The cell is the most complex and most elegantly delineated system. Each cellular process is regulated and executed in a coordinated manner. To perform different functions, the cell has developed an extremely proficient system by which various cell organelles take part to accomplish a task.

1.1 The Mitochondrion: the center for life and death

One of the most important cell organelle is mitochondrion, the powerhouse of cell. Every cellular activity is driven by the hydrolysis of adenosine triphosphate (ATP) to adenosine diphosphate (ADP). Mitochondria synthesize ATP by oxidative phosphorylation (OXPHOS) that occurs along with several protein complexes called the electron transport chain (ETC). Due to an ancestral alpha-proteobacterial endosymbiont origin, it still contains and expresses its own genome, mitochondrial DNA (mtDNA) that encodes a few, but very significant proteins of the mitochondrial OXPHOS system. It plays a major role in several cellular processes such as cell death, energy production, redox metabolism, ion homeostasis, steroid synthesis etc (Green, 1998; Rossier, 2006). It consists of four different compartments- the inner mitochondrial membrane (IMM), the outer mitochondrial membrane (OMM), the inter-membrane space (IMS), and the matrix. The IMM is highly convoluted forming folds which are called cristae. It permits only certain molecules to pass through it and is much more selective than the OMM (Fig. 1.1) (Mannella *et al*., 2001). It functions as a reservoir of suicidal proteins involved in the mitochondria mediated cell death mechanism. In response to cell death stimuli, the outer mitochondrial membrane loses its membrane potential ($\Delta\psi m$) and gets disrupted (Goldstein *et al*., 2000) leading to the release of two vital pro-apoptogenic proteins from inter-membrane space into cytosol viz., cytochrome c and Apoptosis Inducing Factor (AIF) (Susin *et al*., 2000; Joza *et al*., 2001). There has been a heightened interest to understand the mitochondrial biology in terms of energy production, cell survival and cell death, and the association of mitochondrial dysfunction with various diseases.

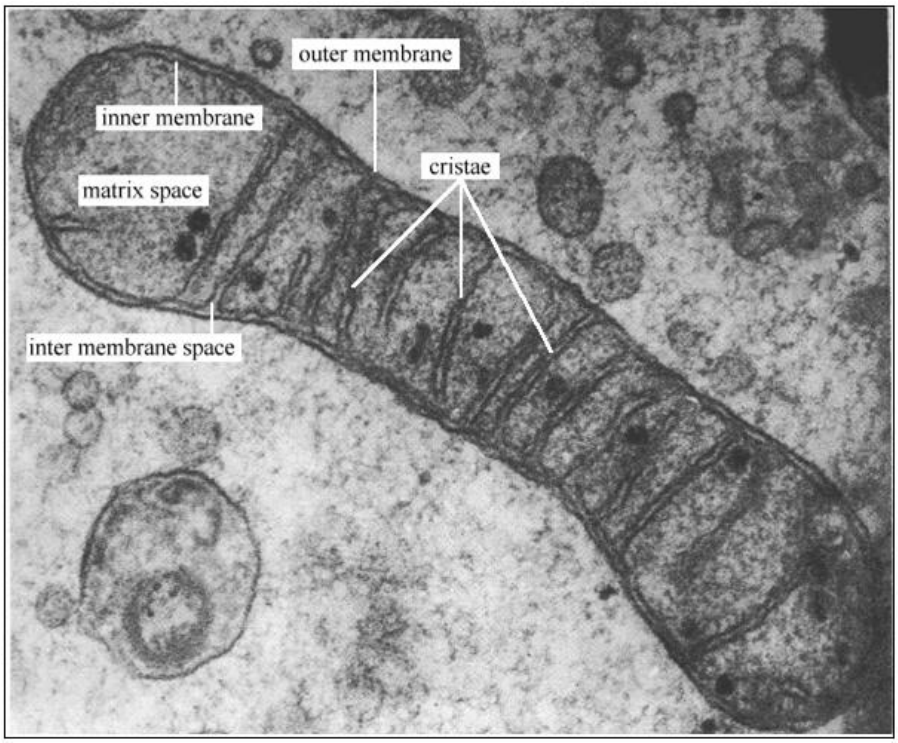

Fig. 1.1: Electron microscopic structure of mitochondrion

[Image credit: Prof. Ruth Bellairs, University College, London WC1E 6BT]

1.2 Apoptosis Inducing Factor (AIF)

AIF, a nuclear encoded mitochondrial protein, was first discovered by Kroemer and his group in 1996. As it conserved the apoptogenic ability in the presence of a pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (z-vad.fmk), the protein was termed as Apoptosis Inducing Factor as one of the soluble proteins released from mitochondria capable to force the nucleus showing apoptotic morphology in a caspase-independent manner (Susin *et al*., 1996).

1.2.1 AIF structure

The AIF gene (2.4 kb) is also known as AIFM1 and PCDC8 and is located on chromosome Xq25–26 region in humans (Daugas *et al*., 2000). Transcription and translation of the ubiquitous nuclear-encoded gene results into a 67 kDa precursor molecule (1-612 aa residues), consisting of the N-terminal Mitochondrial Leading Sequence (MLS), two Nuclear Leading Sequences (NLS), and the Nicotinamide Adenine Dinucleotide (NAD)- and Flavin Adenine Dinucleotide (FAD)- binding motifs and a C-terminal domain (Fig.

1.2) (Vahsen, Ph.D. thesis, 2006). The precursor AIF, a non-native form is synthesized in the cytoplasm and then imported to mitochondria to refold into full-length AIF, acquiring its apoptogenic ability irrespective of FAD binding (Susin *et al*., 1999). The core consensus of the motif of the Rossman fold (GXGXXG/A) is found at two distinct regions of the human AIF sequence (aa 138-143 and 307-312). The N-terminal motif is thought to be implicated in the NADH binding whereas the C-terminal domain is composed of five anti-parallel β-strands followed by two α-helices and binds to FAD (Kleiger and Eisenberg, 2002). The C-terminus of *D. discoideum* AIF homologue has been proposed to have a DNA binding helix-turn-helix motif (Arnoult *et al*., 2001). AIF also has the ability to bind to RNA (Vahsen *et al*., 2006). AIF is thought to bind to DNA through the electrostatic interactions as it has a strong positive electrostatic potential at its surface (Ye *et al*., 2002) which mediates chromatin condensation and DNA fragmentation by increasing the susceptibility of DNA to the endonucleases (Sevrioukova, 2011).

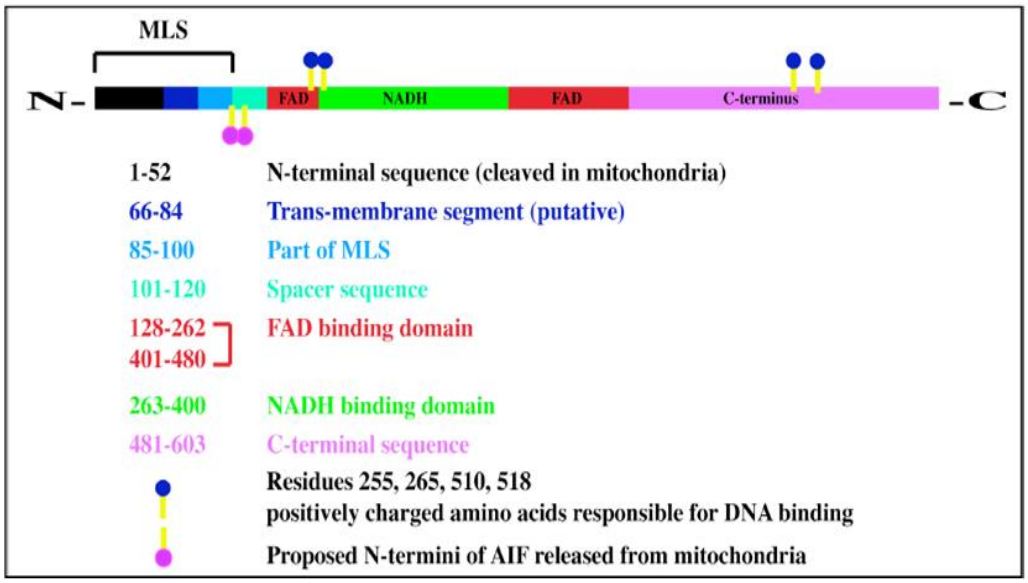

Fig. 1.2: Schematic representation of human AIF protein structure: AIF consists of N-terminal MLS, two NLSs, the NADH- binding motif, FAD-binding motifs and a C-terminal region.

1.2.2 Transcript variants of AIF

1.2.2.1 Membrane-tethered mature AIFΔ1-54

AIF precursor is cleaved by a mitochondrial matrix peptidase at Met54 or Ala55, resulting in the mature form (~62 kDa). Residues 67-85 were identified as the IMS sorting signal and a trans-membrane fragment through which mature AIFΔ1-54 form (Fig. 1.3) is tethered to the inner membrane (Otera *et al*., 2005). Transport of AIF from the mitochondrial matrix into the IMS occurs through the inner membrane channel protein, Tim23 (Herrmann and Hell, 2005). Since the precursor AIF does not contain FAD, proteolytic maturation and import into the IMS appear to be required for the flavin incorporation and protein folding (Susin *et al*., 1999).

1.2.2.2 Soluble apoptogenic AIFΔ1-102/118

Cleavage of the 96-120 segment of AIFΔ1-54 by calpains or cathepsins can lead to the formation of soluble Δ1-102 or Δ1-118 fragments (~57 kDa) (Fig. 1.3) (Polster *et al*., 2005; Churbanova and Sevrioukova, 2008). Upon apoptotic stimuli and permeabilization of the OMM, AIFΔ1-102/118 fragments translocate from the IMS to the nucleus to execute apoptosis.

1.2.2.3 Splice variants AIF-exB, AIFsh, AIFsh2, and AIFsh3

Distantly related homologues of AIF present in humans are called AIF-homologous mitochondrion-associated inducer of death or p53-responsive gene 3 [AMID (apoptosis-inducing factor-homologous mitochondrion-associated inducer of death) /PRG3/AIF2/AIFM2)] and AIF-like (AIFL/AIFM3) (Wu *et al*., 2002; Ohiro *et al*., 2002). An alternatively spliced AIF-exB/AMID is a brain-specific isoform which uses exon 2b instead of 2a which differs from AIF/AIFM1 (Susin *et al*., 1999; Loeffler *et al*., 2001). Exons 2a and 2b are phylogenetically conserved among mammals and their usage is independent of mitochondrial transport of AIF. AIFM2 is predominantly located in the cytosol or loosely associated with the OMM in the cytosol. Though AIFM2 has no recognizable MLS, it is known to be involved in apoptosis (Hangen *et al*., 2010). AIFL (location: chromosome 22) appears to be mainly localized to the mitochondria. However, the mechanisms for its mitochondrial localization are not yet found. Though it is implicated in caspase dependent cell death as it has 2Fe-2S Rieske domain (might be important for apoptosis induction), there is no translocation of AIFL found

during apoptosis induction (Xie *et al*., 2005). Future investigations are needed to understand the underlying cellular mechanisms of AIFM2 and AIFM3. The AIFshort (AIFsh; ~35 kDa) isoform results from an alternative transcriptional start site located at intron 9 whereas AIFsh2 (1-322) and AIFsh3 (87-322) (Fig. 1.3) are produced via alternative usage of exon 9b (Dereeper *et al*., 2008). AIFsh2 and AIFsh3 lack the C-terminal domain and NLS2 and that cannot translocate to the nucleus. As AIF exists in multiple isoforms, the AIF-mediated function and signaling might be regulated at both the transcriptional and post-translational levels (Sevrioukova, 2011).

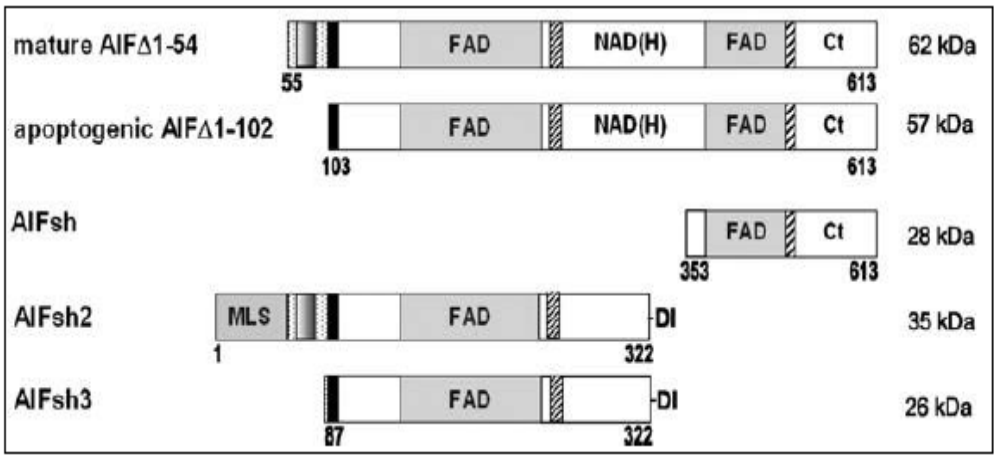

Fig. 1.3 Forms and splice variants of AIF: Mature AIF form is produced upon mitochondrial processing while the truncated apoptogenic form is produced in the IMS upon proteolytic processing. Naturally occurring transcripts corresponding to the AIF precursor are AIFsh, AIFsh2, and AIFsh3. (Sevrioukova, 2011)

1.3 Evolutionary origin and phylogeny of AIF

AIF homologs are conserved throughout the eukaryotic kingdom namely insects, nematodes, fungi, plants and mammalian systems (Cande *et al*., 2002). The strongest homology between FAD domains is observed with several plant ascorbate oxidoreductases, particularly with dehydroascorbate reductase from *Arabidopsis thaliana*, monodehydroascorbate reductase from *Cucumis sativus*, and the ascorbate free radical reductase from *Lycopersicum esculentum* (Lorenzo *et al.,* 1999). AIF also has a significant homology with NADH ferredoxin reductases from Eubacteria and Archaebacteria and putative

oxidoreductases from *Xenopus laevis* (vertebrate), *Drosophila melanogaster* (invertebrate) as well as from *Saccharomyces pombae* (Lorenzo *et al*., 1999). FAD and NAD domains are strongly conserved between AIF and two reductases, namely dihydrolipoamide dehydrogenase from *Pseudomonas putida* and human glutathione reductase (GR), suggesting that AIF possesses an oxidoreductase activity. It displays functional but not structural similarity with bacterial ferredoxin reductases (Mate *et al*., 2002). The oxidoreductase and the apoptogenic activity of AIF are independent as the AIF protein precursor (aa 1-613) does not bind to FAD (Susin *et al*., 1999).

1.4 Apoptotic role of AIF

The apoptotic function of AIF has been studied extensively. Immuno-electron microscopy, immunofluorescence and sub-cellular fractionation experiments have shown that AIF in healthy cells is confined to mitochondria (Daugas *et al*., 2000). Nonetheless, upon an apoptotic insult, it undergoes mitochondrio-nuclear translocation where it interacts with DNA directly to disrupt chromatin structure by recruiting nucleases or proteases and causes chromatinolysis as shown in Fig. 1.4 (Modjtahedi *et al.*, 2006). In order to release AIF from the mitochondria, permeabilization of the OMM activates cysteine proteases to cleave AIF giving rise to the pro-apoptotic form of AIF (~ 57kDa) (Otera *et al*., 2005). AIF cleavage can either happen in a Ca^{2+}-dependent manner involving the calcium-activated protease calpain or in a Ca^{2+}-independent manner (Polster *et al*., 2005) wherein cathepsin B, L, and S process AIF (Yuste *et al*., 2005). An interesting interaction has been observed between AIF and the nuclear protein-modifying and DNA repair enzyme, Poly(ADP-ribose) Polymerase-1 (PARP-1) (Yu *et al*., 2002). PARP-1 mediated cell death appears to be dependent on AIF translocation to the nucleus. PARP-$1^{-/-}$ fibroblasts failed to show AIF mitochondrio-nuclear translocation, suggesting AIF as a downstream effector in PARP-1 induced cell death (Yu *et al.*, 2002). Later, Poly(ADP-ribose) (PAR) binding site was found on AIF and AIF's PARylation triggered the AIF release from mitochondria to the nucleus (Wang *et al*., 2009). The mitochondrio-nuclear translocation of AIF and large scale DNA fragmentation are the hallmark features of caspase independent cell

death in mammalian cells (Cande *et al*., 2002). However, translocation of AIF can be seen in caspase-dependent and independent cell death pathways, depending upon the cell types and apoptotic stimuli, activated one at a time or one after the other in a specific manner (Cregan *et al*., 2004). In contrast to cytochrome c which remains cytosolic after its translocation from mitochondria, AIF moves to the nucleus, connecting to the initial phase of chromatin condensation (Susin *et al*., 1999; Ferri *et al*., 2000). Several reports suggested that AIF release from the mitochondria preceded first that of cytochrome c. However, the release of AIF before cytochrome c is not yet understood (Susin *et al*., 1999; Ferri *et al*., 2000; Daugas *et al*., 2000). On the flip side, AIF neutralization prohibits mitochondrial release of cytochrome c, emphasizing the possibility of AIF being necessary for cytochrome c dependent caspase activation (Cregan *et al*., 2002). In conclusion, the sequence of the release of AIF and cytochrome c appears to depend on the cell types and apoptotic stimuli similar to its dependency on caspases.

The effects of AIF on the nucleus are dependent on the functional and/or physical interaction with certain nuclear proteins such as Cyclophilin A (CypA) and Endonuclease G (EndoG). After translocation of AIF to the nucleus, it induces peripheral chromatin condensation and large-scale (50kbp) DNA fragmentation (Susin *et al*., 1999). AIF binding to DNA is mediated by a strong positive electrostatic potential at the AIF surface (Ye *et al*., 2002). AIF was found to interact with CypA in the nematode and EndoG in the mammals to mediate DNA degradation (Wang *et al*., 2002; Cande *et al*., 2004). Furthermore, in alkylating DNA damage-induced necroptosis (programmed necrosis) AIF contributes to assembling a DNA degradation complex with histone H2AX and CypA (Artus *et al*., 2010).

In addition to the AIF's nuclear apoptotic effect, it also exerts an impact on the mitochondria. Several reports indicate that the reduced cellular AIF levels make the cells highly susceptible to oxidative stress mediated cell death which is characterized by Mitochondrial Membrane Potential (MMP) loss, Phosphatidyl Serine (PS) exposure etc (Schulthess *et al*., 2009). The fact that the effect of AIF on intact cell or isolated organelle cannot be neutralized by

the overexpression of *Bcl-2* or Z-VAD.fmk treatment, clearly states that AIF induced cell death is caspase independent (Susin *et al*., 1999).

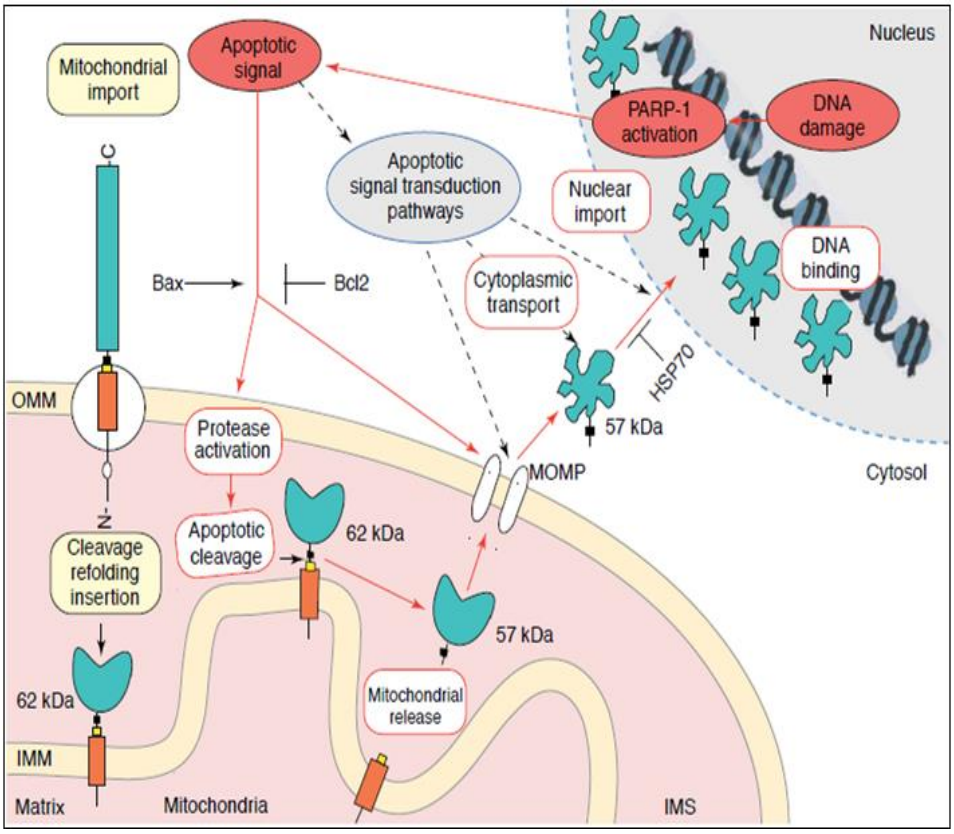

Fig. 1.4 AIF induced cell death: During the processing of AIF, full length precursor AIF is imported to the mitochondrion. After the N-terminal MLS cleavage by a mitochondrial peptidase, it is inserted in the inner mitochondrial membrane (IMM), via its N-terminal transmembrane region (orange), facing towards the inter-membrane space (IMS). In response to genotoxic/apoptotic stress, AIF's activation might be triggered by the PARP-1 signaling pathway. The apoptotic release of AIF from mitochondria to the nucleus requires the inducible activity of mitochondrial outer membrane permeabilization (MOMP) regulated by Bcl-2 family proteins. The cytoplasmic transport of AIF is inhibited by the anti-apoptotic Hsp70 protein. Upon AIF translocation to the nucleus, it binds to the chromatin and induces its condensation (Modjtahedi *et al.*, 2006).

1.5 Non-apoptotic roles of AIF

After cleavage of the MLS by mitochondrial peptidase, prosthetic group (FAD) binding to AIF is necessary for correct protein folding (Mate *et al*., 2002). This mature form of AIF is finally incorporated into the IM with its major part presumably facing the IMS. Under physiological conditions, native AIF exists mainly in a monomeric form but dimerizes upon binding of NADH and subsequent reduction of the flavin moiety (Ferreira *et al*., 2014). The overall structure of AIF comprising various protein domains and particularly its homology to oxidoreductases uncover another role of AIF. The apoptogenic activity is independent of its oxidoreductase function (Loeffler *et al*., 2001). Beyond its role in the cell death, AIF is proposed to have a cell protective role, maintaining mitochondrial homeostasis. Although AIF is well known to be involved in cell death, its cell survival function remains unclear.

1.5.1 Growth and development

Apart from AIF's apoptotic role, it has been shown to have an important role in cell survival in Harlequin (Hq) mice studies. Hq mice with 80% reduced *AIF* exhibited cerebellar and retinal degeneration starting at the age of three months (Klein *et al*., 2002). Muscle or brain specific AIF deficiency also induced cell loss in the tissues with no *AIF* expression (Joza *et al.*, 2005; Cheung *et al.*, 2006). T cell maturation is also regulated by AIF in Hq mice (Banerjee *et al*., 2012). These studies suggest the role of AIF during growth and development. However, the underlying mechanism is still not clear.

1.5.2 Mitochondrial respiration

Various studies appeared reporting a possible connection between AIF and complex I of the ETC. The growth arrest associated with AIF deficiency in fruit flies was characterised by a defective complex I function and the reduced ATP levels (Joza *et al*., 2005). Reduced *AIF* expression manifested impairment in both the complex I and III activities in the retina and brain with reduced expression of complex I subunits at the transcriptional and translational level, signifying AIF's role in the optimum functioning of OXPHOS. In *AIF* mutant mice, the cellular energy demands have been shown to turn their energy metabolism towards glucose utilization through glycolysis and not through oxidative phosphorylation due to its impaired mitochondrial

function (Vahsen *et al*., 2004). The loss of AIF also affected other respiratory complexes apart from complex I and III. A targeted deletion of *AIF* in the mouse heart or liver resulted in an abnormal complex IV activity along with complex I deficiency (Joza *et al*., 2005, Pospisilik *et al*., 2007). The Arginine residue (R201) is a portion of the FAD-binding pocket and the second NAD(H)-binding site of AIF which decides AIF's conformational stability and its dimerization status. Interestingly, deletion of this residue led to mitochondrial encephalomyopathy with significant reduction in complex III activity than complex I in the fibroblast (Ghezzi *et al*., 2010). There are mixed reports connecting AIF and ETC complexes activity. Literature suggests that the redox activity of AIF is necessary to maintain either complex I or complex III activity or both the complex activities. Two hypothetical models have been described for the local action of AIF. i. it may act as an assembly factor of ETC or ii. a maintenance factor of ETC complexes (Fig. 1.5). It is still unknown whether AIF is required for the assembly of all mitochondrial ETC complexes and therefore in its biogenesis, or it participates only in the maintenance of ETC complexes.

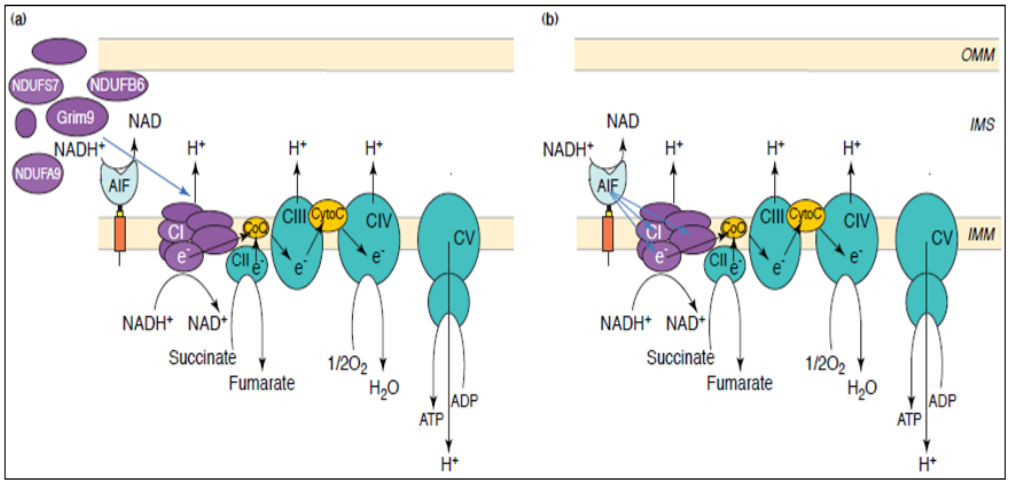

Fig. 1.5 Hypothetical models explaining the local action of AIF: (a) AIF as an assembly factor. As a structural component of the IMM and redox-active enzyme, AIF may be involved in the import or assembly of the complex I and complex III subunits. (b) AIF as a maintenance factor. Depending on the redox activity of AIF, it might be required for the maintenance and stability of complex I and III (Modjtahedi *et al.*, 2006).

1.5.3 Regulation of ROS levels

Loss of *AIF* also seems to alter ROS production because of the complex I impairment. Cerebellar granule cells from Hq mice showed more susceptibility to oxidative stress and increased glutathione and catalase activity, suggesting that loss of *AIF* increases ROS production (Klein *et al*., 2002). In several studies, *AIF* loss has been shown to increase the levels of ROS (Klein *et al*., 2002; Urbano *et al.*, 2005; van Empel *et al.*, 2005; Apostolova *et al.*, 2006; van Empel *et al.*, 2006) which can be minimised by the addition of antioxidants (Urbano *et al.*, 2005). The oxidoreductase domain of AIF may be essential not only for the stabilization of ETC complexes but also for an efficient antioxidant defence (Joza *et al*., 2005). Based on these reports, AIF has been proposed to function as a complex I stabilizer or possibly a ROS scavenger (Klein and Ackerman, 2003; Porter and Urbano, 2006). However, it does not appear to be interacting with any of the mitochondrial redox proteins (Vahsen *et al*., 2004) as it was observed that NAC, a ROS scavenger (N-acetyl cysteine) was unable to rescue *AIF* deficiency (Cheung *et al*., 2006). Additionally, recombinant AIF has been shown to have a very low electron transfer activity and does not have ROS scavenging activity (Churbanova and Sevrioukova, 2008). On the contrary, reports from cancerous cell lines lacking AIF and *AIF* deficient embryonic stem cells did not exhibit any changes in the ROS levels (Apostolova *et al.*, 2006; Pospisilik *et al*., 2007). In line with these data, AIF may act as a sensor for linking metabolism to other cellular processes, not as an electron carrier or ROS scavenger but as a ROS regulator. However, the exact mechanism of AIF in regulating the ROS levels is yet to be unravelled.

1.5.4 Mitochondrial fission-fusion and mitochondrial DNA (mtDNA) content

Mitochondrial metabolism is strongly linked to the mitochondrial structure and morphology. Apart from the role of AIF in stabilising the assembly of mitochondrial ETC complexes, it is also involved in regulating the mitochondrial structure via mitochondrial fission-fusion mechanism and mtDNA content. Mitochondrial fusion helps respiratory active cells to

exchange mitochondrial content, such as metabolites, enzymes, mitochondrial gene products and mtDNA (Nakada *et al*., 2009). The balance between fusion and fission, therefore, is crucial to sustain a proper mitochondrial structure and its function. The altered fission-fusion mechanism can induce mitochondrial fragmentation and a variety of disorders (Chan, 2006). Mitochondrial fission and fusion mechanisms (Fig. 1.6) are mediated by large guanosine triphosphatases (GTPases) in the dynamin family that are conserved between yeast, flies, and mammals. These processes are regulated by proteolysis and posttranslational modifications (Hoppins *et al*., 2007).

In mammalian cells, OMM fusion is mediated by Mitofusin 1 (MFNl) and Mitofusin 2 (MFN2) whereas IMM fusion is mediated by Optical atrophy 1 (OPAl) (Olichon *et al.*, 2002; Eura *et al.,* 2003). Mitochondria undergo fusion to form interconnected structures for the energy production and signal transduction. In order to travel long distances (eg. axons in neurons), mitochondria are divided into smaller segments for efficient and fast trafficking to meet the energy need. Under cellular stress, fusion helps to protect the mitochondrial function by allowing the mixing of membranes, soluble contents and mtDNA (Detmer and Chan, 2007). The importance of mitochondrial fusion during embryogenesis was demonstrated with *MFN1* and *MFN2* knock-out mice, which die in utero at mid-gestation due to a placental deficiency, whereas the *MFN1-MFN2* double knockout mice die even earlier in development (Chen *et al.,* 2003; Chen *et al*., 2010).

Mitochondrial fission is facilitated by a cytosolic dynamin family member [DRP1 (Dynamin related protein 1) in mammals and DNM1 in yeast). Cytosolic DRP1 is recruited to mitochondria to form spirals around mitochondria that compresses to split both inner and outer mitochondrial membranes. Mitochondrial Dynamics Proteins of 49 (MID49), Mitochondrial Dynamics Proteins of 51 (MID51), and Mitochondrial Fission Factor (MFF) recruit DRP1 to mitochondria in mammals whereas Mitochondrial Division Protein 1 (MDV1) recruits DNM1 to mitochondrial fission sites in yeast (Elgass *et al*., 2013). Moreover, mitochondrial fission is crucial for growing and dividing cells to colonize them with enough numbers of mitochondria.

The mitochondrial fission machinery, therefore, has a key role in the cell during physiological situations as well as under stress.

With context to AIF and mitochondrial fission-fusion, OPA1, is one of the mitochondrial fusion proteins found to interact with AIF to maintain OXPHOS and the mitochondrial morphology (Zanna *et al*., 2008). Interestingly, the deletion of an arginine residue (R201) of AIF led to severe mitochondrial encephalomyopathy with almost depleted mtDNA content and fragmented mitochondrial network in the affected patients (Ghezzi *et al*., 2010). Also, neuronal mitochondria from forebrain-specific *AIF* null (tel. AIFΔ) mice were found to be dilated and fragmented with aberrant cristae structure (Cheung *et al*., 2006). Additionally, Hq mice cerebella showed reduced levels of *MFN1*, suggesting that alterations of mitochondrial fusion led to cerebellar degeneration (Chung *et al*., 2011). Besides, the altered fission-fusion also reduced mtDNA copy number and lost membrane potential, compromising ATP synthesis (Chen *et al.*, 2005), proposing its additional role in mtDNA maintenance too. The ETC comprises of ~90 proteins encoded by both the nuclear and mitochondrial genome. All the 13 mtDNA-encoded proteins are vital parts of the ETC and the ATP synthase complex (Malka *et al*., 2006). Consequently, altered mtDNA severely affect mitochondrial OXPHOS. Depletion and mutations of mtDNA cause mitochondrial dysfunctions, resulting in various human diseases such as neurodegeneration and mitochondrial disorders. Conditional deletion of *MFN1* and *MFN2* led to mtDNA depletion and thus mitochondrial dysfunction (Chen *et al*., 2010). Considering these observations, it is important to study the regulatory role of AIF in maintaining the mitochondrial structure through fission-fusion mechanism and mtDNA content.

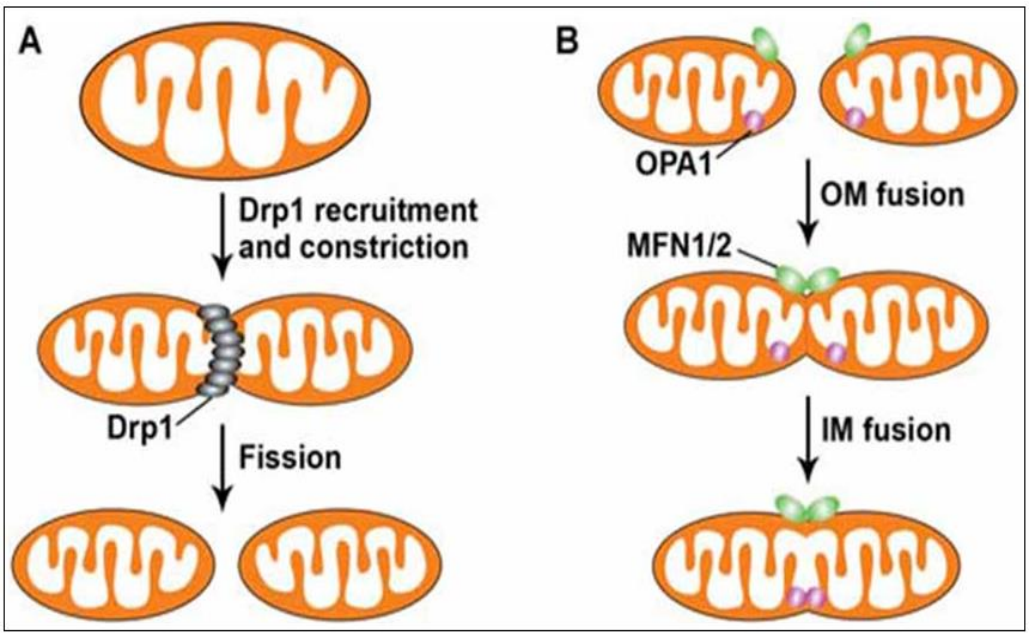

Fig. 1.6 Mitochondrial fission and fusion: Dynamic mitochondria undergo continuous fusion and fission events to mix their lipids and contents. (A) DRP1 regulates mitochondrial fission, which involves two steps: first, cytosolic DRP1 is recruited to the OMM; second, its assembly on the mitochondrial surface leads to mitochondrial constriction, resulting in to the separation of mitochondrion. (B) MFN1/2 at the outer membrane and OPA1 at the inner membrane organizes mitochondrial fusion, which involves MFN1/2-mediated outer membrane fusion of two mitochondria, followed by the OPA1-directed inner membrane fusion. This further leads to elongated and interconnected mitochondria (Cai and Tammineni, 2016).

In view of the existing literature, AIF seems to play a Janus-like role which is involved in cell death as well as cell survival. It is a pro-apoptotic factor but is also requisite for cell survival. Though its apoptotic function is well documented, its non-apoptotic role remains elusive. Thus, the aim of the present study is to understand the role of AIF in cell survival, emphasizing on cell growth, development and mitochondrial functions and structure. As there are no known inhibitors of AIF and lethality due to *AIF* knockout, the present study would decipher AIF's role by downregulating *AIF* using antisense technique in *D. discoideum*.

To address this question, *Dictyostelium discoideum,* a cellular slime mold, has been chosen as a model organism. *D. discoideum* has pathways which are very similar to that found in higher complex organisms. It forms multicellular structures and differentiates into two cell types which will allow investigating the cellular functions of AIF during the unicellular (growth) and multicellular (development) phases of the life cycle of *D. discoideum.* As *Dictyostelium* lacks caspases, it further provides an excellent model system to explore non-apoptotic functions of AIF without any interference of caspases.

1.6 *Dictyostelium discoideum*

D. discoideum is often mentioned as a 'Slime mold' or 'Social amoeba'. It is a haploid organism with 34Mb of genome, compacted into six chromosomes (Eichinger *et al*., 2005). It shows a unique life cycle having both unicellular as well as multicellular phases. It is correctly placed at the crossroad of uni- and multi-cellular stages providing a connecting link between the prokaryotes and the eukaryotes (Eichinger *et al*., 2005). Multicellular morphogenesis makes it a preferred model for various cellular processes like differentiation, development, patterning etc. Moreover, an extensive repertoire of well-developed biochemical and molecular techniques and the accessibility of fully sequenced genome mark it as an attractive model organism. Most of the genes of *D. discoideum* exhibit a high degree of similarity with those of higher organisms (Williams, 2010).

In the presence of adequate nutrients, it remains as undifferentiated unicellular cells which can undergo mitotic divisions at every 10-12 hrs. However, in the nutrient starving conditions, a multicellular developmental stage is induced. In response to the pulsating Cyclic AMP (cAMP) gradients released from aggregation centers, approximately 100,000 cells unite to form an aggregate. These developing structures are encased in an extracellular sheath consisting of protein, cellulose and polysaccharides (Freeze and Loomis, 1977). During aggregation, intracellular cAMP levels are increased by adenylyl cyclase and then cAMP is released out of the cell. The extracellular cAMP functions as a chemotactic agent and the cells move in response to its presence, traveling up its concentration gradient. The cycle of cAMP secretion and amoeboid drive is

continued once every 6 min. Aggregation occurs at 8 to 12 hrs from the time of starvation. Furthermore, the mound or a tight aggregate extends vertically and finally tips over, to form finger-shaped structure and subsequently grows into a slug. The slug finally culminates into mature fruiting body (Escalante and Vicente, 2000).

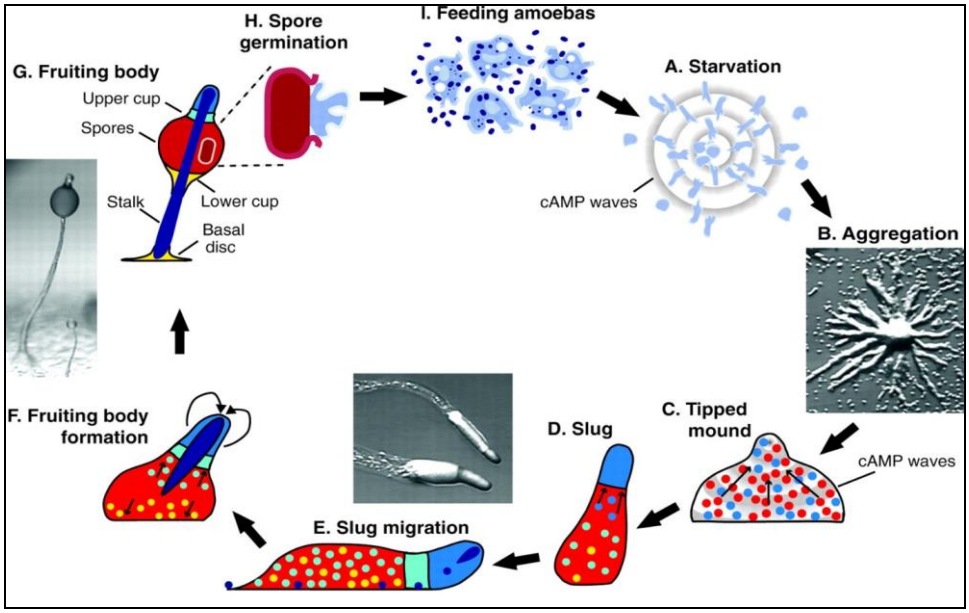

Fig. 1.7: The life cycle of *Dictyostelium discoideum.* Under starvation conditions, the amoebae form a mature fruiting body through different developmental phases such as aggregation, slug and culminants in response to cAMP waves secreted by the cells. Approximately 100 different genes are activated to assemble new biochemical machinery for the formation of multicellular structure from a unicellular structure. (Schaap, 2011)

The slug (2-4 mm long) has an anterior and posterior end and consists of as many as 100,000 cells. Motile slug moves only in the forward direction in response to light and higher temperatures, etc. 20% of the anterior end of slug constitutes prestalk cells which eventually forms the stalk in a mature fruiting body. Furthermore, in a slug stage, pattern formation comprising of the precursors of the two terminally differentiated cell types occurs. The prespore and the prestalk cells are the precursors of the terminally differentiated viable spore and dead vacuolated stalk cells respectively. The posterior cells of the slug (80%) are prespore cells which form the spores of a mature fruiting body

(Fig. 1.7). cAMP plays a major role in cell differentiation and aggregation. Elevated cAMP levels stimulate a morphogen called as DIF (differentiation-inducing factor), a low molecular weight lipid found in the anterior end of the slug and decides prestalk cells. The reorganization of prestalk and prespore cells takes ~8 to 10 hrs to form a mature fruiting body (1-2 mm high). Moreover, the stalk supports the spore mass above the substratum and waits for the proper stimulus to release the spores to the substrate (Watts and Ashworth, 1970; Mir *et al.*, 2007).

1.6.1 Genes responsible for the transition from growth (unicellular) to development (multicellular)

Protein kinase (PKA)

PKA, a cAMP dependent protein kinase plays multiple roles during *Dictyostelium discoideum* development and it is the central component in signal transduction pathway. The inactive form of PKA consists of a catalytic subunit (PKA-C) associated with the regulatory subunit (PKA-R). Exponentially growing *Dictyostelium* cells consists of both subunits as PKA is not required for growth, there is a 5-fold increase in the catalytic subunit in the first 6 hrs of the development and is maintained till culmination. The signal transduction pathway that initiates from binding of cAMP on cell surfaces to the accumulation of transcripts of developmental genes appears to act through PKA (Mir *et al*., 2007).

YAKA

YAKA is a cytosolic protein kinase which phosphorylates itself and enables the exit from the cell cycle and facilitates growth to development transition. It is expressed at a basal level during vegetative phase and peaked at the onset of starvation followed by a dip but is present throughout the developmental phase. Activation of *YAKA* in turn activates the expression of Adenylate Cyclase A (*ACA*), *PKA* and cAMP Receptor 1 (*CAR1*) (Mir *et al*., 2007).

Adenylate Cyclase A (ACA)

ACA, a G-protein-coupled adenylate cyclase is one of the first genes to be expressed upon starvation. It is involved in cAMP production during the initial

aggregation steps (Rodrigues and Sastre, 2016). Three distinct adenylyl cyclases have been observed to be expressed at different stages of *D. discoideum* (Wessels *et al*., 2000).

cAMP

cAMP acts as a chemo-attractant and a morphogen. It via PKA regulates almost all the major transitions including growth to development. Aggregation of amoebae into multicellular structures is systematized by cAMP. Once cAMP reaches the threshold level, it activates the cascade of events (Mir *et al*., 2007).

cAMP receptor 1 (CAR1)

CAR1 is a G-protein coupled receptor that maintains the chemotactic cell movement during multicellular development. It has two major functions: i) synthesizes and relays the cAMP signal; ii) responds to cAMP waves (chemotaxis) (Konijn *et al*., 1967). It is secreted by each cell during aggregation and binds to the high affinity cAMP receptors (cAR) like cAR1 to facilitate chemotaxis (Saxe *et al*., 1993). It binds to extracellular cAMP resulting into a stimulation of adenylyl cyclase thus producing more of cAMP. Four cAR receptors namely cAR1, cAR2, cAR3 and cAR4 are developmentally regulated. The cAR2 and cAR4 are lower affinity receptors that are expressed during the late developmental stages (Louis *et al*., 1994) while cAR3 is expressed simultaneously with cAR1 with a reduced affinity to cAMP than cAR1 (Johnson *et al*., 1993). In addition, endogenous cAMP is able to induce both the spore differentiation (Mehdy *et al*., 1983) and stalk cell differentiation (Brown and Firtel, 1999).

Conditioned Medium Factor (CMF)

CMF is an 80 kDa glycoprotein produced in vegetative cells and released upon starvation irrespective of the cell density. It increases the frequency of pseudopodia formation and is thus required for chemotaxis (Mir *et al*., 2007).

Pre-Starvation Factor (PSF)

During the vegetative growth, amoebae release an autocrine factor known as PSF which is a 68kDa glycoprotein. Its concentration in the cells indicates the

ratio of the cell density to food supply. It induces Discoidin-I and cyclic nucleotide phosphodiesterase (PDE) genes which further activatse the developmental morphogenesis (Watts and Ashworth, 1970; Mir *et al*., 2007).

MYB domain-containing protein (MYB2)

MYB2 is a transcription factor that encompasses 3 MYB repeats, a potential nuclear localization signal, a DNA-binding helix-turn-helix motif, and acidic amino acids-rich & guanine-proline regions of Myb-related transcription factor. It initiates the induction of adenylyl cyclase which is a prime step in the transition from growth to development in *D. discoideum* (Mir *et al*., 2007).

DIAI

DIAI is expressed at 2 hrs after starvation only in response to initial differentiation, reaching a peak at 4 hrs followed by its reduced levels. *DIAI* overexpression affects differentiation and aggregation defects (Mir *et al*., 2007).

Counting factor (CF)

It is a large complex (>450 kDa) of 5 polypeptides (60, 50, 45, 40, 30 kDa) and it's over secretion results in to smaller fruiting bodies. It aids to sense the number of cells in a group and is required for the regulation of organism size. In addition, it also increases cell to cell adhesion and motility (Mir *et al*., 2007).

RegA

It is an intracellular phosphodiesterase that hydrolyses cAMP. It helps for the directional movement during streaming stage in order to travel towards the aggregation center (Wessels *et al*., 2000).

Contact Site A (CsA)

This cell adhesion glycoprotein accumulates in a pre-aggregation phase and is greatly expressed only during aggregation. Loss of *CsA* affects the ability of cells to aggregate and form mature, suggesting its vital role in aggregation (Ponte *et al*., 1998).

Phosphodiesterase A (PdsA)

PdsA is an extracellular phosphodiesterase and controls extracellular cAMP levels by cleaving cAMP as the levels are increased. Altered *PdsA* levels affect aggregation in *D. discoideum,* indicating its importance in the starvation induced development (Weening *et al*., 2003).

1.6.2 *Dictyostelium discoideum*: a model organism

1.6.2.1 Multicellular morphogenesis and pattern formation

In nutrient deprivation situation, unicellular amoebae undergo developmental morphogenesis, involving complex network of gene regulatory mechanisms and organized cell type differentiation and pattern formation, leading to formation of a final mature fruiting body. The accessibility of cellular, biochemical and molecular techniques along with fully sequenced genome has allowed the discovery of complex signaling molecules implicated in the development and differentiation of *D. discoideum* which can be further extrapolated to higher organisms. Cell type specific developmental gene expression and differentiation have been extensively studied in *D. discoideum* which can be helpful for finding a new lead against development related diseases (Watts and Ashworth, 1970).

1.6.2.2 Chemotaxis and signal transduction

Chemotaxis is the directional movement of a cell in response to a chemical gradient. During development, amoebae travel in the direction of the food source. It is a comparable phenomenon to that of immune cells such as macrophages and neutrophils moving towards an invading cell. Since, the overall coordinated cell network including signaling molecules is highly conserved between *Dictyostelium* and mammalian leukocytes, *D. discoideum* proves to be an excellent system for studying chemotaxis, cell-cell interaction, cell proportioning, cell differentiation and signaling pathways and investigating new targets in higher complex organisms (Aubry and Firtel, 1999).

1.6.2.3 Phagocytosis

As many of the similar phagocytic intracellular signaling molecules and regulators seem to be present in mammalian system, it is termed as a

professional phagocyte (Roth *et al*., 2017). Availability of completely sequenced genome and the excellent genetic, cell biology and biochemical tools propose *Dictyostelium*, a model for recognition in lectin type receptor and non-receptor mediated phagocytosis (Vogel *et al*., 1980).

1.6.2.4 Host pathogen interaction

Dictyostelium and human macrophages show fundamental resemblances in their cellular biology which has encouraged the use of *Dictyostelium* as a model host for the study of microbial pathogenesis. It is an attractive system to study a number of human disease-related proteins. Moreover, it can be easily used as screening host for drug testing for pathogenic and non-pathogenic micro-organisms (Steinert and Heuner, 2005).

1.6.2.5 Cell death

Paraptosis is characterized by loss of mitochondrial membrane potential (Ψm), translocation of AIF from mitochondria to the nucleus, and the absence of oligonucleosomal DNA fragmentation (Arnoult *et al*., 2001). *D. discoideum* has a paracaspase gene, neither a metacasapase nor a caspase gene. It shares a similarity with mammalian cell death mechanism and thus, it provides powerful paradigm for studying caspase independent paraptotic cell death.

1.6.2.6 Mitochondrial diseases

D. discoideum has been used as a model organism to study mitochondrial biogenesis and disease conditions (Bozzaro, 2019; Annesley *et al*., 2009). *D. discoideum* cell contains about 200 copies of the mitochondrial genome, double stranded, circular similar to the human mitochondrial genome. The human mitochondrial genome is of 17 kbp while *D. discoideum* has a 55 kbp mitochondrial genome. Many of the nuclear encoded subunits of electron transport chain complexes are found in the mitochondria and hence size is large. At least 33 *D. discoideum* genes are orthologous of disease related genes and this facilitated the use of *Dictyostelium* model to explore the mitochondrial biology and molecular mechanisms underlying various diseases (Francione *et al*., 2011).

In view of the above discussion, *D. discoideum* serves as a perfect system to explore the physiological role of AIF in cellular growth, development and mitochondrial functions along with its structure.

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