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Diversity of electron transport chains in anaerobic protists

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ABSTRACT

Eukaryotic microbes (protists) that occupy low-oxygen environments often have drastically different mitochondrial metabolism compared to their aerobic relatives. A common theme among many anaerobic protists is the serial loss of components of the electron transport chain (ETC). Here, we discuss the diversity of the ETC across the tree of eukaryotes and review hypotheses for how ETCs are modified, and ultimately lost, in protists. We find that while protists have converged to some of the same metabolism as anaerobic animals, there are clear protist-specific strategies to thrive without oxygen.

1. Introduction

Life on Earth is broadly classified into eukaryotes and prokaryotes based on the presence or absence of a nucleus, respectively. The term 'eukaryote' likely invokes images of animals and fungi (opisthokonts) and plants and green algae (archaeplastids). However, the vast majority of eukaryotic biological and phylogenetic diversity exists in the microbial world [1]. Unicellular eukaryotes (protists) are a collection of organisms that display complex morphologies and life cycles and are important community members in many ecosystems on Earth, for example, those environments with low concentrations of oxygen. These environments include the animal gut, freshwater and marine sediments and wastewater treatment facilities [2]. The mitochondrial metabolism of protists that thrive in these environments is drastically different from the metabolism of 'text-book' mitochondria.

2. Mitochondria and related organelles

Eukaryotes evolved from the merger of at least two prokaryotic organisms: an archaeal 'host' and bacterial 'endosymbiont' thought to be related to modern day Asgard archaea and alphaproteobacteria, respectively [3,4], the latter of which ultimately became the mitochondrion. Signatures of this endosymbiosis can be found in the mitochondria of modern eukaryotes that harbour a vestigial genome (mtDNA) that derives from the alphaproteobacterial endosymbiont. Despite the presence of an organelle-localized genome, mtDNA encodes only a fraction of the hundreds of proteins known to function in mitochondria. Most mitochondrially-localized proteins are encoded by the nuclear genome and are translated in the cytoplasm and imported into the organelle post-translationally. In general, the mtDNA encodes for informational processing machinery for mitochondrial protein translation (e.g., transfer and ribosomal RNA, ribosomal proteins) and respiration.

The mitochondrion is best known for its role in ATP biosynthesis via oxidative phosphorylation (OXPHOS; Fig. 1A). In aerobic eukaryotes, glycolysis-derived pyruvate is imported into the mitochondrion and oxidized to acetyl-CoA by the pyruvate dehydrogenase complex (PDC). The resulting acetyl-CoA is fed into the TCA cycle, and, through a series of reduction-oxidation (redox) reactions, generates reducing equivalents which are used by the electron transport chain (ETC) to produce an electrochemical gradient across the inner mitochondrial membrane (Fig. 1A). Electrons from NADH or succinate are transferred to the electron carrier ubiquinone (UQ) by Complex I (CI; NADH dehydrogenase) and CII (succinate dehydrogenase), respectively, to generate ubiquinol (UQH_2), the electrons from which are passed to cytochrome *c* via CIII, and ultimately to oxygen by CIV (Fig. 1A). There are also other UQ-utilizing complexes that can contribute to the UQ/UQH_2 pool such as alternative oxidase (AO) and the electron transferring flavoprotein complex (ETF). However, only CI, CIII and CIV are capable of pumping protons and contributing to the electrochemical gradient. This membrane potential is harnessed by the F_oF_1 ATP synthase (CV) to phosphorylate ADP to ATP. In the absence of oxygen, there is no suitable

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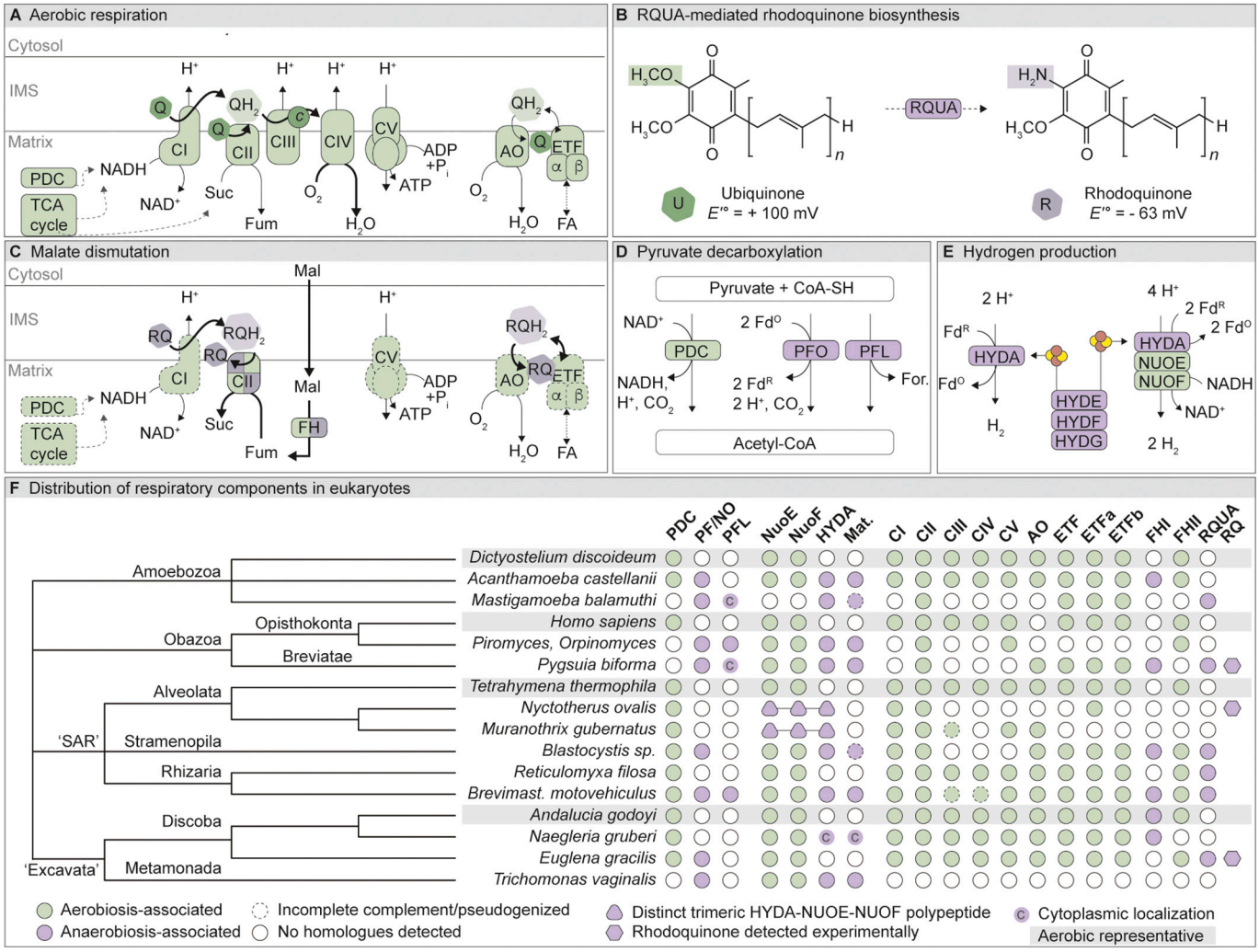

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Fig. 1. Metabolic modules that contribute to the evolution of the respiratory chain. Boxes A–E represent metabolic strategies present in the mitochondria and related organelles in protists. Processes typically associated aerobic and anaerobic lifestyles are shown in green and purple, respectively. A. Oxidative phosphorylation: Electrons from NADH derived from various mitochondrial processes (e.g., the pyruvate dehydrogenase complex (PDC) and tricarboxylic acid (TCA) cycle) and succinate are transferred to ubiquinone (UQ) via Complex I (NADH:ubiquinone oxidoreductase; NUO; CI) and Complex II (succinate dehydrogenase; SDH, CII), respectively, to generate ubiquinol (UQH_2). Electrons from UQH_2 are shuttled to cytochrome *c* (c) via Complex III (ubiquinone:cytochrome *c* oxidoreductase; CIII) and ultimately oxygen via Complex IV (cytochrome *c* oxidase; CIV). The electron transfer reactions from CI, CIII, and CIV transport protons across the inner mitochondrial membrane from the matrix into the intermembrane space (IMS). Electrons from fatty acid (FA) metabolism can be used to reduce UQ to UQH_2 via electron-transferring flavoprotein (ETF) dehydrogenase complex (ETF dehydrogenase; ETF alpha and beta subunits) and alternative oxidase (AO) can reoxidize UQ to UQH_2 with the reduction of oxygen. Suc, succinate; Fum, fumarate. Bolded arrows indicate direction of electron flow. B. UQ (left) is a precursor of rhodoquinone (RQ; right) in some bacteria and this catalysis might, in part, be mediated by RQUA, although other enzymes might be involved (dashed line). C. Malate dismutation: In this example, electrons from NADH are transferred to RQ to generate rhodoquinol (RQH_2) by CI. Malate is imported into the mitochondrial matrix and converted to Fum by a Class I (purple) or Class II (green) Fum hydratase (FH). The RQH_2 is reoxidized by CII functioning in reverse as a Fum reductase ultimately reducing Fum to Suc. All protists predicted to utilize RQ have been shown to contain CII (solid lines) and at least one other quinone-associated complex (depicted here with dotted lines and summarized in F.); however, CV is not always present. D. Pyruvate can be converted to acetyl-CoA by the action of the PDC, pyruvate:ferredoxin (Fd) oxidoreductase (PFO) or pyruvate formate lyase (PFL) generating NADH, reduced Fd and formate (for), respectively. Some protists encode a protein related to PFO named pyruvate:$NADP^+$ oxidoreductase (PNO) that can use $NADP^+$ in place of Fd as an electron acceptor. E. Electrons from Fd generated by PFO are used by a soluble monomeric [FeFe]-Hydrogenase (HYDA, left). Some have speculated that some protists have a soluble, electron-bifurcating, trimeric hydrogenase composed of HYDA and two components of CI (NUOE and NUOF) that couple the unfavourable oxidation of NADH and favourable oxidation of Fd to the reduction of protons. Many protist HYDA proteins require three accessory maturase proteins (HYDE, HYDF, HYDG) for the proper assembly of the Fe—S cluster (red and yellow circles). F. Distribution of respiration machinery across the tree of eukaryotes with an emphasis on non-parasitic lineages. Genes were detected in each of the organisms using standard homology probing (see Supplementary Data File S1) and coloured purple or green based on their association with anaerobic or aerobic processes, respectively. Circles with dotted outlines indicate cases where only some of the components were identified. Those proteins shown to function outside the MRO are labeled with 'c', indicating cytoplasmic localization. The ciliate-specific trimeric HYDA-NUOE-NUOF protein is indicated with linked triangles and those organisms with experimentally determined RQ production are shown with purple hexagons. For each supergroup, one well-characterized aerobic representative is shown in grey shading.

Metabolic modules that contribute to the evolution of the respiratory chain. Boxes A–E represent metabolic strategies present in the mitochondria and related organelles in protists. Processes typically associated aerobic and anaerobic lifestyles are shown in green and purple, respectively. A. Oxidative phosphorylation: Electrons from NADH derived from various mitochondrial processes (e.g., the pyruvate dehydrogenase complex (PDC) and tricarboxylic acid (TCA) cycle) and succinate are transferred to ubiquinone (UQ) via Complex I (NADH:ubiquinone oxidoreductase; NUO; CI) and Complex II (succinate dehydrogenase; SDH, CII), respectively, to generate ubiquinol (UQH_2). Electrons from UQH_2 are shuttled to cytochrome *c* (c) via Complex III (ubiquinone:cytochrome *c* oxidoreductase; CIII) and ultimately oxygen via Complex IV (cytochrome *c* oxidase; CIV). The electron transfer reactions from CI, CIII, and CIV transport protons across the inner mitochondrial membrane from the matrix into the intermembrane space (IMS). Electrons from fatty acid (FA) metabolism can be used to reduce UQ to UQH_2 via electron-transferring flavoprotein (ETF) dehydrogenase complex (ETF dehydrogenase; ETF alpha and beta subunits) and alternative oxidase (AO) can reoxidize UQ to UQH_2 with the reduction of oxygen. Suc, succinate; Fum, fumarate. Bolded arrows indicate direction of electron flow. B. UQ (left) is a precursor of rhodoquinone (RQ; right) in some bacteria and this catalysis might, in part, be mediated by RQUA, although other enzymes might be involved (dashed line). C. Malate dismutation: In this example, electrons from NADH are transferred to RQ to generate rhodoquinol (RQH_2) by CI. Malate is imported into the mitochondrial matrix and converted to Fum by a Class I (purple) or Class II (green) Fum hydratase (FH). The RQH2 is reoxidized by CII functioning in reverse as a Fum reductase ultimately reducing Fum to Suc. All protists predicted to utilize RQ have been shown to contain CII (solid lines) and at least one other quinone-associated complex (depicted here with dotted lines and summarized in F.); however, CV is not always present. D. Pyruvate can be converted to acetyl-CoA by the action of the PDC, pyruvate:ferredoxin (Fd) oxidoreductase (PFO) or pyruvate formate lyase (PFL) generating NADH, reduced Fd and formate (for), respectively. Some protists encode a protein related to PFO named pyruvate:NADP oxidoreductase (PNO) that can use NADP+ in place of Fd as an electron acceptor. E. Electrons from Fd generated by PFO are used by a soluble monomeric [FeFe]-Hydrogenase (HYDA, left). Some have speculated that some protists have a soluble, electron-bifurcating, trimeric hydrogenase composed of HYDA and two components of CI (NUOE and NUOF) that couple the unfavourable oxidation of NADH and favourable oxidation of Fd to the reduction of protons. Many protist HYDA proteins require three accessory maturase proteins (HYDE, HYDF, HYDG) for the proper assembly of the Fe—S cluster (red and yellow circles). F. Distribution of respiration machinery across the tree of eukaryotes with an emphasis on non-parasitic lineages. Genes were detected in each of the organisms using standard homology probing (see Supplementary Data File S1) and coloured purple or green based on their association with anaerobic or aerobic processes, respectively. Circles with dotted outlines indicate cases where only some of the components were identified. Those proteins shown to function outside the MRO are labeled with 'c', indicating cytoplasmic localization. The ciliate-specific trimeric HYDA-NUOE-NUOF protein is indicated with linked triangles and those organisms with experimentally determined RQ production are shown with purple hexagons. For each supergroup, one well-characterized aerobic representative is shown in grey shading.

terminal electron acceptor for the respiratory chain, ultimately leading to an arrest in OXPHOS.

The complement of OXPHOS components retained in protists that experience transient or permanent anoxia varies across the tree of eukaryotes. These organelles are collectively referred to as 'mitochondrion-related organelles' (MROs). MROs can be subclassified based on the presence of different metabolic modules related to hydrogen production (discussed in Section 3.2) and the ETC [5]. For example, organelles that can produce hydrogen and have retained all or some components of the respiratory chain are 'hydrogen-producing mitochondria' (HPMs), while those that can produce hydrogen but have lost the respiratory chain are 'hydrogenosomes'. Organelles that cannot produce hydrogen or ATP are 'mitosomes'. Such terms are helpful for superficially distinguishing between different MROs, however, it is important to recognize that these organelles likely exist on a functional continuum (discussed in [6]). For the purposes of this review, we will examine how the presence of different metabolic modules have influenced respiration in MROs across the tree of unicellular eukaryotes and how these differ from classic 'textbook' mitochondria.

3. Plug-and-play metabolic modules for life without oxygen

3.1. Rhodoquinone biosynthesis and fumarate reduction

Parasitic worms, such as *Ascaris suum* and *Fasciola hepatica*, as well as the soil nematode *Caenorhabditis elegans* encounter both normoxic and low-oxygen environments throughout their life cycles. Under normoxic conditions, the mitochondrial metabolism of these worms proceeds as in a conventional 'aerobic mitochondrion' (Fig. 1A). However, upon infiltration of oxygen-poor environments (e.g., *A. suum* in the host intestine; *F. hepatica* in the host bile ducts) the mitochondrial respiratory chain is markedly different and is composed of only CI, CII and CIV. Under these conditions, the worms utilize malate dismutation to maintain a proton gradient (Fig. 1C; reviewed in [5]). Malate dismutation consists of two main branches: NADH oxidation and fumarate production (Fig. 1C). Here, CI continues to function in order to couple NADH

oxidation to pumping protons into the intermembrane space, however, instead of passing electrons to UQ, electrons are transferred to an alternative quinone species - rhodoquinone (RQ) - ultimately generating rhodoquinol (RQH_2) (Fig. 1B). The second branch of the pathway involves the generation of fumarate. Malate is imported from the cytoplasm and converted to fumarate by the TCA cycle enzyme fumarate hydratase (FH). These two branches converge when CI-derived electrons from RQH_2 are transferred to fumarate via CII functioning as a fumarate reductase (FRD) ultimately regenerating RQ and producing succinate. The lower reduction potential of RQ/RQH_2 ($E'^{\circ} = -63$ mV) relative to UQ/UQH_2 ($E'^{\circ} = +110$ mV) allows for the favourable reduction of fumarate to succinate ($E'^{\circ} = +30$ mV) by CII. Accessory UQ-interacting complexes such as ETF [7] and potentially AO contribute to the RQ/RQH_2 pool.

The presence of RQ is not a prerequisite for FRD activity. For example, bovine CII can act as an FRD at sufficient concentrations of fumarate *in vitro* using artificial electron donors [8] and, following ischemic reperfusion, CII in murine mitochondria can function as a UQH_2:fumarate oxidoreductase, albeit with the production of dangerous reactive oxygen species [9]. This suggests that malate dismutation might be a widespread phenomenon under specific conditions, even in aerobic eukaryotes.

Many RQ-containing animals encode hypoxia-specific subunits of both CI and CII that are predicted to interact with rhodoquinone (CI, NAD7/NDUFS2; CII SDHD) and fumarate (CII, SDHA) [10,11]. These genes are predicted to have arisen by gene duplications independently in different lineages [11]. We identified Class I (FHI) and Class II (FHII) fumarases in eukaryotes (Supplementary Data S1). FHI is considered an 'anaerobic' enzyme based on the presence of an oxygen-sensitive Fe—S cluster and has been well characterized in prokaryotes, although investigations in eukaryotes are limited [12,13]. The malate dismutation system as a whole allows for the maintenance of a proton gradient for the biosynthesis of ATP via CV where fumarate, and not oxygen, is the terminal electron acceptor. In some worms, at least two existing metabolic pathways (i.e., the kynurenine pathway for tryptophan biosynthesis and the ubiquinone biosynthesis pathway) have been co-opted for RQ biosynthesis [14–16].

RQH_2-mediated fumarate reduction likely occurs in some anaerobic protists, although robust experimental characterization is lacking - see sections below. Unlike RQ-containing animals, some protists are predicted to synthesize RQ from UQ using the RQ biosynthesis enzyme, RQUA. This pathway was first elucidated in mutants of the alphaproteobacterium *Rhodospirillum rubrum* that were incapable of synthesizing RQ and found to have a point mutation in the Rru_A3227 gene (*rqua*) [17]. Later studies revealed that the expression of the *rqua* gene allowed for RQ production in species incapable of synthesizing RQ (e.g., *Saccharomyces cerevisiae* and *Escherichia coli* [18]). The *rqua* gene has a narrow distribution among bacteria having only been identified in some lineages of alpha, beta, and gammaproteobacteria. Soon after its discovery in *R. rubrum*, homologues of *rqua* were identified in nearly twenty species of eukaryotes [19]. Much like UQ biosynthesis, RQUA-mediated RQ biosynthesis likely occurs in the mitochondrion, and the RQUA protein been shown to localize to the MROs of at least one RQ-producing protist, *Pygsuia biforma* [19]. Interestingly, some RQUA-containing protists do not encode the capacity for endogenous UQ biosynthesis and are predicted to acquire UQ from exogenous sources (e.g., food prokaryotes) and convert it to RQ using RQUA [19].

3.2. Pyruvate decarboxylation and hydrogen production

In the MROs of protists, at least three methods for pyruvate conversion to acetyl-CoA have been described [20]: via (i) the PDC that generates NADH; (ii) an oxygen–sensitive pyruvate:ferredoxin oxidoreductase (PFO) that generates reduced Ferredoxin (Fd); and (iii) an oxygen-sensitive pyruvate formate-lyase (PFL) that generates formate non-oxidatively (Fig. 1D). The resulting acetyl-CoA is used for ATP biosynthesis via substrate level phosphorylation (reviewed elsewhere [5]). Reduced Fd from the PFO reaction can be reoxidized by a soluble monomeric [FeFe]-Hydrogenase (HYDA; Fig. 1E, left) [21,22]. Some have proposed that protists could also use a soluble, electron-bifurcating, trimeric hydrogenase composed of HYDA and two components of CI (NUOE and NUOF) that couple the unfavourable oxidation of NADH and favourable oxidation of Fd to the reduction of protons (Fig. 1E, right; [23,24]) that has been reported in some bacteria [25]. Many protists that encode HYDA proteins also encode three accessory maturase proteins (HYDE, HYDF, HYDG; [26]) that are predicted to be necessary for the proper assembly of the Fe—S cluster of HYDA. Collectively these systems allow for a CI-independent recycling of NADH (i.e., electron bifurcating HYDA) and/or reduction of NADH-generating pathways (i.e., NAD-independent pyruvate decarboxylation and acetyl-CoA production via PFO and/or PFL).

Below, we outline how the integration of fumarate reduction, PDC alternatives, and hydrogen production have influenced the conventional OXPHOS system of newly-described protists.

4. Examples of anaerobic protist respiratory chains

4.1. Anaerobically functioning mitochondria in protists

The dominant conception of mitochondria as aerobic ATP-generating machines derives largely from early studies of animal mitochondrial physiology, and many protists, including important model systems, such as the ciliate *Tetrahymena* and the social amoeba, *Dictyostelium*, are also inferred to have 'classical' aerobic mitochondria. On the opposite end of the spectrum, many examples of protists with reduced MROs have been described. There are, however, relatively few described protist species that encode components of both aerobic and anaerobic mitochondrial modules, although they provide key insights into the early stages of mitochondrial reduction (below).

The amoeba ***Acanthamoeba castellanii*** (Amoebozoa) and amoeboflagellates ***Naegleria gruberi*** **and** ***Naegleria fowleri*** (Discoba) are facultative anaerobes that are found in soil and freshwater environments. *A. castellanii* is an opportunistic pathogen associated with corneal disease and fatal encephalopathy in humans. Upon exposure to hypoxia, this amoeba has been shown to encyst [27] or replicate at faster rates than under normoxic conditions [28]. *N. gruberi* is a free-living relative of the deadly 'brain-eating amoeba' *N. fowleri*. Extensive biochemical and bioinformatic interrogations of both *A. castellanii* and *Naegleria* species reveal typical aerobic mitochondria complete with the PDC, the TCA cycle and a conventional ETC and other quinone-utilizing subunits (Fig. 1F; Supplementary Data File S1). However, closer inspection of the *A. castellanii* genome and transcriptome revealed the presence of a complete PFO/HYD system and maturase proteins [29,30], some components of which are detectable even under aerobic growth in mitochondria [31]. Similarly, bioinformatic analyses of *N. gruberi* identified genes encoding HYDA and the maturase proteins that were predicted to localize to the mitochondrion [32], however, biochemical studies suggest these proteins may function in the cytoplasm [33,34]. Whether *A. castellanii* or *Naegleria* species can perform malate dismutation or produce RQ has not been investigated, however there is no obvious *rqua* encoded in their genomes. We suspect that, if UQ cannot be regenerated under hypoxic conditions thereby resulting in an arrest of the ETC, these organisms biosynthesize ATP by substrate level phosphorylation via consecutive CoA transfer reactions (reviewed [5]), in contrast to the worms discussed above that continue to use the respiratory chain in some capacity.

The mixotrophic alga ***Euglena gracilis*** (Discoba) grows in freshwater environments as a phototroph or heterotroph. *E. gracilis* encodes the conventional repertoire of proteins necessary for the PDC, ETC and TCA cycle [35] but also RQUA [19] and pyruvate NADP oxidoreductase (PNO) - a homologue of a PFO with a C-terminal cytochrome P450 domain that allows for electrons to be transferred to NADP and not Fd

[36]. Under low-oxygen conditions, the ratio of RQ to UQ in *E. gracilis* mitochondria shifts in favour of RQ [37]. As a result, the mitochondrial metabolism of *Euglena* shifts to malate dismutation, using RQ to generate the succinate necessary for wax ester biosynthesis in the cytosol.

Although only a few protists with the genetic capacity for energy generation using both aerobic and anaerobic systems have been formally described, we suspect that this strategy is far more common among protists than is currently appreciated. Bioinformatic interrogations of transcriptome and genome datasets from across the breadth of eukaryotic biodiversity reveal numerous examples of protists that encode enzymes of both aerobic and anaerobic systems, and frequently express at least some components of each under aerobic conditions (see supplementary tables in [19,20]). It is important to note that many of these datasets indicating the expression of genes encoding anaerobic enzymes, are derived from poorly studied protists that were grown under normoxic conditions. For example, anaerobiosis associated proteins have been identified in transcriptome projects of the diatom *Attheya* sp. (*rqua*) and chromerids (*hyda*, *hyde-g*) that were generated under normoxic conditions. These conditions are known to hinder the expression of oxygen-sensitive transcripts, such as those of the anaerobic energy generation system, indicating that there are systematic biases against the identification of facultatively anaerobic mitochondria in available datasets, which are likely to be ecologically and evolutionarily important.

4.2. Gene gain and pseudogenization drives the loss of CIII and CIV – the point of no return

This next 'step' along the modification of the ETC pathway can be conceptualized as the result of protists having committed to an anaerobic life strategy. Rather than being aerobes that have the capacity to deal with transient hypoxia as is likely the case for *A. castellanii*, *Naegleria* species and *E. gracilis*, the following organisms are likely nascent anaerobes that use the vestiges of their aerobic machinery to tolerate transient normoxia (and may be in the process of losing genes associated with aerobic metabolism).

The earliest stages of evolutionary adaptation to life in anaerobic environments are characterized by molecular changes in genes coding for ETC components or accessory components (e.g., assembly factors), as well as changes in their expression. This is presumably due to relaxation of purifying selection on genes required for aerobic metabolism due to decreased exposure to normoxic environments. Moreover, if proteins containing complex metal centres (e.g., cytochrome *c*, CIII and CIV) are expressed but not assembled efficiently, this could lead to the production of dangerous reactive oxygen species. The first alterations occur in CIII and CIV and can range from subtle (e.g., substitutions in critical amino acid residues) to more drastic (e.g., gene loss or pseudogenization) changes of one or more key subunits. However, other elements of the ETC, such as CI, CII, CV, often remain intact. The maintenance of CV suggests that the proton-pumping mediated by CI alone in these organisms is sufficient to synthesize an appreciable amount of ATP.

An illustrative example of a minimally reduced MRO is the free-living cercozoan ***Brevimastigomonas motovehiculus*** (Rhizaria), first isolated from anoxic freshwater sediments [6]. Analyses of genome and transcriptome data uncovered at least some components of CI-V and the mtDNA along with the capacity for RQ biosynthesis, malate dismutation and PFO/HYD-mediated hydrogen production (Fig. 1F), but also suggest non-functional (or weakly-functional) CIII and CIV. In particular, there is extraordinary sequence divergence of mtDNA-encoded Cob and Cox1, apparent pseudogenization of key genes, like Cox2, and loss of other non-enzymatic subunits and assembly factors. Although CV subunits are robustly expressed in anaerobic conditions, they show potential signs of functional degradation as well. For instance, the otherwise highly conserved F_1 α subunit has 'split' into three distinct, highly divergent polypeptides that have each acquired N-terminal targeting sequences for import into the mitochondrion [6]. Since *B. motovehiculus* also encodes RQUA, we suspect that under low-oxygen conditions, this protist can perform malate dismutation.

Similar metabolic reconstructions have been proposed for a number of other anaerobic microbes that retain CV based on genome and transcriptome sequence data. Among ciliates, both marine (***Muranothrix gubernata*** and *Parablepharisma* sp. [38]) and freshwater (*Cyclidium porcatum* [39]) species are represented. ETC reconstructions demonstrate that *M. gubernatum*, *Parablepharisma* sp., and *C. porcatum* retain CI, CII, and CV. Interestingly, like *B. motovehiculus*, these ciliates also retain an incomplete repertoire of CIII and/or CIV proteins and a cytochrome *c*1 pseudogene, indicating that complete purging of ETC component genes from the genome is a slow and complex process. The selective pressure that maintains CV within some MROs is incompletely understood. Although CV in anaerobes may simply be a 'vestigial' ATP source [6,39,40], some have suggested that in anaerobes, it might serve primarily as a dissipator of the proton motive force [38] or act as a pH regulator [41]. In Section 4.3, we discuss examples of organisms that have retained CV and not CI (e.g., *Orpinomyces* MROs), or CI and not CV (e.g., MROs of *Blastocystis* species and *Nyctotherus ovalis*), suggesting that a link between CI and CV is not mutually exclusive.

Perhaps the most striking feature of these and other anaerobic ciliates compared to other protists is their HYDA; these ciliates possess a HYDA that is fused to NUOE and NUOF-like subunits [38,39,42] thereby encoding the entire trimeric electron bifurcating hydrogenase in one polypeptide. Importantly, phylogenetic analysis of the ciliate HYDA and NUOE/NUOF proteins show that these proteins are not related to other eukaryotic HYDAs or mitochondrial CI subunits, respectively. This suggests that the hydrogenase complex of ciliates has independently converged to the same NADH- and Fd-oxidizing [FeFe]-Hydrogenase of other eukaryotes.

4.3. Differential retention of CI and CV

Following the loss of CIII and CIV, the next stages in ETC reduction results in the loss of CI and CV. As those organisms described in Section 4.2, the following species are generally considered anaerobes that can experience transient oxygen exposure. For example, some stramenopiles (e.g., ***Blastocystis*** species [43], *Opalina* species [44]) and alveolates (e.g., ***Nyctotherus ovalis***) that live in anaerobic environments associated with animals, have lost all components of CIII, CIV and CV, however still retain all genes necessary for CI, CII, and the PDC. *Blastocystis* has also retained other Q-utilizing components (Fig. 1F, Supplementary Data File S1) at least one of which, AO [45], has been characterized experimentally. *Blastocystis* species encode PFO, HYDA, HYDG and RQUA that are predicted to function in the MRO [19,43,45]. *N. ovalis* encodes the ciliate-specific HYDA-NUOE-NUOF fusion but no other anaerobiosis-specific proteins [42,46]. Like other anaerobic ciliates such as *M. gubernata*, *N. ovalis* still encodes a pseudogenized cytochrome *c*1 despite no longer encoding CIII or CIV genes. Although the RQ-biosynthesis enzyme RQUA has not been identified in *N. ovalis*, RQ has been detected experimentally [46]. Assuming that RQUA confers the ability to synthesize RQ in *Blastocystis*, it is likely that the CI and CII of *Blastocystis* species and *N. ovalis* can function as a proton-pumping NADH:RQ oxidoreductase and RQH_2:fumarate oxidoreductase, respectively. However, without a CV, the precise role of generating a proton gradient is unknown. Some have speculated that since the proton gradient maintains a net negative charge of the mitochondrial matrix relative to the IMS, this allows for the electrophoretic import of mitochondrial-targeted proteins that possess a positively charged N-terminal mitochondrial targeting sequence [47].

The chytrid fungi, ***Piromyces* sp. E2**, ***Pecoramyces ruminatium*** (formerly *Orpinomyces* sp. C1A [48]) and *Neocallimastix frontalis* are commensal organisms that live in the anaerobic gut of herbivorous animals. In the past 30 years, numerous genomic and transcriptomic studies have shown that these organisms have lost all traces of the

mtDNA as well as components of the PDC, CI, CIII, and CIV retaining only CII and CV of the ETC. Some studies have illustrated a membrane-bound CI [40], however, we failed to detect any CI subunits beyond NUOE and NUOF (Supplementary Data File S1). Since membrane-integrated CI subunits required for proton-pumping, typically encoded in mtDNA, are apparently absent, we suspect that these chytrid fungi do not encode a traditional proton pumping CI. We could only identify one quinone-utilizing complex (CII) in any of the available genome projects which could suggest that more thorough sequencing is required (Supplementary Data File S1). PFO, PFL, HYDA and the maturase proteins have been identified in most species studied to date, some of which have been characterized experimentally [49,50]. Early functional studies showed that in some chytrid fungi, CV might function in reverse as a ATP-driven proton pump that exports protons from the matrix into the IMS maintaining an alkaline pH necessary for matrix enzymes [41]. As previously suggested, we suspect that these fungi might rely primarily on fermentative metabolism and not CV-mediated ADP phosphorylation for their energy requirements [5].

4.4. Co-ordinated loss of CI and the mtDNA

The free-living marine flagellate ***Pygsuia biforma*** (Breviatae) and freshwater amoeba ***Mastigamoeba balamuthi*** (Amoebozoa) are found in low-oxygen environments and possess hydrogenosomes. Having lost the PDC, the TCA cycle, CI, CIII, CIV, and CV, these organisms retain CII as the only vestige of the ETC. Loss of CI at this stage of reduction is also associated with loss of the mtDNA. This might argue that the inability to efficiently synthesize and import hydrophobic membrane subunits of CI from the cytosol constitutes the only remaining selective pressure for maintenance of mtDNA [51]. Alternatively, the co-location for redox regulation (CoRR) hypothesis suggests that mtDNA-encoded gene expression is regulated by the local redox state of mitochondrion, which in turn, is regulated by the activity of ETC components [52]; the loss of a functional CI would thereby eliminate the selective pressure to retain a localized genome.

P. biforma is predicted to utilize a soluble trimeric [FeFe]-hydrogenase (e.g., HYDA, NUOE, NUOF) with a complete set of maturase proteins [53] within its MRO, while *M. balamuthi* uses a soluble monomeric HYDA with only one maturase protein (HYDE) within its MRO [54].Both organisms rely on PFO and PFL for acetyl-CoA biosynthesis in their MROs and cytoplasm, respectively. Although these protists lack CI, they both encode RQUA, CII and at least one other quinone-utilizing complex. RQ has been detected in *P. biforma* [19]. Neither protist possesses the complement of enzymes necessary to synthesize UQ suggesting that they rely on exogenous sources of UQ that can be used as a precursor for RQ biosynthesis. Since the malate dismutation pathway is fragmented in these protists, we suspect that their CII-mediated RQH_2:fumarate oxidoreduction might be important for an alternative function.

Complete loss of the mtDNA and partial loss of the ETC has also occurred in some aerobes, for example, the dinoflagellate *Amoebophyra ceratii* [55]. In most dinoflagellates, the mtDNA contains only three genes that encode for components of CIII (*cytb*) and CIV (*cox1* and *cox3*). The ETC of *Amoebophyra ceratii*, an aerobic parasite of other protists, is composed of an alternative proton-pumping NAD(*P*)H dehydrogenase, CII, CIV, cytochrome *c* and CV but no evidence for nuclear or mtDNA-encoded CIII subunits. In fact, the typically mtDNA-encoded *cox1* subunit was found to be encoded in gene fragments in the nucleus. In this modified ETC, electrons are funneled via the alternative NAD(*P*)H dehydrogenase and CII to UQ to generate UQH_2 that is reoxidized by AO to reduce oxygen. Other cytochrome *c*-utilizing enzymes (e.g., D-lactate: cytochrome *c* oxidoreductase) are proposed to shuttle electrons to CIV to ultimately reduce oxygen [55]. Although this loss appears to mirror those of anaerobic protists, it is presently unclear whether this metabolic reduction is related to adaptation to anaerobiosis or parasitism.

4.5. Loss of the entire ETC or MRO

There are numerous examples of parasitic and free-living protists across the tree of eukaryotes that have lost all traces the quinone or membrane associated ETC components and mtDNA in their MROs. For example, the human pathogen ***Trichomonas vaginalis*** [56] and some free-living metamonads (e.g., *Carpediemonas*-like organisms [57]) have hydrogenosomes that have lost all traces of CI, CII, CIII, CIV, or CV except for the CI subunits (NUOE and NUOF) associated with their trimeric hydrogenase. Anaerobic parasites such as *Entamoeba histolytica* and *Giardia intestinalis*, possess mitosomes that have lost the entire ETC including the NUOE and NUOF subunits [58,59]. Other mitosome-bearing organisms such as the intracellular parasites *Mikrocytos mackini* and microsporidia have similarly lost all elements of the ETC [60,61] however, their metabolic reduction might be associated to reductive pressures associated with their obligate intracellular lifestyle and not strictly anaerobiosis. The final 'stop' towards mitochondrial reduction can be found in the Oxymonads (Metamonada) where the ETC as well as the mitochondrion has been lost completely and energy conservation occurs exclusively in the cytosol [62,63].

5. Perspectives

While we can trace the origins of aerobic mitochondrial metabolism back to the protomitochondrial symbiont, unraveling the history of genes associated with anaerobic energy biosynthesis in protists is somewhat murkier. Given the widespread prevalence of genes associated with anaerobic metabolism across the tree of eukaryotes, it is reasonable to suggest that these proteins were present in the last eukaryotic common ancestor (LECA) and therefore, the differences in metabolism we see in MROs compared to aerobic mitochondria are the result of numerous differential loss events. However, closer examination of the internal relationships among eukaryotes in phylogenies of anaerobic proteins, often conflict with known organismal relationships, implying there has likely been lateral gene transfer (LGT) among eukaryotes as well (a process called 'eukaryote-eukaryote lateral gene transfer'). This is in striking contrast to the evolution of anaerobic metabolism in animals, which requires tinkering of existing machinery (e.g., CII subunit composition and RQ biosynthesis) instead of gene acquisition (Fig. 2). We have yet to find evidence that the genes typically associated with anaerobic metabolism in eukaryotes were present in the protomitochondrion or host archaeal lineage that gave rise to eukaryotes – that is, these genes are rare in modern representatives of alphaproteobacteria and archaea. In fact, in phylogenetic analyses of some anaerobic proteins, Anoxychlamydiales (i.e., PFO, HYDE, HYDF and HYDG [64]) and Firmicutes (i.e., PFL [65]) are the closest prokaryotic relatives to eukaryotic homologues. Other proteins involved in anaerobic metabolism such as HYDA and RQUA seem to have multiple independent origins in eukaryotes suggesting that these genes have been acquired by LGT in at least some eukaryotic lineages [19,64]. The precise complement of anaerobiosis associated genes that were present in the LECA cannot be determined from present data, but suggests anaerobic eukaryotic metabolism is an evolutionary mosaic. Evidence in favour of gene transfer within and between eukaryotes has been reviewed elsewhere [66], especially with respect to anaerobic proteins [67].

In any given lineage of eukaryotes, the starting point for adaptation to anaerobiosis is likely a facultatively anaerobic mitochondrion, as seen in *Acanthamoeba*, *Naegleria* spp. and *Euglena*. These organisms encode a suite of proteins that allows for the fine-tuned expression of genes involved in respiration and anaerobic fermentation, such that they can thrive in both aerobic and anaerobic ecological niches. The precise factors that favour committal to an anaerobic lifestyle, and the resulting reductive evolution of the ETC, remain mysterious. One aspect that might influence this is the establishment of syntrophic interactions between a protist and epibiotic or endobiotic prokaryotes that can feed on

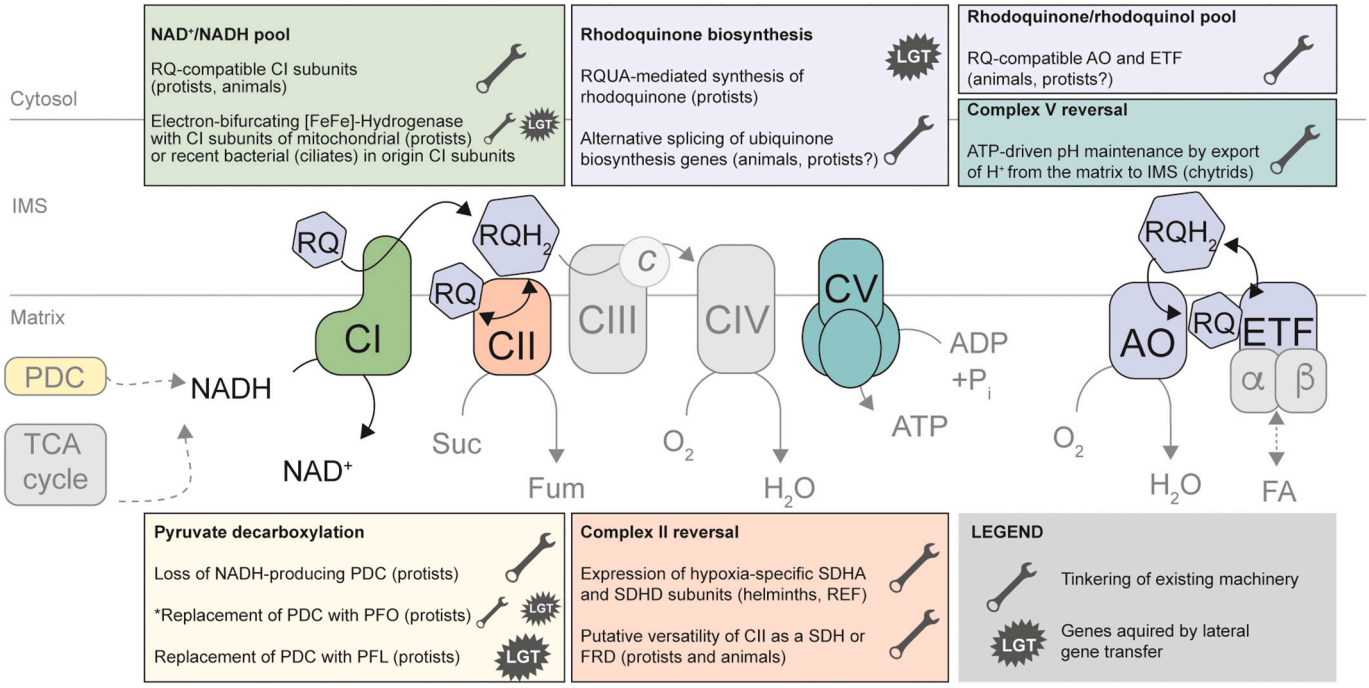

Fig. 2. 'Hacking' the electron transport chain to live without oxygen. Examples of how mitochondrial metabolism in anaerobic eukaryotes differs from aerobic eukaryotes. Abbreviations are as Fig. 1. Coloured text boxes refer to the similarly coloured cartoon enzyme. Differences between specific protists and animals are indicated when applicable. The proposed mechanism of evolution, tinkering of existing machinery and lateral gene transfer (LGT), are shown as wrenches and LGT balloons, respectively.

the metabolic waste products of the protist's anaerobic metabolism (e.g., H_2 and acetate), thereby making the reactions more thermodynamically favourable [38,68–70]. Such interkingdom interactions have been described in a variety of anaerobic protists, including breviates [68], ciliates [2,70], and euglenozoans [69], suggesting that they could be important in driving the transition to an anaerobic lifestyle.

Regardless of what initiated the reduction of the ETC in a given lineage, it is clear that ETC reduction has happened numerous times independently in distantly related groups, including multicellular animals [71]. These reductions have followed similar trajectories in different lineages shaped by the biochemical properties of mitochondrial machinery, although the mechanisms driving this evolution can vary. In animals, the reduction of the ETC seems to be the result of tinkering of existing mitochondrial metabolism. This is in contrast to the situation in protists where both whole-sale acquisition of new functions by LGT and repurposing of existing machinery contributes to ETC reduction. Many of the ETCs discussed herein, lack experimental characterization and therefore future interrogations into the function and enzyme kinetics of these complexes (in particular those ETCs with unusual features such as the CV of *B. motovehiculus* or the minimalist chain of *P biforma* and *M. balamuthi*) or the quinone complement of RQUA-lacking anaerobes (e.g., *A. castellanii*, *N. gruberi*) will no doubt lead to the refinement of our model.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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