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1 **Eukaryotic cellular intricacies shape mitochondrial proteomic**
2 **complexity**

3
4

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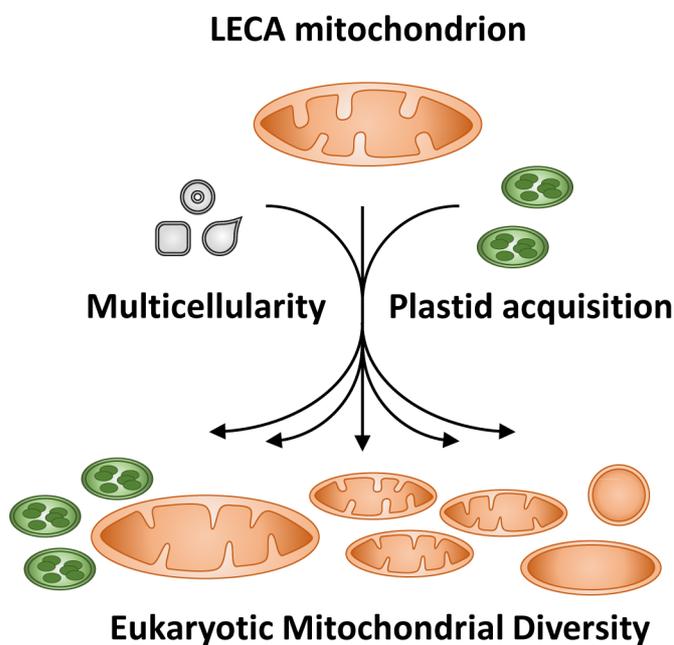
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16



17

18 **Abstract**

19 Mitochondria have been fundamental to the eco-physiological success of eukaryotes since
20 the last eukaryotic common ancestor (LECA). They contribute essential functions to

21 eukaryotic cells, above and beyond classical respiration. Mitochondria interact with, and
22 complement, metabolic pathways occurring in other organelles, notably diversifying the
23 chloroplast metabolism of photosynthetic organisms. Here, we integrate existing literature
24 to investigate how mitochondrial metabolism varies across the landscape of eukaryotic
25 evolution. We illustrate the mitochondrial remodelling and proteomic changes undergone in
26 conjunction with major evolutionary transitions. We explore how the mitochondrial
27 complexity of the LECA has been remodelled in specific groups to support subsequent
28 evolutionary transitions, such as the acquisition of chloroplasts in photosynthetic species
29 and the emergence of multicellularity. We highlight the versatile and crucial roles played by
30 mitochondria during eukaryotic evolution, extending from its huge contribution to the
31 development of the LECA itself to the dynamic evolution of individual eukaryote groups,
32 reflecting both their current ecologies and evolutionary histories.

33

34

35 **Keywords**

36 Mitoproteome; mass spectrometry; photosynthesis; multicellularity; plastid; protist;
37 parasite

38

39 **Abbreviations:** LECA, last eukaryotic common ancestor; TCA, tricarboxylic acid; TOM/TIM,
40 Translocation of the Outer Membrane / Inner Membrane; LOPIT, Localisation of Organelle
41 Proteins via Isotope Tagging

42

43 **Introduction**

44 Mitochondria are regarded as critical components for the process of eukaryogenesis [1, 2],
45 providing the energy and metabolic partitioning necessary for a rapid development of a
46 large contingent of structural and functional genes [3]. In turn this has enabled expansions
47 of both eukaryotic metabolic rates as well as their maximal size constraints [4-6].

48 Accordingly, the eukaryote domain is an extremely diverse group in terms of size, ranging
49 from unicellular organisms less than 1 μm in diameter, to colonial tree populations spread
50 over 40 hectares [7]. Mitochondria contribute multiple essential functions, including
51 oxidative phosphorylation resulting in ATP generation, using NADH_2 and FADH_2 generated
52 through the Tricarboxylic Acid (TCA) cycle and fatty acid oxidation; fermentation, with some
53 organisms also performing the lower half of glycolysis mitochondrially [8]; key steps in the
54 synthesis, degradation and recycling of amino acids [9]; heme [10], fatty acid and iron-
55 sulphur cluster syntheses [11]; and the maintenance of inter-organelle NAD(P)H_2 and ATP
56 homeostasis [12]. In plants and other photosynthetic eukaryotes, mitochondrial respiration
57 has been shown to be important for photosynthetic function [13].

58 This amazing diversity of eukaryotes springs from a single common ancestor, which is
59 believed to have existed between one and two billion years ago [14]. The vast majority of
60 eukaryote lineages comprise protists, which are, mostly distantly, related to plants, animals
61 and fungi, and indeed to one another (**Fig. 1**). Most eukaryotes are aerobes, but a significant
62 minority are facultatively, or even obligatory, anaerobic, typically living either as
63 commensals of other organisms or in deep benthic anoxic habitats [15, 16]. Eukaryotes may
64 derive their energy through autotrophy (photosynthesis) or heterotrophy (predation,
65 saprotrophy, or parasitism). Many photosynthetic unicellular algae and a handful of
66 (carnivorous) plant species also retain the ability to perform heterotrophy, either as
67 phagotrophs or osmotrophs, in combination with photosynthesis (photo-mixotrophy) [17-
68 19]. Despite this diversity in morphology and lifestyle, the vast majority of eukaryotes have
69 retained mitochondria, with even most obligately anaerobic groups retaining mitochondria-
70 derived organelles, such as DNA-lacking hydrogenosomes or non-respiring mitosomes,
71 excepting a small number of unicellular species [20, 21].

72 This all but ubiquitous presence of mitochondria across the eukaryotic tree speaks not only
73 to their importance, but also to their evolutionary history. It is clear that mitochondria were
74 present in the Last Eukaryotic Common Ancestor (LECA), originating from the endosymbiosis
75 of an early-branching alpha-proteobacterium with an archaeon (related to the Asgard-like
76 group of extant Archaea), eventually transitioning to a stable organelle [22]. It remains
77 debated whether other bacteria have participated in this endosymbiotic event, e.g. in a
78 “ménage à trois” or other complicated syntrophic scenario (e.g., a chimeric bacterial
79 symbiont) [23, 24]. To complicate matters further, contemporary mitochondria are
80 supported by a patchwork of proteins coming from the endosymbiont, archaeal host, and
81 other bacteria [25]. Subsequent endosymbiotic events involving photosynthetic bacteria and
82 eukaryotes have yielded a wealth of different plastid-carrying groups, spread across the
83 eukaryotic tree, and like mitochondria, engaged in both fundamental bioenergetic and non-
84 bioenergetic metabolism; although punctuated with frequent instances of secondary loss
85 [26, 27].

86 One of the most useful indicators of an organism’s mitochondrial capacity is the variety of
87 proteins contained within its mitochondria, referred to as the mitochondrial proteome or
88 ‘mitoproteome’. Not surprisingly, animals and fungi have received most attention in regard
89 to mitochondrial proteomic investigations. Comparatively little is known for the
90 mitochondria of plants (with the exception of some model and crop representatives) and
91 protists. In their case, this is due to both their extreme diversity and the relatively few,
92 recent and well-resolved experimental mitoproteome datasets. In this review, we consider
93 mitoproteomes across previously published datasets to gauge mitochondrial evolution (**Fig.**
94 **1**).

95 We describe the mechanisms by which mitochondria have adapted to facilitate major
96 transitions in later stages of eukaryotic evolution, represented by the uptake of
97 photosynthetic plastids, and the development of complex multicellularity. The versatility

98 and biochemical range of mitochondria was vastly reduced in some cases. In contrast,
99 further complexity in mitochondrial pathways also evolved, for instance influenced by
100 photosynthetic metabolism, in many cases arising *via* organelle retargeting events [28]. The
101 independent, repeated advent of multicellularity, both in plants and animals has been
102 consistently supported by metabolic and proteomic partitioning. Overall, this development
103 has reduced mitochondrial complexity in individual cell types, reflecting functional
104 specialisation. Thus, our data illustrate how ancestral constraints have interplayed with
105 novel complexities to give rise to the extensive divergence of mitochondrial metabolism
106 across the eukaryotic tree.

107

108 **Informatic and experimental strategies to identify mitoproteins**

109 The vast majority of mitochondria are supported by their own genomes, reflecting their
110 bacterial origin. However, these genomes only encode a handful of proteins [29], ranging
111 from two in apicomplexans within the alveolates (**Fig. 1**) [30] to 65 in jakobids within the
112 discobans (**Fig. 1**) [31]. These numbers are vastly inferior to the thousands of proteins
113 identified in free-living bacteria, or indeed the hundreds to thousands of proteins
114 documented in different mitochondrial lineages. Hence, the overwhelming majority of
115 mitochondrial proteins (mitoproteins) are encoded by nuclear genes [32], entailing that only
116 limited proteomic knowledge can be acquired by studying these highly reduced
117 mitochondrial genomes (mitogenomes). The mitoproteins encoded by the nuclear genes are
118 typically post-translationally imported into the mitochondria, using dedicated protein
119 import complexes, e.g. Translocation of the Outer Membrane / Innner Membrane (TOM/TIM)
120 that recognise semi-conserved hydrophilic N-terminal targeting sequences.

121 Different strategies exist for defining the likely mitoprotein content of diverse cells and
122 tissues. *In silico* targeting predictors such as Mitofates [33] and TargetP [34], that have been
123 trained on the hydrophilic N-terminal targeting motifs recognised by mitochondrial import
124 complexes, may be used to identify mitochondria-targeted proteins directly from genome or
125 transcriptome sequences. The sensitivity and specificity of *in silico* mitoprotein prediction
126 significantly varies among species and depends on having well-annotated genome or
127 transcriptome data, as well as experimentally validated sequences for calibration. Of note,
128 these may not be able to predict mitoproteins imported using non-conventional targeting
129 pathways (e.g., with ambiguous or dual-targeted sequences; or imported using TOM/TIM-
130 independent pathways, as in the case of many mitochondrial outer membrane proteins). In
131 addition, *in silico* targeting predictors cannot estimate mitoproteome content variations in
132 multicellular organisms (e.g., in different tissues or life stages) except in very specific cases
133 (e.g., where different gene splice-variants or alleles are associated with different samples).

134 Comprehensive tagging studies of organisms possessing well-annotated genomes represent
135 ongoing projects still in their infancy [35, 36], though such efforts continue to demonstrate
136 hundreds of mitochondrial proteins that evade software-based identification, in spite of

137 recent improvements in target peptide detection sensitivity using machine learning
138 methods [37, 38].

139 Mass spectrometric proteomic surveys remain the gold standard to determine
140 mitoproteomes, though such experimental datasets are still costly and labour-intensive,
141 requiring the specific optimisation of purification protocols for individual species.
142 Mitochondrial purification may be particularly complicated in unicellular species by the
143 difficulty of obtaining pure mitochondrial fractions from cell lysate, either as a result of
144 small cell sizes, sensitivity of cells to mechanical disruption, and co-association of the
145 mitochondria with other organelles, such as peroxisomes and chloroplasts [39]. Such issues
146 are being increasingly resolved through the application of next-generation proteomic
147 strategies such as Localisation of Organelle Proteins via Isotope Tagging (LOPIT), which
148 enable the algorithmic identification of probable organelle proteomes from semi-pure
149 organelle fractions [40, 41]. Nonetheless, even the most sensitive experimental
150 reconstructions of mitoproteome content are still likely to miss low abundance proteins
151 below equipment detection thresholds that may be identifiable through *in silico* prediction
152 and green fluorescent protein localisation, such as mitochondrial-targeted RNA polymerases
153 [42].

154 Such complementary strengths and limitations afforded by *in silico* and proteomic
155 approaches may ultimately necessitate hybrid approaches, as seen in a variety of model
156 organisms that have been investigated in such a manner (**Table 1**). These should ideally be
157 grounded in well-resolved experimental proteomes in individual model species and given
158 phylogenomic context through targeted computational reconstructions in others: affording
159 higher levels of insight for tissue-specific and low-abundant mitoproteins, respectively. The
160 recent increase in experimental mitoproteome resolution across eukaryotes has allowed
161 critical insights into the genomic diversity of understudied but important protist lineages
162 [43], which has in turn provided unprecedented glimpses into mitoproteome diversity
163 across the eukaryotes, warranting a timely review of knowledge obtained so far.

164

165 **An elaborate mitoproteome in early eukaryotes?**

166 The first unicellular mitoproteome to be sequenced was that of the fungus *Saccharomyces*
167 *cerevisiae* and initially established 332 proteins [56], which was subsequently elevated to
168 749 [57], and then to 851 [58] (**Fig. 2**). However, as a model for unicellular mitochondrial
169 investigation, *S. cerevisiae* represents a non-ideal organism, having descended from a
170 multicellular ancestor itself, while additionally flocculating under appropriate stimuli.
171 Further experimental mitoproteomes have been completed for protist lineages much more
172 distantly related to animals or fungi, including the alveolates *Tetrahymena thermophila* [59]
173 and *Toxoplasma gondii* [60], the green alga *Chlamydomonas reinhardtii* [61], the
174 amoebozoan *Acanthamoeba castellanii* [46], and the stramenopile *Thalassiosira*
175 *pseudonana* [39], resolving between 325 and 709 mitoproteins each (**Figs. 1, 2**). These

176 findings must be seen in the light of the caveat that many of these organisms still lack the
177 proteomic reanalysis applied to *S. cerevisiae* and other multicellular organisms, which
178 typically improves on previous efforts through increased equipment sensitivity (**Fig. 2**).

179 Comparisons of the mitoproteomes of these distantly related unicellular eukaryotes can
180 provide clues about the mitochondrial complexity of the LECA. While it is difficult to be
181 precise about the kind of mitochondrion that was part of the LECA, given that this organism
182 existed between one and two billion years ago, the picture afforded by considering the
183 proteomes of modern mitochondria is one of surprising complexity. The LECA
184 mitoproteome presumably contained traits that are almost universally represented in its
185 descendants, e.g. oxidative phosphorylation and the TCA cycle, primary metabolic pathways
186 such as iron-sulphur cluster formation, and core biogenesis processes including protein
187 import through the TOM/TIM complexes, mitochondrial division and functional expression
188 of the mitogenome. Moreover, several traits that could have been lost from the
189 mitochondria of most eukaryotes are occasionally detected in the organelles of distantly
190 related and understudied unicellular lineages [81], such as the bacterial secretion system
191 [82] and the signal recognition particle-based machinery [83]. The sporadic distribution in
192 separate protist lineages might imply that they were indeed present in the LECA
193 mitochondrion, although alternative explanations (e.g., later instances of horizontal gene
194 transfer to unicellular recipients [16]) also need to be considered.

195

196 **Reduction, but not disappearance, of the mitoproteome in parasitic protists**

197 Parasitism as a life strategy has evolved multiple times across the eukaryotes, including in
198 several plant lineages [84], and is frequently associated with a shift towards anaerobic
199 metabolism. The streamlining effects on the genomes of parasites are well known, and
200 while most cases represent the effects of neutral evolution, some examples of loss,
201 particularly those in eukaryote parasites, have been shown to provide adaptive advantages,
202 such as energy conservation and spatial efficiency [85]. Mitochondria can likewise be
203 reduced in complexity, particularly in parasites inhabiting anaerobic environments, which
204 leads to simplified organelles of mitochondrial origin in the forms of hydrogenosomes and
205 mitosomes [86]. Their proteomes in turn reflect the extremes of mitochondrial content
206 across unicellular eukaryotes, with fewer than 150 proteins recovered from the non-
207 respiring mitosomes of the discoban *Giardia intestinalis* and the amoebozoan *Entamoeba*
208 *histolytica* [79, 87]. Hydrogenosomes, which can be seen as energy producing intermediates
209 between mitochondria and mitosomes [88], yielded an appropriately intermediate number
210 of proteins (569) within the discoban *Trichomonas vaginalis* [78] (**Fig. 2**).

211 However, parasitism is not inherently reductive for mitochondria, as illustrated by the
212 discoban parasite *Trypanosoma brucei*, from the organelle of which 1,008 proteins were
213 initially recovered [48]. This number was subsequently extended using comparative
214 knockdown studies, establishing 1,120 proteins differentially present upon ablation of the
215 mitochondrion-specific import apparatus [47] (**Fig. 2**). The dual-host lifestyle of *T. brucei* was

216 assumed to explain such organellar complexity, with an intricate, aerobically respiring
217 mitochondrion required in the tsetse fly host midgut, followed by a simplified organelle
218 present in a life stage occupying the bloodstream of the mammalian host. However,
219 profiling both mitoproteomes showed relative parity in proteins found in the insect and
220 mammalian life stages (1,197 vs. 956) [49]. Further investigations into this organism's
221 mitochondrion have established expanded respiratory complexes [71], extensive RNA
222 editing [89], a complex protein import machinery [90] and protein-rich ribosomes [91] with
223 a complex assembly pathway [92].

224

225 **Complex evolutionary histories of mitoproteomes in photosynthetic protists**

226 The specific changes undergone by mitochondria in response to the uptake of
227 photosynthetic plastids, and plastids in general, remains a mostly underexplored area.
228 Chloroplasts have been acquired many times across the eukaryotic tree through either the
229 two recorded endosymbiotic uptakes of photosynthetic bacteria (primary endosymbiosis) or
230 the numerous uptakes of eukaryotic algae (secondary, tertiary, etc. endosymbioses) [26,
231 93]. This acquisition of a photosynthetic organelle, with its associated metabolism, may
232 dramatically impact the ways in which mitochondria subsequently function. Accordingly,
233 photosynthetic eukaryotes may have highly complex mitoproteomes. These include over
234 1,000 verified proteins in the mitochondria of the plants *Arabidopsis thaliana* and *Solanum*
235 *tuberosum*, and *in silico* predictions of as many as 3,000 in *A. thaliana* [69] (**Fig. 2**). An
236 unprecedented complexity of 1,756 proteins resolved experimentally in the mitoproteome
237 of the photosynthetic discoban *Euglena gracilis* [53] further challenges naïve assumptions
238 about organellar composition within “simple” unicellular organisms. Future exploration of
239 the mitoproteomes of photosynthetic eukaryotes with large and highly diversified genomes,
240 e.g. dinoflagellates of the alveolates, may set further records in mitoproteome complexity.

241 Comparisons of the mitoproteomes of photosynthetic and non-photosynthetic eukaryotes
242 reveals an intricate dance of mitoprotein gain, loss and replacement upon the acquisition of
243 the chloroplast. Certain mitoproteins that may be widespread across non-photosynthetic
244 species seem to be lost in photosynthetic lineages because they become redundant to
245 chloroplast functions. For example, heme biosynthesis typically occurs in the mitochondria
246 of non-photosynthetic eukaryotes *via* the condensation of glycine and succinyl-CoA to
247 produce δ -aminolevulinic acid. In contrast, in photosynthetic eukaryotes δ -aminolevulinic
248 acid is typically synthesised in the chloroplast *via* glutamyl-tRNA, although exceptions are
249 known [94].

250 Other endogenous mitoproteins may be replaced by equivalents of chloroplast origin. This
251 has frequently occurred in the case of organellar aminoacyl-tRNA synthetases, which in
252 photosynthetic organisms are typically represented as single-copy enzymes that are dual-
253 targeted to the chloroplast and mitochondria, and which may derive from either the
254 mitochondrial or chloroplast ancestor [95-97] (**Fig. 3**). The outsourcing of chloroplast
255 metabolic pathways may particularly contribute to the bloated *E. gracilis* mitoproteome,
256 which includes sulphate assimilation and cysteine synthesis pathways of plastid origin [98],

257 as well as at least 185 proteins that are co-enriched from the *E. gracilis* plastid proteome
258 [99], suggesting their dual-localisation.

259

260 **Organelle-organelle crosstalk shape mitoproteome complexity**

261 The metabolic and physiological interactions between chloroplasts and mitochondria may
262 further necessitate elaborations of mitochondrial metabolism in plastid-containing
263 eukaryotes. Typically, mitochondria form close physical associations with chloroplasts [100,
264 101], even in the case of non-photosynthetic plastids, e.g. in apicomplexans [102]. In
265 photosynthetic species, mitochondria perform elaborate metabolic pathways not known in
266 non-photosynthetic relatives that are directly essential in supporting photosynthetic
267 activity, particularly the recycling of photosynthesis waste products. Key examples include
268 photo-respiration, in which 2-phospho-glycolate generated through the wasteful oxidase
269 activity of RuBisCo is recycled through mitochondrial glycine and serine intermediates [103,
270 104] (**Fig. 3**), as well as the presence of the γ -aminobutyric shunt in the TCA cycle of the *E.*
271 *gracilis* mitochondrion [53] (**Fig. 3**), which in other organisms enables metabolic conditions
272 more amenable to photosynthesis [105, 106]. An interesting example of chloroplast-
273 mitochondrion dynamics, most extensively described in diatoms but potentially widespread
274 among photosynthetic algae, is the dissipation of excess photosynthetic reducing agents
275 (e.g., NADPH) *via* mitochondrial oxidative respiration [12, 107] (**Fig. 3**). The exact
276 mechanisms by which this interaction occurs remain poorly resolved, but likely include
277 amino acid (e.g., aspartate and alanine) shunts [108] (**Fig. 3**), and potentially the diversion of
278 succinyl-CoA into lipid synthesis [109]. This latter interaction depends on the formation of a
279 metabolic intermediate, propionyl-CoA, by methylmalonyl-CoA mutase (which uses vitamin
280 B₁₂ as a co-factor), giving rise to an additional auxotrophy in the mitochondria of
281 photosynthetic species.

282 Mitochondria may even play roles in the efficient generation of primary metabolites in
283 photosynthetic species. This involves the management of storage compounds, or the
284 supplementation of the carbohydrate stores generated *via* photosynthesis and the
285 glyoxylate cycle, employed within specialised peroxisomes (termed glyoxysomes) of photo-
286 mixotrophic plants. By contrast, *E. gracilis* directly employs a glyoxylate cycle within its
287 mitochondrion for this purpose [53] (**Fig. 3**). Furthermore, mitochondria may have indirect
288 roles in facilitating photosynthetic carbon assimilation, e.g. *via* the presence of
289 mitochondria-targeted carbon concentrating machinery enzymes [110] or C₄-type
290 photosynthetic metabolism in some photosynthetic algae [111], although the exact
291 functions of these enzymes remain to be experimentally confirmed (**Fig. 3**).

292 Mitochondria are even influenced by chloroplast metabolism even after the loss of
293 photosynthesis, for example in amino acid recycling and breakdown [9, 109]. This mostly
294 under-explored aspect is brought to light through the study of the mitoproteomes of
295 secondarily non-photosynthetic algae, which may retain plastids that perform essential, but

296 photosynthesis-independent, metabolic pathways. For example, the recently completed
297 genome of the non-photosynthetic stramenopile *Nitzschia putrida* notably encodes
298 complete mitochondria-targeted urea and glyoxylate cycles, and a mitochondrial glutamate
299 shunt, which may be linked to the retention of most of its plastidial amino acid synthesis
300 pathways [112, 113]. In a further example, the secondarily non-photosynthetic stramenopile
301 *Paraphysomonas* has retained at least ten amino-acyl tRNA synthetases of apparent
302 plastidial evolutionary origin, despite the complete loss of plastid DNA from this species.
303 These tRNA-synthetases, which were presumably ancestrally dual-targeted to the
304 *Paraphysomonas* chloroplast and mitochondria, have now been relocalised to uniquely
305 support the mitoproteome [96].

306

307 **Multicellularity reveals mitochondrial heterogeneity through mass spectrometry**

308 In terms of major evolutionary developments affecting the eukaryote landscape, the
309 acquisition of further endosymbiotic organelles is joined by the development of true
310 multicellular lineages. Multicellularity is known to have arisen at least nine times
311 independently in organisms as diverse as animals, plants, fungi, seaweeds and slime moulds
312 [114] (**Fig. 1**). Truly multicellular organisms have been distinguished by their protein
313 content, but also through diversification of cell types (i.e., into different tissues and the
314 partitioning of germline and somatic cells). Much is known about the mitoproteome content
315 of model multicellular plants (*A. thaliana*) and animals (*Homo sapiens*, *Mus musculus*), for
316 which multiple proteomic surveys have been conducted [51, 54].

317 Mass spectrometry studies have identified 1,005 mitoproteins in *A. thaliana* [51], and 1,204
318 and 1,330 mitoproteins in mouse and human cells, respectively [54, 55] (**Fig. 2**). *In silico*
319 predictions for animals estimate approximately 1,500 mitoproteins to be present in animals
320 [54] against 3,000 for certain plants [69]. It remains to be determined which of these plant
321 mitochondrial innovations differ from those of unicellular photosynthetic algae [115], i.e.
322 may be connected specifically to multicellularity, alongside general photosynthesis-
323 associated mitoproteome innovations, such as the presence of glycine decarboxylase and
324 thioredoxins involved in photo-respiration [116].

325 Since their inception, mass spectrometry studies have validated that mitochondria of
326 multicellular organisms vary dramatically depending on tissue type in plants [117, 118] and
327 in animals [73, 119]. Mitochondria can even vary within the same cell type, with striated
328 muscles in mammals exhibiting two distinct forms of the organelle, characterised by
329 differences in structure, function and proteome [120, 121], similar to the heteroplastidy
330 observed in certain plants, whose proteomically distinct chloroplasts enable C4
331 photosynthesis to be performed in a single cell [122].

332 Tissue-specific mitoproteomes may arise *via* multiple mechanisms. For example, some
333 mitoproteins (e.g., glutaminase in humans) undergo alternative splicing, with different

334 splicing variants accumulating in different cells [123]. Some of these alternative splicing
335 variants may show tissue-specific localisation (e.g. glutamine synthetase, which is uniquely
336 targeted to mitochondria in liver tissue) [124]. Certain mitoproteins, such as cytochrome c in
337 many animals, may be encoded by multiple paralogous genes, with specific isoforms
338 expressed in certain tissues only [125, 126]. Most dramatically, entire mitochondrial
339 metabolic functions may be confined to a given tissue, as is the case of tobacco root cells,
340 which are capable of reducing nitric oxide, a trait absent from leaf cell mitochondria [127],
341 or the first steps of the urea cycle occurring only in liver and kidney mitochondria [72]. An
342 important part of this specialisation may be determined by different isoforms of complexes
343 involved in mitochondrial protein import, as “gatekeepers” of the entire mitoproteome. This
344 can be achieved *via* differential accumulation of common mitochondrial protein import
345 subunits in different tissues over entire developmental cycles (as observed, for example, in
346 *A. thaliana* [128]). Alternatively, expression of tissue-specific isoforms of core mitochondrial
347 import complex subunits, or tissue-specific protein import motifs, as has been observed
348 with plant chloroplasts [129, 130], may also play roles.

349 Recent investigations have illuminated which tissue types, generally, show the greatest
350 contrast in mitoproteome contents. MitoCarta, the most comprehensive organelle
351 proteomics study at a tissue-specific level, surveyed the mitochondria of 14 tissues and
352 organs, categorising 1,158 proteins across both human and mouse [72]. Furthermore, a
353 Mito-tag screen in mouse showed significant enrichment of 511 proteins in hepatocytes
354 [55], while additional Mito-tag lines allowed generation of separate mitoproteomes of
355 Purkinje cells, astrocytes and granule cells, with 5-fold enrichment of 18, 114 and 51
356 mitoproteins, respectively [131].

357 Reanalysing these mitoproteomes beyond enrichment, we can also highlight proteins
358 absent from each tissue. While it is expected that some low abundance peptide may
359 manifest as “absent” proteins, and will eventually be detected in future investigations, it
360 remains notable that at present, fewer than one third of the mouse MitoCarta proteins
361 (380/1,158) were recorded in all 14 tissues, with a variety of proteins unique to, or uniquely
362 absent from, each tissue [72] (**Fig. 4A**). These include 20 mitoproteins confined to testes,
363 pointing to specific mitochondrial requirements in male gametes such as increased energy
364 generation, and nearly 30 uniquely absent from the heart compared to all other tissue types
365 (**Fig. 4A**).

366 Such tissue-specific trends are additionally observed at a cellular level. Mito-tagged cerebral
367 tissue reveals 41, 56 and 7 Mito-Carta verified proteins that were absent in Purkinje, granule
368 and astrocyte cells, respectively [131] (**Fig. 4B**). Mito-tag-based analysis of mouse
369 hepatocytes additionally shows 177 proteins recovered solely from their mitochondria,
370 while 58 “typical” mitoproteins were absent [55] (**Fig. 4C**). It remains to be determined
371 which of them are among the key drivers in the differentiation of specific mouse tissues.

372 One of the manifest advantages arising from multicellularity has been the specialisation of
373 cell types to best suit their designated function. The corollary observation is that such an
374 important organelle as the mitochondrion has to follow a similar trajectory in multicellular
375 organisms. This is due to the fact that the key mitochondrial advantages, such as efficient
376 ATP generation and pathway redistribution, as well as sequestration of toxic metabolic by-
377 products, are hard-wired in eukaryotic cells. In the case of multicellular model organisms,
378 we indeed observe the specialisation of mitochondria through a reduction of protein
379 complexity. Proteomic variance has equally been observed in the plastids of different plant
380 tissues [132]. We thus expect this trend to be observed beyond key model organisms, in the
381 full multitude of multicellular lineages. As is so often the case, and again as illustrated
382 above, the tissue diversity of mitoproteomes has been much more extensively studied in
383 animals than in plants. However, an interesting overview of the variation in plant
384 mitochondrial composition observed for different tissues, especially in root mitochondria
385 challenged by adverse soil conditions can be found in [133].

386

387 **Mitochondrial modifications and evolutionary transitions**

388 While the mitochondrion's most important evolutionary contribution might have been to
389 enable the development of the LECA, our survey stresses subsequent, somewhat neglected
390 evolutionary developments (at least from a mitochondrial perspective). Indeed, plastid
391 acquisitions (whether following primary or secondary endosymbiosis) on the one hand, and
392 the transition to multicellularity on the other, have been supported by further organelle
393 remodelling and protein redistribution.

394 In protists, mitochondria appear to have responded to several dynamic factors. Certain
395 environmental niches and survival strategies strongly shift resources, enabling some
396 organisms to simplify their mitochondria, or to even lose them entirely, as seen in some
397 anaerobic protists [134]. However, the reasons for the apparent loss of complexity in
398 mitochondria of multicellular eukaryotes (**Fig. 2**) remain to be determined. If the LECA
399 mitochondrion was indeed complex, as seems highly likely, one might speculate that the
400 mitoproteome "splits up" across different tissues. Equally, a certain level of mitochondrial
401 functionality is presumably necessary for the evolution of multicellularity, such as aerobic
402 metabolism to allow sufficient ATP production to support complex tissues, although its long-
403 term retention in multicellular eukaryotes turns out to be non-universal [135]. Moreover,
404 mitochondria of multicellular organisms presumably should have developed more functions
405 in signalling and stress responses than their unicellular predecessors. It remains to be
406 determined what kind of mitochondrial incarnation, with regard to protein complexity,
407 capacity for ATP production, and signalling, is best suited to make the jump towards tissue-
408 specific mitochondrial function in multicellular species.

409 Finally, we note that similar "power sharing" relationships as documented between
410 mitochondria and chloroplast proteomes have also occurred with other cellular organelles,

411 including ones probably present in the LECA. For example, the mitochondrial fatty acid
412 degradation pathway (β -oxidation) has been relocated to peroxisomes either partially (e.g.
413 only the breakdown of very long-chain fatty acids in mammals) or completely, as in many
414 fungal species and in plants (in their guise of glyoxysomes) [136, 137]. This might be related
415 to overall radical oxygen species (ROS) control [138]. Further dynamic connections link the
416 mitochondria to the nucleus (e.g., iron-sulphur cluster biosynthesis and ROS signalling) and
417 endomembrane system (e.g., glycoprotein import and fatty acid synthesis) in eukaryotic
418 lineages. Probing the diversity of interactions between mitochondria and other organelles
419 across the eukaryote tree may give insights as to how different eco-physiological constraints
420 impact their co-evolution and provide new levels of understanding regarding the synthetic
421 potential of organelle proteomes working in partnership.

422

423 **Conclusions**

424 Especially the further investigation of protists holds promise to better understand the
425 evolutionary dynamics discussed in this review. The relative ease of isolating mitochondria
426 from *E. gracilis* recommends algal protists as a potentially superior alternative for the study
427 of photosynthetic trends within mitochondria, contrasting with the traditionally much less
428 tractable plant mitochondria (**Fig. 2**). The advent of next generation proteomics tools such
429 as LOPIT holds the potential to reliably separate dual-targeted proteins from mere
430 contaminants. Genome-wide localisation studies through tagging, while unwieldy and
431 expensive, are suited to investigations of unicellular organisms to identify low abundance
432 proteins currently not detected *via* mass spectrometry. While tissue-specific investigations
433 serve to catalogue multicellular specialisations, accounting for life cycle-specific
434 mitochondrial changes is underexplored amongst unicellular organisms [49]. Indeed,
435 determination of mitoproteomes in different protist life stages will allow us to functionally
436 characterise mitoproteins that undergo changes. Finally, a broader resolution of the
437 eukaryote domain will serve to highlight features likely possessed by the LECA and provide a
438 backdrop to better understand the mitochondrial specialisations coinciding with
439 multicellularity. We hope that the data and questions presented in this study will encourage
440 future endeavours within this broad space of the eukaryote domain, representing, by some
441 accounts, the large majority of its diversity [139]. This will deepen understanding of this
442 absolutely crucial organelle and its evolutionary history, as influenced by cell differentiation
443 according to body plan and organellar crosstalk.

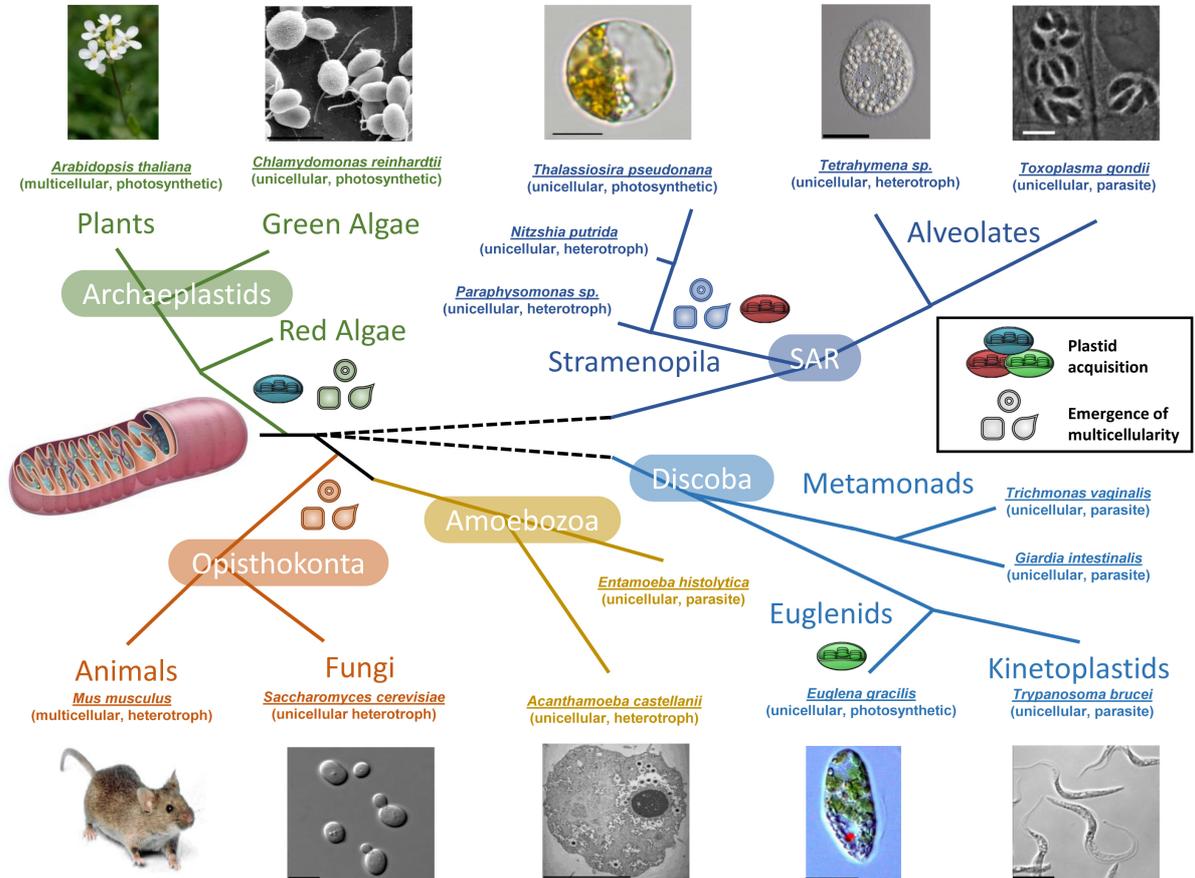
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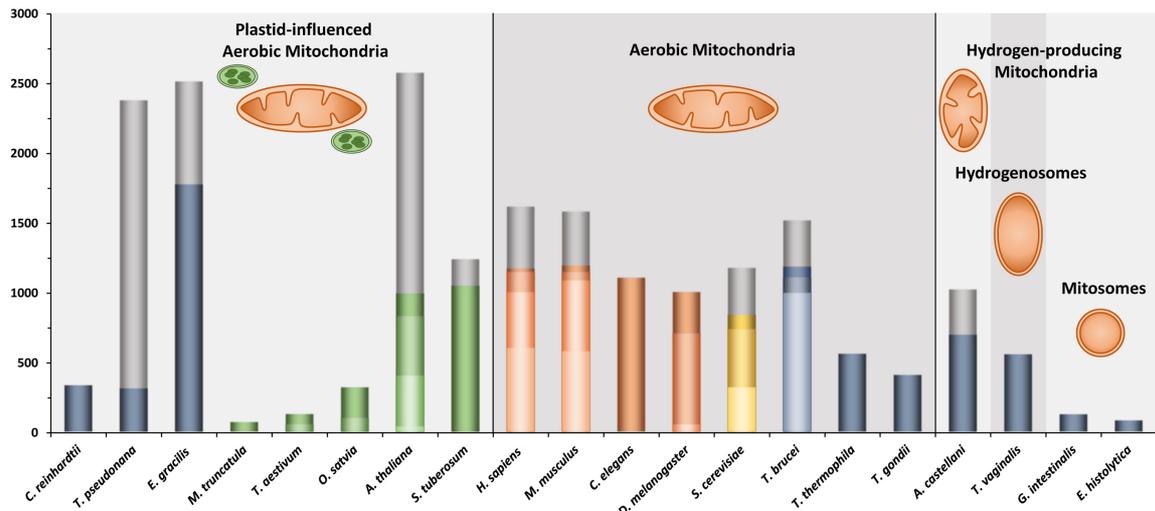
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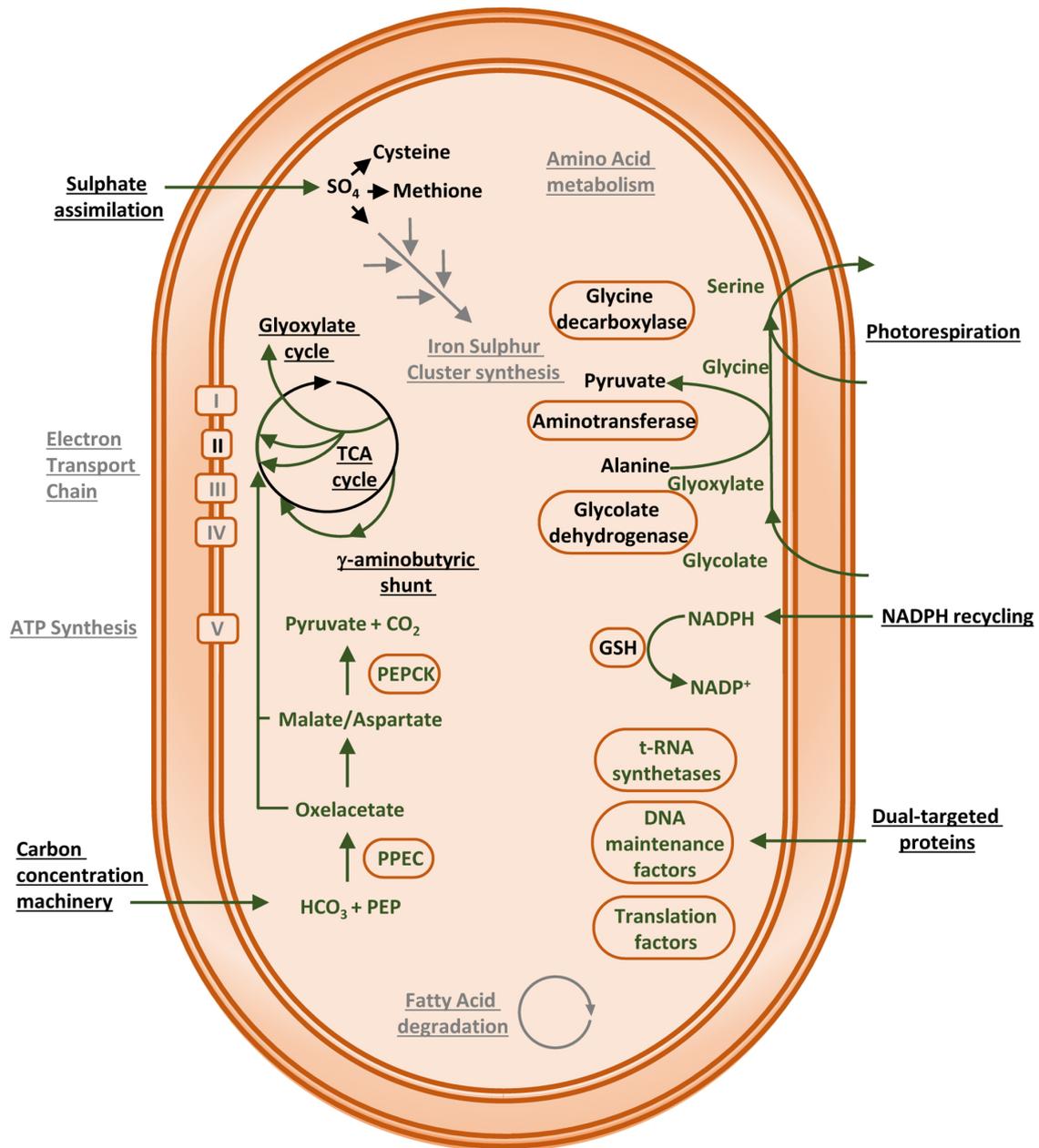
451 **Figure legends**





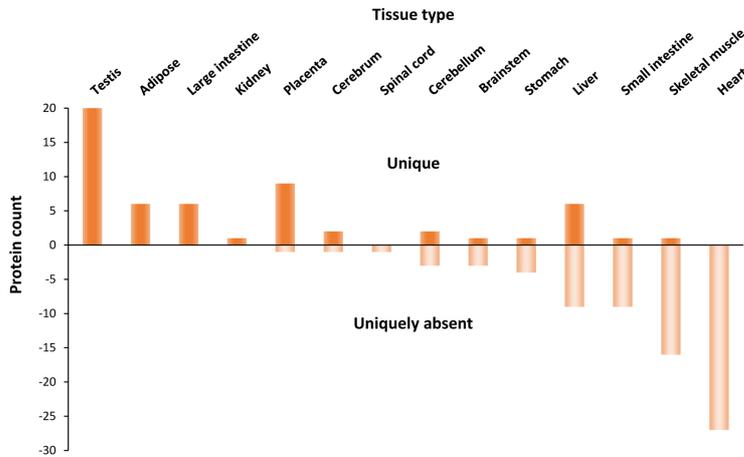
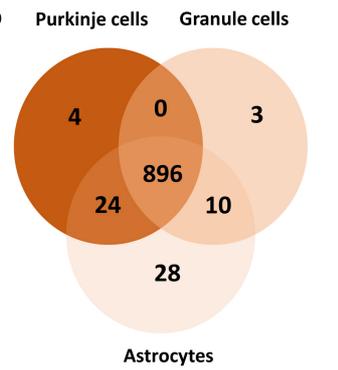
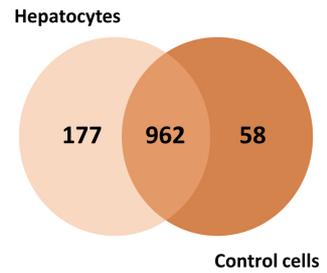
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460 **Figure 2:** Mitoproteomes (>50 proteins) of surveyed organisms including plants (green), animals
 461 (orange), fungi (gold) and protists (blue) grouped by mitochondrial form. Grey regions indicate *in*
 462 *silico* predictions based on molecular data and experimental yields. Different proteomic studies
 463 (listed in Supplementary File S1) are represented by colour shaded regions. Species include:
 464 *Thalassiosira pseudonana* [39], *Chlamydomonas reinhardtii* [61], *Euglena gracilis* [53], *Medicago*
 465 *truncatula* [62], *Triticum aestivum* [63, 64], *Oryza sativa* [65, 66], *Arabidopsis thaliana* [50, 51, 67-69],
 466 *Solanum tuberosum* [52], *Homo sapiens* [54, 70-72], *Mus musculus* [54, 71-73], *Caenorhabditis*
 467 *elegans* [74], *Drosophila melanogaster* [75-77], *Saccharomyces cerevisiae* [44, 45, 56-58],
 468 *Trypanosoma brucei* [47-49], *Tetrahymena thermophila* [59], *Toxoplasma gondii* [41, 60],
 469 *Trichomonas vaginalis* [78], *Giardia intestinalis* [79], and *Entamoeba histolytica* [80].



470
 471 **Figure 3:** Schematic of pathways and proteins found in the mitochondria of various photosynthetic
 472 organisms. Metabolites, proteins and pathways of chloroplast origin indicated in green.
 473 Core mitochondrial pathways indirectly affected by chloroplast influence indicated in grey.

474

A**B****C**

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Figure 4: Mitochondrial protein datasets generated from MitoCarta2.0 [72], showing number of proteins unique to one tissue or uniquely absent (A). Comparison of mitochondrial proteins in Mito-tagged mouse cerebral tissues [131] (B) and in mouse hepatocytes vs. controls [55] (C).

479

480 Table legends

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Table 1: Model organisms with mitoproteomes predicted in silico as well as experimentally determined within the last decade. Species listed include *Saccharomyces cerevisiae* [44, 45], *Acanthamoeba castellanii* [46], *Trypanosoma brucei* [47-49], *Arabidopsis thaliana* [50, 51], *Solanum tuberosum* [52], *Euglena gracilis* [53], *Thalassiosira pseudonana* [39], *Homo sapiens* [54] and *Mus musculus* [54, 55].

486 Contributions:

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489

JL, DS and MH conceived the manuscript. All authors provided ideas and topics of interest. MH and RD performed the research and wrote the manuscript. MH generated figures. JL and DS edited the manuscript.

490 References

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