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Review

Dictyostelium, a microbial model for brain disease ☆

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ABSTRACT

Background: Most neurodegenerative diseases are associated with mitochondrial dysfunction. In humans, mutations in mitochondrial genes result in a range of phenotypic outcomes which do not correlate well with the underlying genetic cause. Other neurodegenerative diseases are caused by mutations that affect the function and trafficking of lysosomes, endosomes and autophagosomes. Many of the complexities of these human diseases can be avoided by studying them in the simple eukaryotic model *Dictyostelium discoideum*.
Scope of review: This review describes research using *Dictyostelium* to study cytopathological pathways underlying a variety of neurodegenerative diseases including mitochondrial, lysosomal and vesicle trafficking disorders.
Major conclusions: Generalised mitochondrial respiratory deficiencies in *Dictyostelium* produce a consistent pattern of defective phenotypes that are caused by chronic activation of a cellular energy sensor AMPK (AMP-activated protein kinase) and not ATP deficiency *per se*. Surprisingly, when individual subunits of Complex I are knocked out, both AMPK-dependent and AMPK-independent, subunit-specific phenotypes are observed. Many nonmitochondrial proteins associated with neurological disorders have homologues in *Dictyostelium* and are associated with the function and trafficking of lysosomes and endosomes. Conversely, some genes associated with neurodegenerative disorders do not have homologues in *Dictyostelium* and this provides a unique avenue for studying these mutated proteins in the absence of endogeneous protein.
General significance: Using the *Dictyostelium* model we have gained insights into the sublethal cytopathological pathways whose dysregulation contributes to phenotypic outcomes in neurodegenerative disease. This work is beginning to distinguish correlation, cause and effect in the complex network of cross talk between the various organelles involved. This article is part of a Special Issue entitled Frontiers of Mitochondrial Research

1. Introduction — the value of the *Dictyostelium* model

D. discoideum is a social amoeba or cellular slime mould that has long been regarded as a valuable eukaryotic model organism for the study of many signalling processes, including those leading to chemotactic motility and regulation of the actinomyosin cytoskeleton. The reasons for *Dictyostelium*'s success as a model are numerous. Firstly the complete genome of *D. discoideum* has been sequenced [1], it is genetically tractable, readily grown clonally as a eukaryotic microorganism and is highly accessible for biochemical, cell biological and physiological studies. These properties are shared with other microbial model organisms. What sets *Dictyostelium* apart from the other systems is its unique lifecycle with motile unicellular and multicellular stages and multiple cell types (Fig. 1). These offer for study an unparalleled variety of phenotypes which serve as accessible "readouts" of the associated signalling pathways.

Dictyostelium lives as an amoeba in the soil of temperate forests, feeding by phagocytosis of microorganisms (laboratory strains can also survive by macropinocytosis of liquid nutrients) [2]. When the food source is depleted the amoebae differentiate and begin to emit pulses of cAMP to which they are now attracted, resulting in the aggregation of approximately 100,000 cells. These cells then undergo a multicellular developmental programme in which the cells differentiate into two main cell types, prestalk and prespore cells that are predestined to form the stalk and spores of a multicellular fruiting body (Fig. 1A).

Aggregation initially produces a mound of cells that then forms a motile organism called a slug, which is composed of multiple cell types organised into different tissues recognisable on the basis of differential gene expression [3,4] (Fig. 1B). From the rear to the front of the slug these are

1. a small rearguard region (*ca.* 5% of the cells) with a concentration of anterior-like cells (ALC) predestined to form the basal disc,
2. a prespore region in most of the posterior portion of the slug (*ca.* 70% of the cells), and

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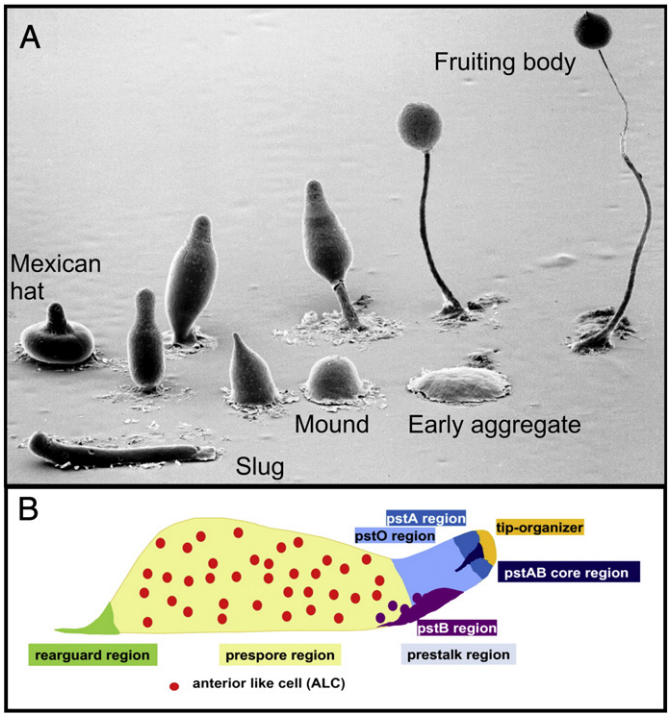

Fig. 1. Life cycle of *Dictyostelium discoideum*. A. Starvation triggers the initiation of a multicellular developmental programme in *Dictyostelium discoideum*. Individual amoebae begin to emit pulses of cAMP and they become attracted to it which results in the formation of an aggregate containing about 100,000 cells. The cells then further differentiate into prestalk and prespore cells and form a finger. This finger then falls over and forms a slug which is motile for a variable period of time. The slug is phototactic and thermotactic which enables the organism to move to a more favourable location. The slug ceases migration and further differentiates into a fruiting body consisting of two main cell types forming the long slender stalk and spore cells encased in a sorus atop the stalk. Each spore can then germinate to release a vegetative amoeba and renew the life cycle. Modified with permission from http://dictybase.org/Multimedia/LarryBlanton/index.html. Copyright, M.J. Grimson & R.L. Blanton, Biological Sciences Electron Microscopy Laboratory, Texas Tech University. B. Cell types in the motile *Dictyostelium* slug. Most of the cells are prespore cells (destined later to become spores in the sorus) and occupy the rear 2/3 of the slug. The distribution of the prestalk cell subtypes at the front as well as the tip, rearguard and anterior like cells are shown. From Fig. 3 of Gaudet et al. [4] published under the terms of the Creative Commons Attribution Licence (http://creativecommons.org/licenses/by/2.0/).

3. an anterior prestalk region (*ca.* 20% of the cells) which is subdivisible into the pst0 (prestalk 0), pstB (prestalk B), pstAB (prestalk AB), pstA (prestalk A) and tip regions.

A reserve of anterior-like cells are scattered through the prespore region and a recently identified subset of them, the pstU (prestalk U) cells [5] are predestined to localise to the upper cup at the very top of the fruiting body above the spores. Recently, new cell types were discovered - an epithelial layer of cells on the outside of the slug [6] and S (sentinel) cells scattered through the slug that serve a function in phagocytic immunity [7]. More than anything else, these emerging complexities reinforce the fact that *Dictyostelium* exhibits true multicellularity that evolved independently of the better known forms of multicellularity in animals and plants.

Not only does the slug exhibit complex patterns of regulated cell differentiation, but it also exhibits complex motile behaviours, including thermotaxis and phototaxis which enable it to move to a more desirable location for subsequent spore dispersal. After a variable migration time, the slug stops and forms a fruiting body with a long slender stalk whose cells have undergone autophagic cell death. Atop the stalk is a sorus, a droplet of spore cells which can be dispersed to sites where food may be more abundant and where they germinate releasing the single celled amoebae (Fig. 1). It is the diverse phenotypic traits offered by this life cycle, that have allowed the experimentally tractable *Dictyostelium* to become a valuable model for studying mitochondrial diseases and other disorders affecting the central nervous system.

Because multicellularity evolved independently in the *Dictyostelium* lineage and is achieved by aggregation of postmitotic cells rather than by adhesion of dividing cells as occurs in metazoan embryogenesis, the tissues and cell types it produces do not have direct homologues in humans. Most neurological disorders selectively affect specific regions of the brain and specific neuronal subtypes. In the absence of directly homologous cell types and tissues, the *Dictyostelium* model cannot, on its own, explain this selectivity. Yet without a deep knowledge of the underlying molecular pathology that is shared by all cell types, we will not understand how this pathology can be constrained to specific cells or tissues.

Dictyostelium lends itself to the study of these shared pathological mechanisms affecting ancestral, cellular functions including central metabolism, cell division, growth, endocytosis, secretion, motility, autophagy, macromolecule and vesicle trafficking, intracellular signalling. These and other cellular processes are conserved in diverse eukaryotic lineages, tissues and cell types, including the human brain. Their dysfunction in different organisms or in different tissues within the same organism can produce similar outcomes at the cellular and molecular level, but appear superficially very different at the level of the whole organism. For example, *Dictyostelium* mutants with impaired phagocytosis are unable to grow with bacteria as a food source, while mutants with defective chemotaxis cannot aggregate to undergo multicellular development. Genetically equivalent phagocytic and chemotaxis defects in human macrophages produce immune dysfunction eg. Chediak–Higashi Syndrome [8,9] and Wiscott–Aldrich Syndrome [10,11]. Similarly, genes whose disruption is not inimical to cell viability, can nonetheless be embryonic lethal in multicellular animals because of the dependence of embryogenesis on normal cell signalling, movement and differentiation. Such defects are not lethal in *Dictyostelium* because the viability of the organism does not depend on multicellular morphogenesis. Examples discussed later in this review include the presenilins and huntingtin.

In the case of mitochondrial disease, the primary genetic cause is mutations in mitochondrial DNA or nuclear encoded mitochondrial genes. In humans these mutations result in a range of phenotypic

outcomes which do not correlate well with the underlying genetic defect, but which commonly include neurological dysfunction and neurodegeneration. In the case of mutated mitochondrial DNA the proportion of mutant mitochondrial genomes can differ in different individuals, in different tissues in the same individuals and in the same tissues at different ages. Combined with tissue-specific differences in energy requirements and expression of nuclear-encoded mitochondrial proteins, this can result in similar or very different phenotypic outcomes depending on the proportion and distribution of the mutant mitochondria [12,13]. Thus the same underlying molecular pathology of mitochondrial disease can cause blindness if it occurs in photoreceptor cells in the retina, while in the *substantia nigra* it can produce parkinsonism [12,13].

These complexities are avoided in *Dictyostelium*, because the unicellular, amoeboid form represents a single, clonally grown, haploid, totipotent stem cell type while multicellular development is initiated by aggregation, includes no sexual stage, involves comparatively few cell types and occurs without the overlaid complications of cell growth and division. We and others have found that mitochondrial mutations which affect oxidative phosphorylation result in common defective phenotypes regardless of the underlying mutation [14]. Previously the phenotypic outcomes of mitochondrial disease were thought to result from a depletion of ATP, however work in *Dictyostelium* has shown that diverse cytopathological outcomes are due to chronic activation of a cellular energy sensor AMPK [15,16]. AMPK is activated by low ATP levels and high AMP/ADP levels. Its role in otherwise healthy cells is to restore ATP levels homeostatically by turning off energy consuming pathways and turning on energy producing pathways. Chronic dysregulation of these signalling pathways is what leads to the phenotypic outcomes of mitochondrial disease. Since this discovery in *Dictyostelium*, AMPK has been shown to be activated chronically in several mammalian neurodegenerative disorders that involve mitochondrial dysfunction [17–19]. In at least some of these, it appears to play a cytopathological role, as predicted from the *Dictyostelium* work.

The pathological role of chronic AMPK dysregulation in *Dictyostelium* and in the human brain provides an example of the relationship between disease mechanisms in these two very different organisms. Neuronal cell death in neurodegenerative diseases is frequently accompanied by increased elaboration within the cell of autophagic vacuoles and this autophagic cell death can be the dominant neuronal death type in these diseases [20]. However it has been difficult to discern whether the elevated levels of autophagy are a cause or a survival-promoting companion of death, a situation made more complicated by cross talk between autophagic cell death, apoptotic and other cell death pathways. The *Dictyostelium* prestalk/stalk differentiation pathway is also a form of autophagic cell death, uncomplicated by the presence of apoptosis [21]. *Dictyostelium* mutants unable to undergo autophagy provided clear-cut genetic evidence that autophagy is not essential for death [22]. In mitochondrially diseased *Dictyostelium*, chronic AMPK hyperactivity causes more cells to enter this pathway, so that thickened, misshapen stalks form in the final stages of morphogenesis [15]. The induction of autophagy by AMPK is conserved in mammalian cells, so that some of the molecular events involved in *Dictyostelium* stalk morphogenesis are shared by neuronal death pathways in brain disease.

In *Dictyostelium* other disease phenotypes caused by mitochondrial disease include AMPK-dependent defects in growth, increased susceptibility to the intracellular bacterial pathogen, *Legionella pneumophila*, as well as aberrant slug phototaxis and thermotaxis [15,16]. In addition to these AMPK-dependent phenotypic outcomes which result from generalised OXPHOS defects (when respiratory complexes are coordinately affected), other AMPK-independent signalling pathways are activated when individual respiratory complex subunits are mutated. These defects are specific to the protein and complex concerned and will be discussed in detail in the later sections [23,24].

In humans, mitochondrial diseases commonly cause neurological and neurodegenerative defects [12,13]. On the other hand many non-mitochondrial brain disorders are also characterised by mitochondrial pathology, including Parkinson's and Alzheimer's Disease, the two most common neurodegenerative diseases. In the case of Parkinson's Disease (PD) several of the known genetic causes of familial PD are mutations in genes encoding mitochondrial proteins [25]. Although not normally considered to be mitochondrial proteins, even α-synuclein and Aβ, the hallmark polypeptides of PD and Alzheimer's Disease (AD) respectively, are found in the disease state in the mitochondria where they exert pathological effects [25,26]. Thus the *Dictyostelium* mitochondrial disease model may also shed light on non-mitochondrial diseases affecting the brain, including PD and AD.

Regardless of the underlying genetic cause, PD and AD and many other neurodegenerative diseases are characterised not only by mitochondrial dysfunction but also by the accumulation of intracellular protein aggregates and autophagosomes. That all of these features are frequently found together in the same disorder reflects extensive cross talk between the dysregulated signalling pathways involved. Elevated levels of autophagosomes and protein aggregates indicate a failure or dysregulation of autophagy, a conserved organellar and protein quality control mechanism involving the biogenesis and trafficking of lysosomes, endosomes, autophagomes and other classes of intracellular vesicle. Many neurodegenerative diseases are caused by mutations affecting the function and trafficking of lysosomes and other vesicles. The *Dictyostelium* model has been deployed to study a number of these disorders.

Many of the proteins involved in neurodegenerative diseases have homologues in *Dictyostelium* and as such these proteins can be mutated or the expression levels altered in *Dictyostelium* in order to study their functions. Alternatively some proteins associated with neurological disorders do not have homologues in *Dictyostelium*. These human proteins can be expressed in *Dictyostelium* where they can be studied in the absence of endogeneous copies of the gene which could interfere with the analysis. For example, the cytotoxicity of normal and disease-causing mutant forms of human alpha-synuclein has been studied in *Dictyostelium* [27] and is discussed in later sections.

The remainder of this article will describe work which has been done in *Dictyostelium* that has led to it becoming a useful model for the study of mitochondrial and neurological disorders, despite the absence of a nervous system. We have focused on diseases with mitochondrial, lysosomal or autophagosomal involvement, but *Dictyostelium* has also been used to successfully study other neurological disorders or their treatment, including lissencephaly [28] as well as the mechanism of action of lithium and valproate in the treatment of epilepsy and bipolar disorder [29–32].

2. Mitochondrial disease in the *Dictyostelium* model

A large variety of mutations affecting the mitochondria have been studied in *Dictyostelium*. They fall into several categories — generalised OXPHOS defects in which multiple respiratory complexes are coordinately affected, isolated OXPHOS defects in which only a single respiratory complex is affected and defects not known to affect respiratory oxidative phosphorylation. The downstream consequences and underlying mechanisms differ in these different categories, depending on the underlying genetic defect, and can be AMPK-dependent or AMPK-independent.

2.1. Generalised mitochondrial OXPHOS defects result in common defective phenotypes in Dictyostelium

To study mitochondrial disease in *D. discoideum*, mitochondrial respiratory dysfunction has been produced by mitochondrial DNA depletion with ethidium bromide treatment [33]; by antisense inhibition of expression of chaperonin 60 (Cpn60), an essential nuclear-encoded

mitochondrial protein [34]; or by disruption of mitochondrial genes in a subset of the mitochondrial genomes in the cell — heteroplasmic gene disruption [35–37]. Knock down of chaperonin 60 causes a general defect in the folding of mitochondrial proteins, while mitochondrial DNA depletion or disruption of any of the mitochondrial genes reduces the expression of the entire mitochondrial genome [33,37]. In each of these situations, a generalised respiratory dysfunction would result that coordinately affects at least Complex I, III, IV and V, all of which include subunits encoded in the mitochondrial genome.

To determine if the disease phenotypes were consistent regardless of which mitochondrial gene was targeted, Francione and Fisher [37] studied heteroplasmic disruptions of 9 different genes distributed around the mitochondrial genome (each red-boxed gene in Fig. 2 except *rps4*). Because the entire *Dictyostelium* mitochondrial genome is transcribed unidirectionally from a single promoter, one might expect sequences downstream of the targeted gene to be affected preferentially. However, there was no evidence for a polar effect on mitochondrial gene expression and the relative levels of expression were not correlated with the position of the targeted gene in the mitochondrial disruptants [37]. Instead, quantitative northern hybridisation analyses revealed a general reduction in expression of genes spanning the entire mitochondrial genome relative to cellular mRNA levels in the mutants compared to the wild type strain. Such a reduction could indicate that the plasmid insertions either cause greater instability of the RNA transcripts or decreased rates of transcription.

The aberrant phenotypes that resulted from heteroplasmic mitochondrial gene disruptions in *Dictyostelium* included impaired slug phototaxis (Fig. 3) and thermotaxis, aberrant multicellular development (Fig. 4) and slow growth, both on plates and in liquid medium [37]. That thermotaxis as well as phototaxis was impaired is not surprising, since the signalling pathways for these behaviours converge soon after signal reception, so that most genes involved in phototaxis are also involved in thermotaxis [38,39]. This pattern of phenotypic outcomes was similar irrespective of the location of the gene relative to the single transcription start site and regardless of whether the targeted gene product was known to be essential for energy production. Similar patterns of phenotypes were also observed in response to antisense inhibition of Cpn60 expression [34,37], and ethidium bromide inhibition of mtDNA replication [33].

Humans with mitochondrial diseases exhibit increased susceptibility to infectious diseases, particularly of the upper respiratory tract, the reasons for this are unknown. Since *Dictyostelium* provides a well established model for studying pathogenesis by various microbes including *Legionella pneumophila* [40], it was apposite to examine the effects of mitochondrial disease on *Dictyostelium* susceptibility to *Legionella* infection and intracellular proliferation [16]. *Legionella*-containing vesicles had been observed previously to associate temporarily with mitochondria in infected human macrophages [41,42] and mitochondrial genes had been shown to be upregulated in infected *Dictyostelium* cells [43]. Francione et al. [16] found that mitochondrially diseased *Dictyostelium* strains support greater intracellular proliferation of *L. pneumophila* than the wild type *Dictyostelium* strain (AX2) (see Fig. 5). The initial uptake of *Legionella* during the infection process was unaltered. Once again the same phenotype was observed in response to either chaperonin 60 knock down or heteroplasmic disruption of any 9 different mitochondrial genes.

The *Dictyostelium* model has thus revealed regularities in the underlying cell biology of mitochondrial disease that are presumably obscured in humans by the overlaid complexities of mammalian development. As noted in the Introduction, the genotype-phenotype linkage in human mitochondrial disease is broken in the sense that clinical outcomes cannot be predicted from knowledge of the underlying genetic defect. By contrast, the existence of a consistent pattern of mitochondrial disease phenotypes in *Dictyostelium* suggests a common cytopathological mechanism that underlies all generalised defects in mitochondrial respiratory function and is not a specific consequence of

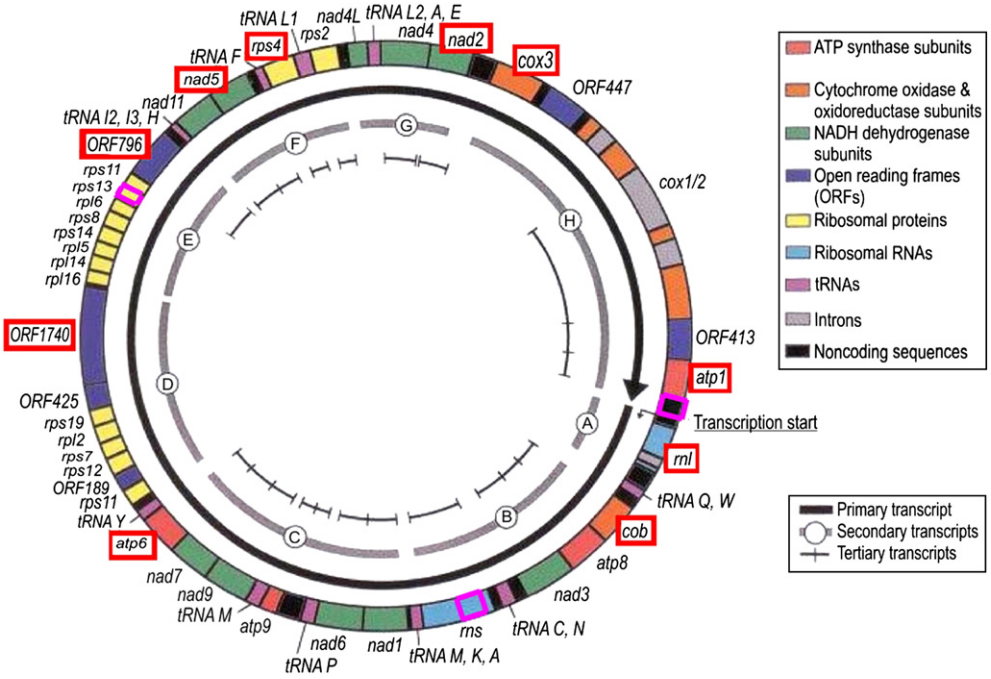

Fig. 2. Map of the *Dictyostelium discoideum* mitochondrial genome, highlighting genes targeted for heteroplasmic disruption. Transcription proceeds from a single transcription start site and the large primary transcript, which spans the entire genome, is cotranscriptionally processed into secondary and tertiary transcripts [233]. The mitochondrial genes targeted for disruption are boxed in red. Six genes encode subunits of respiratory chain enzymes — *atp1*, *atp6*, *cob*, *cox3*, *nad2* and *nad5*; one encodes the large subunit ribosomal RNA — *rnl*; one encodes a small ribosomal subunit protein — *rps4*; two encode the open reading frames *ORF1740* and *ORF796*–ORF1740 encodes a ribosomal S3 C-terminal domain-containing protein, whereas the function of the ORF796 product is unknown. Pink boxes indicate the sites whose transcription was measured in quantitative northern blots in mitochondrial gene disruptants [37]. Figure modified from Francione and Fisher [37] and Barth et al. [233].

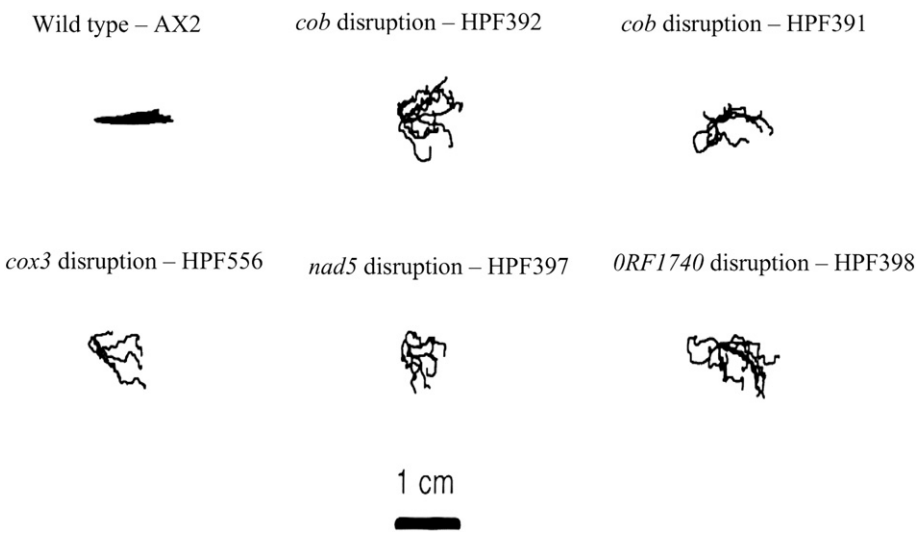

Fig. 3. Qualitative phototaxis of wild type (AX2) and heteroplasmic mitochondrial gene disruptants. Digitised trails from various strains of *Dictyostelium discoideum* slugs migrating in the presence of a light source positioned at the right of the figure. The wild type strain migrated directly towards the light source whereas the phototaxis mutant strains were highly disoriented as shown for HPF391, HPF392, HPF397 and HPF398. Other mutants such as HPF556 did not migrate from the inoculation site as represented by the dot. From Francione and Fisher [37].

disrupting specific genes. As described in the next section, this mechanism was subsequently revealed to be dysregulation of intracellular signalling caused by chronic activation of an energy-sensing protein kinase, AMPK (AMP-activated Protein Kinase).

2.2. The role of AMPK in diseases involving mitochondrial dysfunction

The primary cause of the pathological features of mitochondrial disease was previously assumed to be insufficient supplies of ATP [44,45]. However, Bokko et al. [15] reported that diverse mitochondrial disease phenotypes in *Dictyostelium* are actually caused by chronic activation of AMPK. These authors showed that knocking down expression of the catalytic α subunit of AMPK could suppress ('rescue') the aberrant phenotypes produced in mitochondrially diseased cells by antisense inhibition of chaperonin 60 expression. Conversely the mitochondrial disease phenotypes were phenocopied by overexpression of the catalytic domain of AMPKα in otherwise healthy *Dictyostelium* cells. Using the same approach, Francione et al. [16] went on to show that chronic AMPK activation also caused the increased proliferation of *Legionella* observed within mitochondrially diseased *Dictyostelium* cells.

AMPK is a highly conserved serine/threonine protein kinase that plays a crucial role in maintaining cellular homeostasis in healthy and diseased cells [46]. It is activated by various metabolic stresses which result in energy depletion or consumption of ATP and consequently a rise in AMP. Upon activation, AMPK inhibits anabolic processes such as lipid synthesis and promotes ATP-generating pathways including mitochondrial biogenesis [47]. There are a large variety of downstream pathways regulated by AMPK including fatty acid oxidation and

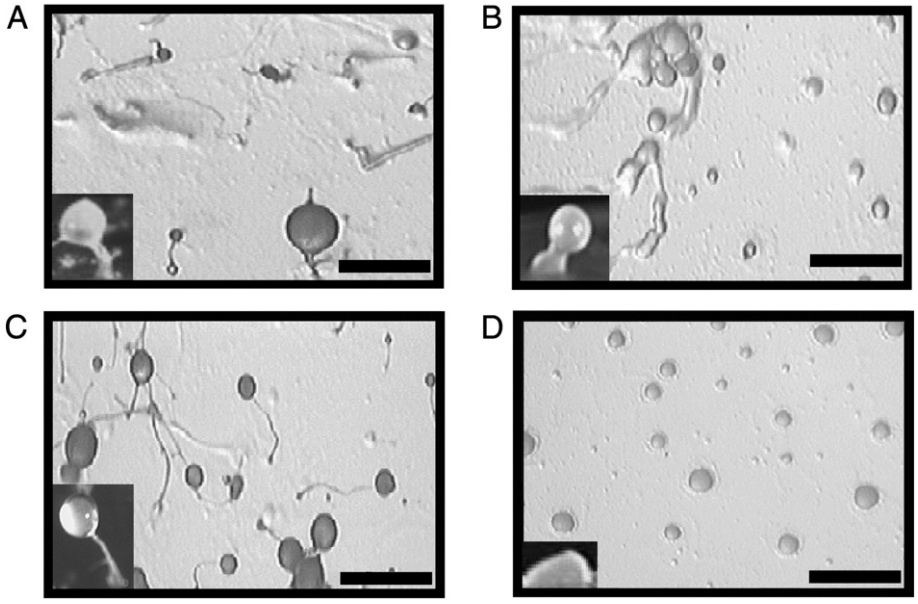

Fig. 4. Effect of heteroplasmic, mitochondrial gene disruption on multicellular morphogenesis. Photographs were taken of morphologies displayed by *Dictyostelium* heteroplasmic mitochondrial gene disruptants grown at 21 °C on *E. coli* B/2. (A) a *nad2* disruptant, HPF561. (B) a *cox3* disruptant, HPF556. (C) wild type (AX2) and (D) a *cob* disruptant, HPF395. The sparse aggregates, short thick stalks and relatively enlarged sori exhibited by HPF561 and HPF556 are typical in the mitochondrial disease strains. In addition to the abnormal fruiting bodies, HPF556 displayed a "broken stream effect" as shown by Panel B of Fig. 3, which may be indicative of defective signal transduction in the aggregation stage. In some strains, such as HPF395, development arrested at the mound stage. The scale bars represent 1 mm. From Francione and Fisher [37].

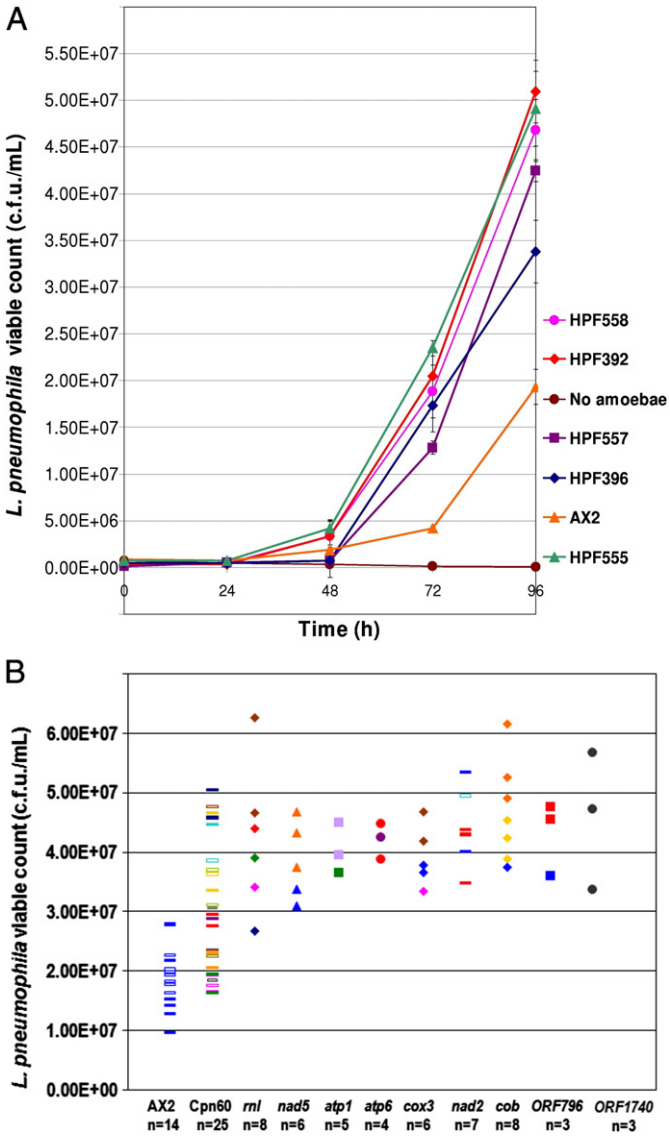

Fig. 5. Mitochondrial disease enhances susceptibility to *Legionella* in *Dictyostelium*. The cells of all strains were infected with the *L. pneumophila* Corby strain over a 96 h period. The wild type (AX2) strain was used as a control. A negative control in which the *Legionella* were plated with no host amoebae was also included. A: Bacterial viable counts are plotted as a function of time. Viable counts were performed in duplicate or triplicate. Error bars are standard errors reflecting viable count errors within the experiment. The strains with mitochondrial dysfunction carry heteroplasmic mitochondrial gene disruptions of *nad2* (HPF558), *nad5* (HPF396), *cob* (HPF392), *atp6* (HPF557) and *cox3* (HPF555). B: *Legionella* viable counts on the 5th day (after 96 h) of infection of normal (AX2) or mitochondrially diseased cells. Each point represents a separate experiment and each different symbol indicates a different, independent mutant. The X axis indicates the mutant class — either chaperonin 60 antisense inhibition (Cpn60) or the mitochondrial gene targeted for disruption. The extent of *Legionella* proliferation in the mitochondrially diseased strains was significantly greater than in AX2 according to the results of Kolmogorov–Smirnov, Kruskal–Wallis and Student's t two-sample (unequal variance) tests ($p < 0.05$). From Francione and Fisher [37].

glycolysis as well as mTOR-regulated protein synthesis and autophagy [48,49].

AMPK is a heterotrimer comprised of a catalytic α subunit, a regulatory γ subunit and a β subunit that works as a scaffold for the other subunits (Fig. 6). In mammalian cells, there are three isoforms of the γ subunit and two isoforms of each of the α and β subunits which assemble in different combinations in a tissue specific manner, whereas the *Dictyostelium* genome encodes one isoform only of each of the AMPK subunits [15].

The activation of AMPK results from the binding of AMP to the two Bateman domains on the γ subunit, releasing its inhibition of the catalytic α subunit. This exposes the threonine residue (Thr188 in *Dictyostelium* and Thr172 in mammalian α1) in the activation loop of the catalytic α subunit which is then phosphorylated by upstream kinases known as AMPK kinases (AMPKK) triggering a 50–100 fold increase in AMPK activity [50]. The activated AMPK also becomes resistant to dephosphorylation by phosphatases. Recent work has suggested that ADP can also act as an activator of AMPK [51]. Since ATP competitively inhibits AMPK by binding to the same sites as AMP and ADP, AMPK activation is effectively regulated by the ratio of ATP to AMP and ADP.

It was clear as early as 1987 that AMPK could be activated by an upstream kinase in rat liver [52] and this was subsequently shown also to be the case in yeast [53,54]. However it was not until 2003 that the first mammalian AMPK kinases were actually identified in genetic screening studies in yeast [55–57]. From these studies, three AMPKKs were identified — LKB1, a tumour suppressor [58]; CaMKK2 (also known as CAMKKβ), Ca^{2+}/calmodulin-dependent protein kinase [59] and TAK1, transforming growth factor-β (TGFβ)-activated kinase-1 [60].

LKB1 is the main upstream kinase of AMPK. It also functions as a master kinase upstream to 12 other human protein kinases (AMPK-related kinases in the CAMKL subfamily) that fall on the same branch as AMPK by phylogenetic analyses [61]. Apart from LKB1 and AMPK

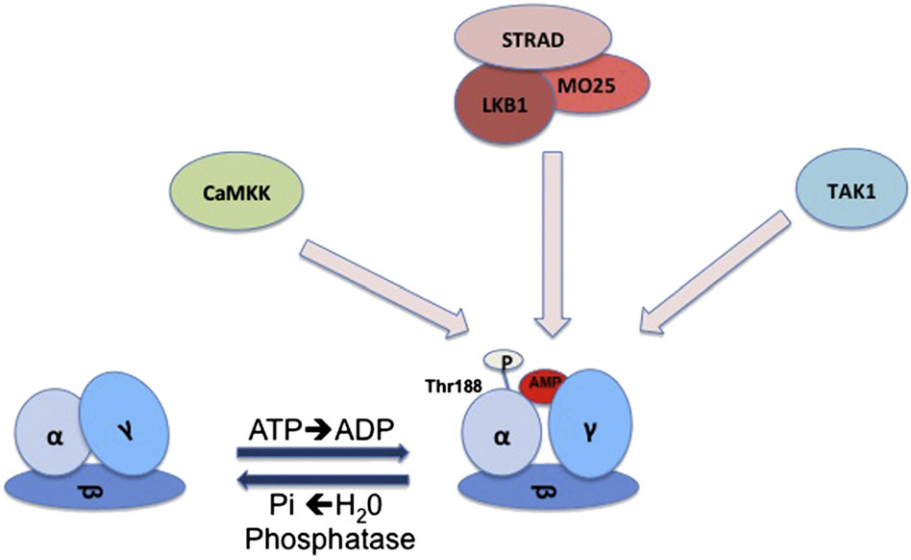

Fig. 6. The activation of AMPK by upstream kinases. AMPK activation occurs when AMP binds to the two Bateman domains on the γ subunit creating a conformational change in the AMPK complex and exposing the threonine residue (Thr188 in *Dictyostelium*) in the activation loop of the catalytic α subunit. ATP competes with AMP for binding, so that activation of AMPK is regulated by AMP/ATP ratios, with high ATP levels rendering it inactive. In the active form, the activating threonine is phosphorylated by upstream kinases — LKB1-STRAD-MO25 complex, TAK1 or CaMKK2. CAMKK2 can activate AMPK independently of AMP in response to elevated Ca^{2+} levels. Activated AMPK is also resistant to dephosphorylation by phosphatases.

itself, *Dictyostelium* has 6 kinases in this group which are therefore all potential substrates for LKB1 [62]. LKB1 is a serine/threonine kinase whose loss of function can lead in humans to Peutz–Jeghers syndrome, an autosomal dominant disease characterised by benign gastrointestinal tumours (hamartomas) [63]. Mutations in LKB1 have also been associated with diverse sporadic cancers, particularly in non-small lung cancer [64]. LKB1 is normally found in its active form as a complex with two other subunits, STE20 related adapter (STRAD, a pseudokinase) and Mouse Protein 25 (MO25), in a 1:1:1 ratio to form the LKB1-STRAD-MO25 complex [65]. The kinase domain of LKB1 binds to the pseudokinase domain of STRAD, and this interaction is stabilised by the scaffolding protein MO25 in a conformation optimal for phosphorylation of substrates [66].

LKB1 in *Dictyostelium* exhibits a 46% amino acid identity to human LKB1, while *Dictyostelium* MO25 and STRAD alpha show respectively 52% and 53% identity to their homologues in humans. A study conducted by Veeranki et al. [67] revealed that like its mammalian counterpart, *Dictyostelium* LKB1 phosphorylates and activates AMPK under osmotic and oxidative stresses. Knocking down LKB1 not only abrogated the phosphorylation of AMPK in response to these stresses, it also severely impaired starvation-induced aggregation and subsequent multicellular development. This phenotype is not dissimilar to that caused by AMPK knockdown in strains with very high copy numbers of an AMPK antisense construct [15]. These results are consistent with a possible role for LKB1 in *Dictyostelium* as the main upstream kinase responsible for AMPK activation in mitochondrially diseased cells. Tissue-specific deletion analyses of LKB1 in mice have shown AMPK activation to be mostly mediated by LKB1 in almost every tissue type studied [68].

Unlike in most other cell types and tissues, in neurons and T cells AMPK seems to be particularly activated by CAMKK2 [68]. A study using LKB1-knockouts in neurons displayed phosphorylated levels of AMPK similar to that in wild-type cells under normal physiological conditions [69]. This finding led to the characterization of another upstream kinase of AMP, CAMKK2. Interestingly, CaMKK2 activates AMPK in response to increases in intracellular calcium and can do so in an AMP-independent manner [56,70]. Unexpectedly AMPK activation by CAMKK2 is also stimulated by ADP [51]. CAMKK in *Dictyostelium* shares 41% sequence identity to human CaMKK, but has not yet been studied functionally.

The third AMPKK candidate originally identified in the yeast genetic screens was Transforming Growth Factor-β (TGFβ)-activated kinase-1 (TAK1), a member of the mitogen-activated protein kinase kinase kinase family [57]. The least studied of the three AMPK kinases, TAK1 activates AMPK in LKB1-deficient HeLa cells [57] and knockout of TAK1 in cardiomyocytes resulted in the inhibition of AMPK T172 phosphorylation [60]. However, more research is needed to further understand the role of TAK1 in regulating AMPK under normal physiological conditions.

Since the discovery of AMPK's cytopathological role in mitochondrially diseased *Dictyostelium* cells, research in other systems has been published which supports the hypothesis that chronic activation of AMPK can cause cytopathological outcomes. Mitochondrial dysfunction is a feature of many neurodegenerative diseases and in some of these – Amyotrophic Lateral Sclerosis (ALS), Alzheimer's and Huntington's Disease – activation of AMPK has been confirmed to occur as predicted from the *Dictyostelium* model [17–19].

Not only is AMPK activity elevated in these neurodegenerative diseases, but chronic AMPK overactivity is neurotoxic. This is most tellingly revealed in the pathology of a rare human genetic disorder, AICA-ribosiduria, in which an inactive AICAR (5-aminoimidazole-4-carboxamide ribonucleotide) transformylase causes the AMPK activator ZMP (AICA-riboside) to accumulate in cells [71]. In addition to dysmorphic features, affected patients suffer from severe neurological dysfunction, epilepsy and congenital blindness, features that are highly reminiscent of mitochondrial disease.

Neuropathological roles for chronically active AMPK have also been implicated in more common neurodegenerative disorders in which mitochondrial dysfunction plays a role. Although AMPK activation has not been examined directly in Parkinson's Disease patients, increased levels of active AMPK are observed in cultured cells treated with MPTP, a pharmacological inducer of Parkinson's Disease that inhibits Complex I [72]. Overexpression of AMPK in neuronal cells inhibits neurite extension and causes increased accumulation and phosphorylation of α-synuclein, the major component of Lewy Bodies seen in all PD patients [73]. High lactic acid levels occur in PD brains as a result of mitochondrial respiratory dysfunction, and lactic acid is a known activator of AMPK. Exposure of neuronal cells to lactate causes an AMPK-dependent

accumulation of monomeric, oligomeric and phosphorylated forms of α-synuclein in the cytoplasm [73].

The α1 isoform of the AMPK catalytic subunit is enriched in the brains of HD patients and a mouse model (R6/2) of HD [74]. In the mouse model, expression of poly-Q-expanded huntingtin polypeptide enhanced the kinase activity of CaMKII, activating and promoting the nuclear translocation of AMPK-α1. This resulted in a reduction of Bcl-2 expression and increased cell death. AMPK has also been implicated in ALS, with knock down of AMPK in a SOD1 ALS mouse model resulting in motor neuron protection [19]. In similar vein, CAMKK2 activation of AMPK phosphorylation of Tau on S262 has been proposed to mediate the synaptotoxic effects of the Aβ42 peptide in Alzheimer's Disease [75,76]. On the other hand, short term treatment with the hormone leptin activates AMPK and protects rat neurons against lipid toxicity [77]. As a result of such studies, there is now a growing consensus that although acute AMPK activation is a protective response in stressed cells, chronic high levels of activation can be neuropathological [78] as predicted from the *Dictyostelium* model.

2.3. TOR Complex I may mediate diverse AMPK-dependent phenotypes

The TOR (Target of Rapamycin) protein is a conserved protein kinase that regulates diverse cellular functions including growth and cell division, cell differentiation, autophagy, chemotactic motility, phagocytosis and pinocytosis [79]. The protein is found in two distinct protein signalling complexes, TORC1 and TORC2, each of which has its own distinct subunit composition, accessory proteins, downstream targets and functions [80–82].

Named after its component protein kinase TOR, TORC1 is a downstream target of AMPK in mammalian cells, but is not yet explicitly implicated in mitochondrial disease cytopathology. In other organisms, AMPK inhibits TORC1 both directly by phosphorylating Raptor and indirectly by phosphorylating Tsc2, an indirect regulator of TORC1 [79,83,84]. Raptor is a key subunit of TORC1, and serves an essential role in the complex as a scaffolding protein that binds both downstream targets and upstream regulators of the complex. Because of its unique and distinctive role in TORC1, Raptor function serves as the critical control point for the complex. It is regulated by both activatory and inhibitory phosphorylations at multiple sites by several upstream kinases. One of these is AMPK, which phosphorylates mammalian Raptor at two unique sites S722 and S792 and thereby inhibits TORC1 signalling [79,84,85]. Chronic inhibition of TORC1 by AMPK could be responsible for diverse cytopathologies in mitochondrial disease for the following reasons:

1. AMPK inhibits TORC1 in mammals by two different pathways — by phosphorylating Raptor and by regulating the Tsc1/Tsc2 complex, an upstream inhibitor of Rheb which in turn activates TORC1. The former of these appears to be the eukaryotic ancestral mechanism, while the latter appears to have been a later addition to the original pathway [81]. Consistent with this, *Dictyostelium* has no recognizable Tsc1, but does have a Tsc2 homologue [15]. Furthermore the *Dictyostelium* Tsc2 lacks homologous regions to some parts of the mammalian protein including the reported AMPK phosphorylation sites [86].
2. In other organisms, TORC1 exerts transcriptional and translational control to activate cell growth, proliferation and to inhibit autophagy and mitochondrial biogenesis [83,87,88]. These are amongst the phenotypes that are deranged in *Dictyostelium* mitochondrial disease in an AMPK-dependent manner [15].
3. We searched for the consensus motif for AMPK phosphorylation sites [89] in the *Dictyostelium* Raptor sequence and found a single site with a 100% match to the motif. This putative AMPK phosphorylation site contains two potentially phosphorylatable serines (Ser886 and Ser885). It is the same site identified by Gwinn et al. [85] as matching the sites in mammalian Raptor whose phosphorylation by AMPK inhibits TORC1 signalling and causes cell cycle arrest at the G2/M checkpoint.
4. Phagocytosis and pinocytosis are not regulated by TORC1 [90] or by AMPK [15]. However phagocytosis is regulated by Tsc2 and Rheb in *Dictyostelium* through TORC2 [90]. If AMPK regulated Tsc2 and Rheb, its knock down or hyperactivity should also affect phagocytosis, but this is not observed [15].
5. *Dictyostelium* growth in liquid medium is slowed by Raptor knock down [90] and AMPK hyperactivity [15].

TORC2 is unlikely to be involved in AMPK-mediated disease pathways in *Dictyostelium*, because TORC2 subunits are not phosphorylated by AMPK in other organisms and TORC2 regulates phenotypes that are independent of AMPK [15,90].

2.4. Defects in specific respiratory complexes can result in AMPK-independent Complex-specific phenotypes as well as AMPK-dependent phenotypes

While the mitochondria play many important physiological roles within eukaryotic cells, their primary responsibility is to provide energy in the form of adenosine triphosphate (ATP), via aerobic respiration involving oxidative phosphorylation (OXPHOS) (Fig. 7) [91]. This pathway is inherently complex with each of the five participating protein complexes composed of multiple structural subunits and requiring a host of assembly factors for biogenesis and functionality. In many organisms, including humans, there is the added complexity of tissue specific isoforms [92] and tissue-specific thresholds of minimal respiratory activity [93–95].

Mutations within structural and/or assembly proteins of specific OXPHOS complexes can result in reduced respiratory capacity [96]. Complex I (CI) is the largest of the five enzymatic complexes, consisting of 45 structural subunits in mammalian cells and 11 assembly factors. Of these, the *Dictyostelium* genome encodes homologues of all 14 core subunits, 14 out of 31 accessory subunits and 7 out of 11 assembly factors [14]. Mutations affecting Complex I subunits or assembly factors are observed in 40% of all known human mitochondrial disease cases where they produce either isolated CI deficiency (affecting Complex I only) or combined CI deficiency (affecting other respiratory complexes as well as Complex I) [24,97]. *Dictyostelium* shares with humans 20 of the 22 subunits known to be associated with human disease (14 core subunits, 2 accessory subunits and 4 out of 6 assembly factors) [14]. Mutations causing specific or combined deficiencies have also been observed with all of the other OXPHOS complexes [98].

As described in the foregoing sections, generalised mitochondrial deficiencies in *Dictyostelium* produce reproducible defective phenotypes mediated by chronic activation of AMPK rather than ATP deficiency. These phenotypes included defective slug phototaxis and thermotaxis, impaired fruiting body morphology and growth defects. Since any genetic impairment of mitochondrial respiratory function should compromise ATP production and chronically activate AMPK, it was a surprise to discover that mutations affecting individual subunits of Complex I or II can produce both AMPK-dependent and AMPK-independent phenotypes.

The first isolated respiratory complex deficiency to be studied in *Dictyostelium* was a knockout of the CI assembly protein MidA, a methyl transferase that interacts with the core CI subunit NDUFS2 [23,99,100]. In the absence of MidA the frequency of mitochondrial fission events is significantly reduced, while the frequency of mitochondrial fusions is unchanged [101]. Both in the *D. discoideum* knockout strain and in siRNA-treated human cells, the absence of the assembly protein MidA resulted in a specific defect in CI activity and assembly. The ensuing CI deficiency resulted in aberrant AMPK-dependent phenotypes in *Dictyostelium* - defective slug phototaxis and thermotaxis that were rescued by knockdown of AMPKα. In addition, AMPK-independent phenotypes, defective phagocytosis, pinocytosis and growth were observed. These results suggested that in the *mid*A knockout the

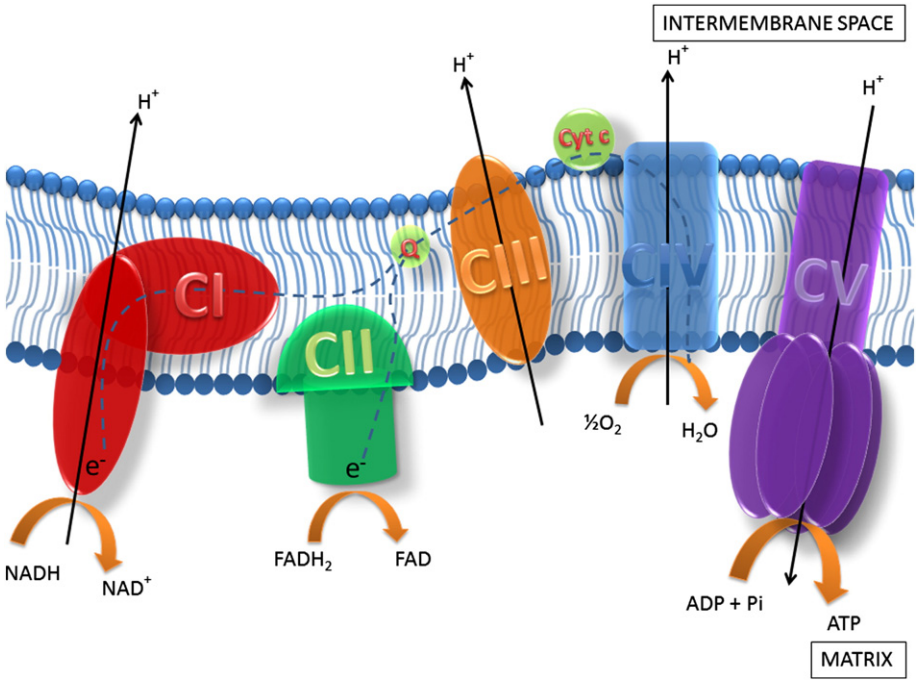

Fig. 7. *Dictyostelium discoideum* derives its energy primarily through aerobic respiration via the mitochondrial oxidative phosphorylation (OXPHOS) system. This pathway consists of five multisubunit complexes (CI–CV) which are embedded in the lipid bilayer of the inner mitochondrial membrane (IMM). The electron transport chain (ETC) is constituted by complexes (CI–CIV). The flow of electrons (e^-) in the ETC is initiated following the oxidation of substrates NADH and $FADH_2$ by CI and CII resulting in the release and transfer of elections to CIII via coenzyme Q_{10} (Q). The electrons are then transferred to CIV via cytochrome c (Cyt c), where the terminal electron acceptor oxygen is reduced to water. The energy derived from the transport of electrons is utilised by complexes I, III and IV to pump protons (H^+) from the mitochondrial matrix across the IMM to the intermembrane space (IMS) generating a proton gradient. CV (ATP synthase) then allows controlled retrograde flow of these protons back into the matrix and in the process utilises the proton motive force to generate ATP.

signalling dysregulation includes both chronic AMPK activation and additional defects in the signalling pathways controlling endocytosis and growth [23]. Whereas expression of the wild type MidA protein suppressed all of the defective phenotypes in the MidA null mutant, this functional rescue was not possible with mutant forms of MidA carrying point mutations in the putative methyl transferase catalytic domain.

Further surprises ensued when another CI assembly factor NDUFAF5, also a methyl transferase, was knocked out in *Dictyostelium* [24]. The phenotypic outcomes of this were similar in many respects to those observed in the MidA knockout, including defects in phototaxis, thermotaxis, endocytosis and growth. However there were some unexpected differences. In contrast to MidA, none of the phenotypic defects including slug phototaxis and thermotaxis could be rescued by knockdown of AMPK. It thus appears that chronic activation of AMPK is not responsible for the phenotypic outcomes in the NDUFAF5 mutant.

Other differences between these two isolated CI deficiencies are observed. In both cases there was an increase in mitochondrial mass compared to wild type cells, 68% in the NDUFAF5 null cells and 30% in the MidA null cells [24]. However these increases in mitochondrial mass were accompanied by a 60% *increase* in total cellular ATP in the NDUFAF5 mutant but a 30% *decrease* in the MidA mutant. As AMPK is known to be inhibited in the presence of high ATP levels, the increased ATP in the NDUFAF5 null mutant may explain why no AMPK-dependent phenotypes were observed. Conversely, the decreased ATP in the MidA null mutant may be consistent with chronic AMPK activation and could explain the AMPK-dependent phenotypes as well as the increased mitochondrial biogenesis in this strain. Despite the apparently similar roles of these two proteins in CI assembly and function, the divergent phenotypes suggest that these two proteins play additional roles in mitochondrial function separate from CI respiratory activity or regulation [24].

2.5. The phenotypic consequences of mitochondrial mutations which do not affect oxidative phosphorylation

The preceding sections summarised data showing that generalised deficiencies in OXPHOS in *Dictyostelium* produce consistent AMPK-dependent phenotypic outcomes regardless of the underlying genetic cause, while isolated deficiencies in Complex I or Complex II can produce additional AMPK-independent phenotypic defects. Mutations in mitochondrial genes that do not cause OXPHOS deficiencies are not expected to activate AMPK and, as expected, they display a range of phenotypes different to those associated with OXPHOS deficiency. Several mitochondrial proteins which are not known to be involved in the OXPHOS pathway have been studied in *Dictyostelium*. These include FtsZs, Tortoise, CluA and TRAP1. Even though these proteins are not involved directly in the production of ATP they still play important roles in maintaining mitochondrial morphology and biogenesis.

In bacteria there are about a dozen cell division proteins and FtsZ is one of the most important. FtsZ is a GTPase and is associated with invaginating the inner margin of the bacterial cell membrane [103,104]. A protein related to FtsZ has also been shown to play a role in the division of organelles in certain eukaryotes. In *Arabidopsis thaliana* FtsZ is essential for chloroplast division with antisense RNA inhibition of either of two versions of FtsZ (AtFtsZ1-1 and AtFtsZ2-1) resulting in failure of chloroplast division [105]. Similar results were obtained with disruption of chloroplast FtsZ in the moss *Physcomitrella patens* [106]. A role for FtsZ in mitochondrial division was shown in *Dictyostelium* [107].

Dictyostelium has two nuclear encoded FtsZs, FszA and FszB both of which are targeted to the inside of the mitochondria [107]. Knockout of *fszA* and/or *fszB* causes a change in mitochondrial morphology from mitochondrial spheres and rods into tubules. The two proteins have different distributions within the mitochondria. FszA localises as a belt around the mitochondria and also in a punctuate fashion at multiple places along mitochondria which may be future sites of division [107].

FszB localises to submitochondrial bodies (SMBs) which are electron dense structures found inside the mitochondrion near one pole. In addition to the differences in localisation knockout strains display different phenotypes. FszB knockout mutants grew slowly on bacterial lawns and displayed surface dependence for growth while FszA knockout mutants grew normally on bacterial lawns, suggesting involvement of FszB but not FszA in controlling cell growth. The differences in mitochondrial localisation and phenoytpes in the null mutants suggest that the two FtsZ proteins play divergent roles in mitochondrial morphology and biogenesis in *Dictyostelium* [107].

The SMBs in *Dictyostelium* also contain another mitochondrial protein Tortoise (TorA), which is required for efficient chemotaxis [108]. The overexpression of TorA resulted in the formation of SMB-like clusters (round electron dense masses) in the mitochondria. TorA null cells appear to be able to detect external chemical gradients but cannot properly coordinate extension of their pseudopods [108]. As a result of this, mutants grew slowly on bacterial lawns and showed surface dependent growth similar to FszB null mutants. The TorA null cells also displayed larger and rounder mitochondria than those of wild type cells [108]. These studies indicate that TorA is required to maintain normal mitochondrial morphology.

CluA (Clueless) is another protein first discovered in *Dictyostelium*, where it was shown to be important for normal distribution and morphology of the mitochondria [109]. Homologous sequences to CluA have been found encoded in the genomes of *Saccharomyces cerevisiae*, *Caenorhabditis elegans, Arabidopsis thaliana* and other organisms including humans [110–113]. In *Dictyostelium* CluA null cells the mitochondria are clustered near the cell centre and loss of the CluA homologue in *Saccharomyces, Drosophila* and *Arabidopsis* produces similar mitochondrial clustering. Recent work showed that *Dictyostelium* CluA is also required for both mitochondrial fusion and fission events, more so for the former than the latter [101]. *Drosophila* Clueless associates with the Parkinson's Disease protein Parkin and the null mutants exhibit a shorter life span and sterility in both males and females, similar phenotypes to those observed in *Drosophila* as a consequence of the loss of Parkin. The *cluA Dictyostelium* null cells show an increased number of multinucleated cells, suggesting an impairment in cytokinesis [109].

CluA is not the only protein known to be important for the normal distribution of mitochondria in cells. In animal cells, Miro (also called RhoT1/2) and its cognate adaptor protein Milton (also called TRAK1/2) form a complex with the microtubule motor proteins Kinesin-1 and the dynein/dynactin complex [114]. *Dictyostelium* possesses homologues of all of the proteins in this complex except for Milton, whose role is to connect Miro to the microtubule motor complex. Knocking out Miro in *Dictyostelium* had no effect on mitochondrial trafficking [115] and the absence of Milton may explain why. Since yeast and plant Miros are also not needed for mitochondrial trafficking, this role of Miro appears to be an evolutionary innovation in the animal lineage. Nevertheless Miro is associated with *Dictyostelium* mitochondria and the null mutants grow slowly with decreased steady state ATP levels [115]. These Miro mutant phenotypes in *Dictyostelium* reveal a more ancient and presumably conserved role for Miro in mitochondria that may be obscured in studies in animal models by its trafficking function.

Dd-TRAP1 is a *Dictyostelium* homologue of human TRAP-1, tumour necrosis factor (TNF) receptor associated protein 1 [116]. The TRAP-1 protein is a mitochondrial molecular chaperone related to members of the Hsp90 family and has a mitochondrial localisation sequence at its N-terminus. TRAP-1 plays a role in many cellular processes. It has been shown to interact with the retinoblastoma protein (Rb) and the tumour suppressors EXT1 and EXT2 and as such has a role in cell cycle regulation [117,118]. Other roles for TRAP-1 have been found in differentiation and mitochondrial apoptosis [119,120].

Dictyostelium provided the first molecular genetic model of the cytopathological consequences of TRAP1 deficiency [119,121,122]. Dd-TRAP1 was found to play a role in the prestarvation response which is triggered by high cell densities and conditioned medium [119,121]. Under normal conditions Dd-TRAP1 is found in the cortex of growing cells, but during the prestarvation response Dd-TRAP1 translocates to mitochondria. Dd-TRAP1 is thus a dual-targeted protein that relocates to the mitochondria in response to prestarvation stress. This may help reconcile the unresolved paradox of mammalian TRAP1's observed mitochondrial localization with its known association with the plasma membrane-associated cell death receptor TNFR [123]. The knockdown of Dd-TRAP1 by RNAi resulted in reduced growth rates and delayed development, whilst overexpression of Dd-TRAP1 resulted in precocious early development with increased aggregation, but development did not progress past aggregation. These cells could develop into tight mounds and then arrested at that stage under high cell densities and submerged conditions [121]. The knockdown strains also showed defects in sporulation and reduced heat shock resistance consistent with the protein's role as a molecular chaperone [122]. Since Dd-TRAP1 is localised to the cell cortex and not the mitochondria during growth, it appears not to be essential for OXPHOS function.

Like many other organisms (animals, plants and protists, but not humans), *Dictyostelium* possesses an alternative, cyanide (CN) resistant respiratory pathway mediated by alternative oxidase (AOX) which can replace Complexes III and IV in reducing molecular O_2 to H_2O without pumping protons [124,125]. Null mutants exhibit normal growth and development under normal culture conditions. However their development is completely abolished by 5 mM KCN, whereas wild type cells develop normally even in the presence of 10 mM KCN [126]. These results suggest that CN-resistant respiration might compensate for the absence of Complex IV in oxidative phosphorylation, allowing electron flow from NADH and FADH through Complexes I and II to molecular oxygen. This would allow continued succinate dehydrogenase activity of Complex II in the TCA cycle and continued, albeit less efficient generation of a mitochondrial membrane potential by Complex I. Animal cells, being naturally deficient in AOX, may be more susceptible to the effects of Complex III/IV deficiency than cells of other organisms.

3. *D. discoideum* as a model for the study of neurological disorders

Different cell types and tissues have different energetic needs that are reflected in differences in the number of mitochondria [127,128]. Neurons and cardiac and skeletal muscle cells have high energy demands and therefore contain a high mitochondrial content compared to other cell types [129,130]. These cells are also more sensitive to energy-dependent defects that arise from mitochondrial damage. Accordingly, patients with mitochondrial disease display clinical features that are frequently associated with dysfunction in neurons and muscle cells such as neurological seizures, ataxia, progressive muscle weakness, stroke-like episodes and cognitive impairment [131]. These diseases typically strike in infancy, but the age of onset and the severity of the disease vary widely and correlate with the degree of deficiency in ATP generation [132].

Neurological disorders are characterised by a set of cognitive, behavioural and motor alterations that develop due to the dysfunction and/or death of specific neurons [133]. There are many lines of evidence that neurological disorders are often associated with an underlying mitochondrial defect. For example, over 188 separate genetic loci have been associated with inherited forms of diverse neurological and neurodegenerative disorders, including Alzheimer's disease (AD), Charcot-Marie-Tooth disease, Huntington's disease (HD), optic atrophy and Parkinson's disease (PD) [134]. About one third of the identified genes are known to be associated with mitochondrial function.

Defects in the trafficking of mitochondria have been associated with various neurodegenerative diseases. Mitochondria are dynamic organelles which are able to anchor or detach themselves to other organelles such as the ER, endocytic vesicles and the plasma membrane. They are enriched in the presynaptic axonal termini and also the postsynaptic ends of dendrites [135]. If their trafficking to these sites is defective,

localised respiratory deficiencies could result in these regions of the neuron, even if the total OXPHOS capacity of the cell is unchanged. Altered trafficking of mitochondria has been shown in AD, PD and HD [136,137]. In addition motor neurons from ALS mice have reduced mitochondrial transport capabilities [138,139]. Trafficking of mitochondria is also important for cells to be able to remove dysfunctional and structurally damaged mitochondria in order to protect the cells from excessive oxidative stress and cell death [140]. The autophagic removal of damaged mitochondria is referred to as mitophagy. Mutations to genes involved in autophagy and reduced autophagic activity are associated with numerous neurodegenerative disorders including PD, AD, HD [141,142].

Autophagy is important not only for the removal of damaged mitochondria but also for the removal of protein aggregates, accumulation of which is a hallmark of many neurodegenerative disorders. The cytotoxicity of these protein aggregates is still debated [143]. Some studies argue that the oligomers are the neurotoxic forms, while the macroscopic aggregates could have a protective role by trapping the neurotoxic species in specific subcellular compartments destined for removal by the ubiquitin-proteasome system (UPS) and the lysosome-mediated systems. On the other hand, the large protein aggregates might physically obstruct axonal transport and thus contribute to neurotoxicity [144,145]. Their negative effects can also be caused by the sequestration of proteins that are essential for neuronal survival or by altering the intracellular trafficking of molecules and organelles. The removal of protein aggregates through autophagy is neuroprotective in neurological disorders characterised by accumulation of these aggregates.

In normal brain cells autophagosomes are usually found in low numbers, but in AD patients the increased numbers of autophagosomes indicates an enhanced activation of autophagy with impaired maturation or fusion with the lysosome [146]. Autophagy also plays an important role in the pathogenesis of HD, as polyglutamine sequences, the hallmark of HD, are poor substrates for proteasome degradation resulting in the build-up of highly ubiquitinated aggregates of huntingtin in the endosomal-lysosomal compartment of neurons in patients with PD [147].

The cellular organelle and protein quality control systems are eroded with age, favouring the accumulation of aberrant and misfolded proteins, partly a consequence of an age-related decline in autophagic and lysosomal activity [148]. Mitochondrial DNA mutations also accumulate with age as does the consequential mitochondrial damage. It is not surprising then that many neurological disorders are associated with ageing and age-related mitochondrial dysfunction combined with the failure of the autophagic pathway to remove the damaged mitochondria. *Dictyostelium* is being used as a model to study a variety of these neurological and neurodegenerative disorders that are not overtly mitochondrial (*ie*. the mutant gene does not encode a mitochondrial protein).

3.1. Alzheimer's Disease

Alzheimer's Disease (AD) is a progressive neurodegenerative disorder in which mitochondrial dysfunction is implicated [149]. It is characterised by two pathological aggregates — extracellular senile plaques, composed largely of amyloid-beta (Aβ) and intracellular neurofibrillary tangles (NFTs), containing abnormal hyperphosphorylated Tau protein [150]. AMPK is chronically activated in AD and is one of the upstream kinases that phosphorylates Tau, contributing to its aggregation and toxicity [151].

Aβ is formed via the proteolytic processing of its parental molecule, the amyloid precursor protein (APP) [152]. It is believed that the overloading of Aβ ultimately results in the other pathological features of AD such as the hyperphosphorylation of Tau protein, oxidative damage to neurons, synaptic dysfunction and eventually mass neuronal loss [153]. Inappropriate processing of Aβ in familial Alzheimer's disease (FAD) is largely due to alterations in proteins which process APP including γ secretase [154].

D. discoideum has orthologues of all subunits of γ secretase, with single genes for Nct, Aph1 and Pen2 as well as two presenilin genes [155]. Presenilin forms the catalytic subunit of γ secretase and mutations in presenilin genes are associated with FAD [156]. The two presenilin genes exhibit different patterns of expression during development, suggesting different roles in the *Dictyostelium* life cycle. Even though *D. discoideum* lacks APP and some other known substrates of γ secretase such as Notch [157], *Dictyostelium* γ secretase is able to process ectopically expressed human APP resulting in the production of Aβ peptide [155].

Despite its significance in AD, the normal cellular functions of γ secretase and the products of its activity are not well understood. The importance of such an understanding is highlighted by the stopping of a clinical trial of a γ secretase inhibitor, because it worsened disease outcomes [158]. APP is not the only substrate of mammalian γ secretase. There at least 91 different known substrates, most of which are receptors or single transmembrane domain proteins with a large ectodomain and a cytoplasmic C-terminal domain involved in intracellular signalling [159]. The functions of γ secretase cleavage of these substrates are not well studied, but have been suggested to include diverse cellular activities, including protein sorting, transcription regulation, cell growth, cell adhesion, cell fate determination and endocytosis.

Although the presenilin mutations that cause FAD were thought to involve novel gain of function, more recently it has become clear that loss of presenilin function contributes to AD cytopathology [158,160]. To study this, null mutants have been isolated for each of the *Dictyostelium* γ secretase subunits except Pen2, as well as a series of double null mutants [155,161]. The mutant phenotypes showed that active γ secretase plays a cell autonomous role in prespore differentiation and cell fate determination, so that in mixtures with wild type cells, γ secretase deficient cells enter the stalk differentation and programmed cell death pathway [155]. The knockout mutant phenotypes also revealed that γ secretase is essential for phagocytosis, consistent with its proposed but unproven role in endocytosis in mammalian cells.

Glycogen synthase kinase 3 (GSK3) is one of the signalling proteins which interacts with presenilin which may function as a scaffold [162–164]. Although direct interactions between GSK3 and presenilins in *Dictyostelium* have not been reported, the cellular functions of *Dictyostelium* GSK3 have been extensively investigated. GSK3 null mutants have been found to exhibit abnormal mitotic spindle dynamics, precocious early development and increased commitment of cells to the stalk differentiation pathway, the *Dictyostelium* form of autophagic programmed cell death [165,166]. Some of these functions of GSK3 are reminiscent of those of AMPK – knock down of AMPK inhibits early differentiation and subsequent aggregation while its overexpression causes increased numbers of cells to enter the stalk differentiation pathway [15]. These results suggest that GSK3 may be a downstream target for inhibition by AMPK phosphorylation and/or *vice versa*. In fact, in mammalian cells GSK3β and AMPK have been found to mutually inhibit one another [167,168]. Chronically active AMPK could thus exert cytotoxic effects in AD both by phosphorylating Tau and by inhibiting GSK3β. On the other hand, GSK3β is itself one of the upstream kinases able to mediate Tau phosphorylation and tangle formation [169]. It may be possible to clarify some of these issues by genetically manipulating GSK3 and AMPK levels in *Dictyostelium* and studying their effects on the cytotoxicity and phosphorylation of heterologously expressed human Tau.

Mitochondrial signalling pathways are thought to be disrupted during the progression of AD [149]. Mitochondrial dysfunction is thought to result from the accumulation of Aβ and play a crucial role in cognitive decline [170]. Postmortem brain specimens from patients have shown Aβ interacts with the mitochondria which are particularly concentrated at the synapses of neurons [170]. Lower ATP production

and release of free radicals due to mitochondrial damage may be responsible for synaptic loss leading to cognitive failure [171]. The activity of a number of mitochondrial enzymes such as cytochrome *c* oxidase, α ketoglutarate dehydrogenase and pyruvate dehydrogenase are affected [171]. Aβ has been shown to directly inhibit complex IV of the OXPHOS pathway resulting in leakage of electrons from the ETC and increased ROS production leading to oxidative stress [26]. The accumulation of mutations in mitochondrial DNA due to ageing may be responsible for the increased production of Aβ and the reduction of cytochrome oxidase activity [170]. More studies are needed to reveal the exact role of the mitochondria in AD pathogenesis and *D. discoideum* may provide a tractable model for understanding this. Heterologous expression of toxic forms of human Aβ in *Dictyostelium* would provide a means to investigate the downstream cytotoxic pathways in the absence of interference from endogeneous Aβ.

3.2. Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease. It is characterised by the loss of dopaminergic neurons in the substantia nigra and formation of Lewy Bodies [25]. The manifested symptoms include progressive rigidity, bradykinesia, resting tremor and postural instabilities [172,173]. Mitochondrial dysfunction has been found to be involved in the pathogenesis of PD. For example mitochondrial complex I activity has been shown to be reduced in patients with PD [174], and Complex I inhibitors, like rotenone, induce Parkinson's Disease-like features in rats [175].

To date eight genes have been shown to be associated with PD. These genes are listed in Table 1. Included in this list are genes encoding PINK1, a molecular chaperone, and HTRA2, a serine protease. These proteins aid in the correct assembly and folding of mitochondrial proteins. Mutations in these genes in mice result in elevated levels of ROS and accumulation of misfolded proteins in the mitochondria of brain cells [176–179].

As seen in Table 1 many of the genes associated with PD have homologues in *D. discoideum*. This implies that the signal transduction pathways have been conserved throughout evolution and studying these proteins in *D. discoideum* may aid in our understanding of the pathology of PD.

One of the PD-associated genes LRRK2, is a member of the Roco protein kinase family first identified as such in *D. discoideum* [180]. Roco kinases contain several conserved domains: a leucine-rich repeat domain (LRR) for protein-protein interactions, a Ras GTPase protein domain (Roc), a C-terminal of Roc domain (COR), and a MAP3 kinase domain (MAPKKK) [181]. Various mutations in the LRR domain (R1441C, R1441G, R1441H), the COR domain (Y1699C), and the kinase domain (G2019S, I2020T) have been shown to be involved in PD, while mutations outside of the catalytic domains are not related to PD. This implies that the kinase and GTPase activities of LRRK2 are crucial in the pathogenesis of PD [182]. In *D. discoideum*, there are 11 Roco family members and 5 of them have been studied functionally. However, the regulation of these Roco family proteins remains poorly understood with the exception of GbpC/Roco1 and Pats/Roco2.

GbpC, also called Roco1 is a large multidomain protein involved in cGMP-mediated chemotaxis in *D. discoideum* [183]. In addition to the conserved Roco family domains, GbpC contains a RasGEF domain, which accelerates the GDP/GTP exchange of the Roc domain, and two cGMP-binding domains, which stimulate the binding of GbpC to GTP (Fig. 8). GbpC null mutants or mutants lacking the RasGEF, Roc, or kinase domain show defects in chemotaxis [181].

In resting cells, GbpC is localised in the cytoplasm, then translocates to the cell cortex upon stimulation with cAMP or under osmotic stress conditions in a cGMP- and guanylyl cyclase-independent manner [181,184]. A mutation in the GRAM domain, which is responsible for lipid-binding, disrupted both the membrane association and the activation of GbpC function during chemotaxis. Therefore it was proposed that extracellular cAMP-stimulation elicits an intracellular cGMP-mediated activation of kinase activity during chemotaxis and separately induces a GRAM-dependent translocation of GbpC [184]. This is analogous to LRRK2 in mammalian cells which is localised in the cytoplasm, while the membrane-associated dimeric form is physiologically active and able to exert its kinase and GTP binding activities [185].

Pats1 or Roco2, has been shown to play a role in cytokinesis. Roco2 null mutants grow faster than wild-type cells yet aggregate more slowly and form larger aggregates compared with wild type cells. The null cells also contain many nuclei [181,186,187]. Roco2 is also important for pseudopod extension during chemotaxis and random cell motility. This may be due to its interaction with the actin binding protein filamin which has been shown to be involved in cell polarisation and extension of the pseudopod [188] as well as photosensory signalling in the slug stage of the life cycle [189,190]. Filamin is a scaffolding protein and has been shown to bind many proteins including AMPK, thus supporting a link between PD and the signalling pathways involved in mitochondrial dysfunction in *Dictyostelium* [15,189,190]. In mammals filamin 1 (FLN1) is highly expressed in the developing brain [191] and is essential for neuronal migration [192]. The FLN1 gene is located on the X chromosome and loss of function mutations to FLN1 are embryonic lethal in males and result in periventricular heterotopia (PH) in females. This disorder is termed a cell autonomous mosaic phenotype due to the random inactivation of the X chromosome. Neurons expressing the mutation fail to migrate presumably due to a failure of FLN1 to crosslink actin filaments and form a leading edge. The common symptoms of PH are epilepsy and cerebellar abnormalities [192].

Other Roco family members are involved in growth and morphogenesis. For example, *D. discoideum* cells lacking QkgA/Roco3 proliferate rapidly [187] while Roco4 and Roco11 are required for correct morphological development. During the formation of the fruiting body, the prestalk cells of roco4-null mutants produce less cellulose, which leads to instability of the stalk and a failure of the

Table 1
PD-associated genes and their homologues from Dictyostelium.

Locus	Gene	Inheritance	*Dictyostelium* homologue	Blast result		
				Identity (%)	Positive (%)	Gap (%)
PARK1/4	a-synuclein	AD	None			
PARK2	PARKIN	AR	MH			
PARK5	UCH-L1	AD	UCH1	44	61	3
PARK6	PINK1	AR	MH			
PARK7	DJ-1	AR	DJ-1	26	45	10
PARK8	LRRK2	AD	MH			
PARK9	ATP13A2	AR	MH			
PARK13	HTRA2/OMI	AD	HTRA2	28	46	8

KEY: AD, autosomal dominant; AR, autosomal recessive; MH, multiple homologues.

Fig. 8. Domain architecture of *D. discoideum* GbpC protein. Apart from the Roco family conserved domains (LRR, Roc, COR, and MAPKKK), GbpC also contains a Ras nucleotide exchange motif - N-GEF, DEP, RasGEF, and two cyclic nucleotide-binding domains with the phospholipid-binding GRAM domain inserted in between [180].

stalk to support the sorus. Mutants lacking Roco11 form larger fruiting bodies than the parental wild type strain [181].

At least one protein associated with PD has no homologues in *Dictyostelium* - α-synuclein. This provides a unique avenue for study of this protein as it and various mutations thereof can be expressed in *Dictyostelium* and their function analysed without interference from endogenous isoforms. α-Synuclein is the major component of the characteristic Lewy Bodies seen in PD patients [193]. Hyperexpression or mutations of the α-synuclein gene are believed to be associated with mitochondrial abnormalities and onset of familial PD. In order to study the function of α-synuclein, the wild type and two disease-associated mutant forms of human α-synuclein have been expressed in *Dictyostelium* [27]. All three forms were cytotoxic in that they impaired phagocytosis and growth on bacterial lawns. Expression of the two mutant forms also impaired phototaxis in the multicellular slug stage of the life cycle in an AMPK-dependent manner consistent with mitochondrial dysfunction.

However, further phenotypic characterisation showed that the pattern of aberrant phenotypes caused by α-synuclein (impairment of phototaxis, phagocytosis and growth on bacteria, but not pinocytosis, growth in liquid or multicellular morphogenesis) is distinct from that observed in generalised mitochondrial disorders in *Dictyostelium*. Furthermore, α-synuclein expression protected cells from *Legionella* infection, whereas mitochondrial dysfunction causes increased *Legionella* proliferation inside infected cells [27]. These results indicate that there are distinct α-synuclein cytotoxicity pathways that are different from those associated with AMPK-mediated cytopathology in mitochondrial dysfunction.

Since α-synuclein is believed to specifically inhibit mammalian Complex I and isolated Complex I deficiencies in *Dictyostelium* can cause AMPK-independent cytopathologies (see earlier sections) it remains possible that α-synuclein cytotoxicity in *Dictyostelium* is mediated by an isolated Complex I deficiency. To shed further light on this, immunofluorescence and confocal microscopy were used to examine the subcellular localization of α-synuclein. These experiments revealed that in *Dictyostelium* α-synuclein is present throughout the cytoplasm but more concentrated close to the plasma membrane. Both in the cytoplasm and the cell cortex the wild type and point mutant forms of α-synuclein were present in small punctoid aggregates, while the C-terminally truncated form produced large aggregates in the cytoplasm. There was no evidence for any association of α-synuclein with the mitochondria. Similar subcellular localisation was observed in two types of mammalian cell lines (Vero and LN18) transfected with the three forms of α-synuclein [27]. Despite the absence of any visible association of α-synuclein with the mitochondria in human or *Dictyostelium* cells, it is possible that only a very small fraction of the α-synuclein molecules need to bind to Complex I to inhibit its function. Be that as it may, α-synuclein cytotoxicity in the *Dictyostelium* model clearly involves more than a simple inhibition of mitochondrial oxidative phosphorylation.

3.3. Huntington's Disease

Huntington's Disease (HD) is another neurodegenerative disease in which mitochondrial dysfunction and elevated AMPK activity are believed to play cytopathological roles [19,74,194]. HD is caused by abnormally large expansions (>35 copies) of the nucleotide triplet CAG in the coding sequence of the huntingtin protein. The resulting expanded polyQ form of huntingtin is neurotoxic and the clinical severity correlates with the number of repeats. Patients exhibit progressively worsening motor dysfunction as well as psychiatric and cognitive abnormalities. How the toxic forms of huntingtin damage cells is not well understood because the normal functions of the protein are still unknown. To study this, the *Dictyostelium* gene encoding the huntingtin homologue has been disrupted and its phenotype studied [195–197]. The mutants exhibit slow growth, impaired cytokinesis, poor chemotaxis, sensitivity to hyposmotic stress, delayed development, small fruiting bodies, inability to complete spore differentiation, and cytoskeletal abnormalities (reduced association of myosin II with the actin cytoskeleton and in the cleavage furrow during cytoskinesis).

Dictyostelium huntingtin lacks glutamines in the divergent N-terminal region, this being a feature that appears only in deuterostome animals beginning with 2 glutamines in sea urchins and expanded further in other lineages [198]. The N-terminus of huntingtin from diverse organisms, including *Dictyostelium*, was anti-apoptotic when expressed in *htt*-null cells [198]. The molecular basis for this anti-apoptotic action must be upstream and independent of the apoptotic machinery itself, since *Dictyostelium* lacks apoptosis. However, only the N-termini from deuterostomes were able to restore N-cadherin-mediated neural cell-cell adhesion in *htt*-null cells [198]. One function of the N-terminus thus appears to be ancient and ubiquitous, while another appears to be an evolutionary innovation in deuterostomes. The *Dictyostelium* model has helped to distinguish between these functions and should assist in the future in defining their molecular nature more clearly.

3.4. Autophagy and ESCRTs

The accumulation of autophagosomes and protein aggregates are hallmarks of many neurodegenerative diseases including AD, PD, polyglutamine expansion diseases and prion-related diseases among others [140,199,200]. These visible cytopathologies arise because of dysregulation or dysfunction of the cellular processes of organelle and protein quality control, a major part of which involves autophagic removal of protein aggregates and damaged organelles, including defective mitochondria. In *Dictyostelium*, mitochondrial dysfunction causes a misdirection of cells into the autophagic cell death (or stalk differentiation) pathway, so that morphogenesis becomes abnormal with the formation of fruiting bodies with shorter, thicker, misshapen stalks [14]. In isolated Complex I deficiencies *Dictyostelium* cells exhibit accumulation of autophagosomes [23,24]. However there is no increase in the level of mitophagy, indicating that the increase represents a general upregulation of autophagy not an increased recognition and detection by the autophagic system of damaged mitochondria.

One protein believed to play a role in the interactions between damaged mitochondria and the autophagic pathway is p97, also known as VCP (Valosin-Containing Protein) or CDC48, TER ATPase, TER94 (in *C. elegans*), Cdc48p (in yeast) or VAT (in archaea) [201,202]. Dominant point mutations leading to single amino acid changes in p97 (eg. R155C) are responsible for human IBMPFD (Inclusion Body Myopathy with early onset Paget's disease of Bone and Frontotemporal Dementia), HSP (Hereditary Spastic Paraplegia), and a form of ALS (Amyotrophic Lateral Sclerosis). p97 is a ubiquitin-binding AAA-ATPase that disassembles protein aggregates and complexes, participating in their degradation in proteasomes and ERAD (Endoplasmic Reticulum-Associated Degradation). Energised by ATP hydrolysis, p97 is believed to extract proteins from the ER (in cooperation with Ufd1-Npl4) or the mitochondrial outer membrane (in cooperation with Vms1-Npl4) into the cytosol for proteasomal degradation [202]. A recent study of p97

function in *Dictyostelium* revealed mutually inhibitory genetic interactions between p97 and ATG9, a transmembrane protein required for formation of autophagosomes from Golgi-derived membranes [203]. The normal and R155C point mutant forms were ectopically expressed in wild type and ATG9 knockout cells. Like its homologues in other organisms, *Dictyostelium* p97 formed hexamers. Consistent with the dominance of the point mutation in human disease, the *Dictyostelium* R155C mutant protein formed heteromeric hexamers with wild type p97 and inhibited its ability to bind to protein aggregates formed in the ATG9 knockout cells. The phenotypic analysis of the various mutant strains suggested that p97 and ATG9 are key players in mutually inhibitory protein quality control mechanisms — ATG9-dependent autophagy and p97-dependent proteasomal protein degradation. The affected downstream phenotypes included phototactic slug migration, multicellular morphogenesis and growth. The combined power of genetic manipulation and phenotypic analysis available in *Dictyostelium* has thus begun to unravel the poorly understood interactions between autophagy and other protein/organelle quality control mechanisms in brain disease.

Whether autophagy serves beneficial or pathological roles in the autophagic cell death observed in neurodegenerative diseases has been controversial. Its study has been complicated by the presence of and cross talk between multiple forms of programmed cell death in mammalian cells, including apoptosis which is by far the most intensively studied. The study of autophagy and autophagic cell death in simple model organisms, including *Dictyostelium*, avoids many of these complications [102,204]. *Dictyostelium* does not possess the machinery for apoptotic cell death, but the process of autophagosome formation in *Dictyostelium* exhibits strong similarities with that in mammalian cells [102]. Six conserved autophagy genes (encoding ATG1, ATG5, ATG6, ATG7, ATG8 and ATG9) have been knocked out and characterised functionally [205–208]. The mutants collectively exhibit, to varying extents, reduced survival during nitrogen starvation, impaired endocytosis and growth, altered capacity to take up and support proliferation of the pathogen *Legionella pneumophila*, reduced protein degradation/recycling during development, aberrant or arrested morphogenesis and defective spore differentiation. As well as their function in autophagy itself, these results indicate roles for autophagy genes in endocytic pathways and cell differentiation, but not in cell type choice.

Another group of proteins that play essential roles in both the autophagic and endocytic pathways are the components of the ESCRT (endosomal sorting complex required for transport) machinery (Fig. 9). Reduced activity of the ESCRT subunits leads to the accumulation of autophagosomes and failure to clear intracellular protein aggregates that have been marked for degradation by polyubiquitin tags. The ESCRTs were first identified as class E Vacuolar protein sorting (Vps) proteins in yeast. They form four heteromeric complexes -0, -I, -II, -III, each of which includes several Vps proteins. Genetic and biochemical studies in many different model systems have shown that the ESCRTs have a crucial role in sorting ubiquitinated membrane proteins into the MVB (multivesicular body) compartment. The ESCRT machinery is functionally conserved among eukaryotic lineages and has ancestral forms in Archaea, indicating that it existed and played its role in degradative pathways in the first eukaryote [199].

Dictyostelium has contributed significantly to understanding the evolutionary function of the ESCRTs as well as its role in the autophagic pathway. The Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) and STAM (signal transducing adaptor molecule) subunits (part of the ESCRT-0 complex) are absent in plants and *Dictyostelium*, whereas homologues of all other ESCRT components are found in these organisms. This observation suggested that in the absence of Hrs/STAM, sorting of ubiquitinated proteins in plants and *Dictyostelium* might rely on other ESCRT complexes or alternative components. A study conducted by Blanc et al. [209] demonstrated that the protein Tom1 (Target of Myb1) in *Dictyostelium* (Dd-Tom1) provides the same function as mammalian Tom1 in interacting with ubiquitin and clathrin and in recruiting Tsg101 (Tumour susceptibility gene 101, a component of the ESCRT-I). Dd-Tom1 was also implicated as a participant in an ancestral ESCRT-0 complex as an alternative component to Hrs/STAM [209]. Thus the Tom1 proteins could be a component of an ancestral complex contributing to the invagination and sorting of ubiquitinated proteins into the MVB compartment.

Dictyostelium has been used as a model to investigate the function of Alix (Apoptosis-linked gene-2 interacting protein X) and ALG-2 (the product of Apoptosis-linked gene-2) proteins. Alix and its binding partner ALG-2 are widely conserved in eukaryotes. Alix is involved in a number of processes including apoptosis (by binding to ALG-2), regulation of cell adhesion, protein sorting (via the ESCRTs), adaptation to stress conditions, and budding of HIV (human immunodeficiency virus) [210]. In *Dictyostelium*, Alix (Dd-Alix) is encoded by a single gene and is expressed during vegetative growth and multicellular development [211]. Studies conducted by Mattei et al. [211] have shown that *Dictyostelium alx* null strains fail to complete their developmental programme. The *alx* null strains show impaired morphogenesis leading to aberrant structures containing vacuolated and undifferentiated cells but no spores. The ALG-2 disruptants in this study remained unaffected. Furthermore, knockdown of the *Dictyostelium* homologue of Tsg101 was shown only to have a subtle effect on the development cycle with slightly smaller fruiting bodies [211].

In another study Ohkouchi et al. [210] disrupted Dd-Alix by homologous recombination and showed that Dd-Alix is essential for development under low Ca^{2+} conditions, suggesting that Dd-Alix is involved in Ca^{2+} signalling during development. The absence of Dd-Alix also caused developmental defects in alkaline pH environments.

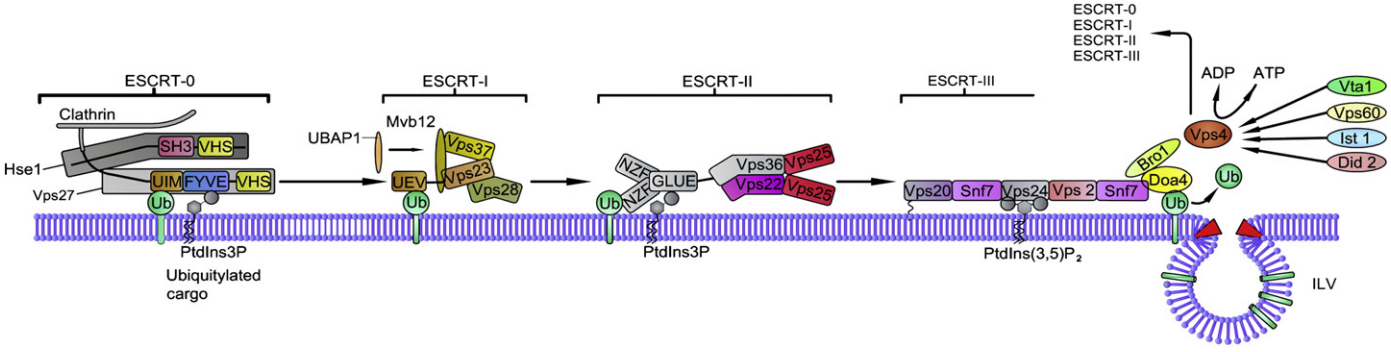

Fig. 9. Composition and molecular interactions of the ESCRTs. Interactions between the four ESCRTs are indicated, as are interactions with ubiquitylated cargo, accessory molecules such as PtdIns(3)P, deubiquitylating enzymes (DUBs), Bro1 and the ATPase Vps4. Yeast protein names have been used but the figure is a composite of data obtained from studies of many model systems containing the ESCRTs. In *Dictyostelium* the yeast nomenclature has been adopted. Note that *Dictyostelium* uses TOM1 to provide the ESCRT-0 function (see text). Figure from Ilievska et al. [199].

Table 2
Diseases of lysosomal function and trafficking studied in *Dictyostelium*.

Human Disease	Dictyostelium Gene/Protein	Cellular function	Disease phenotype		Reference
			Humans	Dictyostelium	
Tay–Sachs/Sandhoff	nagA/ N–acetylhexosaminidase	Lysosomal enzyme	Neurodegeneration, bindness, death in infancy	Small aggregates.	216–217
Familial Visceral Amyloidosis	alyA/Lysozyme	Lysosomal enzyme	Episodic internal bleeding, renal failure	Initial slow growth on bacteria, increased phagocytosis.	215
Alzheimer's Disease	psenA, psenB/ Presenilin A and B	Caatalytic subunit of g–secretase – ER and lysosomal protease	Neurodegeneration, cognitive defects, death	Slow growth on bacteria, impaired phagocytosis, psenA null mutants arrest development at the slug stage, psenA null cells are restricted to the prestalk region in chimeras*.	155
X–linked mental retardation	apg1/AP–1 (Adaptor Protein–1 Complex) g subunit; apm1/AP–1 m subunit	Large subunit of AP–1 transporting lysosomal cargo from the trans Golgi network to endosomes (and contractile vacuole in Dictyostelium).	X–linked mental retardation from g subunit mutations. (Mutations in the m and g subunit s are embryonic lethal in mice.)	apm1 null mutants exhibit slow growth, delayed fruiting body formation, small fruiting bodies/aggregates, reduced spore viability.	219
Hermansky–Pudlak–Syndrome Type 2 (HSP2)	apm3/AP–3(Adaptor Protein–3 Complex) m subunit	Secretion from secretory lysosomes.	Albinism, bleeding, immunodeficiency.	Exocytosis defect. Other phenotypes not reported.	220
Chediak–Higashi Syndrome (CHS)	lvsB/LvsB or LYSTor Beige	Secretory lysosome maturation. Preventing promiscuous fusion amongst endosomes by inhibiting Rab (Rab14 in Dictyostelium).	Partial albinism, immunodeficiency and infection susceptibility, episodic bleeding, neurodegeneration, ataxia, weakness, sensory deficits.	Exocytosis defect. Other phenotypes not reported.	9,221–225
Action Myoclonus–Renal Failure Syndrome	lmpA, lmpB, lmpC/ LmpA, LmpB, LmpC (LIMPII/Limp–2 in humans)	PIP2–binding protein, targets specific proteins to the lysosome (b–glucocerebrosidase in humans), endocytosis.	Epilepsy and renal failure	LmpA null mutants – defective macropinocytosis and growth in suspension, normal phagocytosis, growth on bacteria, fewer smaller fruiting bodies, reduced spore viability. LmpB/C mutants not studied.	226–228
Neuronal Ceroid Lipofuscinosis (NCL)	catD/Cathepsin D	Lysosomal enzyme	Neurodegeneration, motor and mental deterioration, visual loss, seizures, ataxia and reduced life span. Cathepsin D deficiency produces the most severe form with extreme brain atrophy, and death soon after birth.	Slight reduction in growth rate, slightly delayed development.	213, 214, 218
Mucolipidosis Type IV	mcln1/Mucolipin or MCOLN1	Lysosomal Ca^{2+} channel	Growth and psychomotor retardation, severe visual impairment attributed to corneal clouding, and elevated blood gastrin levels caused by achlorhydria.	Knockout – increased lysosome exocytosis; aberrant Ca2+ homeostasis.	229–232

*Null mutants lacking the Aph1 or Nct subunits of γ-secretase exhibit similar phenotypes to the *psenA* mutant.

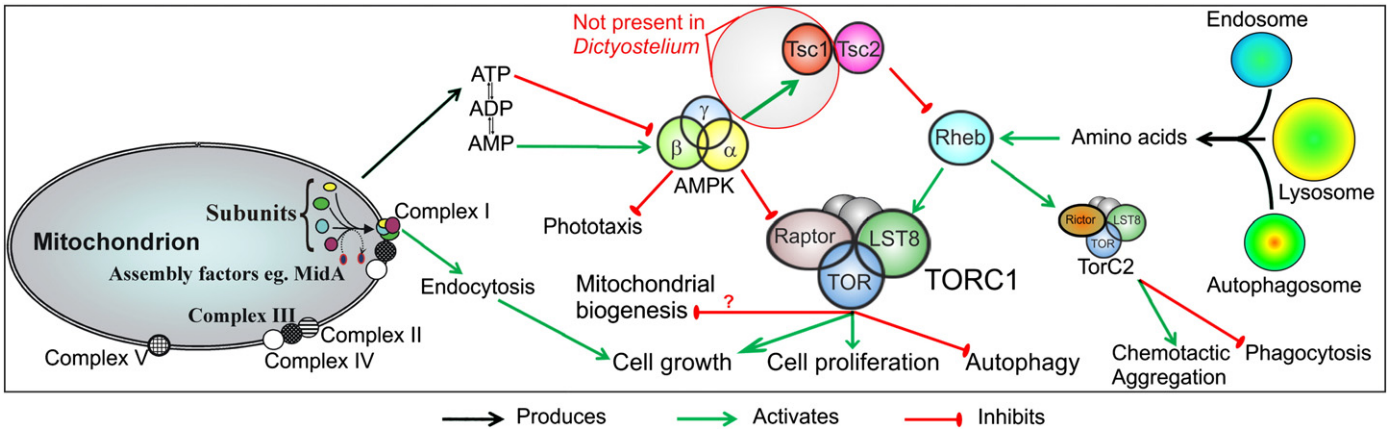

Fig. 10. A partial model for dysregulated signalling pathways in the *Dictyostelium* model for brain disease. The model shown accounts partially for the aberrant phenotypic outcomes of genetic defects in *Dictyostelium* that in humans cause neurodegenerative diseases. The model centres around two central stress-sensing protein kinases – AMPK sensing energy stress and TOR sensing nutrient stress (amino acid starvation). These pathways are interconnected through AMPK phosphorylation of Raptor and (in mammals but not in *Dictyostelium*) Tsc2. The phenotypic outcomes in *Dictyostelium* include alterations in phototaxis (and thermotaxis), macropinocytosis, phagocytosis, cell growth and proliferation on bacterial lawns and/or in liquid medium, chemotactic aggregation, autophagy and entry into the *Dictyostelium* autophagic cell death pathway (stalk differentiation). In the human central nervous system, dysregulation of the same conserved signalling pathways would produce outcomes that may be similar at the cellular level but produce different sequelae at the tissue and organismal level eg. increased autophagic cell death producing excessive stalk formation in *Dictyostelium* but loss of neurons and cognitive function in Alzheimer's Disease. Although AMPK activation leads to increased mitochondrial biogenesis [15], it is not yet clear whether this effect is caused by the inhibition of TORC1 in *Dictyostelium* (indicated by the question mark in the Figure). In yeast and animal cells TOR inhibition results in increased mitochondrial gene expression and enhanced mitochondrial respiration [87,88,234], but there are also reports to the contrary (eg. [235]).

The fruiting body formation efficiency of the Dd-Alix deletion mutants at pH 9.0 was significantly lower than that of wild-type cells [212].

3.5. Lysosomal disorders

The recycling of defective organelles and proteins in cells requires fusion of autophagosome-derived vesicles with the lysosome in order to deliver the lysosomal degradative enzymes and the autophagosomal cargo into the same cellular compartment. A number of lysosomal diseases have been studied in *Dictyostelium* [157] and most of them have adverse central nervous system sequelae [155,213–232] (Table 2). Depending on the protein and its role in lysosomal function and trafficking, the disease phenotypes in *Dictyostelium* are varied and not yet well understood in terms of how the lysosomal defects produce the resulting growth and developmental abnormalities. Phenotypic analysis of the mutants has revealed multiple effects on both growth and endocytosis. An example is provided by knockout of the gene encoding lysozyme (*alyA*), mutant alleles of which are a cause of Neuronal Ceroid Lipofuscinosis [213–215]. The absence of lysozyme (or other protein-degrading enzymes) in the lysosomal lumen may be expected to reduce the amino acid supply. Interestingly, the growth retardation in this mutant is accompanied by an increased rate of phagocytosis in an apparently homeostatic response to the decreased supply of amino acids from phagolysosomes. It is possible that this homeostatic feedback is mediated by TOR Complex 2 which suppresses phagocytic nutrient uptake [90] and in other organisms is activated indirectly by amino acids.

Lysosomes play a central role in the trafficking of endosomes and one protein involved in this trafficking is mucolipin, a lysosomal Ca^2 channel, believed to play roles in the Ca^{2+}-mediated regulation of vesicle fusion events involving lysosomes and their trafficking derivatives. Defects in mucolipin are responsible for a devastating childhood neurodegenerative disease, mucolipidosis Type IV (Table 2). A *Dictyostelium* knockout mutant lacking mucolipin was recently reported to exhibit defective exocytosis, lower intralysosomal Ca^{2+} levels and increased resistance to Ca^{2+} deprivation [229].

4. Conclusion

The cytopathological correlates and molecular causes of neurodegenerative diseases in humans have been difficult to disentangle. Mitochondrial dysfunction, elevated levels of protein aggregates and autophagic vacuoles are common features regardless of the underlying genetic causes. These reflect cross talk between these dysfunctional intracellular organelles that is difficult to disentangle. *Dictyostelium* offers the opportunity to distinguish cause and effect, and to dissect the organellar cross talk in a single system with reproducible, measurable phenotypic outcomes. Fig. 10 shows a speculative partial model of cross talk between dysregulated signalling pathways in mitochondrial and lysosomal disorders that may explain some of the observed phenotypic patterns. Thus, none of the lysosomal disorders would cause impaired phototaxis in *Dictyostelium*, a phenotype that is characteristic of OXPHOS defects and with one exception (NDUFAF5 knockout) is caused by chronic AMPK activation. On the other hand, the mitochondrial disorders and many of the lysosomal disorders cause reduced growth rates, a phenotype which may be mediated by converging pathways that inhibit TORC1 (TOR Complex I) signalling. In some lysosomal defects, phagocytosis is upregulated even though growth on bacteria is slower (Table 2). This phenotypic pattern may be explicable by homeostatic feedbacks via TORC2 that stimulate phagocytosis. Further genetic dissection of mitochondrial and neurodegenerative diseases in the *Dictyostelium* model promises to unravel the complex cause-effect relationships and cross talk that underlie conserved elements of the cytopathology of these diseases in humans.

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