both as a free radical scavenger as well as a promoter of cell death according to the location of the protein. Parallels can be drawn between AIF and cytochrome c: both are important for cell viability when they are located in mitochondria, but when either is released from the mitochondria, they activate death programs. PARP activators, including the DNA-alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine, hydrogen peroxide (H_2O_2) and NMDA induce the translocation of AIF from mitochondria to the nucleus, nuclear condensation, annexin-V staining, dissipation of the mitochondrial membrane potential and cell death. Each of these events is caspase independent as broad-spectrum caspase inhibitors do not block these events (Yu *et al.*, 2002). Neutralizing antibodies to AIF block PARP- mediated cell death (Yu *et al.*, 2006). Taken together, these results indicate that AIF is an essential downstream effector of the cell-death programs initiated by PARP. Pharmacological agents that hinder the translocation of AIF or inhibit the promotion of DNA fragmentation induced by AIF might have tremendous therapeutic potential (Ravagnan *et al.*, 2001). Our previous results suggest that under various stress conditions PARP activation induces mitochondria-nuclear translocation of AIF that is followed by cell death. However, there are no known inhibitors of AIF. The broad-spectrum cytoprotective molecules such as Bcl-2 and a member of the heat shock protein 70 family can delay or prevent AIF mediated toxicity. In this study an attempt was made to down regulate AIF by anti sense.

8.1.1. Strategy for targeted down-regulation of *aif*

Gene sequence of AIF

DDB0187853 [DNA coding sequence] gene: *aif* on chromosome: 5 position 1306170 to 1307857

ATGATAAGAAATTTAACAAAATTAACAAAATTTACAATTGGAAATAGATTTTACCAATCC

T

TTAACA

AAATCACTTTGGGCTACTAAAGATGATAATGTTGTAAATACTTTAAATTTCTCTGATTGG

TCTGGTAAAAAACAAAATTTATTATATGAACAAGAATCAGCATATGGTAATGAAATTTTA

CAATTTATTAGAACTAAAAAAGTTATTGATTTACATATTGATGAAAAATTAGTATTATTA

AATGATGGTAAATTAATTCGTTATGACAAATGTTTAATTGCAACAGGTGGTGAACCACGT

CAATTAAAATTCACATCAACCAATGATAAAAAGATTTCAACCTATAGAACCGTTGAAGAT

TTTAGAAAACTTTATGAAGTTGTAAAGGATGGTGGTAAACATGTCACCGTTTTAGGTGGT

GGTTTCTTAGGTAGTGAATTAACTTGTGCAATCAATTCAAACTTTCAAGATAAAAACATT

AAAATCGATCAAATTTTCCCAGAATCTGGTGTATTAAGTACTTTATTCCCAGACTATCTT

AGTAAATATGCAACTGAAGAAATCATTAAGAGTGGTGTTAATGTTCACACTGGTACTTTA

ATTAAAGATGTAGTCGATAACTCTGAAAATGGTAGATTAACTGTCACTTTAAACAATGGT

AAAACCTTTGAAACTGATCATGTTGTTGTTGCCGCCGGTATCATTCCAAATACAAATGTA

GTTAAAAGTACAACATTAGAAATAGATCCAATAAATGGTGGTTATGTCGTCAATCCAGAA

CTTCAAGCTCGTACCGATCTATATGTCGCTGGTGATGTCGCATCCTATTATGATTTCAGT

TTAGGTGTACGTCGTCGTGTTGAACATCATGACCATGCACGTGCCACTGGTGAAATGGCT

GGTTCAAATATGTCAACTAAGGATACACCAGCTCCATACACATATCAACCATTCTTTTGG

TCAGATTTAACACCAGGTGTTGGTTTTGAAGCTGTTGGTAATACTAGCTCAAAATTAAAA

ACATTTTCAGTTTGGGAGAAGCCATCCTCTGATGAAACTAAACAATCCTACACCAAAGGT

AACATCTATTACTTGAATGATAACAATAATGTCGTTGGTGTCTTATGTTATGGTAACTAT

GGTAAAATGGATACTGCTCGTGACCTCATCTTAAAACGTAGAACTATCGAAGATTTAAAT

CAATTACAACATGCTATCGATTTCGATGAACATCATTAA

Figure 8.3: *aif* gene sequence of *D. discoideum*. Blue highlighted region depicts part of the N terminal region amplified using PCR.

As predicted from the domain information (http://dictybase.org; http://www.expasy.ch/tools), the conserved region of N terminal domain of *aif* gene was used as template to design primers for antisense since N terminal targeting antisense is known to be most effective for downregulation. Primer designing was done with the help of online software, Primer 3 as well as manually. Two restriction sites (*viz. Kpn*I and *BamH*I) were incorporated within the primers to facilitate directional cloning of the amplicon in the vectors of choice. The predicted amplicon sequence (353 bp) was pre-checked for the absence of internal recognition sites for these two restriction endonucleases with the help of an online tool Restriction mapper (http://www.restrictionmapper.org). Figure 8.4 below clearly depicts the region of the gene targeted for the construction of antisense.

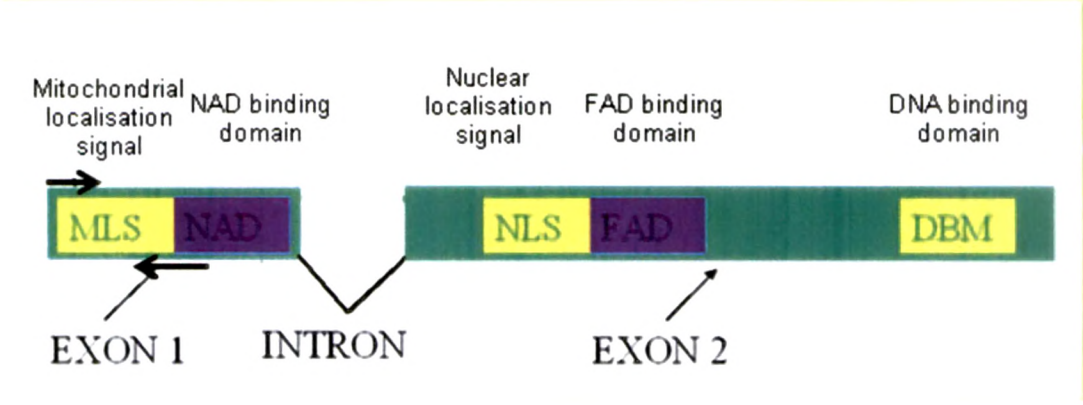


Figure 8.4: Schematic representation of the target for antisense construction. Expected amplicon size: 353 base pair

(i) pTX-GFP

[Procured from dictybase] (http://dictybase.org)

- GFP expression under control of the actin15 promoter
- Constitutive expression
- MCS flanking GFP
- Geniticin & ampicillin resistance as selectable markers.

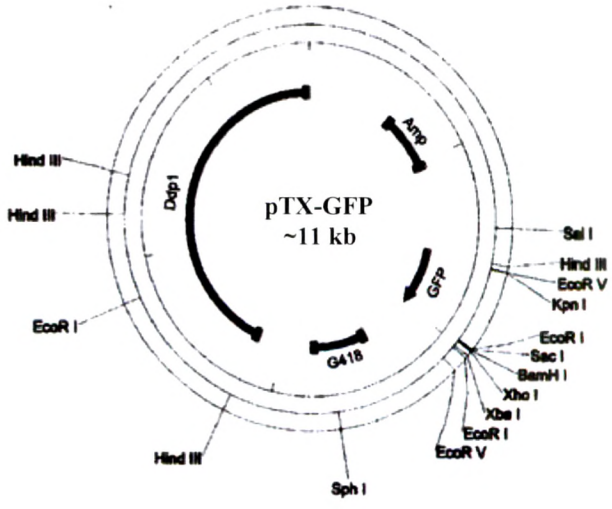


Figure 8.5: Restriction maps of pTX-GFP.

8.2. Results

8.2.1. Cloning of AIF antisense and downregulation of AIF

DNA from a log phase culture of *D. discoideum* was extracted as mentioned earlier in materials and methods section. It was then further used as a template for the PCR amplification in order to get the 5' region of *aif* using specific primers (PCR conditions as mentioned in materials & methods) and the resultant 353 bp amplicon is shown in Fig. 8.6. The confirmed amplicon was subjected to gel elution and subsequently purified DNA fragment was ligated to *EcoR*V digested pBluescript (pBKS) plasmid. The ligation mixture was transformed in *E. coli DH5α* and the transformants were subjected to blue white selection on Luria agar containing ampicillin antibiotic. Randomly selected white colonies were screened for the presence of recombinant plasmids (named A1 and A30) which was confirmed by restriction digestion pattern (Fig. 8.7). Thus obtained recombinant plasmid A1 was used as an intermediate plasmid for further cloning into the target *Dictyostelium* expression vectors.

Intermediate clones (A1) that gave a release of 390 bp upon *Bam*HI digestion was directionally subcloned into (constitutive *Dictyostelium* expression vector) pTX using *Xba* I and *Kpn* I restriction enzymes. The backbone of pTX-GFP and the insert released from pBKS backbone were gel eluted and purified and subjected to ligation. *E.coli DH5α* was transformed with the ligation mixture and ampicilin resistant colonies obtained were randomly selected. Restriction enzyme digestion patterns of *Bam*H I and *Sal* I (Fig. 8.8) confirmed required orientation of the clone. *BamH* I and *SalI* digestion gave a release of 390 bp and pTX-GFP showed linearised DNA with *BamHI* digestion confirming the required subcloning. The confirmed clone containing AIF antisense, pTX-AIF, was used for transformation of *D. discoideum* cells.

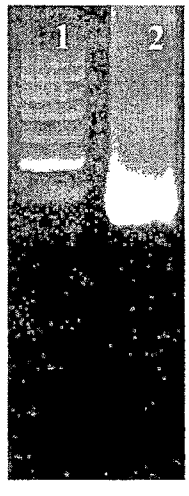


Figure 8.6: PCR amplification of 5' region of *aif*. Lane 1: 100bp ladder; Lane 2: amplicon obtained after PCR with primers corresponding to 5' region of *aif*.

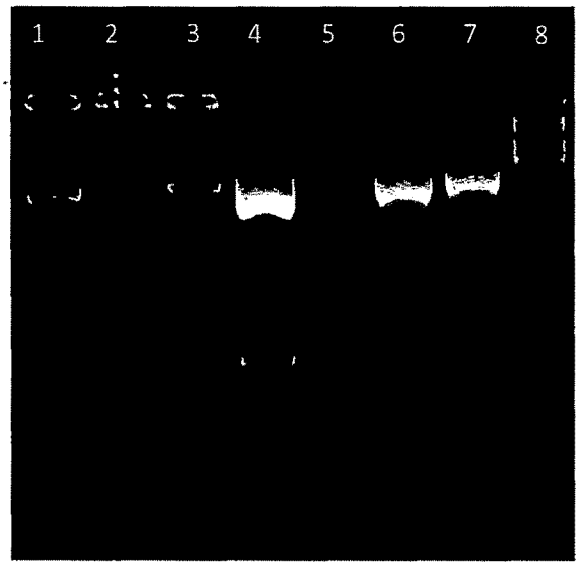


Figure 8.7: Confirmation of the orientation of inserts in clones: A1 and A30.

Lane 1: Linearization of pBKS by *BamH* I

Lane 2: Linearization of A1 by *Kpn*I

Lane 3: A1 clone undigested

Lane 4: ~390 bp release by *Bam H* I digestion of A1 clone

Lane 5: 100 bp ladder

Lane 6: ~350bp release by *XbaI* & *Kpn*I digestion of A1

Lane 7: Linearization by *BamH* I digestion of A30

Lane 8: pBKS vector

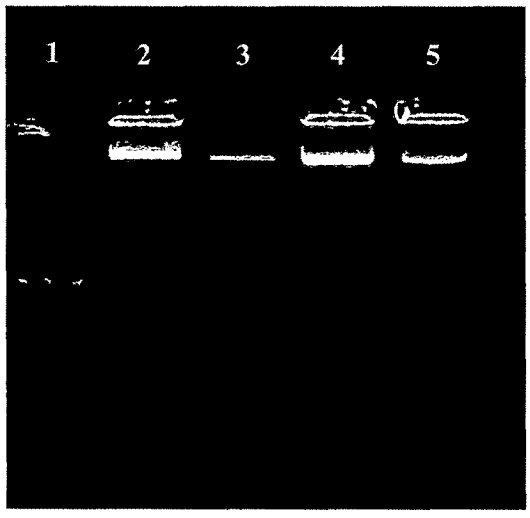


Figure 8.8: Confirmation of insert orientation in pTX-AIF As clone

Lane 1: 100 bp ladder

Lane 2: *Bam H*1 & *Sal* 1 digestion of A1clone

Lane 3: Linearisation of clone by *BamH*1

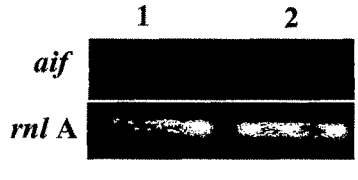
Lane 4: *Xba* 1& *Kpn* 1 digested pTX vector

Lane 5: Linearization of pTX vector by *BamH*1

Cells transformed transformed with the clone failed to sustain the highest G418 concentration i.e., 20 ug/ml, whereas positive controls grew to confluency in same selection pressure. Out of curiosity cells were harvested after selection at a lower concentration of G418 (~8 ug/ml) and subjected to development and quantitation of AIF mRNA. AIF mRNA was found to be reduced by 70% (Fig. 8.9).

Figure 8.9: RT-PCR of *aif* and constitutive gene *rnl*A from *D. discoideum*

Lane 1: Control cells, Lane 2 AIF downregulated cells



8.2.2. Effect of AIF dR on development

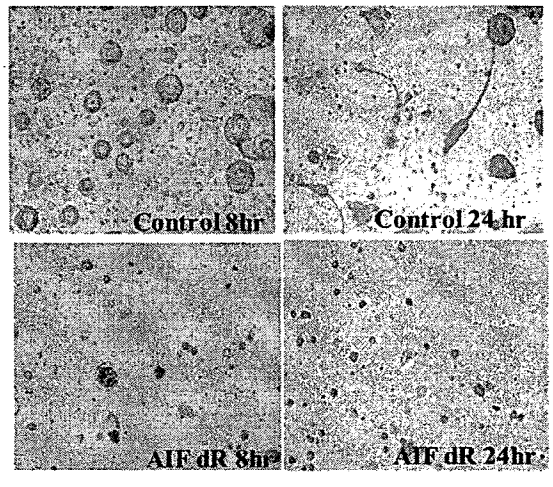


Figure 8.10: Developmental arrest in *D. discoideum* cells with constitutive down-regulation of PARP. The development was monitored after 24 hours. The photographs have been taken under 10X objective.

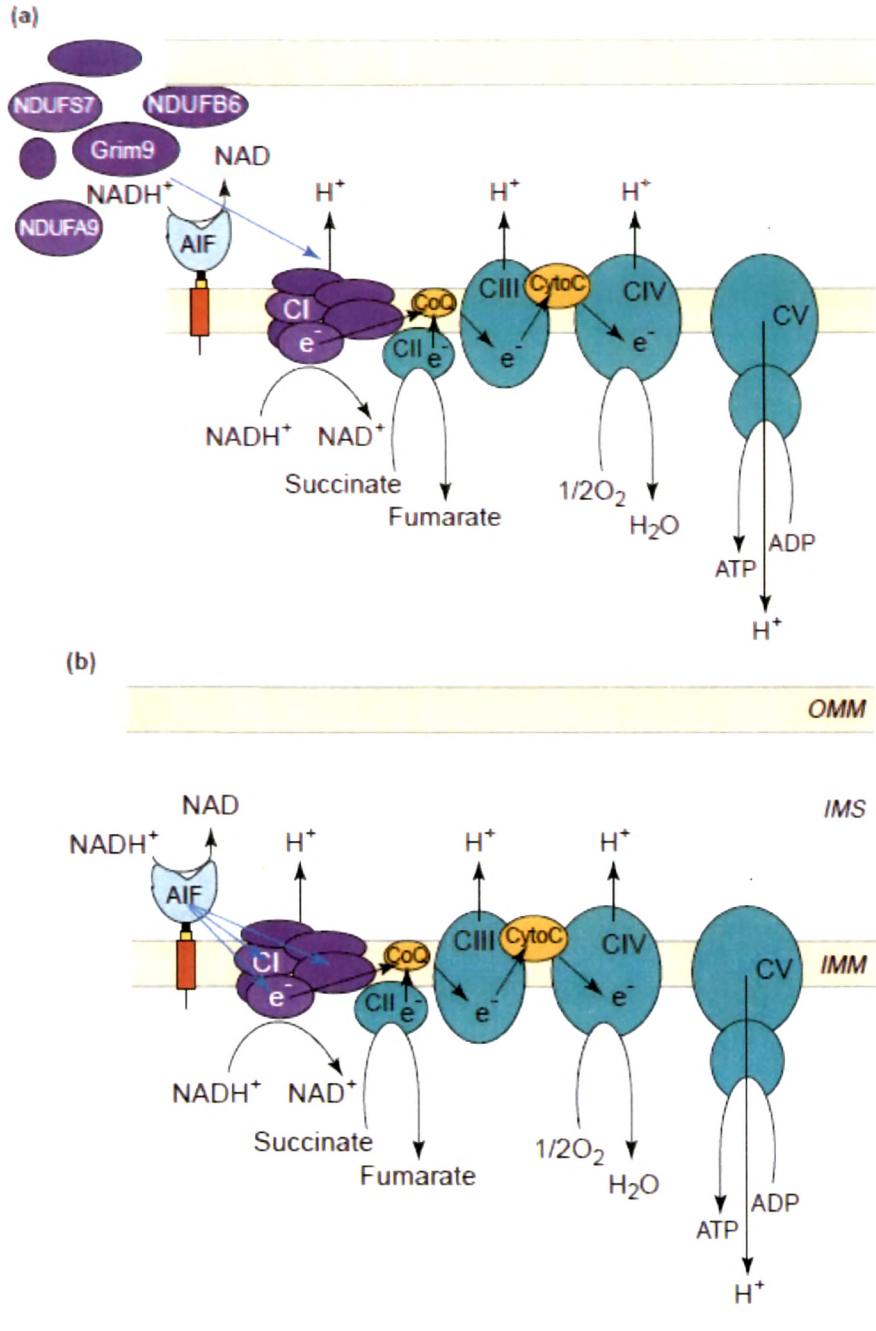
Cells were harvested two weeks after transformation and were subjected to development. Also these cells were unable to enter development as shown in figure 8.10.

8.3. Discussion

Caspases are not indispensable for programmed cell death. The observation that cells do exhibit alternate pathways to undergo cell death which are caspase independent has evoked interest in PARP and AIF and their role in these alternate pathways of cell death. Much interest has been emerging to understand the precise mechanism by which PARP and AIF mediate different types of cell death. We have addressed the role of PARP with the use of benzamide and also by antisense mediated down regulation. Our results on PARP inhibition by benzamide suggested that UV-C induced paraptotic and necrotic cell death are PARP mediated. Our antisense results also suggest that PARP down-regulation has delayed the oxidative stress induced paraptotic cell death (Rajawat, 2010). Our results also suggest that *D. discoideum* necrotic cell death that occurs in an orchestrated manner *via* PARP overactivation is also shifted to paraptotic type in PARP down-regulated cells. PARP down-regulated *D. discoideum* cells get arrested at loose aggregation stage when subjected to development however, no effect was observed on the growth of unicellular *D. discoideum* (Rajawat, 2010). These results support the idea that complex development and differentiation in *D. discoideum* may require PARP. Presence of PARP in *D.*

discoideum and also in *A. nidulans* (filamentous fungi) signifies its role in multicellularity (Semighini *et al.*, 2006). However, further studies are needed to confirm the link between PARP and multicellularity.

AIF is another key player of caspase independent cell death; however inhibitors for AIF are not available. AIF plays an important role in the acute neurotoxicity induced by trauma, hypoglycemia, transient ischemia and chronic neurodegenerative diseases (Hong *et al.*, 2004). AIF nuclear- mitochondrial translocation was studied in several experimental models of neurodegeneration, including the death of photoreceptors induced by retinal detachment (Hisatomi *et al.*, 2002), the *in vivo* neuronal death induced by brain trauma (Zhang *et al.*, 2002) and cerebral ischemia (Zhu *et al.*, 2003), the death of cortical neurons induced *in vitro* by exposure to heat inactivated *Streptococcus pneumoniae* (Braun *et al.*, 2001), hydrogen peroxide, peroxynitrite (Zhang *et al.*, 2002), the topoisomerase I inhibitor-camptothecin, infection with a p53-expressing adenovirus (Cregan *et al.*, 2002), the striatal cytotoxicity model of Huntington's disease (Wang *et al.*, 2003) and the excitotoxin NMDA (Yu *et al.*, 2002). Under excitotoxic conditions, the NMDA-induced mitochondrial release of AIF is PARP-dependent but caspase-independent, and neutralization of AIF by an AIF-specific antibody prevents cell death (Wang *et al.*, 2003; Yu *et al.*, 2002). AIF inactivation abolishes cavitations, an apoptosis-mediated process indispensable for early embryonic morphogenesis (Joza *et al.*, 2001). Regardless of the presence or the absence of NAD(P)H and/or FAD (which is the essential prosthetic group of the oxidoreductase), AIF can induce nuclear apoptosis (Loeffler *et al.*, 2001; Miramar *et al.*, 2001). These studies suggest that the oxidoreductase function of AIF is not required for its apoptogenic action. Considering all these studies it was expected that AIF down-regulated cells would show survival benefit as compared to control cells in the presence of inducer of paraptosis. However, the results presented in this study show that down-regulation of AIF has lethal effect on *D. discoideum* cells i.e. AIF down-regulated *D. discoideum* cells could not survive till the highest concentration of G418 (20 ug/ml) i.e. till maximum down-regulation. This effect could be owing to its vital role in electron transport chain and its short half life (~ 5 h) (Desmots *et al.*, 2008). AIF deficient *S. cerevisiae* (Wissing *et al.*, 2004) and *D. melanogaster* (Joza *et al.*, 2008) show growth defects. AIF dR *C. elegans* (Wang *et al.*, 2002) show slower growth and smaller brood size whereas AIF knock outs are associated with embryonic lethality (Brown *et al.*, 2006).



Modjtahedi *et al.*, 2006

Figure 8.11. Vital functions of AIF. Two hypothetical models describing the local action of AIF. (a) AIF as an assembly factor: as a structural component of the inner mitochondrial membrane and redox-active enzyme, AIF might be involved in the import or assembly of the 46 complex I subunits (as well as those of complex III, not shown). (b) AIF as a maintenance factor: depending on its redox-activity (NADH oxidase or another), AIF might be necessary for the maintenance and stability of complexes I and III. These hypothetical models do not suggest the direct implication of AIF in the respiratory chain activity.

A few reports suggest that AIF down-regulated cells could exhibit a reduction in the expression of complex I subunits i.e., p17, p20 and p39, coupled to a reduction in complex I activity (Vahsen *et al.*, 2004 and Urbano *et al.*, 2005). Also *aif* gene silencing studies by siRNA shows partial complex III defect in embryonic stem cells and HeLa cells (Vahsen *et al.*, 2004) and hence survival defects. Thus respiratory defect in complexes I and III is linked to the reduction in the abundance of many proteins participating in these complexes (Vahsen *et al.*, 2004). This reduction is not seen in mRNA levels, indicating that a post-transcriptional mechanism is responsible. On the other hand cortical neurons from wild-type and Hq mice (mice in which AIF expression is reduced markedly) are equally sensitive to hydrogen peroxide-induced injury. This is because sufficient AIF is expressed in Hq neurons to mediate AIF-dependent cell death and maintain electron transport chain (ETC). This effect is tissue specific which could be due to redundant gene function (Modjtahedi *et al.*, 2006).

Local redox activity of AIF is required to sustain complex I activity, however, it is unknown whether AIF is necessary for an optimal assembly of complex I, and therefore in its biogenesis, or it participates in the maintenance of complex I (Fig. 8.11) (Modjtahedi *et al.*, 2006). Although AIF might function as a free-radical scavenger to prevent apoptosis under normal physiological circumstances, it is also evident that AIF is an important factor for apoptosis.

As mentioned earlier *D. discoideum* cells could not sustain till 20 ug/ml of G418 selection pressure when the down-regulation of AIF would be at its peak, cells selected at a lower G418 concentration were harvested and subjected to development. It was alarming that these cells which showed 70% reduction in AIF mRNA levels were unable to enter development. These results suggest that AIF is crucial for survival and in order to study its role in *D. discoideum* cell death one must play with its DNA binding domain without affecting its oxidoreductase function. Also different results obtained in different experimental models and conditions as discussed above tell us to be cautious while drawing conclusions for the contribution of AIF during cell death and in the functioning of electron transport chain.

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