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The *Dictyostelium* model for mitochondrial biology and disease

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ABSTRACT The unicellular slime mould *Dictyostelium discoideum* is a valuable eukaryotic model organism in the study of mitochondrial biology and disease. As a member of the Amoebozoa, a sister clade to the animals and fungi, *Dictyostelium* mitochondrial biology shares commonalities with these organisms, but also exhibits some features of plants. As such it has made significant contributions to the study of eukaryotic mitochondrial biology. This review provides an overview of the advances in mitochondrial biology made by the study of *Dictyostelium* and examines several examples where *Dictyostelium* has and will contribute to the understanding of mitochondrial disease. The study of *Dictyostelium*'s mitochondrial biology has contributed to the understanding of mitochondrial genetics, transcription, protein import, respiration, morphology and trafficking, and the role of mitochondria in cellular differentiation. *Dictyostelium* is also proving to be a versatile model organism in the study both of classical mitochondrial disease e.g. Leigh syndrome, and in mitochondria-associated neurodegenerative diseases like Parkinson's disease. The study of mitochondrial diseases presents a unique challenge due to the cryptic nature of their genotype-phenotype relationship. The use of *Dictyostelium* can contribute to resolving this problem by providing a genetically tractable, haploid eukaryotic organism with a suite of readily characterised phenotype readouts of cellular signalling pathways. *Dictyostelium* has provided insight into the signalling pathways involved in multiple neurodegenerative diseases and will continue to provide a significant contribution to the understanding of mitochondrial biology and disease.

KEY WORDS: *Dictyostelium, mitochondrial biology, mitochondrial disease, AMPK, neurodegeneration*

Introduction

Mitochondria are double-membrane-bounded organelles present in all nucleated eukaryotic cells, with few exceptions. Beyond their primary role of ATP synthesis via oxidative phosphorylation, mitochondria are also involved in many cellular processes such as calcium signalling, cellular differentiation and proliferation, and programmed cell death (Bertero and Maack, 2018, Umemoto *et al.*, 2018, Vakifahmetoglu-Norberg *et al.*, 2017). The mitochondrion is thought to have originated from an endosymbiotic event between a primitive eukaryote and a α-proteobacterium (Leger *et al.*, 2015). The strongest evidence of this is that mitochondria possess their own genome, and like bacteria, this genome is circular in structure, while the proteins it encodes are more closely related to bacterial than to eukaryotic counterparts. Indeed, eukaryotic mitochondria have retained a division system akin to that of their prokaryotic ancestors (Leger *et al.*, 2015). Over time, many of the requisite genes for the proper functioning of mitochondria have migrated to the nuclear genome, a process that has continued more or less independently in the different phylogenetic lineages. The result is that the gene composition and size of the mitochondrial genome is different in each eukaryotic lineage, with the human mitochondrial genome being one of the most severely reduced in size (13 protein-encoding genes, 16 kb in size) (Wallace, 2005), while the least reduced is that of the protist *Reclinomonas americana* whose 69 kilobases encode 65 proteins (Burger *et al.*, 2013).

In humans, mutations affecting either the mitochondrial genome or nuclear genes encoding mitochondrial proteins can cause complex diseases whose diverse clinical presentations break the nexus between genotype and phenotype (Stenton and Prokisch, 2018). Thus, the same genetic defect can cause very diverse clini-

Abbreviations used in this paper: ETC, electron transport chain; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species.

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cal outcomes in patients and similar disease phenotypes can be produced by multiple genetic causes (Sofou *et al.*, 2018). Because of this complexity, the study of mitochondrial biology and disease has relied upon contributions from several model organisms, including the yeast *Saccharomyces cerevisiae*, and the cellular slime mould *Dictyostelium discoideum*.

D. discoideum belongs to the Amoebozoa, a sister clade to the Opisthokonta, the group containing fungi and animals. *Dictyostelium* mitochondria share many similarities with all eukaryotes, but have some features found in either animals or plants. *D. discoideum* has been used extensively as a eukaryotic model organism in the study of cellular processes such as chemotaxis, thermotaxis, motility, programmed cell death, cell differentiation, signal transduction, and human disease modelling (Maniak, 2011, Steinert and Heuner, 2005). It is also a well-established model organism for the investigation of mitochondrial genetics and in mitochondrial disease modelling (Barth *et al.*, 2007; Francione *et al.*, 2011). Due to its biology including several cellular processes more akin to higher eukaryotes e.g. phagocytosis, chemotaxis, and differentiation, *Dictyostelium* is an ideal model for the study of conserved genes not found in yeast (Torija *et al.*, 2006). A fully sequenced nuclear and mitochondrial genome, tractability to genetic modification, and a life cycle that provides numerous phenotypic readouts of cellular processes make *D. discoideum an* attractive model for mitochondrial biology and disease. This review aims to provide an overview of *D. discoideum* mitochondrial biology and its utility as a model in understanding human mitochondrial disease mechanisms.

Mitochondrial biology

Mitochondrial genetics

The mitochondrial genome of *D. discoideum* is fully sequenced and is 55,564 bp in length with an A + T content of 72.6% (Ogawa *et al.*, 2000). The genome is circular and each cell contains approximately 200 copies of the mitochondrial genome. This A + T richness is a feature shared with other protozoa, fungi, nematodes, and invertebrates (Nakao *et al.*, 2002, Stewart and Beckenbach, 2005). Despite this, unlike other organisms with similar A + T richness, *D. discoideum* appears to use the universal genetic code in its translation of mitochondrial mRNAs (Angata *et al.*, 1995). The mitochondrial genome contains 61 genes, the majority of which, like all mitochondrial genomes, encode gene products related to respiration and translation (Ogawa *et al.*, 2000). Interestingly, all genes are encoded on the same DNA strand, in the same orientation, and the genome is transcribed from a single start site (Le *et al.*, 2009). This primary transcript is processed into eight major polycistronic transcripts which are additionally further processed into di- and tri-cistronic transcripts (Barth *et al.*, 1999, Barth *et al.*, 2001). *D. discoideum* mtDNA encodes 33 proteins, 2 ribosomal RNAs, and 18 transfer RNA genes with many of these genes ortho-/homologues to *Homo sapiens* mtDNA genes (Table 1). Notably, in humans the genes encoding ribosomal proteins have all transferred to the nuclear genome. However, the majority of common respiratory chain constituents remain encoded in the mtDNA. As expected from this "loss" of genes, the human mitochondrial genome is substantially smaller at 16,569 bp (Anderson *et al.*, 1981). As many of the requisite proteins for mitochondria are now encoded on the nuclear genome, many required proteins are imported into the mitochondria, including those required for transcription.

Mitochondrial transcription

Mitochondrial DNA in fungi, plants, and animals is transcribed by a nuclear-encoded mitochondrial RNA polymerase (mtRNAP) that has a high sequence similarity to bacteriophage RNA polymerases (Filée *et al.*, 2002, Gustafsson *et al.*, 2016, Ringel *et al.*, 2011). Unlike bacteriophage mtRNAPs, eukaryotic mtRNAPs require additional proteins for efficient transcription, known as mitochondrial transcription factors (Falkenberg *et al.*, 2002). Consisting of two families, mitochondrial transcription factors A & B (mtTFA; mtTFB), together with mtRNAP, form the machinery required for mitochondrial transcription. *D. discoideum* similarly has a nuclear-encoded phage-like mitochondrial RNA polymerase, RpmA. The *rpmA* gene consists of a continuous open reading frame of 2,850 nucleotides in length and codes for a protein of 950 amino acid residues, within which all catalytically essential residues are conserved (Le *et al.*, 2009). While being shown to initiate transcription, RpmA, did so inefficiently, suggesting that, like other eukaryotes *D. discoideum* requires mitochondrial transcription factors for mtDNA transcription. Manna *et al.,* (2013) identified and characterised a *D. discoideum* mitochondrial transcription factor B. Identified as *tfb1m*, this gene is 1,458 bp in size, with a corresponding amino acid sequence 485 residues in length, and approximately 56 kDa in molecular weight. Tfb1m was shown to localise to the mitochondria and function both as a rRNA adenosine dimethyltransferase, but also to activate mitochondrial transcription in concert with RpmA. The dual role of methyl transferase activity and mitochondrial transcription initiation is concordant with the human homologues of Tfb1m (Cotney and Shadel, 2006). While RpmA expressed without Tfb1m

TABLE 1

A COMPARISON OF *DICTYOSTELIUM DISCOIDEUM* AND *HOMO SAPIENS* MITOCHONDRIAL GENES RELATING TO RESPIRATION AND TRANSLATION

	Dictyostelium discoideum	**Homo sapiens**
Respiratory chain	NADH-dehydrogenase: *nad1, 2, 3, 4, 4L, 5, 6, 7, 9, 11* Apocytochrome b: *cob* Cytochrome oxidase: *cox1/2, cox3*	NADH–dehydrogenase: MT-ND1, 2, 3, 4, 4L, 5, 6 Cytochrome *b*: MT-CYB Cytochrome *c* oxidase: MT-CO1, 2, 3
Oxidative phosphorylation	F_0- ATPase: *atp6, atp8, atp9* F_1- ATPase: *atp1*	F_0- ATP synthase: MT-ATP6, 8
Ribosomal proteins	Small subunit: *rps2, rps4, rps7, rps8, rps11, rps12, rps13, rps14, rps19* Large subunit: *rpl2, rpl5, rpl6, rpl11, rpl14, rpl16*	*nDNA encoded
Ribosomal RNAs	Large subunit: *rnl* Small subunit *rns*	Large subunit: MT-RNR2 Small subunit: MT-RNR1
Transfer RNAs	A, C, E, F, H, 11, 12, 13, K, L1, L2, M, N, P, Q, R, W, Y	A, C, D, E, F, G, I, K, L1, L2, M, N. P, Q, R, S1, S2, T, V, W, Y

D. discoideum mtDNA data from (Ogawa *et al.*, 2000). Human mitochondrial data from (Anderson *et al.*, 1981, Capt *et al.*, 2016).

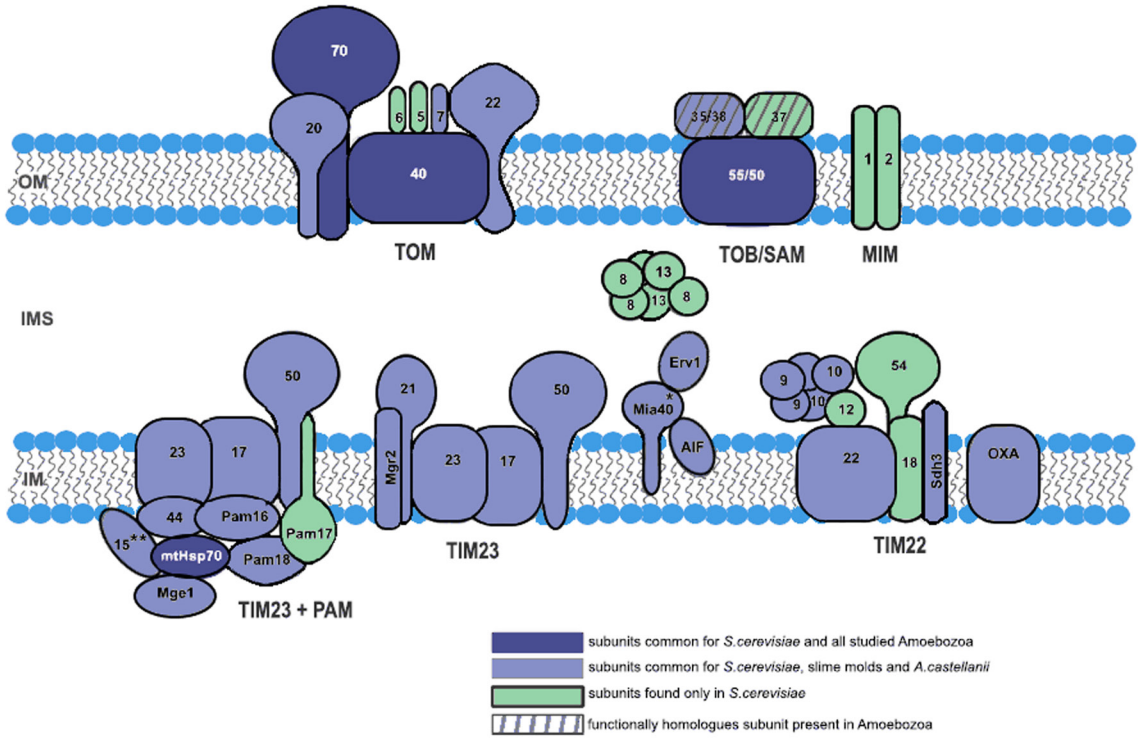

Fig. 1. The mitochondrial protein import complexes of the Amoebozoa supergroup. D. discoideum *(and other Amoebozoa) share a highly similar organisation of their protein import complexes to the canonical Saccharomyces protein import complexes and other members of the Amoebozoa. Diagram reused under the terms of the Creative Commons Attribution 4.0 International License. Diagram modified from Wojtkowska* et al., *(2017).*

produced undetectable amounts of transcripts, coexpression with Tfb1m improved transcription efficiency dramatically, to the point where it was suggested that Tfb1m may be the sole transcription factor necessary for mitochondrial transcription in *D. discoideum* (Manna *et al.*, 2013). As illustrated above, mitochondrial function is dependent on nuclear-encoded protein import and this is facilitated by the TOM/TIM complexes in mitochondria.

Protein import

As most mitochondrial proteins are encoded in the nuclear genome rather than the mitochondrial genome, mitochondria rely upon protein import and subsequent assembly for correct functioning and biogenesis. After early controversy, the demonstration that mitochondrial protein import could occur posttranslationally *in vitro* resulted in a long-held belief that this was the predominant mechanism, with protein synthesis occurring in the cytosol and subsequent being imported (Ahmed and Fisher, 2009, Fox, 2012). However, *Dictyostelium* provided an early counter demonstration that some proteins are imported into the mitochondria posttranslationally and others cotranslationally as a result of selective trafficking of the mRNA to the mitochondrial surface (Ahmed *et al.*, 2006). It is now clear that both mechanisms are used in parallel: approximately half of the proteins that make up the mitochondrial proteome are translated at the surface of mitochondria, with the remainder being synthesized in the cytosol and posttranslationally imported (Fox, 2012). Most mitochondrial inner membrane proteins were found to be cotranslationally targeted in *Saccharomyces* (Williams *et al.*, 2014). This localised translation of proteins is presumed to facilitate their correct import and assembly, however why not all mitochondrial proteins are cotranslationally imported is not fully understood (Fox, 2012). Localised synthesis of proteins coupled with their simultaneous transport is not mitochondria-specific (Aviram and Schuldiner, 2017). Similar cotranslational import of proteins also occurs in the endoplasmic reticulum, and chloroplasts (Dudek *et al.*, 2015, Króliczewski *et al.*, 2016). Cotranslational import of proteins now appears to be a commonplace occurrence not limited to any particular organelle, and a system used in concert with posttranslational import (Allen *et al.*, 2019, Wiedemann and Pfanner, 2017).

Whether it occurs cotranslational or posttranslationally, the import of proteins into mitochondria is a particularly challenging problem that eukaryotes needed to "solve", due to the complex architecture of the mitochondria, requiring the bridging of two membrane delineated spaces i.e. the intermembrane space and matrix (Wojtkowska *et al.*, 2015). The reference model of mitochondrial import machinery is that of *Saccharomyces cerevisiae* (Demishtein-Zohary and Azem, 2017). The complete protein import system consists of three outer membrane (TOM, TOB/SAM, MIM), four inner membrane (TIM22, TIM23, TIM23+PAM, OXA) and three mitochondrial intermembrane space assembly (MIA) complexes. Comparative sequence analysis of genome and transcriptome data by Wojtkowska *et al.,* (2017) indicates that in *D. discoideum*, protein import complexes of the canonical *Saccharomyces* appear to be conserved with the exception of MIA (albeit with some subunit variation/loss) (Fig. 1).

TOM and TOB/SAM (topogenesis of the mitochondrial outer membrane β-barrel proteins/sorting and assembly machinery) are present and are responsible for preprotein recognition and translo-

cation, and protein sorting and assembly, respectively (Wenz *et al.*, 2015). TIM22 & 23 (translocase of the inner membrane) mediate precursor protein import through the IMM, dependant on the location of the protein's import targeting signals (within sequence or N-terminus, respectively). PAM (presequence translocase-associated motor) functions to drive translocation of proteins into the matrix. OXA (oxidase assembly factor) mediates insertion of proteins from the matrix into the inner membrane (Wenz *et al.*, 2015).

Most protein import from the cytosol through the outer mitochondrial membrane (OMM) is facilitated by the TOM complex (Rapaport, 2002). In *D. discoideum*, the TOM complex consists of Tom 7, 20, 22, 40, 70 (Macasev *et al.*, 2004, Wojtkowska *et al.*, 2017). Tom22 and Tom40 are conserved in all eukaryotes with the addition of Tom7 after the divergence of the Excavates supergroup (Mani *et al.*, 2016).

Harbauer *et al.,* (2014) suggest that the mitochondrial import machinery should be considered a regulatory hub as impairment of protein import activity acts as a sensor for mitochondrial health and regulates biogenesis and mitochondrial cycling. Dysfunction in protein import can have deleterious effects on mitochondrial membrane potential and cause the accumulation of misfolded proteins in the intermembrane space inducing the mitochondrial stress response (Haynes *et al.*, 2013). This triggers retrograde signalling to the nucleus and upregulation of molecular chaperones, proteases, import complex subunits and proteins in an attempt to alleviate mitochondrial stress and restore mitochondrial homeostasis (Haynes *et al.*, 2013).

Due to its intrinsic role in cellular and mitochondrial health, and association with mitochondrial disease, a complete understanding of mitochondrial import may prove critical in the management of neurological disorders and mitochondrial diseases. Dysfunction of mitochondrial protein import mechanisms is associated with several neurological disorders. Accumulation of amyloid precursor protein in mitochondrial import channels of Alzheimer's patient brains results in the inhibition of the transport of cytochrome c oxidase subunits into mitochondria, decreased cytochrome c oxidase activity and increase ROS production (Devi *et al.*, 2006). Oligomeric α-synuclein (the putative toxic form of the pathological agent of Parkinson's disease) binds to the TOM20 subunit of the translocase of the outer membrane (TOM), impairing protein import and causing dysfunction of respiratory Complex I (Di Maio *et al.*, 2016).

Mitochondrial morphology and trafficking

Previously (still currently in undergraduate textbooks) depicted to be discrete bean-shaped organelles free in the cytoplasm of cells, mitochondria are now known to be more complex and dynamic in their morphology, arrangement and subcellular distribution (Tilokani *et al.*, 2018, Wai and Langer, 2016). Mitochondria undergo continuous cycles of fusion and fission and are redistributed within the cell to areas requiring energy production (Eisner *et al.*, 2018). Mitochondrial movement, fusion and fission are dependent on the cell cytoskeleton, both actin and microtubular, and mediated by actin and microtubule motor proteins (Woods *et al.*, 2016).

D. discoideum mitochondria have been reported to be present in various proportions of spherical (66 %), rod (24 %), and tubular (10%) forms in fixed cells (Gilson *et al.*, 2003). Live cell imaging via laser scanning confocal imaging, also showed they are in the main, spherical (Schimmel *et al.*, 2012). Gilson *et al.,* (2003) reported that *D. discoideum* has two homologues of the α-proteobacterial bacterial fission protein, FtsZ, FszA and FszB. Homologues are also found in other eukaryotic lineages, but not in metazoa, fungi or higher plants (which do however retain plastid homologues of the cyanobacterial FtsZ). When FszA and/or FszB are knocked out, the proportions of the mitochondrial shape classes change greatly, the tubular shape being promoted, consistent with a conserved role in mitochondrial fission. Submitochondrial localisation patterns of FszA suggest that it plays a direct role in mitochondrial division with the distribution of FszA along tubular mitochondria at sites of recent or likely future divisions. The nature of FszB's role in mitochondrial division is unclear but may be indirect.

While mutation of FszA and/or FszB impaired mitochondrial fission it did not cause the complete cessation of mitochondrial division (Gilson *et al.*, 2003). Other eukaryotes use dynamin-like proteins during mitochondrial fission and two *D. discoideum* dynamin orthologues, DymA and DymB were suggested as candidate proteins for involvement in fission. Electron micrographs of *Dictyostelium dymA* knockouts show the accumulation of mitochondria into dense clusters and most mitochondria taking an interconnected tubular, branching form (Wienke *et al.*, 1999). These defects were attributed to defects in membrane trafficking processes, rather than defects in mitochondrial division directly. DymB, while being targeted to the mitochondria and playing other diverse roles in the cell, such as stabilisation of actin filaments in cytokinesis, has no obvious role in mitochondrial division (Masud Rana *et al.*, 2013, Rai *et al.*, 2011). Indeed, using a live cell fission and fusion assay and laser scanning confocal microscopy, Schimmel *et al.,* (2012) demonstrated that fusion/fission rates were not affected by loss of DymA, DymB or MidA (Schimmel *et al.,* (2012), a mitochondrial Complex I assembly factor whose loss causes a specific Complex I respiratory defect (Carilla-Latorre *et al.*, 2010). Thus *dymA- D. discoideum* cells have aggregated spherical mitochondria, with tubular structures, but not altered fusion or fission rates, while the mitochondria in *dymB-* cells also approximated wildtype fission/fusion rates. It appears that uniquely in *D. discoideum* (thus far), DymA and DymB are not essential for mitochondrial replication.

However, mitochondrial fission and fusion are affected by loss of CluA, a protein first discovered in *Dictyostelium* and found to be essential for normal localization of mitochondria in the cell (Zhu *et al.*, 1997). Knocking out *D. discoideum cluA* caused clustering of the mitochondria in the perinuclear region at the centre of the cell along with some moderate growth and viability defects (Zhu *et al.*, 1997). CluA has homologues present as open reading frames in all sequenced eukaryotic organisms (Fields *et al.*, 2002). The mammalian homologue, CLUH, binds a specific subset of mRNAs for nuclear encoded mitochondrial proteins and its loss not only causes clustering of the mitochondria, but reduced mitochondrial biogenesis (Gao *et al.*, 2014). Knocking out the *Drosophila* homologue causes morphologically abnormal mitochondria, reduced ATP levels and clustering in germ line cells that suggested unequal inheritance (Cox and Spradling, 2009, Sen *et al.*, 2013). When the *Saccharomyces cerevisiae* homologue (*CLU1*) was expressed in *cluA- D. discoideum*, the aberrant mitochondrial localisation and growth defects were remedied, indicating that *CLU1* and *cluA* encode functional homologues (Fields *et al.*, 1998). Despite a similar result of interconnected mitochondria as in the case of *dymA-* mutants, this seemed not to be due to cytoskeletal disruption, but to the late blocking of mitochondrial outer membrane fission (Fields *et al.*, 2002). Previously hypothesised to function as a mitochondria-

cytoskeleton linker protein, CluA has since been demonstrated not to be required for mitochondrial motility but does play an as yet unidentified role in mitochondrial fusion and fission (Woods *et al.*, 2016). While it is known that FszA, FszB, and CluA are all essential for mitochondrial fusion and fission, it is not known whether these can account for all of the mitochondrial division activity, since there are no triple mutants available.

Pharmacological disruption of microtubules demonstrated that they are the primary cytoskeletal element involved in *D. discoideum* mitochondrial movement and distribution (Woods *et al.*, 2016). Actin filaments appear to play a secondary role, affecting the number of mitochondria moving at one time, but not their movement speed or cellular distribution (Woods *et al.*, 2016). The Ras-related GTPase Miro was shown in humans to play a role in the calcium-dependent regulation of mitochondrial transport along microtubules. Miro was found to play a similar role in mammals and other metazoans such as *Drosophila.* However in *Saccharomyces*, *Arabidopsis,* and *D. discoideum*, Miro is involved in mitochondrial morphology, inheritance and homeostasis rather than trafficking (Yamaoka and Hara-Nishimura, 2014). The *D. discoideum* orthologue of the human Miro gene is *gemA*. When knocked-out, *gemA*- mutants exhibited significant growth defects, particularly in liquid media, attributed to reduced mitochondrial mass and ATP content. Mitochondrial O_2 consumption was increased in *gemA*- mutants, however glucose consumption and mitochondrial membrane potential remained equivalent to wildtype. Mitochondrial mass and morphology were unaffected, and mitochondrial trafficking *in vivo* showed no impairment (Vlahou *et al.*, 2011). Given the commonalities in function in plants, yeasts and amoebae, Miro appears to have an ancestral function in the homeostasis of mitochondria that has been later modified in metazoans for mitochondrial transport (Yamaoka and Hara-Nishimura, 2014). An understanding of the regulation of mitochondrial dynamics is crucial, as they are essential for key cellular processes in *D. discoideum* such as differentiation and multicellular development.

Mitochondrial involvement in development and differentiation

Mitochondria have functions in regulation of the growth/differentiation transition, cell-type determination, taxis and pattern formation. *D. discoideum* cells with depleted mtDNA had a delayed entry into differentiation and a cessation of development at the slug stage (Chida *et al.*, 2004). The proportion of prestalk cells was significantly increased with concomitant reduction in prespore cells, in addition, prespore/prestalk cell patterning was disrupted (Chida *et al.*, 2004). Similar findings of shortened fruiting body stalks indicating an increase in the proportion of prestalk cells were made in mutant strains in which expression of the essential mitochondrial protein chaperonin 60 was knocked down (Bokko *et al.*, 2007, Kotsifas *et al.*, 2002).

Further evidence of mitochondria's role in differentiation is that the expression of mitochondrial ribosomal protein S4 (mt-RPS4) in response to starvation is essential for the initiation of differentiation (Inazu *et al.*, 1999, Maeda and Chida, 2013). Disruption of *mrps4* within mitochondria impaired differentiation. Knockouts of *mrps4* do not aggregate after 16 h, but do grow normally, suggesting that mt-RPS4 is not needed for growth but is essential for differentiation. Perhaps serendipitously, mt-RPS4 has several nuclear localisation signals in *D. discoideum* and was shown to localise to the nucleus in *mrps4* ectopic overexpression models. Surprisingly, this overexpression of mt-RPS4 in the nucleus enhanced early differentiation, while stable nuclear expression of *mrps4* antisense RNA produced the opposite outcome. The mechanisms for these extramitochondrial actions of the mitochondrially encoded mt-RPS4 are unknown. Although not required for mitochondrial fusion/fission, the Complex I assembly factor MidA is essential for correct cellular and mitochondrial function and development (Torija *et al.*, 2006). *MidA* knockout in *D. discoideum* leads to diverse phenotype outcomes: cell growth, size, macropinocytosis and phagocytosis were all reduced. In addition, multicellular development was arrested at the end of the slug stage. While oxygen consumption remained similar to wildtype, a significant reduction of mitochondrial ATP generation in *midA*- mutants was noted but without any defect in mitochondrial membrane potential or in mitochondrial number, indicating MidA involvement in ATP synthesis. Later work determined that MidA interacts with, and is required for mitochondrial Complex I function (Carilla-Latorre *et al.*, 2010). Further discussion of MidA's role in mitochondrial respiration is discussed in the mitochondrial disease modelling section of this document.

In mitochondrially diseased *D. discoideum*, multicellular morphogenesis is disrupted, in particular fruiting body stalk and basal disk formation are abnormal, generally having shortened, thickened stalks (Kotsifas *et al.*, 2002). Stalk-cell differentiation in *D. discoideum* is coordinated by a group of chlorinated hexaphones known as differentiation-inducing factors (DIF) (Kubohara *et al.*, 2017, Thompson and Kay, 2000). These DIFs also are involved in chemotaxis in *D. discoideum,* a role that has created interest in these compounds for their potential as anti-metastatic cancer drugs. There are three DIF factors: DIF-1, DIF-2, and DIF-3. DIF-1 is the primary inducer of stalk cell differentiation, with DIF-2 playing a lesser role, and DIF-3 (the first metabolite resulting from the degradation of DIF-1) having effectively no activity on stalk differentiation induction (Thompson and Kay, 2000).

While their phenotypic outcomes have been well examined, the signalling pathways the DIFs act upon remain to be understood. It has been proposed that DIF-1 may function via increases in cytosolic calcium or proton concentrations (Kubohara *et al.*, 2017). As mitochondria are integral to intracellular calcium homeostasis, they appear to be good candidate targets for DIF activity (Paupe and Prudent, 2018). A fluorescently labelled DIF-1 was shown to localise to mitochondria under early starvation or after a 21 hour incubation with cAMP (Kubohara *et al.*, 2017). This may indicate that DIF-1 interacts with the mitochondria in its induction of stalk cell differentiation. DIFs have been shown to act as a mitochondrial uncoupler in mammalian cells (Kubohara *et al.*, 2015). When *D. discoideum* mitochondria were uncoupled pharmacologically using CCCP (2-[2-(3-Chlorophenyl)hydrazinylyidene]propanedinitrile) or DNP (2,4-dinitrophenol), a significant but small induction of stalk cell formation was observed. This suggests that uncoupling of the mitochondria by DIF-1 only partially explains its induction of stalk cell differentiation (Kubohara *et al.*, 2017).

DIF-1 was also shown to bind to and inhibit malate dehydrogenase (MDH) in HeLa cells (Matsuda *et al.*, 2010). Importantly, this inhibition was isolated to the mitochondrial malate dehydrogenase (mMDH) not the cytoplasmic isoform. MDH is an essential enzyme of the tricarboxylic acid cycle and malate-aspartate shuttle pathway. Inhibition of mMDH by DIF-1 lowered cellular ATP levels and inhibited proliferation (Matsuda *et al.*, 2010). Interestingly, DIF-3 was able to bind to mMDH, but did not inhibit mMDH enzymatic

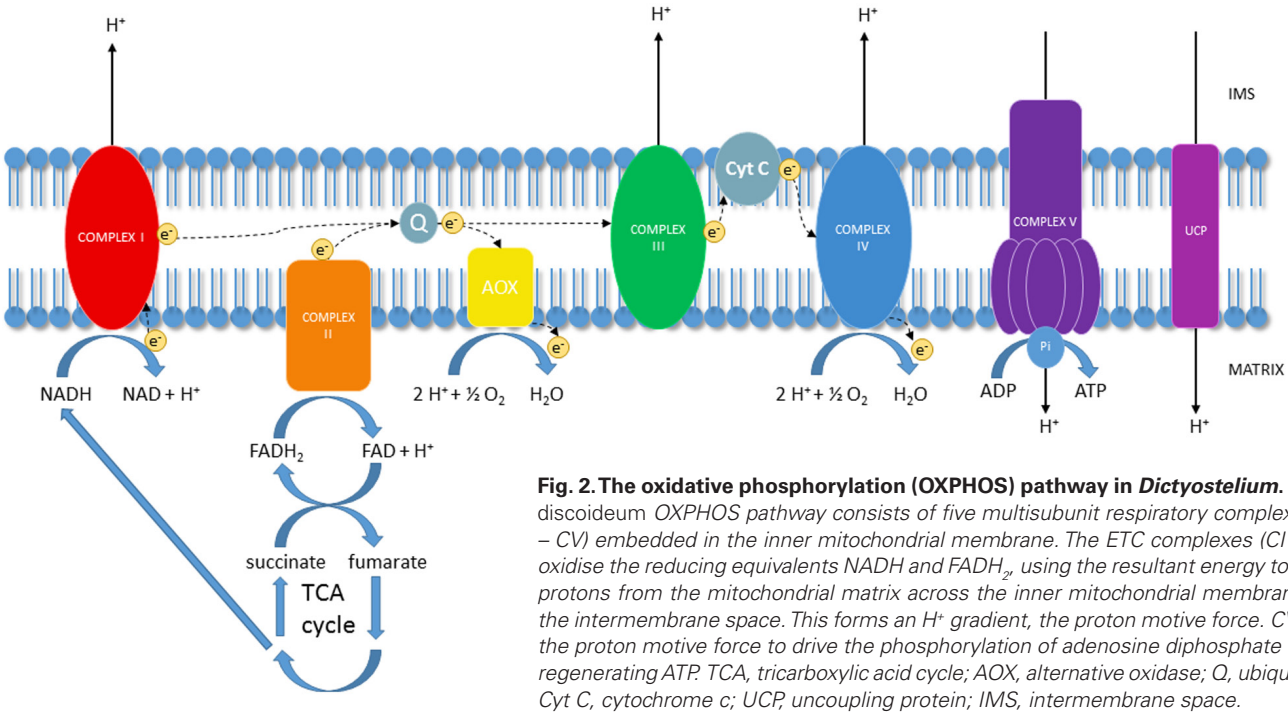

Fig. 2. The oxidative phosphorylation (OXPHOS) pathway in *Dictyostelium*. *The* D. discoideum *OXPHOS pathway consists of five multisubunit respiratory complexes (CI – CV) embedded in the inner mitochondrial membrane. The ETC complexes (CI – CIV) oxidise the reducing equivalents NADH and $FADH_2$, using the resultant energy to pump protons from the mitochondrial matrix across the inner mitochondrial membrane into the intermembrane space. This forms an H^+ gradient, the proton motive force. CV uses the proton motive force to drive the phosphorylation of adenosine diphosphate (ADP), regenerating ATP. TCA, tricarboxylic acid cycle; AOX, alternative oxidase; Q, ubiquinone; Cyt C, cytochrome c; UCP, uncoupling protein; IMS, intermembrane space.*

activity nor reduce cellular ATP levels, despite inhibiting HeLa cell proliferation. This parallels the observation that the DIF analogues play different roles in *D. discoideum*, i.e. DIF-3 is not a strong inducer of *D. discoideum* stalk cell differentiation whereas DIF-1 is. While it is currently unknown whether DIF-1 interacts with *D. discoideum* mitochondrial malate dehydrogenase, it appears to be a valid target for the understanding of DIF-1 induction of the stalk cell differentiation pathway and its interaction with mitochondria.

The fact that DIF-3 was able to inhibit HeLa cell proliferation without affecting ATP steady-state levels may make it a more attractive candidate as an anti-cancer compound than DIF-1 (Matsuda *et al.*, 2010). Fluorescently labelled DIF-3 localised to the mitochondria and had deleterious effects on mitochondrial morphology and activity in HeLa cells (Kubohara *et al.*, 2013). DIF derivative compounds also inhibited the migration of metastatic murine osteosarcoma cells *in vitro* (Kubohara *et al.*, 2015). DIF-3 promotes mitochondrial fission, induces autophagy, and induces caspase-independent cell death in human leukaemia (K562 CML) lines. K562 cells treated with DIF-3 showed mitochondrial morphology transitioning from the typical network of interconnected mitochondria, to individual organelles with disrupted cristae after 3 - 6 hours, and complete absence of mitochondria after 24 hours. This had significant deleterious effects on ATP production, induced superoxide production and depleted the mitochondrial membrane potential. DIF-3 induces autophagy in K562 cells by rapid inhibition of mTOR pathway signalling as a result of dephosphorylation of mTOR at Ser2481 (Dubois *et al.*, 2016). DIF-3 triggered caspase-independent cell death through the induction of Ca^{2+} influx into the cytoplasm, and the subsequent recruitment of DRP1 to the mitochondria. K652 tumours implanted in mice were inhibited by DIF-3 treatment in food. Beneficially, the inhibition of tumour growth was not apoptotic in nature, so could bypass the apoptotic drug resistance frequently encountered in tumour cells (Dubois *et al.*, 2016). It appears that the DIFs have significant interactions with mitochondria both in *D. discoideum* and human cancer cells and further pursuit of this relationship may provide some insight into mitochondrial roles in cellular morphogenesis.

Mitochondrial respiratory function

Oxidative phosphorylation

The primary function of the mitochondria is the production of adenosine triphosphate (ATP) by oxidative phosphorylation (OXPHOS) via the electron transport chain (ETC). The OXPHOS pathway in *D. discoideum* is fundamentally the same as its mammalian counterpart, aside from the presence of an additional alternative oxidase (discussed in the following section) (Fig. 2). As the primary means of ATP production, OXPHOS function is central to cellular health and dysregulation of respiration is a common indicator of many mitochondrial diseases and disorders.

The first and largest respiratory complex in the OXPHOS pathway is NADH:ubiquinone oxidoreductase or Complex I (CI), consisting of 45 subunits in mammals with a combined weight of 970 kDa. Fourteen of these are considered “core” subunits as they comprise the minimal functional CI conserved in bacteria, the remainder, so-called “accessory” subunits, in mammals have unknown roles (Fiedorczuk *et al.*, 2016). *D. discoideum* possesses proteins homologous to the core subunits, and a subset of the mammalian accessory proteins. Three subunit genes that are now encoded on the nuclear genome in mammals are encoded on the mitochondrial genome in *D. discoideum.* CI couples the oxidation of NADH to the reduction of ubiquinone, and the pumping of protons. This complex is the primary contributor to respiratory electron transport and creation of the mitochondrial membrane potential. As

a side effect of this, it is also the main site of production of reactive oxygen species which form by leakage of electrons directly to molecular O_2 at the point where electrons are normally transferred via ubiquinone to Complex III. A common cause and indicator of mitochondrial disease, respiratory complex I dysfunction has been successfully modelled in *D. discoideum,* as discussed in a later section of this article.

Alternative oxidase and uncoupling protein

In addition to the classic pathway, the *D. discoideum* OXPHOS pathway has both a cyanide-resistant ubiquinol alternative oxidase (AOX), similarly to many plant and fungi mitochondria, and uncoupling proteins (UCPs) as do most eukaryotes (Jarmuszkiewicz *et al*., 2002, McDonald, 2008). *D. discoideum* AOX (AoxA) is a monomeric oxidase able to accept electrons generated via the electron transport chain, reduce oxygen to water, and generates heat from the dissipation of free-energy (McDonald, 2008). This decreases the yield of oxidative phosphorylation overall but provides a cyanide-resistant alternative pathway for mitochondrial respiration to continue if the cytochrome *c* oxidase pathway is inhibited. UCP also reduces oxidative phosphorylation yield by dissipation of the H^+ gradient generated by the ETC.

AOX and UCPs are used for thermogenesis in plants and mammals, respectively. However, in unicellular eukaryotes, like *D. discoideum*, any thermal differential with the environment would be quickly dissipated. Therefore, their purpose in *D. discoideum* is likely that of an energy-dissipation system for modulation of the reduction of ETC components. AOX and UCP both decrease the formation of reactive oxygen species (ROS), so may protect mitochondria from DNA damage, and the initiation of programmed cell death by preventing the over-reduction of the ETC chain (McDonald, 2008, Popov *et al*., 1997). AoxA was also suggested to play a role in multicellular differentiation of *D. discoideum,* since pharmacological inhibition of AoxA in starved amoebae triggered differentiation into stalk-like sterile cells (Kimura *et al*., 2010). However, AoxA null strains developed normally under unstressed conditions. This apparent contradiction suggests that the phenotypes exhibited during pharmacological inhibition may not be due solely to the inhibition of AoxA (Kimura *et al*., 2010). It is possible that the AOX inhibitor also caused oxidative or some other stress in the treated cells, in addition to inhibiting AoxA. When stressed with hydrogen peroxide, the development of AoxA null strains was significantly impaired, in that the mutants were unable to aggregate (Kimura *et al*., 2010).

Since AoxA is needed in oxidatively stressed cells for the transition from growth to aggregation, it must be expressed at that time. Jarmuszkiewicz *et al.,* (2002) used antibodies against plant AOX to assay expression of *Dictyostelium* AoxA. They found that the protein is expressed continuously in the lifecycle of *D. discoideum*, but its expression levels changed significantly during development. AoxA expression was significantly higher during exponential growth than during stationary phase (20-fold) (Jarmuszkiewicz *et al*., 2002). However, inducing aggregation of exponentially growing cells by starvation caused the maintenance of AoxA levels at 50% of exponential growth. Maintenance of AoxA levels during starvation-induced development may be necessary for the correct developmental stalk/spore ratio in the fruiting body stage (Jarmuszkiewicz *et al*., 2002).

A similar approach to studying *D. discoideum* UCP expression revealed it to be constant throughout growth and development, suggesting a constant need for its activity whatever the life-stage (Jarmuszkiewicz *et al*., 2004). While its expression level remained constant, *D. discoideum* UCP was activated by free fatty acids, and consequently dissipated the mitochondrial membrane potential. Doing so should reduce the generation of ROS and so may provide a "safety valve" for the cell, with UCP acting indirectly as an antioxidant (Jarmuszkiewicz *et al*., 2004).

More recent bioinformatic analysis indicates that *Dictyostelium* has three uncoupling protein isoforms: UcpA, UcpB, and UcpC (Satre *et al*., 2007). Due to their similar size (33 kDa, 31 kDa, and 35 kDa, respectively) it is unclear whether an individual isoform or the sum of all their expression was detected using the plant-derived antibodies. Therefore, it is possible that individual isoforms may differ in their expression during growth and development, and further investigation of this is warranted. Overall, AOX and UCP appear to provide a means of relieving ROS generation during high metabolic activity e.g. during exponential growth, and additionally AOX has a role in multicellular development in *D. discoideum.*

Mitochondrial disease modelling in *Dictyostelium*

Mitochondrial disease characteristics

Diseases involving mitochondrial dysfunction can be broadly divided into two groups: diseases that arise from a direct primary mitochondrial cause, typically mutations directly affecting the activity or expression of mitochondrial proteins, and those that originate elsewhere but have effects on the mitochondria. Primary mitochondrial diseases in humans cause a range of diverse outcomes including myopathy, diabetes, kidney disease, epilepsy, autism, blindness, deafness, heart disease, and cancer (Kalyanaraman *et al*., 2018). Diseases of nonmitochondrial origin that involve mitochondrial dysfunction include many neurodegenerative and neurological disorders such as retinitis pigmentosa, Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis (Onyango *et al*., 2017, Park *et al*., 2006, Reddy and Beal, 2005).

A primary mitochondrial disease may be caused by inherited or acquired mutations in mitochondrial or nuclear-encoded mitochondrial genes and manifest phenotypically by impacting the ATP production of mitochondria (Alston *et al*., 2017). As mitochondrial genomes exist in multiple copies in each mitochondrion, and each mitochondrion is one of hundreds in each cell, mutations in the mtDNA may occur in only a proportion of the mitochondrial population, a state known as heteroplasmy (Stefano and Kream, 2016). The proportions of mutant mitochondrial genomes can vary between individuals, between tissues in an individual, and change with age. Phenotypic outcomes of mitochondrial diseases are determined by how large a proportion of the mitochondrial population is affected (mutant load) (Stewart and Chinnery, 2015). The appearance of a phenotype requires a mutant load threshold to be reached. For example, mutations in ATPase 6 (m.8993T>G) will cause neuropathy, ataxia and retinitis pigmentosa (NARP) when heteroplasmy is between 60 and 90% and develop Leigh syndrome when >90% (Claeys *et al*., 2016). If the same position is mutated differently, i.e. m8993T>C then Leigh syndrome develops in homoplasy and a >90% heteroplasmy will cause NARP (Weerasinghe *et al*., 2018). Furthermore, Leigh syndrome can also be caused by more than 75 other mutations in entirely separate genes e.g. loss of function of a subunit of Respiratory Complex II, *sdhA* (Lake *et al*., 2016). Defects

in the same respiratory complex can also give rise to multiple disorders. Dysfunction of Respiratory Complex I can cause a number of diseases including MELAS syndrome, Parkinsonism, Leber's disease, and Leigh syndrome (Rodenburg, 2016). In addition to mutant load, cells at particular risk of mitochondrial dysfunction are those that have high energy requirements such as the cerebrum, muscles, and neurons (Zhao *et al.*, 2019). Therefore, in some tissues where energy requirements are lower, the cells are not diseased, whereas in others with high ATP demand, the mutant load may be high enough to cause disease e.g. retinitis pigmentosa (Petit *et al.*, 2018). The complexity and difficulty in studying mitochondrial diseases emphasises the need for a simpler model organism such as *Dictyostelium discoideum* to clarify these complex phenotypes and provide the basis for investigating the signalling pathways involved and the downstream cellular consequences.

Dictyostelium *models of mitochondrial disease*

D. discoideum provides a well-established model for the study of mitochondrial disease. Presented here is a discussion of some mitochondrial disease modelling from all areas of mitochondrial cellular involvement. *D. discoideum* mitochondrial disease models have been developed that fall into two categories: those with direct primary involvement of mitochondrial genes and proteins, and those where there is mitochondrial involvement, but the disease aetiology is not mitochondrial.

The first *D. discoideum* model of mitochondrial disease was created via the mutation of the mitochondrially encoded large ribosomal RNA subunit gene, *rnl* (Wilczynska *et al.*, 1997). This created a heteroplasmic mitochondrial mutant strain with aberrant phototaxis. Further investigation of heteroplasmic mitochondrial mutations in *D. discoideum* revealed that a common phenotypic signature was shared across strains (Francione, 2008, Wilczynska *et al.*, 1997). This signature phenotype includes impaired growth, both on bacterial lawns and in liquid media, deranged phototaxis and thermotaxis, defective multicellular morphogenesis with shortened and thickened fruiting body stalks, and susceptibility to *Legionella* proliferation within the amoeba (Francione *et al.*, 2009). Interestingly, the antisense inhibition of the *D. discoideum* chaperonin 60 gene, a protein required for the correct folding of proteins within mitochondria, caused the same aberrant phenotypes (Bokko *et al.*, 2007). Chaperonin 60 knockdown causes an overall knockdown in respiratory activity without specific respiratory complex deficiency (Jasim, 2018). This is not unexpected as chaperonin 60 has a generalised role in mitochondrial protein folding (Henderson *et al.*, 2013). These phenotypes are similar to phenotypes observed when the mitochondrial genome is depleted by ethidium bromide treatment (Chida *et al.*, 2004).

How might these phenotypes arise? Kotsifas *et al.,* (2002) suggested that signal transduction is more readily disrupted by energy depletion than cellular activities. Signal transduction is energetically expensive and the most obvious *a priori* prediction of mitochondrial genome dysfunction in mitochondrial disease is that many of the manifested phenotypes would be due to a shortfall in the production of ATP. The depletion of ATP would cause disease through disruption of cellular activities, and those activities/tissues with high energy requirements would be most affected. However, it has been shown in *D. discoideum*, that mitochondrial disease phenotypes in these cases are attributable to chronic AMP-activated protein kinase (AMPK) signalling rather the direct result of ATP insufficiency (Bokko *et al.*, 2007, Francione *et al.*, 2009). AMPK is an energy-sensing protein kinase responsible for maintaining ATP homeostasis by inhibiting anabolic processes, such as growth and proliferation, and promoting ATP-generating pathways, such as mitochondrial biogenesis and fatty acid oxidation. The net result is that ATP homeostasis in maintained by chronic activation of AMPK with the downstream phenotypic consequences of this activation being a permanent feature. Antisense inhibition of AMPK in the *D. discoideum* mitochondrial disease models rescued the defective phenotypes where the respiratory activity of the mitochondria was broadly affected (Bokko *et al.*, 2007).

Bolstering the case for AMPK's role, similar phenotypic consequences were observed when specific respiratory complexes were impaired. As previously discussed, MidA plays a role in *D. discoideum* development. MidA is the *D. discoideum* orthologue of human NDUFAF7 protein, an arginine methyltransferase that is required for the assembly and function of mitochondrial complex I (CI) (Carilla-Latorre *et al.*, 2010, Hameed *et al.*, 2018). *D. discoideum midA* knockout disrupted phototaxis and thermotaxis, growth and multicellular development. The defects in phototaxis and thermotaxis were found to be entirely and the growth defects partially mediated by chronic activation of AMPK (Carilla-Latorre *et al.*, 2010). Analysis of respiration in *midA* null and *midA* catalytic site point mutants by Seahorse respirometry confirmed the critical and specific role of MidA in proper functioning of CI, with a significant reduction of protonophore-uncoupled, whole cell O_2 consumption attributable to CI, unaccompanied by defects in other respiratory complexes measured (CII & CV). The proportion of basal O_2 consumed for ATP production was unchanged in the *midA* mutants, as expected if AMPK is chronically activated to maintain ATP homeostasis (Hameed *et al.*, 2018).

The second category of *D. discoideum* mitochondrial dysfunction models are those that have mitochondrial involvement not directly related to respiration. While not considered classical mitochondrial diseases, there is evidence that mitochondrial dysfunction is involved in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) (Viscomi *et al.*, 2016). Several known genetic mutations in familial Parkinson's (PD) affect human mitochondria-associated proteins such as PRKN, PINK1, DJ-1, HTRA2, and LRRK2 with corresponding mitochondrial dysfunction (Kalinderi *et al.*, 2016, Narendra, 2016). Attractively, *D. discoideum* does not endogenously express some of these proteins so they can be studied in a naïve cell to determine their putative interacting partners and possible cytotoxic mechanisms. Alternatively, where native homologues of human disease related proteins exist in *D. discoideum*, they can be readily manipulated and analysed.

Discussed here are two recent *D. discoideum* models that have been created to study the involvement of mitochondrial dysfunction in neurodegenerative diseases.

Respiratory Complex I defects have been reported in post-mortem PD brain samples (Larsen *et al.*, 2018). Chronic exposure to pesticides, such as rotenone, a respiratory Complex 1 inhibitor, has been associated epidemiologically with PD (Betarbet *et al.*, 2000). Treatment of rats with rotenone resulted in the depletion of dopaminergic neurons and induced PD-like symptoms (Alam and Schmidt, 2002). When *D. discoideum* cells were treated with rotenone, it disrupted the cytoskeletal trafficking of mitochondria, inhibited mitochondrial fusion, and increased ROS levels without

affecting ATP levels (Chernivec *et al*., 2018). Disrupted mitochondrial dynamics and ROS damage were implied to possibly contribute to the loss of neurons in PD due to their high energy requirement and reliance on mitochondrial movement (Chernivec *et al*., 2018).

HTRA2 is a nuclear encoded serine protease which, when mutated, can cause late-onset Parkinson's disease (Fu *et al*., 2017). HTRA2 is localised to the mitochondrial intermembrane space in normal conditions and is believed to perform a positive, protective role against cellular stress and also function as a transducer of mitochondrial stress signals (Strauss *et al*., 2005). Exposure to cellular stresses causes the release of HTRA2 to the cytosol, where it binds to inhibitors of apoptosis, deactivating their inhibition of caspases, initiating programmed cell death (Martins *et al*., 2002). HTRA2 deletion causes mitochondrial dysfunction and PD-like features (Martins *et al*., 2004). PD patient-associated mutations in HTRA2 caused the deactivation of the protease activity of HTRA2 when replicated in neuroblastoma SH-SY5Y-derived cell lines (Strauss *et al*., 2005). In addition, some of these mutant cell lines were more susceptible to staurosporine-induced cellular stress (Strauss *et al*., 2005). Furthermore, HTRA2 interacts with PINK1, a protein kinase associated with early onset PD, and forming part of the same stress-sensing pathway. HTRA2 phosphorylation is PINK1-dependent and occurs adjacent to a site of a known PD-associated mutation (Plun-Favreau *et al*., 2007). However, it is not entirely understood how HTRA2 mutations cause PD, and whether this is due to a reduction or increase in its serine protease activity or related to its role in cell death.

The single *D. discoideum* homologue of human HTRA2, also called HTRA2, appears to play a conserved cytoprotective role (Chen *et al*., 2018). Antisense inhibition and overexpression of a catalytically inactive form of *D. discoideum* HTRA2 both resulted in defects in morphogenesis and growth but did not affect phototaxis. This suggests that HTRA2 inhibition does not affect mitochondrial ATP production and therefore does not chronically activate AMPK (Chen *et al*., 2018). The protective role of HTRA2 appears contingent on its expression level. Overexpressed wildtype HTRA2 was cytotoxic in *D. discoideum*, even without translocation to the cytosol, evidently due to its serine protease activity, since a protease-dead mutant form could be overexpressed. Overexpressing active HTRA2 may cause the depletion of proteins essential to mitochondrial function and thereby have triggered cell death pathways (Chen *et al*., 2018). An alternative hypothesis is that overexpression may have caused an accumulation of active, cytotoxic protease in the cytosol by breaching the maximum capacity for mitochondrial import of the protein. However, the protease-dead GFP-tagged HtrA did not accumulate in the cytosol when overexpressed but was correctly targeted to the mitochondria.

Conclusion

It is clear that the study of mitochondrial biology and disease in *D. discoideum* has yielded valuable insight into the inner workings of eukaryotic mitochondria and begun to tease out the complexities of human mitochondrial disease. Some of the proteins with conserved roles in mitochondrial biology, notably MidA and CluA, were first discovered and studied in *Dictyostelium*. In other cases, *Dictyostelium* has helped reveal ancient protein functions, such as the role of Miro in mitochondrial homeostasis or FszA in mitochondrial fission, that have been conserved in some eukaryotic lineages but modified or lost in metazoa.

Both classical mitochondrial disease and mitochondria-associated neurodegenerative disease models have been successfully created in *D. discoideum.* Mitochondrial disease modelling in *D. discoideum* first revealed that the deficiency in ATP production may not directly cause disease phenotypes by inhibition of cellular activities, rather, that they may be caused by the chronic activation of AMPK in an attempt to restore energy homeostasis. As no effective therapies currently exist for mitochondrial disorders, this knowledge may be critical in the treatment or alleviation of mitochondrial disease symptoms. By bridging the gap between yeast models and higher eukaryotes, *D. discoideum* is well placed to continue contributing further in our understanding of mitochondrial biology, and by extension, eukaryotic cell biology.

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