Biol. Rev. (2021), pp. 000–000.
doi: 10.1111/brv.12794

The convoluted history of haem biosynthesis

Luděk Kořený[1]*, Miroslav Oborník[2,3], Eva Horáková[2], Ross F. Waller[1] and Julius Lukeš[2,3]

[1]*Department of Biochemistry, University of Cambridge, Hopkins Building, Tennis Court Road, Cambridge, CB2 1QW, U.K.*
[2]*Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Branišovská 31, České Budějovice (Budweis), 370 05, Czech Republic*
[3]*Faculty of Sciences, University of South Bohemia, Branišovská, České Budějovice (Budweis), 31, Czech Republic*

ABSTRACT

The capacity of haem to transfer electrons, bind diatomic gases, and catalyse various biochemical reactions makes it one of the essential biomolecules on Earth and one that was likely used by the earliest forms of cellular life. Since the description of haem biosynthesis, our understanding of this multi-step pathway has been almost exclusively derived from a handful of model organisms from narrow taxonomic contexts. Recent advances in genome sequencing and functional studies of diverse and previously neglected groups have led to discoveries of alternative routes of haem biosynthesis that deviate from the 'classical' pathway. In this review, we take an evolutionarily broad approach to illuminate the remarkable diversity and adaptability of haem synthesis, from prokaryotes to eukaryotes, showing the range of strategies that organisms employ to obtain and utilise haem. In particular, the complex evolutionary histories of eukaryotes that involve multiple endosymbioses and horizontal gene transfers are reflected in the mosaic origin of numerous metabolic pathways with haem biosynthesis being a striking case. We show how different evolutionary trajectories and distinct life strategies resulted in pronounced tensions and differences in the spatial organisation of the haem biosynthesis pathway, in some cases leading to a complete loss of a haem-synthesis capacity and, rarely, even loss of a requirement for haem altogether.

Key words: tetrapyrrole, porphyrin, metabolic pathways, evolution, eukaryogenesis, mitochondrion, chloroplast, photosynthesis, iron metabolism

CONTENTS

I. INTRODUCTION

The incredible activities and complexity of all cellular life forms are enabled by their ability to channel and transform free energy harvested from light or chemical compounds. Such transformations are largely achieved by a multitude of redox reactions in which electrons are passed from electron donors to accepting substrates. The chemical properties of transition metals make them ideal mediators in such electron transport cascades. Iron is the most utilised metal by biological systems which may be rooted in the abundance and availability of the soluble ferrous (Fe^{2+}) ions in the primordial

* Address for correspondence (Tel: +44 (0)1223 768942; E-mail: lk360@cam.ac.uk)

ocean (Lill, 2009; Ilbert & Bonnefoy, 2013). In protometabolic reaction networks iron likely served as a catalyst and played a pivotal role in the origin of life. These early iron riches lasted until the great oxygenation event approximately 2.4 billion years ago (Gumsley *et al.*, 2017) when most of the iron in the biosphere oxidised into the insoluble ferric (Fe^{3+}) form. This change required organisms to evolve more sophisticated mechanisms to continue utilising iron (Imlay, 2006) or to switch to alternative transition metals such as copper (De la Rosa *et al.*, 2002). Today iron is rarely used as a cofactor in its atomic form but instead is built into coordination complexes with sulphur, as in the case of iron–sulphur (Fe–S) clusters, or into a porphyrin ring in haem (Ilbert & Bonnefoy, 2013). Fe–S clusters are likely to have formed readily and spontaneously in the primaeval ocean (Imlay, 2006; Bonfio *et al.*, 2017). Abiotically formed haem might also have been available to the protometabolism of the earliest life forms, as suggested by recent experiments simulating conditions on primordial volcanic islands, despite the more complex structure of the tetrapyrrole ring (Lindsey, Ptaszek & Taniguchi, 2009; Pleyer, Strasdeit & Fox, 2018). The preponderance of proteins that utilise both of these iron cofactors throughout all domains of life implies that these were key components of the most recent common ancestor of Bacteria, Archaea and eukaryotes, commonly referred to as the last universal common ancestor (LUCA).

The widespread use of haem in the cellular electron transport chains lies in the ability of this molecule, when attached to various proteins, to cover a wide range of biologically relevant redox potentials (−0.4 to +0.4 V). The redox potential of a given haemoprotein, usually called a cytochrome, can be fine-tuned by several factors. These include: (*i*) the identity of the axial ligands, the amino acid groups of the apoprotein that are involved in iron coordination; (*ii*) the protein environment such as local charges and electrostatics, haem distortion, and the extent of solvent access to haem; and (*iii*) the pH of the solvent (Liu *et al.*, 2014). As well as being a constituent of the electron transport chains (e.g. in the respiratory cytochromes), haem is involved as an electron transfer component in numerous specialised redox reactions including the anabolic metabolism of fats, steroids and other secondary metabolites, and the catabolism of xenobiotics and compounds of endogenous metabolism (Schenkman & Jansson, 2003). Haem peroxidases catalyse one-electron oxidation of a variety of structurally diverse organic compounds, while haem catalases convert hydrogen peroxide into water and are therefore critical for preventing damage caused by oxidative stress (Chelikani, Fita & Loewen, 2004; Bonifacio *et al.*, 2011; Kraeva *et al.*, 2017). In addition to haem's function as an electron carrier, haem iron has the capacity to bind diatomic gases. As part of haemoglobin or myoglobin, haem transports oxygen in animals and is responsible for the red colour of their blood (Hardison, 1998; Chen, Ikeda-Saito & Shaik, 2008). It also functions as a nitric oxide sensor in signal transduction pathways, where the binding of NO to the haem-containing domain of the soluble guanylyl cyclases activates the cyclase domain (Montfort, Wales & Weichsel, 2017). Finally, haem is an important regulatory molecule reversibly binding proteins such as transcription factors and ion channels and, in so doing, modulating their functions (Hou *et al.*, 2006).

Some haem functions are universal to all life forms, as is the presence of some haem-containing proteins. However, there is a continuing debate about the timing and nature of ancestral haem utilisation and acquisition. Although horizontal gene transfer (HGT) among lineages has likely contributed to the wide distribution of some haemoproteins, several phylogenetic analyses allow us to argue that they emerged prior to the Archaea/Bacteria divergence and, thus, are proposed to represent 'pre-LUCA' enzymes (Ducluzeau & Nitschke, 2016). A contrary proposal suggests a haem-free LUCA based on fundamental differences in the ways archaea and bacteria synthesise haem (Lane & Martin, 2012; Sousa *et al.*, 2013). This is consistent with other major metabolic distinctions between the two principal branches of life, which led to a proposal that LUCA was a geochemically confined replicating entity with a complexity less than that of the free-living prokaryotes and from which Bacteria and Archaea arose in parallel (Martin & Russell, 2003; Martin, Sousa & Lane, 2014). This scenario does not exclude the possibility of LUCA utilising abiotically formed haem recruited by haemoproteins that evolved before the Archaea/Bacteria split. It is also possible that one type of haem biosynthesis is ancestral and pre-dates LUCA, while the other one is an evolutionary innovation of one of the two main branches (Ducluzeau & Nitschke, 2016).

While the origins of haem biosynthesis and utilisation remain contested, the presence of extant pathways throughout prokaryotes and eukaryotes show that haem biosynthesis has undergone extensive remodelling throughout subsequent evolutionary time. This review considers the events and processes that have led to the remarkable diversity of cellular haem pathways, starting with multiple contested origins in prokaryotes, and then focusing on eukaryotes where the flexibility of the pathways centred around this key molecule for life is best represented.

II. HAEM SYNTHESIS IN BACTERIA AND ARCHAEA: WHICH PATHWAY CAME FIRST?

Until 2010, only one haem biosynthetic pathway was known, now called the classic or the protoporphyrin-dependent (PPD) pathway. It is present in traditional models of both prokaryotes and eukaryotes, including *Escherichia coli*, yeasts, animals, and plants, and thus was presumed to be universal for all organisms capable of synthesising haem. This view of a common conserved pathway was revised following the discoveries of alternative pathways of haem biosynthesis in Archaea and Gram-positive bacteria (Dailey *et al.*, 2017). Three distinct haem biosynthesis pathways are currently known, and while they share some common steps, they differ in the entry and exit points, their molecular intermediates, and the enzymes catalysing their conversion (Fig. 1).

The universal first intermediate of haem biosynthetic pathways is 5-aminolevulinic acid (ALA). In Archaea and most bacteria, ALA is synthesised from the five-carbon skeleton of transfer RNA (tRNA)-bound glutamate (glutamyl-$tRNA^{glu}$) in the so-called C5 pathway. Glutamyl-$tRNA^{glu}$ is converted into ALA by two consecutive enzymatic steps executed by glutamyl-tRNA reductase (GTR) and glutamate 1-semialdehyde aminotransferase (GSA-AT) (Jahn, Verkamp & Söll, 1992). Glutamyl-$tRNA^{glu}$ is from the same pool as that used for protein synthesis. The involvement of tRNA and tRNA-like molecules in chemical reactions such as this is thought to be a legacy of the hypothetical 'RNA world' of self-replicating and catalytic ribonucleic acid molecules before the origin of translation and enzymatic catalysis based on proteins (Francklyn & Minajigi, 2010; Katz *et al.*, 2016). Unique amongst bacteria, α-proteobacteria use an alternative path to ALA, the so-called C4 (or Shemin) pathway. In a single enzymatic step succinyl-CoA and glycine are condensed into ALA by ALA-synthase (ALAS).

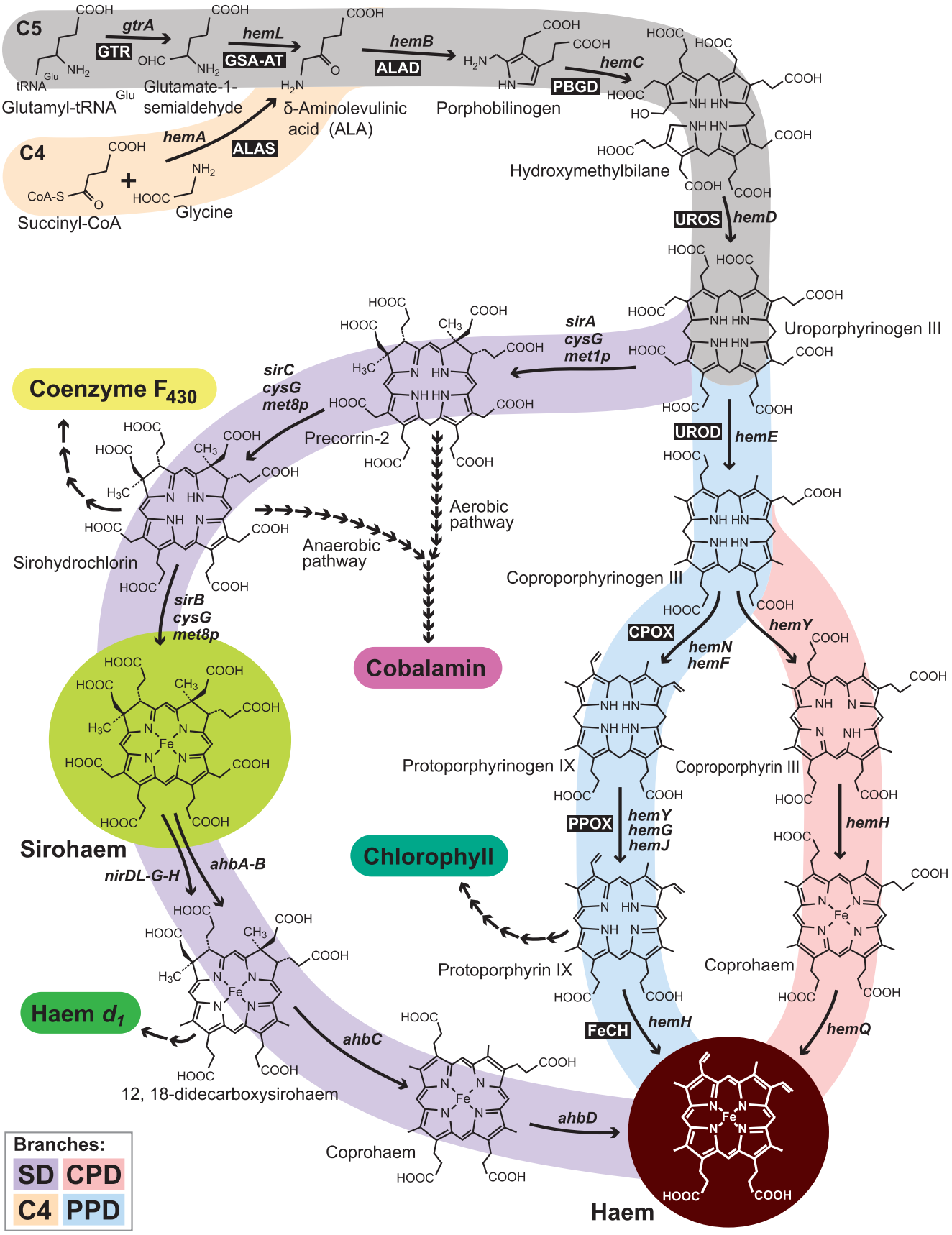
From the two entry points to ALA, C5 and C4, the next three reactions leading to the first cyclic tetrapyrrole (or porphyrin) are universal for all forms of life where they have been examined. ALA dehydratase (ALAD) condenses two molecules of ALA to produce the monopyrrole porphobilinogen (PBG), and four molecules of PBG are in turn condensed into the linear tetrapyrrole hydroxymethylbilane (HMB) by PBG deaminase (PBGD). Uroporphyrinogen-III synthase (UROS) then catalyses the closure of the linear HMB to form an asymmetric tetrapyrrole ring called uroporphyrinogen III (Uro-III). Uro-III is the last universal intermediate utilised in all types of pathways for haem synthesis, and it also serves for the syntheses of other modified tetrapyrroles such as chlorophyll, vitamin B_{12}, sirohaem and coenzyme F_{430} (Bryant, Hunter & Warren, 2020).

The originally well-characterised PPD (or classic) pathway then modifies the side chains of Uro-III by uroporphyrinogen decarboxylase (UROD) to produce coproporphyrinogen III (CPIII), which then gets decarboxylated by coproporphyrinogen oxidase (CPOX) to form protoporphyrinogen IX (PPG), subsequently converted to protoporphyrin IX (PPIX) by protoporphyrinogen oxidase (PPOX). The final reaction involves the insertion of ferrous iron into PPIX catalysed by ferrochelatase (FeCH), leading to the formation of haem (Dailey *et al.*, 2000; Layer *et al.*, 2010). In phototrophic organisms, PPIX is also used for the synthesis of chlorophylls and bacteriochlorophyll. Most steps of the PPD pathway leading from ALA to haem are catalysed by homologous enzymes in all organisms utilising this type of pathway, consistent with its single origin. Exceptions exist for the two penultimate steps where alternative enzymes of independent origins are found. HemF is an aerobic form of CPOX, whereas HemN is an anaerobic form of this enzyme. Similarly, HemG, HemY, and HemJ are all alternative versions of the PPOX enzyme (Kobayashi *et al.*, 2014).

An alternative and potentially more ancient type of bacterial haem synthesis, the coproporphyrin-dependent (CPD) pathway, was discovered through studies of the Firmicutes and Actinobacteria. Initially, both groups were thought to possess HemN of the PPD pathway; however, these were later identified as other members of the radical S-adenosyl-L-methionine (SAM) enzyme superfamily (RSS). Lack of the alternative enzyme, HemF in these groups means that none of the known CPOX enzymes are found in the Gram-positive bacteria. This absence of CPOX was accounted for by discovering another enzyme essential for haem biosynthesis in Gram-positive bacteria, HemQ, which defines the CPD haem pathway that deviates from the PPD pathway after the synthesis of CPIII (Fig. 1). Here CPIII is oxidised to coproporphyrin III, followed by the insertion of iron to make coprohaem. This iron porphyrin is then decarboxylated by HemQ to form protohaem (Dailey *et al.*, 2010). Even though the involvement of HemQ is specific for the CPD branch, the first two steps are catalysed by enzymes homologous to those found in the PPD pathway, although here they serve the synthesis of different intermediates. The oxidation of CPIII is catalysed by an enzyme homologous to the HemY form of PPOX, and the coproporphyrin ferrochelatase belongs to the same family of chelatases as the protoporphyrin ferrochelatase (HemH) used in the PPD pathway (Hansson & Hederstedt, 1994).

The third type of haem biosynthesis called alternative haem biosynthesis or the sirohaem-dependent pathway (SD), was found in Archaea, and the denitrifying and the sulphate-reducing bacteria. It follows a different path starting from Uro-III, with sirohaem as an intermediate (Fig. 1). All four enzymes (AhbA-B, C, and D) that catalyse sirohaem conversion into protohaem are specific for the SD pathway. Even though the final step of the SD pathway (coprohaem decarboxylation to protohaem) is the same as in the CPD pathway of the Gram-positive bacteria, each pathway's enzymes, AhbD and HemQ, respectively, evolved independently and there is no similarity between them (Celis & DuBois, 2015).

The SD pathway is clearly the ancestral haem biosynthetic pathway of Archaea, but its proposed origin prior to the bacterial PPD and CPD pathways and, therefore, before the Archaea/Bacteria split remains questionable (Dailey *et al.*, 2017). While this pathway is also found in the sulphate-reducing and the denitrifying bacteria, this could be explained by gene gain *via* HGT, and the SD pathway might have evolved specifically in the Archaea lineage after the split. On the other hand, an argument for the early origin of the SD branch of haem biosynthesis compared to the CPD/PPD routes is the utilisation of the SD pathway intermediates in the formation of other essential tetrapyrroles. The first intermediate of the SD synthesis from Uro-III is precorrin-2, which is also used to synthesise cobalamin (vitamin B_{12}). Cobalamin synthesis occurs in only a small subset of Bacteria and Archaea (Raux, Schubert & Warren, 2000), yet organisms across all domains of life require this cofactor for amino acid and fatty acid metabolism (Guo & Chen, 2018). In the following reaction of the SD pathway, precorrin-2 is transformed into sirohydrochlorin, which is then the substrate of sirohydrochlorin ferrochelatase in the formation of



(Figure legend continues on next page.)

Table 1. List of abbreviations

Abbreviation	Definition
ABCB	ATP binding cassette transporter B
ACO	Aconitase
ADP	Adenosine diphosphate
ALA	δ-Aminolevulinic acid
ALAD	ALA dehydratase
ALAS	ALA synthase
ATP	Adenosine triphosphate
CPD	Coproporphyrin-dependent pathway
CPIII	Coproporphyrinogen III
CPOX	Coproporphyrinogen oxidase
EGT	Endosymbiotic gene transfer
FAD	Flavin adenine dinucleotide
FECA	First eukaryotic common ancestor
FeCH	Ferrochelatase
FLVCR	Feline leukaemia virus subgroup C receptor
FUM	Fumarase
FXN	Frataxin
GSA-AT	Glutamate-1-semialdehyde aminotransferase
GTR	Glutamyl-tRNA reductase
GUN	Genomes uncoupled
HGT	Horizontal gene transfer
HMB	Hydroxymethylbilane
HRG	Heme response gene
LECA	Last eukaryotic common ancestor
LUCA	Last universal common ancestor
Mtfrn	Mitoferrin
NAD	Nicotinamide adenine dinucleotide
PBG	Porphobilinogen
PBGD	Porphobilinogen deaminase
PCD	Programmed cell death
PPD	Protoporphyrin-dependent pathway
PPG	Protoporphyrinogen IX
PPIX	Protoporphyrin IX
PPOX	Protoporphyrinogen oxidase
ROS	Reactive oxygen species
RSS	Radical SAM enzyme superfamily
SAM	S-adenosyl-L-methionine
SAR	Stramenopiles, Alveolates, Rhizaria
SD	Sirohaem dependent pathway
TCA	Tricarboxylic acid
tRNA	Transfer RNA
TIM23	Translocase of the inner membrane 23
TOM	Translocase of the outer membrane
URO	Uroporphyrinogen
UROD	Uroporphyrinogen decarboxylase
UROS	Uroporphyrinogen synthase
UroIII	Uroporphyrinogen III

sirohaem. Sirohydrochlorin is also the starting point of the newly described pathway for the synthesis of coenzyme F_{430} in the methanogenic and the methanotrophic Archaea (Zheng *et al.*, 2016; Moore *et al.*, 2017). Coenzyme F_{430} is responsible for biological methane production with an immense effect on the biosphere's carbon cycle and Earth's climate (Allen, Wegener & White, 2014). Sirohaem itself has been a crucial player in the evolution of life as a cofactor of sulphite and nitrite reductases that facilitate the biological assimilation of sulphur and nitrogen. Furthermore, sirohaem is also a precursor for the synthesis of haem d1, a cofactor of nitrite reductase catalysing a key reaction in the nitrogen cycle, which is found in many species of denitrifying bacteria (Bali *et al.*, 2011).

Some of the aforementioned tetrapyrroles that are synthesised by the SD branch have been suggested to have first appeared earlier in the evolution of life than haem, and indicate a possible ancient origin of at least part of the SD pathway compared to the CPD and PPD branches of tetrapyrrole biosynthesis (Holliday *et al.*, 2007; Bryant *et al.*, 2020). A further argument for the SD pathway's antiquity derives from the utilisation of the radical SAM chemistry for oxidation steps catalysed by RSS. These RSS enzymes function under anaerobic conditions and it was suggested that they likely evolved earlier than the oxygen-requiring enzymes of the PPD and CPD branches (Bali *et al.*, 2014). The RSS has been proposed to be an ancient protein superfamily based on its involvement in the biosynthesis of over half of the known organic cofactors with many of the reactions that RSS catalyse being fundamental to all types of life (Holliday *et al.*, 2018). However, this argument is challenged by the presence of anaerobic RSS enzymes also in the PPD pathway. While the HemF version of CPOX and the HemY version of PPOX require molecular oxygen for catalysis, the alternative RSS enzymes for these two oxidation reactions (HemN and HemG, respectively) are widely utilised by Gram-negative bacteria.

The synthesis of at least some tetrapyrroles likely pre-dates the Archaea/Bacteria split and at least part of the biosynthetic pathway, which now also serves for the synthesis of haem, was present in LUCA. However, it is possible that the later steps of the synthesis leading to haem evolved in both major groups independently. If LUCA did synthesise haem, it was more likely to do so *via* the SD pathway. In either case, bacteria have apparently evolved their own haem

(Figure legend continued from previous page.)
Fig. 1. Different tetrapyrrole biosynthetic pathways leading to haem. The first bacteria likely used the coproporphyrin-dependent (CPD) pathway, while the protoporphyrin-dependent (PPD) branch, which also serves the synthesis of chlorophyll, evolved somewhat later in Gram-negative bacteria and is also used by eukaryotes. α-proteobacteria use the C4 pathway (ALAS enzyme) to form ALA and this is found in mitochondria also. The archeal sirohaem dependent (SD) pathway forms an alternative route to haem. The branching pathways for the syntheses of other tetrapyrroles, including the number of enzymatic steps involved (arrows), are highlighted in different colours. The names of genes encoding the individual enzymes are in italics. Note that there are alternative genes for some of the reactions, and some of them evolved independently. The traditional abbreviated enzyme names are shown in black rectangles for the 'classical' pathway that involves the PPD branch. See Table 1 for a list of abbreviations.

synthesis pathways (CPD and PPD) (Dailey *et al.*, 2017). The CPD branch was proposed to be older than the PPD synthesis due to the broad distribution of HemQ among bacterial lineages, which at least in some phylogenetic studies are earlier-branching than bacteria that utilise protoporphyrin (Jun *et al.*, 2010; Lang, Darling & Eisen, 2013; Dailey *et al.*, 2015, 2017; Hug *et al.*, 2016). However, the root of the bacterial tree is still debated, and any conclusion based on the taxonomic distributions of genes is complicated by the extensive HGT events that are known to occur in bacteria.

The relationship of haem synthesis to oxygen casts further light on the evolution of the bacterial pathways. It is likely that the first bacterial pathway for haem synthesis pre-dates the formation of the oxygenic atmosphere (Ponka, 1997). Thus, similarly to the SD pathway in Archaea, the ancestral pathway of Bacteria would be expected to have been catalysed by anaerobic enzymes. Indeed, the oxygen-independent enzymes of the PPD branch of the Gram-negative bacteria (HemN and HemG) are more widespread, suggesting they evolved earlier than their counterparts that require oxygen as an electron acceptor (HemF and HemY) (Cavallaro, Decaria & Rosato, 2008; Kobayashi *et al.*, 2014). However, only the oxygen-dependent form of the enzyme catalysing the oxidation of CPIII to coproporphyrin III (HemY) was found in bacteria that utilise the presumably older CPD branch. A number of these organisms are capable of haem synthesis under anaerobic conditions, and hence the existence of a so-far unidentified anaerobic version has been suggested (Dailey *et al.*, 2017).

Nevertheless, it is also possible that bacteria were able to utilise molecular oxygen for oxygen-dependent chemical reactions (like those in the PPD and CPD pathways) much earlier in their evolution than previously thought, well before the great oxygenation event, as suggested by the recent phylogenetic study of all known oxygen-utilising and -producing enzymes (Jabłońska & Tawfik, 2021). Consistent with this, other recent studies argue that biological production of oxygen began soon after the origin of life, but it did not start to accumulate in the atmosphere until much later once the vast pools of reduced compounds on early Earth were exhausted. For instance, the time-resolved molecular evolution of the photosynthetic machinery by Oliver *et al.* (2021) points to the origin of photosynthetic water oxidation closer to LUCA. Furthermore, there are other means of biological oxygen production that occur in the dark and could pre-date the origin of oxygenic photosynthesis (Ettwig *et al.*, 2012). Interestingly, HemQ of the CPD pathway belongs to the same protein family as the oxygen-generating enzyme chlorite dismutase (Celis & DuBois, 2015). This enzyme uses haem as the cofactor to convert chlorite to chloride and molecular oxygen, while HemQ utilises coprohaem as both the substrate and the cofactor to form haem (Hofbauer *et al.*, 2014; Dailey & Gerdes, 2015). This link between haem synthesis and oxygen generation might be significant for the evolution of haem biosynthetic pathways. In addition to chlorite dismutase, other haem-containing enzymes such as catalase or the putative nitric oxide dismutase generate molecular oxygen (Kraeva *et al.*, 2017; Zhang *et al.*, 2018). Furthermore, haem in globins reversibly binds oxygen and could have facilitated oxygen-dependent chemical reactions by locally increasing oxygen concentrations in the conditions before the great oxygenation event (Traut, 2008).

The evolution of haem and oxygen metabolisms might, therefore, have been interlinked. Even today, most haem functions relate directly or indirectly to oxygen and there is relatively little use for haem in strictly anaerobic organisms (Pyrih *et al.*, 2014). As the PPD pathway is the only type of haem synthesis also used to synthesise chlorophylls, the origin of this branch coincides with the origin of oxygenic photosynthesis. The coupling of chlorophyll and haem synthesis through oxygen might have contributed to the initiation of the atmosphere's oxygenation. Furthermore, haem as a redox moiety was likely favoured in the aerobic conditions compared to the presumably more ancestral Fe–S clusters, which are very unstable in the presence of oxygen (Imlay, 2006; Boyd *et al.*, 2014). In fact, the decomposition of Fe–S clusters is one of the factors behind oxygen toxicity, and many prokaryotes abandoned some of the least stable Fe–S containing electron-transfer pathways when adapting to the aerobic habitats (Imlay, 2006; Liu *et al.*, 2014).

The last piece of the puzzle of the evolution of the tetrapyrrole synthesis in prokaryotes returns us to the start of the pathway with the C4 formation of ALA. ALAS, which combines glycine and succinyl-CoA to make ALA, seemingly originated in the ancestor of α-proteobacteria. It was suggested that ALAS evolved directly from GSA-AT, the second enzyme of the C5 pathway of ALA synthesis, as these two proteins belong to the same superfamily (Schulze *et al.*, 2006). While ALAS is relatively restricted within the prokaryotic domain, this enzyme went on to play a major role in the formation of eukaryotes and the ongoing balance of power for control of haem synthetic routes between the metabolic compartments of these complex cells.

III. HAEM SYNTHESIS IN PRIMARY HETEROTROPHIC EUKARYOTES

The complex cellular history of eukaryotes has led to further variations of the haem biosynthetic pathways, mostly through convoluted recombinations of the prokaryote-derived pathways and enzymes. It is widely accepted that all extant eukaryotes arose from a symbiotic relationship between an α-proteobacterial predecessor of mitochondria as an endosymbiont, and a pre-eukaryotic host cell related to the recently discovered Asgard group of Archaea, specifically to Heimdallarchaeota (Williams *et al.*, 2020). Several extant eukaryotic groups previously considered amitochondrial were later revealed to harbour mitochondria-derived organelles such as hydrogenosomes and mitosomes, and there is only one documented group of protists that completely lost this organelle (Karnkowska *et al.*, 2016).

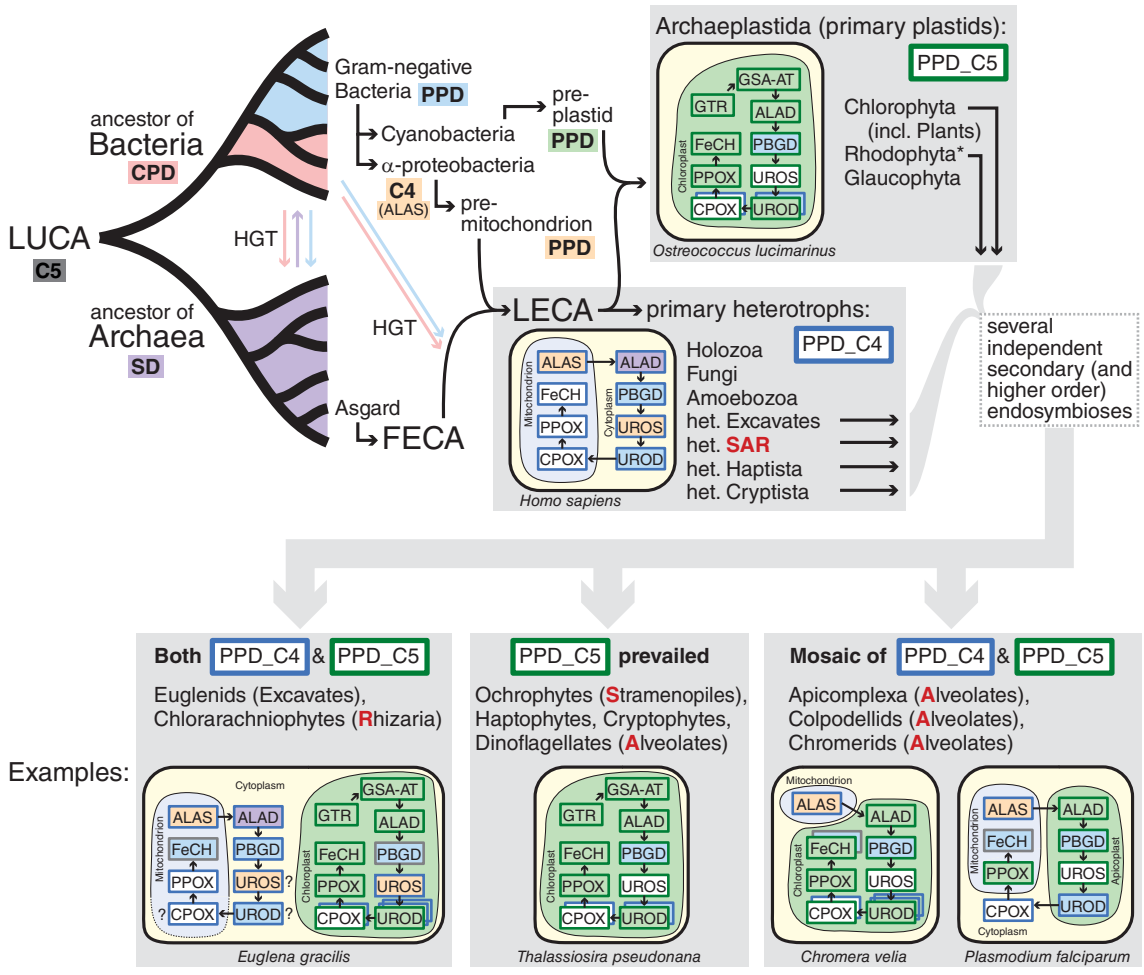


Fig. 2. Evolution of the haem biosynthetic pathways. While the type of haem biosynthesis or even its presence in LUCA is unknown, it is predicted that the ancestor of archaea used the SD-type and the first bacteria utilised the CPD pathway. The PPD branch likely evolved later in the evolution of Gram-negative bacteria, which also include the cyanobacterial predecessor of plastids and the α-proteobacterial predecessor of mitochondria. The first eukaryotes inherited ALAS (the C4 pathway) from the mitochondria while the second enzyme (ALAD) likely represents the pre-eukaryotic component shared with archaea. The other enzymes either originate *via* non-endosymbiotic HGT from various Gram-negative bacteria or their origin is not obvious from the phylogenetic reconstructions (white-filled boxes). This mosaic arrangement of the haem pathway in LECA (PPD_C4) can be found in primary heterotrophic eukaryotes across different eukaryotic supergroups. Archaeplastida inherited most of their enzymes from the cyanobacteria-derived primary plastids (PPD_C5). All other plastid-bearing eukaryotes originated from higher level endosymbiotic events that involved a heterotrophic host and an eukaryotic endosymbiont that already had a plastid. These events had three principal outcomes (schematics at the bottom of the figure). Enzyme box infill colour indicates prokaryotic source of the enzyme, and box margins indicate the ancestral state in either eukaryotes (LECA, blue) or Archaeplastida (green). White infill denote undefined origin, grey margins denote HGT from bacteria. See Table 1 for a list of abbreviations. het., heterotrophic.

The gain of the proto-mitochondrion significantly enhanced the efficiency of energy production for the host cell. It has been argued that this single key evolutionary event allowed eukaryotes to achieve cell complexity far beyond that found in prokaryotes (Lane & Martin, 2010; Lane, 2014), although this has been questioned recently (Hampl, Čepička & Eliáš, 2019). In the early phase of endosymbiosis, both partners possessed their own tetrapyrrole pathways, resulting in biochemical and genetic redundancy for haem synthesis. From the first eukaryotic common ancestor (FECA, the archaeon at the start of eukaryogenesis) to the last eukaryotic common ancestor (LECA, the fully fledged eukaryote which gave rise to all extant eukaryotic lineages), both aspects of this redundancy were resolved by losing genes and the contraction into a single biosynthetic pathway. Yet, this process of reconciliation ultimately resulted in increased complexity and diversity of the cellular pathways for haem synthesis.

The establishment of endosymbiotic associations typically entails the transfer of genetic control from the endosymbiont to the host through the process of endosymbiotic gene transfer (EGT) (Archibald, 2015). The resolution of genetic redundancy that results from EGT can either occur

by: (*i*) losing the endosymbiont's version of the gene, or (*ii*) losing the host's gene in favour of the endosymbiont-derived version (Timmis *et al.*, 2004; Kleine, Maier & Leister, 2009). The genes encoding components of the haem biosynthetic pathway are especially amenable to such EGT replacements as there are seemingly no compatibility issues with the enzymes involved, which may hamper EGT for multi-protein complexes and interactive regulatory networks. The autonomous behaviour of haem synthesis enzymes was demonstrated by experiments in yeast where the whole haem pathway was successfully replaced with the genes from either the bacterium *Escherichia coli*, the plant *Arabidopsis thaliana*, or humans (Kachroo *et al.*, 2017). Phylogenetic reconstruction of genes' evolutionary histories has been instrumental in revealing the ancestry of haem pathways enzymes – from either the endosymbiont or the host – in primary heterotrophic eukaryotes providing a picture of this pathway in the LECA. Several studies indicate that a combination of the EGT-driven gene replacements, retention of host genes, and also HGT from other bacteria have played an essential

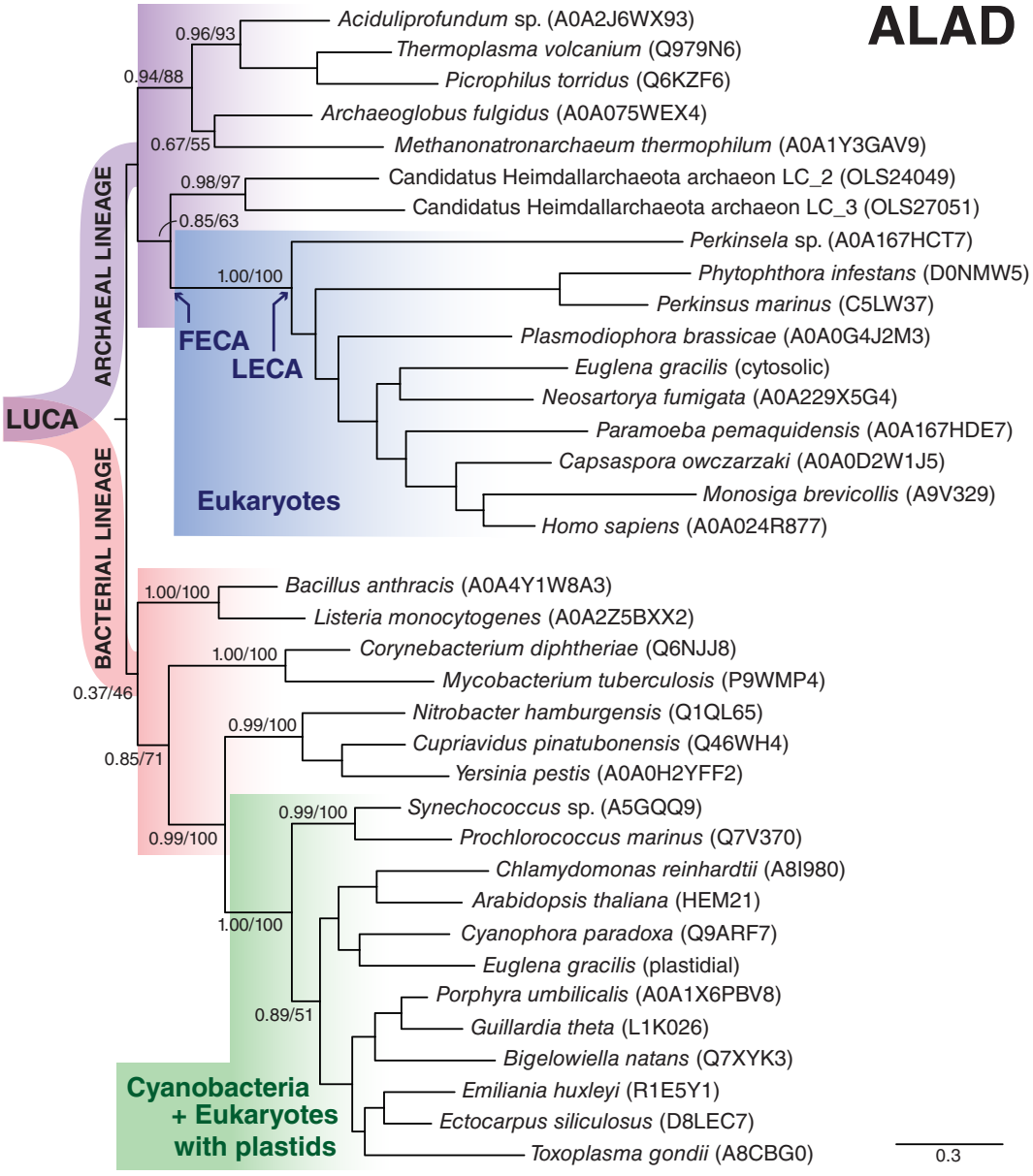


Fig. 3. Maximum likelihood phylogenetic tree of δ-aminolevulinic acid dehydratase (ALAD) protein sequences calculated in PhyML 3.1. Numbers at nodes are SH-like aLRT (Shimodaira–Hasegawa approximate likelihood-ratio test)/bootstrap (1000 iterations) branch support values and are only shown for the relevant major groups. UniProt entries for each protein sequence are shown in parentheses except for the two sequences from *Euglena gracilis*, taken from Kořený & Oborník (2011). FECA, first eukaryotic common ancestor; LECA, last eukaryotic common ancestor; LUCA, last universal common ancestor.

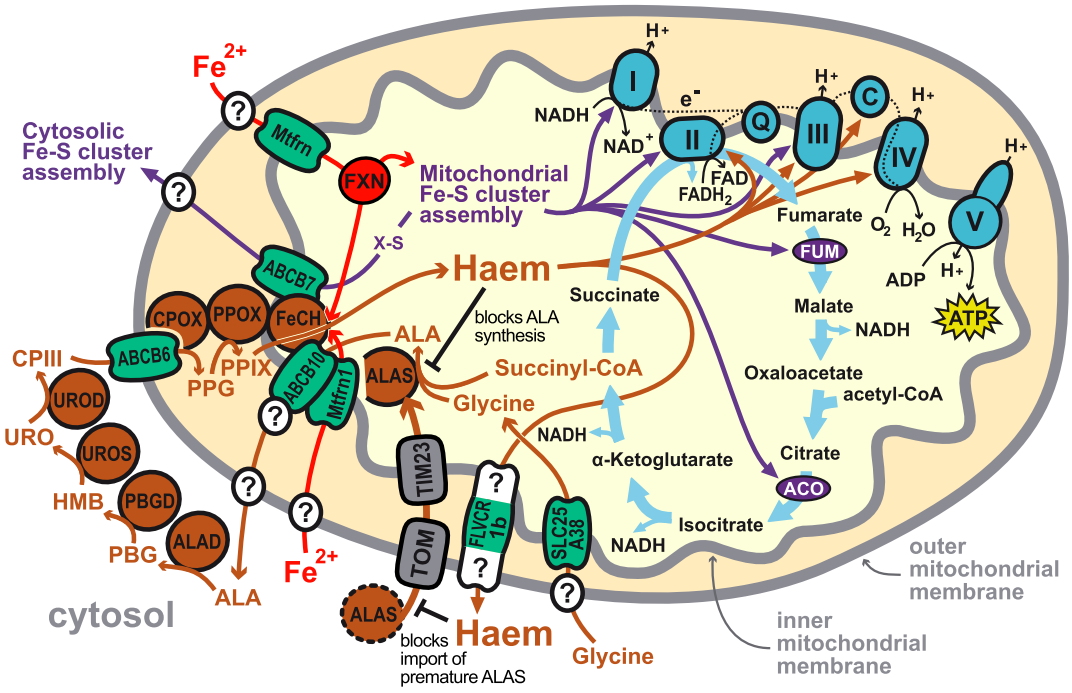


Fig. 4. Haem biosynthesis, trafficking, regulation and interaction with mitochondrial iron and energy metabolism in an animal cell as an example. ALAS catalyses the condensation of glycine with succinyl-CoA, which is the first step of haem synthesis. Import of this enzyme into the mitochondrion is inhibited by cytosolic haem which binds to its targeting pre-sequence. Haem in the mitochondrion also directly inhibits ALAS activity. Glycine must be imported into the mitochondrion for the formation of ALA which, in turn, is exported to the cytosol where the next four steps of the pathway occur. Coproporphyrinogen III (CPIII) is than imported back to the mitochondrion, where it is converted into haem in three enzymatic reactions. Haem is synthesised on the matrix side of the inner mitochondrial membrane and most of it is used for the formation of respiratory cytochromes. Haem intended for use outside of the mitochondrion is exported *via* FLVCR1b but it is yet to be identified which mitochondrial membrane this transporter localises to. ABCB10 was previously also suggested to be involved in haem export but more recent data suggest a role in an early stage of haem synthesis such as the export of ALA. This transporter interacts with mitoferrin-1 (Mtfrn1) which supplies iron specifically for the formation of haem by FeCH, another interaction partner of ABCB10. Frataxin (FXN) is an alternative iron supplier for FeCH as well as for the mitochondrial Fe–S cluster assembly. FeCH also interacts with ABCB7 which exports a yet unknown sulphur-containing compound utilised by both the mitochondrial and the cytosolic Fe–S cluster assembly pathways. FeCH might therefore be important for the regulation of the syntheses of both types of the iron-containing electron carriers that are utilised in mitochondrial respiration. The identity of some of the mitochondrial transporters is currently unknown (indicated by question marks) but the passage of iron and the small uncharged molecules across the outer mitochondrial membrane is likely allowed by the large porin channels. See Table 1 for a list of abbreviations.

role in the evolution of eukaryotic haem biosynthesis, resulting in pathways of mosaic origin (Oborník & Green, 2005) (Fig. 2). Moreover, this genetic mosaicism also established a pathway whose location spans both endosymbiont and host compartments, the boundaries of which have remained fluid through subsequent eukaryotic radiation.

The enzyme for the first step of haem synthesis in primary heterotrophic eukaryotes, ALAS of the C4 type of ALA synthesis, was likely inherited from the α-proteobacterial predecessor of the mitochondrion. Following EGT of the ALAS gene to the nucleus, the enzyme was targeted back to the organelle (Dailey, Woodruff & Dailey, 2005). Mitochondrial localisation of this enzyme makes one of its substrates, succinyl-CoA, readily available although the cosubstrate glycine has to be imported from the cytosol (Lunetti *et al.*, 2016). The origins of the subsequent genes for haem synthesis in the LECA have been less evident from earlier phylogenetic studies, but most of these were carried out before Asgard archaea's relevance to eukaryotic evolution became obvious and corresponding sequences became available. To test whether any of these genes found in extant eukaryotes might represent the host's original pre-eukaryotic genes, we analysed the haem-biosynthesis genes from the available Asgard genomes. Indeed, we found that the genes encoding ALAD (the second enzyme of the pathway) of Heimdallarchaeota and the primary heterotrophic eukaryotes, are grouped together in the phylogenetic tree (Fig. 3). ALAD is located in the cytoplasm, in accordance with its origin in the pre-eukaryotic cell. Thus, the first intermediate of the pathway, ALA, must be exported from the mitochondrion *via* an as yet unidentified transporter, although there are indications that ATP binding cassette transporter 10 (ABCB10) might be involved (Bayeva *et al.*, 2013) (Fig. 4).

The genes for the three subsequent steps that also take place in the cytosol (PBGD, UROS, and UROD) group in phylogenies with homologs of various Gram-negative bacteria pointing to a pre-LECA origin *via* HGT (Fig. 2). UROS might originate from the mitochondrion by EGT as the genes of primary heterotrophic eukaryotes are specifically related to homologs in α-proteobacteria (Kořený *et al.*, 2011; Kořený & Oborník, 2011). The genes for the last three steps (CPOX, PPOX and FeCH) also likely originate from the pre-LECA HGT from bacteria, although no specific and well-supported relationships with any particular bacterial group have been resolved for these genes (Kořený *et al.*, 2011; Kořený & Oborník, 2011). All three enzymes localise to mitochondria in mammals, with CPOX found between the inner and outer mitochondrial membranes (Figs 2 and 4) (Elder & Evans, 1978; Ferreira *et al.*, 1988; Medlock *et al.*, 2015). The ABCB6 transporter has been proposed to facilitate the transport of CPIII across the outer mitochondrial membrane (Krishnamurthy *et al.*, 2006). In yeast, on the other hand, CPOX is cytosolic, and, indeed, the yeast sequence lacks a mitochondrion-targeting N-terminal extension present in mammals (Camadro *et al.*, 1986). We found similar putative targeting extensions in the CPOX sequences of the majority of heterotrophic eukaryotes suggesting that the mitochondrial localisation is the ancestral state and that the re-location to cytosol has occurred multiple times independently (e.g. in ciliates and different lineages of fungi). Furthermore, some eukaryotes seem to have replaced this 'ancestral' pre-LECA gene with different bacterial homologs *via* subsequent HGTs. One such event occurred in choanoflagellates and amoebozoans, and others in heterotrophic euglenozoans, namely diplonemids, phagotrophic euglenids, and kinetoplastids of the genus *Perkinsela* (Cenci *et al.*, 2016; Lakey & Triemer, 2017). Most of these CPOX sequences lack the N-terminal extension suggesting cytosolic localisation of their protein products, except for some diplonemids where a relatively short extension is seen, and the localisation, therefore, remains uncertain.

Contrary to the variable localisation of CPOX, the last two steps (PPOX and FeCH) seem to be confined to the mitochondrion in all examined taxa of primary heterotrophs. These two enzymes interact with each other across the inner mitochondrial membrane, and they form a complex with ALAS and with certain mitochondrial membrane transporters (Medlock *et al.*, 2015) (Fig. 4). In a typical aerobic heterotrophic eukaryote, the majority of haem is required for the respiratory cytochromes. These electron carriers are located on the mitochondrial cristae (Pánek *et al.*, 2020) and have rapid turnover rates due to damage caused by electron leakage-induced oxidative stress (Karunadharma *et al.*, 2015). The stable organellar localisation of the two terminal enzymes of haem synthesis might be important for the effective transport and delivery of haem, specifically into the essential mitochondrial respiratory complexes. However, haem is also required in other cellular compartments and must therefore be exported out of the mitochondrion. The ABCB10 transporter of the inner mitochondrial membrane was initially suggested to be responsible for haem export but this was later disputed (Bayeva *et al.*, 2013). In a recent study, the ATPase domain of purified ABCB10 was stimulated explicitly by the haem analogue Zinc-mesoporphyrin. However, the relevance of these findings under physiological conditions and whether ABCB10 transports haem or other porphyrins remains to be investigated (Martinez *et al.*, 2020). There is currently better experimental support for the involvement of feline leukaemia virus subgroup C receptor 1b (FLVCR1b) in mitochondrial haem export (Swenson *et al.*, 2020) (Fig. 4). This is a shorter isoform of FLVCR1a which is the haem exporter localised in the plasma membrane. It is currently unknown which of the two mitochondrial membranes this transporter localises to and a co-involvement of another transporter is likely needed for haem export out of mitochondria (Fig. 4). Importantly, our knowledge of the membrane transporters of haem and the intermediates of haem synthesis relies mainly on the findings from the animal models, and different proteins might have been utilised in the other eukaryotic lineages.

The mitochondrial co-localisation of both the first and the last steps of the pathway allows a tight regulation of haem biosynthesis. This is essential as the accumulation of free haem, as well as the porphyrin intermediates, leads to the generation of reactive oxygen species (ROS) (Kumar & Bandyopadhyay, 2005). The direct inhibition of ALAS activity by haem was shown to be one of these regulatory mechanisms (Yamauchi, Hayashi & Kikuchi, 1980). Another one is the haem-induced inhibition of the import of new ALAS into the mitochondrion (Kolluri *et al.*, 2005). Also, the use of tricarboxylic acid (TCA) cycle-intermediate succinyl-CoA as a precursor for haem synthesis in heterotrophic eukaryotes couples haem biosynthesis directly with mitochondrial respiration (Fig. 4). The mitochondrial location of both the start and the end of haem synthesis makes sense for the aforementioned reasons, but the question remains why does the middle part of the synthesis occur in the cytosol? This might reflect that this was the ancestral location of the pathway. The need for mitochondrion-located regulation and haem utilisation might have required only these terminal parts of the pathway to locate to the organelle leaving the cytosolic middle portion as an evolutionary 'footprint' of the enzymes' past. Alternatively, this spatial separation might be functionally important due to the reactivity of porphyrin intermediates such as uroporphyrin and coproporphyrin that can cause organelle damage (Collin, 2019; Maitra *et al.*, 2019). Escaping electrons from the respiratory chain in the mitochondrion combined with these porphyrin intermediates could potentially lead to unmanageable dangerous levels of ROS production.

Most groups of primary heterotrophic eukaryotes show general conservation of haem biosynthesis genes and their pathways resemble that of LECA. However, there are cases of lineage-specific HGTs from various bacteria for most of these genes (as for the already mentioned CPOX). These instances typically involve free-living bacterivorous protists, that in some cases also harbour bacterial endosymbionts (e.g. in *Acanthamoeba* spp. and other free-living amoebae) and had many opportunities for further gene replacements

(Iovieno *et al.*, 2010; Keeling *et al.*, 2014; Wang & Wu, 2017). Other examples of lineage-specific gene replacements are found in jakobids, kinetoplastids, and in recently available genomic data from marine diplonemids (Cenci *et al.*, 2016; Flegontova *et al.*, 2016).

IV. HAEM SYNTHESIS IN PLASTID-BEARING EUKARYOTES

(1) Primary endosymbiosis

All eukaryotic plastids – the generic term for chloroplasts – can be traced back to a single endosymbiotic event involving the ancestor of Archaeplastida and an engulfed cyanobacterium (with the rare exception of *Paulinella* spp., see below) (Gould, Waller & Mcfadden, 2008; Archibald, 2009, 2015; Maréchal, 2018). This so-called primary plastid was vertically inherited and maintained in red and green algae, including plants, and glaucophytes, and this endosymbiosis provided a new chapter of haem biosynthesis pathway acquisition and reconfiguration.

Phylogenetic analyses revealed that the haem biosynthetic pathway of most Archaeplastida groups comprises genes that predominantly originated from the primary plastid (i.e. cyanobacterial) genome (Fig. 2). These include the C5 pathway for ALA synthesis that redefined the input molecules for haem synthesis in these eukaryotes. These plastid genes were transferred to the nucleus, and the pre-existing LECA-derived genes were mostly lost. Three genes, however, do not convincingly group with their counterparts in cyanobacteria, but phylogenies still indicate that they are specific to Archaeplastida and, therefore, the plastid of the common ancestor. For two of these genes (CPOX and UROS) poor phylogenetic resolution obscures their original source (Kořený *et al.*, 2011; Kořený & Oborník, 2011). Curiously, however, the gene for PBGD of red and green algae (including plants) groups specifically with α-proteobacteria. While this could suggest a mitochondrial origin of this gene previously acquired by the host, paradoxically, the predicted LECA PBGD was acquired from cyanobacteria by HGT, presumably before the original plastid endosymbiosis (Oborník & Green, 2005; Kořený *et al.*, 2011; Kořený & Oborník, 2011) (Fig. 2). PBGD of the glaucophyte *Cyanophora paradoxa* also groups with cyanobacteria in the phylogenetic trees but somewhat more closely compared to the PBGD of primary heterotrophs (and LECA), suggesting that glaucophytes could have acquired this gene from the chloroplast. Perhaps the ancestor of Archaeplastida had several versions of PBGD that were then independently lost in different lineages, with glaucophytes keeping the plastid-derived gene and the rest of Archaeplastida retaining the one from an α-proteobacterium (Oborník, 2021). Whether it is a mitochondrial-derived version of PBGD that persisted until the gain of primary plastid, or if this was an independent HGT from another α-proteobacterium is currently not possible to discern. However, evidence of ancestral gene persistence is seen in some members of Archaeplastida that additionally retain the original eukaryotic (pre-LECA) genes for CPOX and UROD, and, therefore, possess two variants for each (Fig. 2).

Consistently with its predicted origin, in the well-studied members of Archaeplastida such as plants and the green alga *Chlamydomonas reinhardtii*, the entire haem biosynthesis pathway occurs within the plastid (Fig. 2) (Moulin & Smith, 2005; van Lis *et al.*, 2005; Tanaka & Tanaka, 2007). Thus, the pre-existing LECA versions of CPOX and UROD must have been re-targeted into this organelle. The plastidial localisation of the tetrapyrrole pathway in phototrophs makes biological sense given that it also produces chlorophyll, the required amount of which is ∼50 times higher than that of haem in photosynthetic organisms (Papenbrock *et al.*, 1999). Furthermore, a substantial amount of the plastidial haem is also needed for the photosynthetic machinery, including cytochromes and the light-harvesting bilin pigments (Castelfranco & Jones, 1975). This shift in the intracellular distribution of tetrapyrroles, and the tendency to eliminate redundant pathways, led to the selection of the plastid as the sole site of haem synthesis, and the original eukaryotic mitochondrial–cytosolic pathway being lost. Consequently, the pool of haem needed elsewhere in the cell must be exported from the plastid, although the identity of the membrane transporters involved remains unknown. Differential demands for the plastidial and mitochondrial tetrapyrroles required the evolution of sophisticated regulatory mechanisms, including feedback loops for both haem and chlorophyll, multiple regulatory proteins and coupling to the circadian clock (Brzezowski, Richter & Grimm, 2015). The plant tetrapyrrole-binding GUN (genomes uncoupled) proteins that are involved in the regulation of both haem and chlorophyll synthesis are among the best studied systems of retrograde signalling from the plastid to the nucleus (Wu & Bock, 2021). So far, studies of the complex regulation of the tetrapyrrole biosynthesis in phototrophs are confined to a few model species (Brzezowski *et al.*, 2015).

Even though the origin and localisation of the haem pathway to the plastid are shared by the majority of Archaeplastida, there are some notable exceptions and modifications. *Rhodelphis*, a newly discovered basal branch of the Archaeplastida, is secondarily heterotrophic but still preserves a non-photosynthetic plastid where most of the haem biosynthetic enzymes are located (Gawryluk *et al.*, 2019). However, it retains the mitochondrial-localised ALA synthesis of the C4 pathway, presumably a relic of the ancestral haem pathway. The common ancestor of rhodophytes and *Rhodelphis* might have represented a transitional phase when both mitochondria and plastids still contributed to haem synthesis. Rhodophytes adopted this first step of haem synthesis as the plastid-located form, while *Rhodelphis* maintained the mitochondrion as the start point of the pathway. By extension, this suggests that C4–C5 redundancy was also present in the early chlorophytes and glaucophytes. Within rhodophytes, however, subsequent reversion of the end of the haem pathway back to the mitochondrion occurred, and this seemingly coincided with

the acquisition, *via* HGT, of a new proteobacterial gene. In the single-celled rhodophyte *Cyanidioschyzon merolae*, FeCH localises to the mitochondria (Watanabe *et al.*, 2013), consistently with a previous *in silico* prediction (Oborník & Green, 2005). Moreover, the mitochondrial localisation was also predicted for its UROD, CPOX, and PPOX (Oborník & Green, 2005; Watanabe *et al.*, 2013), although the power of current prediction tools to distinguish between the plastid and the mitochondrion in rhodophytes is relatively weak. In any case, this demonstrates the re-targeting of at least some elements of the haem biosynthesis pathway back to the mitochondrion in rhodophytes and, as for *Rhodelphis*, a biosynthesis pathway that straddles both organelles. The tight physical association of the plastid and mitochondrion in *C. merolae* (Miyagishima, Kuroiwa & Kuroiwa, 2001) might facilitate the exchange of intermediates. However, whether this dual location of haem biosynthesis is the case in other rhodophytes is currently unknown.

Unlike *Rhodelphis* and *C. merolae*, chlorophytes retain the whole pathway (starting with C5) in the plastid even after the loss of photosynthesis. This is seen in diverse chlorophyte taxa including the free-living *Prototheca wickerhamii* (Borza, Popescu & Lee, 2005), the parasitic *Polytomella* spp. and *Helicosporidium* sp. (De Koning & Keeling, 2004; Atteia, Van Lis & Beale, 2005; Smith & Lee, 2014), and was also suggested for the parasitic plants *Rafflesia* spp. (Ng *et al.*, 2018). Interestingly, *Polytomella*, and possibly also *Rafflesia*, lack the plastid genome (Molina *et al.*, 2014; Smith & Lee, 2014). This means that the tRNA for Glu cannot be transcribed in the plastid and even though its role in plastidial translation is now redundant, it is likely still required in the C5 pathway for haem biosynthesis, an explanation put forward for the baffling persistence of the plastid genome in other algae with colourless plastids (Barbrook, Howe & Purton, 2006). It follows that in the DNA-free plastids the haem pathway could only remain functional through import of the nuclear-encoded tRNA for Glu from the cytosol (Smith & Lee, 2014) although this still has to be experimentally demonstrated.

Despite the universal presence of the entire pathway in the plastids of all thus far investigated chlorophytes and plants, several of the terminal enzymes are additionally seen in the mitochondrion of some of the higher plants. The production of haem in the plant mitochondrion would particularly be relevant in the non-photosynthetic tissues, such as roots where no chlorophyll is required. In this respect, these cells are similar to the heterotrophic eukaryotes, with the last two or three steps localised in the mitochondrion. This mitochondrial branch of the pathway likely evolved together with the origin of multicellularity and tissue specialisation in plants since the related unicellular chlorophytes do not possess these additional plant-specific enzyme variants, and the entire pathway is located to the chloroplast (van Lis *et al.*, 2005). The plant mitochondrial variants of CPOX and FeCH were created by plant-specific duplications of the plastid-derived genes, whereas the second (i.e. mitochondrial) PPOX was introduced into the plant genome *via* HGT from a proteobacterium (Kořený, Lukeš & Oborník, 2010).

Some controversy about the mitochondrial localisation of the haem pathway's terminus in plants remains. While PPOX was convincingly detected in both mitochondrion and chloroplast of *Spinacia oleracea* and *Nicotiana tabacum* (Narita *et al.*, 1996; Lermontova *et al.*, 1997; Watanabe *et al.*, 2001), and there is evidence for the same dual distribution of CPOX in some plants (Williams *et al.*, 2006), the suggested confinement of one of the plant FeCH isoforms to the mitochondrion remains highly contested (Chow *et al.*, 1997, 1998; Roper & Smith, 1997; Lister *et al.*, 2001; Cornah *et al.*, 2002; Suzuki *et al.*, 2002; Masuda *et al.*, 2003; Woodson, Perez-Ruiz & Chory, 2011; Espinas *et al.*, 2016; Hey *et al.*, 2016). It is difficult, however, to imagine what the function of PPOX in the plant mitochondria would be in the absence of FeCH, other than a harmful accumulation of protoporphyrin. Alternatively, if both plant FeCH variants localise exclusively to the chloroplast as several studies suggested, perhaps another enzyme is responsible for the mitochondrial FeCH activity, e.g. the sirohydrochlorin ferrochelatase of the sirohaem biosynthesis pathway (Masuda *et al.*, 2003). Another proposed candidate for this activity supported by experimental evidence is frataxin that ordinarily provides iron during the Fe–S cluster formation (He *et al.*, 2004; Yoon & Cowan, 2004; Bencze *et al.*, 2007; Lill, 2009). Alternatively, could there be a role for PPOX in plant mitochondria not associated with haem biosynthesis? In animals and amoebae, protoporphyrin triggers programmed cell death (PCD) by disrupting mitochondrial membranes leading to the release of cytochrome *c* (Marchetti *et al.*, 1996; Arnoult *et al.*, 2001). A similar mechanism was also shown to function in plants, where PCD plays an essential role in leaf senescence (Yao *et al.*, 2004; Yao & Greenberg, 2006). Directing the haem pathway's intermediates to mitochondria, specifically in the plant tissues that are shed, could be an elegant mechanism for triggering PCD. These uncertainties associated with the mitochondrial functions in plants should be addressed by future research.

Despite the seminal role of Archaeplastida in the primary plastid gain, there is an unrelated and minor eukaryotic lineage that harbours a photosynthetic organelle acquired from a different cyanobacterium. This independent primary endosymbiosis took place much more recently (60 to 200 million years ago) in *Paulinella*, an amoeboid rhizarian protist (Marin, Nowack & Melkonian, 2005; Nowack, Melkonian & Glöckner, 2008). This so-called chromatophore resembles a free-living cyanobacterium much more than the extant Archaeplastida plastids (Lhee *et al.*, 2019). It also retains a larger genome derived from its prokaryotic progenitor, and this contains the genes for tetrapyrrole biosynthesis (Cihlář, Füssy & Oborník, 2019). The available transcriptome of *Paulinella chromatophora* (Nowack *et al.*, 2016) revealed both a chromatophore-encoded pathway and also evidence of the mitochondrial–cytosolic pathway of the host cell. Indeed, we found genes for ALAS, ALAD, PBGD, UROD, PPOX and FeCH that share higher similarity with the primary heterotrophic eukaryotes than with the chromatophore. Thus, *P. chromatophora* remains at an early stage of endosymbiotic

integration, and while there is evidence of some EGT to the nucleus (Nowack *et al.*, 2016; Nowack & Weber, 2018), the redundancy of two haem biosynthetic pathways continues.

(2) Complex endosymbioses

The constructively disruptive influences on haem biosynthetic pathways of the endosymbiotic cell mergers that resulted in mitochondria and primary plastids have continued in those eukaryotes that acquired secondary and higher-order (complex) plastid endosymbionts (Füssy & Oborník, 2018; Oborník, 2019). A wide range of photosynthetic, as well as non-photosynthetic, plastid-containing eukaryotes evolved one of the following three broad types of haem pathway reconfiguration: (*i*) retention of both the host and symbiont pathways; (*ii*) a second replacement of the host pathway by the plastid symbiont pathway; and (*iii*) further multi-location mosaics of host and symbiont steps to haem biosynthesis (Fig. 2).

Two secondary endosymbioses of green algae occurred in two unrelated groups resulting in photosynthetic euglenids (Euglenozoa) and chlorarachniophytes (Rhizaria) (Rogers *et al.*, 2007; Archibald, 2015). In both cases, seemingly complete independent pathways for haem biosynthesis have been retained in their original locations: a C4 pathway in the host mitochondrion, and a C5 pathway in the plastid, presumably serving the tetrapyrrole requirements of each compartment independently (Fig. 2) (Kořený & Oborník, 2011; Cihlář *et al.*, 2016). This redundancy is unusual and might represent an intermediate stage in the process of the endosymbiont's integration with the host. Indeed, these two endosymbioses are considered relatively recent and the chlorarachniophytes still retain a remnant of the green algal nucleus termed the nucleomorph. On the other hand, extensive EGT occurred in both protist groups with the majority of algal genes being relocated to the host nucleus and targeted back to the endosymbiont (Archibald, 2015). Hence, the lack of genetic integration is an unlikely explanation for this persistent redundancy. An alternative explanation postulates that the secondary endosymbionts are surrounded by extra membranes (three and four membranes in the plastids of euglenids and chlorarachniophytes, respectively), representing a significant barrier. The evolution of transport systems for haem across these extra membranes might be a limiting factor to a common haem source located to just a single compartment. Yet again, examples of such multi-membrane haem transport are described below for other systems, so they cannot be considered an insurmountable barrier. The puzzle of a duplicated haem pathway in these two eukaryotic groups thus remains unsolved, but it is also possible that this is simply a stable and advantageous novel configuration of haem biosynthesis.

Red algal-derived secondary, tertiary and potentially even higher-order endosymbioses are found in diatoms, kelps, haptophytes, dinoflagellates, and cryptomonads (Oborník, 2018). In these organisms, most of the original host genes for haem biosynthesis have been lost, whereas the newly acquired symbiont-derived genes were relocated into the host nucleus and their products are targeted back to the plastid. This evolutionary scenario is reminiscent of the one found in Archaeplastida, another case of the symbiont's haem pathway replacing that of the host cell. Moreover, in both systems, one or few of the host-derived enzymes (most commonly UROD) were retained, acquired the plastid-targeting N-terminal presequences, and consequently contribute to haem synthesis in the plastid (Fig. 2). Indeed, these ancestral genes are sometimes retained along with the endosymbiont-derived genes, and in some dinoflagellates, UROD from the primary symbiont (cyanobacterium), secondary symbiont (red algae) and host cell coexist (Kořený *et al.*, 2011; Cihlář *et al.*, 2016). The benefits of such a parallel arrangement and genetic mosaicism are unclear but must serve a purpose. The alga *Guillardia theta* (Cryptophyta) has also retained a host-derived FeCH which, however, likely retains its original mitochondrial localisation (Cihlář *et al.*, 2016).

Some dinoflagellates went through several rounds of endosymbioses, with the original peridinin pigment-containing plastid being replaced by new endosymbionts of diverse algal origin (Cihlář *et al.*, 2016; Waller & Kořený, 2017). *Lepidodinium* replaced the ancestral peridinin plastid with a chlorophyte-derived organelle, while *Karenia* obtained its new plastid from an engulfed haptophyte (Matsumoto *et al.*, 2011). However, unlike in other cases of a rhodophyte-derived plastid gain, in both instances, the haem biosynthesis genes obtained from the newly acquired endosymbiont have been lost and the products of the peridinin plastid-derived genes were redirected to the new organelle (Cihlář *et al.*, 2016, 2019). Another group of dinoflagellates, referred to as dinotoms, gained novel plastids *via* a tertiary endosymbiosis with diatoms. In these cases, no protein-targeting from the host to symbiont has been established, so no productive EGT has occurred. The diatom symbiont retains its plastid-located haem pathway, but so does the dinoflagellate in a residual cryptic form of the peridinin plastid. Since this plastid is no longer photosynthetic, it is likely that one of its main purposes is to provide haem for the dinoflagellate host cell. This is the only known example of an organism with two plastids of distinct endosymbiotic origin, both maintaining autonomous haem biosynthesis (Hehenberger *et al.*, 2014; Cihlář *et al.*, 2019).

In the plastid-bearing lineages, the loss of photosynthesis and conversion to obligate heterotrophy has occurred many times (Gawryluk *et al.*, 2019). The secondary heterotrophic euglenid *Euglena longa* simply eliminated the plastid-localised C5 pathway for haem and chlorophyll synthesis and produces haem solely with the mitochondrial–cytosolic C4 pathway that pre-dates the acquisition of the chloroplast. Nevertheless, *Euglena longa* still possesses a (colourless) plastid, which produces phospholipids, glycolipids, tocopherols and phylloquinone derivates and contains a linearised Calvin–Benson pathway that also includes RuBisCO (Füssy *et al.*, 2020). Other eukaryotes with complex plastids that became heterotrophic did so after the complete or partial loss of the mitochondrial–cytosolic pathway. Thus, many secondary heterotrophs such

as the diatom *Nitzschia* sp. Nitz4, the cryptophyte *Cryptomonas paramecium* and the dinoflagellate *Crypthecodinium cohnii* now depend on their plastids for haem synthesis (Hadariová *et al.*, 2018), similarly to the heterotrophic members of Archaeplastida mentioned earlier. In the free-living bacterivorous dictyochophytes *Pteridomonas* spp., haem biosynthesis remains one of the last essential functions of the plastid and the expression of the only essential gene encoding $tRNA^{glu}$ (the substrate for the C5 pathway) appears to be the sole reason for the retention of its highly reduced genome (Kayama *et al.*, 2020).

A conspicuous example of the transformation from phototrophy to heterotrophy is in the sister lineage of dinoflagellates that includes *Plasmodium* and other apicomplexan parasites, as well as their free-living relatives, the colpodellids (Fig. 2). Despite their secondarily acquired heterotrophy, these protists almost invariably retain the non-photosynthesis plastid termed the apicoplast in this group (Janouškovec *et al.*, 2019; Mathur *et al.*, 2019; Oborník, 2020). They contain the ancestral mitochondrial ALAS of the host C4 pathway, unlike their dinoflagellate relatives that use the plastid C5 pathway (Nagaraj *et al.*, 2009*a*,*b*, 2010*a*,*b*). However, this difference does not seem to be driven solely by the absence or presence of photosynthesis because their free-living photosynthetic relatives, chromerids (Füssy & Oborník, 2017), also use mitochondrial ALAS, while the rest of the tetrapyrrole pathway is predicted to take place in their plastids (Kořený *et al.*, 2011; Kořený, Oborník & Lukeš, 2013; Füssy, Faitová & Oborník, 2019). These steps are mostly composed of the plastid enzymes of endosymbiotic origin or, as for the other primary and complex plastids, UROD and CPOX variants derived from the host and re-directed to the plastid (Kořený *et al.*, 2011). While this split of the pathway between the mitochondrion and the plastid is reminiscent of *Rhodelphis* (Archaeplastida, see Section IV.1), chromerids are the only phototrophs in which the pathway starts in the mitochondrion and ends in the plastid where chlorophyll synthesis is expected to drive the major demand (Fig. 2). This must have implications for the mitochondrial metabolism as succinyl-CoA must be diverted from the TCA cycle for chlorophyll production. Moreover, the mechanisms regulating both chlorophyll and haem biosynthesis must differ from other organisms where ALA synthesis is a critical control point (Hamza & Dailey, 2012). In green algae and plants, this occurs at the C5 enzyme GTR controlled *via* a negative feedback loop from both branches of the tetrapyrrole synthesis in the chloroplast (Vavilin & Vermaas, 2002; Czarnecki & Grimm, 2012). In the primary heterotrophs, ALAS is haem-inhibited (Fig. 4), however in chromerids another haem control system must be in place to account for the separation of the start and end of the pathway (Kořený *et al.*, 2011).

In the apicomplexan parasites a further version of pathway mosaicism is seen. Here the apicoplast imports ALA from the mitochondrion and retains the next four steps of the haem biosynthetic pathway, while the last two steps are again located in the mitochondrion, preceded by the cytosolic CPOX (Fig. 2). These enzymes share the origins of the plastid-localised CPOX, PPOX and FeCH of *Chromera velia*, suggesting they have been relocated after apicomplexans lost photosynthesis (Kořený *et al.*, 2011). This was likely a response to the shift in the balance of tetrapyrrole needs, where photosynthetic pigments and cytochromes gave way to the needs of mitochondrial respiration of these new secondary heterotrophs. In an act of evolutionary poetry, through two endosymbiotic events, the circuitry of haem synthesis in apicomplexans returned to that resembling the original primary heterotrophic eukaryotic state. The only difference is that the middle part of the pathway now locates to the apicoplast instead of the cytosol. Haem biosynthesis is known to be essential in *Toxoplasma gondii* and *Plasmodium* spp., although the blood stage of *Plasmodium* is able to scavenge sufficient haem from the host-derived haemoglobin (Shanmugan *et al.*, 2010; Yeh & DeRisi, 2011; Ke *et al.*, 2014; Tjhin *et al.*, 2020). Thus, the requirement for haem synthesis that partially localises to the apicoplast is likely one of the key reasons why this organelle has been only rarely lost by apicomplexans and other eukaryotes that secondarily returned to heterotrophy (Sato *et al.*, 2004; Nagaraj *et al.*, 2009*a*; Kalanon & McFadden, 2010).

Similar to apicomplexans, many lineages of dinoflagellates are non-photosynthetic and retain a reduced plastid which still retains haem biosynthesis. Consequently, it has been argued that this metabolic function contributes to the relevance of this organelle even after this dramatic change of trophic mode (Janouškovec *et al.*, 2017). An unusual exception is the parasitic dinoflagellate *Hematodinium* sp. that still synthesises haem *via* the original eukaryotic pathway split between the mitochondrion and the cytosol (Van Dooren, Kennedy & Mcfadden, 2012). This suggests that in *Hematodinium* this pathway was yet to be lost in favour of the plastid pathway before its photosynthetic lifestyle was abandoned. Furthermore, *Hematodinium* represents one of the rare examples of plastid loss (Gornik *et al.*, 2015) and the alternative site for haem biosynthesis is likely one of the reasons that enabled it. The only legacy of the plastid's presence in this pathway is the replacement of the cytosolic UROS by the plastid-derived enzyme, analogous to some of the enzyme swapping that has occurred in other plastid-containing eukaryotes (Fig. 2), possibly pre-dating the loss of photosynthesis.

V. LOSS OF HAEM BIOSYNTHESIS IN EUKARYOTES

Some prokaryotes seem to be devoid of cytochromes and might even have no need for haem. Examples include certain methanogenic Archaea, the homoacetogenic bacteria of the class Clostridia, and some of the strict fermenters such as the Thermotogales (Sousa *et al.*, 2013; Ducluzeau & Nitschke, 2016). However, these are exceptions from the rule since most organisms require haem for a number of essential functions, although they do not necessarily have to synthesise

it themselves. The absence of *de novo* haem synthesis occurs widely in symbiotic, pathogenic, and strictly anaerobic bacteria, and the strategies for haem acquisition in the bacterial pathogens have been extensively studied (Choby & Skaar, 2016).

All extant eukaryotes descended from an ancestor that benefitted from the oxygen-dependent energy production of its mitochondrion, as well as from other types of oxidative metabolism facilitated by a plethora of haem-containing proteins (Hamza & Dailey, 2012). However, many eukaryotes have secondarily adapted to anaerobic conditions, and as a consequence have lost their dependency on (many) haemoproteins including those operating in oxidative phosphorylation. This reduced demand for haem triggered a loss of the pathway for its synthesis. All major eukaryotic groups except Archaeplastida contain at least a few anaerobic members, e.g. *Cryptosporidium* and *Gregarina* (Apicomplexa), *Giardia* and *Trichomonas* (Metamonada), *Blastocystis* (Stramenopila), *Nyctotherus* (Ciliata), *Entamoeba* (Amoebozoa), *Nosema* (Microsporidia) and many other fungi as well as a number of metazoans (Stairs, Leger & Roger, 2015).

Despite the reduced need for haem in the absence of oxygen, eukaryotic anaerobes typically possess some haemoproteins (Kořený *et al.*, 2013; Pyrih *et al.*, 2014; Rafferty & Dayer, 2015). So why is it that the adaptation to anoxic environments typically results in the loss of haem synthesis? One possible reason is that the eukaryotic PPD pathway uses oxygen-dependent enzymatic steps, namely HemF as CPOX and HemY as PPOX. Although the oxygen-independent enzymes for the same reactions exist and are widespread in bacteria, they are not common among eukaryotes (Heinemann, Jahn & Jahn, 2008). Moreover, in species where the genes for these enzymes are found, their involvement in haem biosynthesis has not been demonstrated. For example, a homolog of the anaerobic CPOX (HemN) has been studied in vertebrates, including humans. Biochemical and genetic experiments revealed that this enzyme does not retain CPOX activity and is instead proposed to function as a chaperone facilitating the insertion of haem into cytochromes (Hunt, 2006; Haskamp *et al.*, 2018). Furthermore, we are not aware of any eukaryote with genes for both oxygen-independent enzymes, HemN and HemG. While the anaerobic protists could potentially have acquired these genes from bacteria as reported for other genes for anaerobic metabolism (Stairs *et al.*, 2015), the fact that none have done this for the above enzymes suggests that there is not a strong selection pressure to do so.

Given the wide incidence of haem synthesis loss, options for scavenging haem from external sources must occur. For parasites, this might be relatively straightforward given their access to host metabolites. Indeed, even some aerobic parasites with high haem requirements for mitochondrial respiration have lost the capacity to synthesise haem *de novo* (Tripodi, Menendez Bravo & Cricco, 2011). For example, the African parasite *Trypanosoma brucei* lives in vertebrate blood and uptakes the host's haemoglobin *via* a specific receptor HpHbR (Vanhollebeke *et al.*, 2008). Paradoxically, the 'blood stage' of this parasite requires very little if any haem as it generates the majority of its ATP by glycolysis, and many haemoproteins including the respiratory cytochromes are silenced or downregulated (Zíková *et al.*, 2017). However, trypanosomes need the haemoproteins in the insect vector where their mitochondria are fully functional and employ a dedicated haem transporter, HRG (heme response gene) (Merli *et al.*, 2016; Horáková *et al.*, 2017). The tick *Boophilus microplus* also acquires high quantities of haemoglobin with its blood meal diet and lost the genes for haem synthesis. Haem auxotrophy in ticks and some other animals also requires trafficking from the site of uptake into all tissues and cells, so cell-to-cell transport of haem must occur (Lara *et al.*, 2005; Perally *et al.*, 2008; Perner *et al.*, 2016, 2019).

For other eukaryotes that lost the capacity to synthesise haem the only other source is their bacterial prey, as is the case in the free-living kinetoplastid flagellate *Bodo saltans* and the nematode *Caenorhabditis elegans* (Rao *et al.*, 2005). Alternatively, some eukaryotes harbour endosymbiotic bacteria that may supply them with haem or its precursors. This was convincingly demonstrated in several trypanosomatids that can be cultured *in vitro* without haem. However, if their β-proteobacterial endosymbionts are killed by antibiotics, haem must be supplemented in the media for the growth to continue (de Souza & Motta, 1999). The α-proteobacterial symbionts of the genus *Wolbachia* are also suggested to supply their host, the parasitic nematode *Brugia malayi*, with haem (Foster *et al.*, 2005). The amoeboflagellate *Naegleria gruberi* harbours a diverse bacterial fauna (Michel *et al.*, 2000; Fritz-Laylin *et al.*, 2010), however, as this protist also feeds on bacteria, it remains to be established from where it takes haem.

While many parasites and endosymbiont-bearing eukaryotes cannot synthesise haem *de novo*, they often retain genes for either the last one or three steps of haem synthesis. This suggests that the intermediates of this pathway might be utilised instead of haem. The trypanosomatid parasites *Herpetomonas roitmani* and *Phytomonas* spp., as well as the nematode *Brugia malayi* and the tick *Ixodes ricinus* all possess only FeCH (Alves *et al.*, 2011; Wu *et al.*, 2013), while the parasitic *Leishmania* spp. and *N. gruberi* possess genes for the last three steps. Thus, for some of the haem auxotrophs it might be easier to access the later intermediates of the synthesis rather than its final product. The location of haem intermediates might drive this, as for example, the intracellular *Leishmania* might extract the cytosolic CPIII from its vertebrate host's macrophage more easily than the mitochondrial haem (Fig. 4) (Kořený *et al.*, 2010, 2013; Laranjeira-Silva, Hamza & Pérez-Victoria, 2020). Furthermore, *Leishmania* can be cultivated with PPIX in place of haem. However, since FeCH was shown to be non-essential in *Leishmania* this parasite can also apparently access host haem directly (Sah *et al.*, 2002; Akilov *et al.*, 2007; Orrego *et al.*, 2019). In this human parasite, haem uptake has been proposed to be driven by a newly identified membrane transporter, which is a homolog of the animal plasma membrane haem importer FLVCR2 (Cabello-Donayre *et al.*, 2020). Finally, further evidence of parasites' flexible ability to utilise haem pathway intermediates comes

from secondary acquisitions of these terminal enzymes *via* HGTs from γ-proteobacteria, as is the case in *Leishmania* and *Angomonas* (Kořený *et al.*, 2010; Alves *et al.*, 2011).

The widespread persistence of FeCH as the only enzyme of the pathway in haem auxotrophs might also have alternative explanations. The free-living nematode *C. elegans* possesses a homolog that lacks the canonical FeCH activity, and it was suggested that this orphaned enzyme plays a role in membrane transport (Sinclair & Hamza, 2015). Porphyrins likely cross the outer mitochondrial membrane *via* the ABCB6 transporter (Krishnamurthy *et al.*, 2006) (Fig. 4), but it is unknown how they pass the inner membrane. In haem autotrophs, FeCH was proposed to facilitate porphyrin or haem import (Khan & Quigley, 2011). Furthermore, FeCH has been shown to form a complex with mitoferrin, as well as with two different ATP-dependent transporters, ABCB10 and ABCB7 (Chen *et al.*, 2009; Chen, Dailey & Paw, 2010; Maio *et al.*, 2019) (Fig. 4), and may have a role in modulating the stability and function of these transporters. The function of ABCB10 is not clear, but it has been suggested to be involved in exporting ALA or haem from the mitochondrion (Shirihai *et al.*, 2000; Chen *et al.*, 2010; Khan & Quigley, 2011; Bayeva *et al.*, 2013). While the molecule transported by ABCB7 is also yet to be identified, this transporter is essential for cytosolic Fe–S cluster synthesis (Lill, 2009; Horáková *et al.*, 2015). Mitoferrins deliver iron for both Fe–S cluster assembly and the final step of haem synthesis. Thus, FeCH is possibly bridging both iron pathways (Chen *et al.*, 2010) and these interactions might represent regulatory processes for iron-compound molecular transport that have been retained even after haem biosynthesis has been lost. These eukaryotes that cannot synthesise haem *de novo* yet possess the final enzyme(s) of the pathway might serve as useful experimental models for elucidating the potential additional functions of these proteins.

VI. EUKARYOTIC LIFE WITHOUT HAEM

Until quite recently, eukaryotic life without haem was not known. Some anaerobic protists possess only a handful of haemoproteins of currently unknown function. The potential non-essentiality of these proteins would render such organisms haem-independent. Where these organisms can be cultured, however, they require a medium with animal serum present so might yet be utilising exogenous haem. Moreover, the best-studied anaerobic species are obligatory parasites that may scavenge haem from their hosts (Kořený *et al.*, 2013). One of the most haemoprotein-reduced eukaryotes is the intracellular parasite *Encephalitozoon intestinalis* (Microsporidia) that has a highly reduced metabolism that relies on host nutrients and metabolites (Corradi *et al.*, 2010). It possesses a reduced respiratory chain lacking haem-containing cytochromes (Williams *et al.*, 2010) and is incapable of sterol synthesis, typically executed by haem-containing enzymes (Katinka *et al.*, 2001). Only a single known haemoprotein is encoded in their genomes, namely a member of the cytochrome b_5 family, yet its function is unknown (Kořený *et al.*, 2013). Cytochromes b_5 are ubiquitous haemoproteins that contribute to various redox enzymes, such as nitrate reductase, sulphite oxidase, or fatty acid desaturases (Schenkman & Jansson, 2003). It cannot be currently excluded that *E. intestinalis* still requires haem for this single protein.

The only eukaryote that was shown to thrive in the total absence of haem is the kinetoplastid parasite of plants *Phytomonas serpens* (Kořený *et al.*, 2012). While this is surprising for an organism in an aerobic environment, similar to the 'blood form' of African trypanosomes it lives in a sugar-rich environment and generates sufficient ATP by glycolysis (Sanchez-Moreno *et al.*, 1992). The final electron acceptor in the rudimentary mitochondrial electron transport chain is still oxygen, but the respiration generates no energy. Not even trace amounts of haem have been detected in cellular extracts of *P. serpens*, excluding the possibility of cryptic endosymbionts supplying the host cell with haem. Analysis of the draft genomes of three *Phytomonas* species also revealed the lack of most haemoproteins and the enzymes for haem biosynthesis (Porcel *et al.*, 2014). Moreover, biochemical experiments confirmed that haem plays no role in the mitochondrial respiratory chain, oxidative stress defence, or desaturation of fatty acids (Kořený *et al.*, 2012). This implies some unique metabolic adaptations that have allowed this rare bypass of all requirements for haem.

Phytomonas's haem independence demonstrates that haem is not necessarily indispensable in eukaryotes. Indeed, many processes in which haem typically partakes can be substituted with non-haem analogues. For example, many peroxidases are haemoproteins, but there are also non-haem peroxidases which use other cofactors (Bonifacio *et al.*, 2011). While eukaryotes generally require cytochrome b_5 for the desaturation of fatty acids, some bacteria and plants use ferredoxin as an electron carrier (Domergue *et al.*, 2003). Haem is a critical component of the electron transport chains of mitochondria and plastids, however, many anaerobic as well as some aerobic eukaryotes generate ATP primarily through glycolysis and hence do not need oxidative phosphorylation.

The only process that depends entirely on haem and is present in most eukaryotes is the demethylation of lanosterol during the synthesis of sterols. This reaction is catalysed by lanosterol 14α-demethylase, a member of the cytochrome P450 family, and there are no documented substitutions with an analogous non-haem enzyme (Lepesheva & Waterman, 2007). Sterols such as cholesterol in animals, ergosterol in fungi and some protists, or the plant sterols, are fundamental structural compounds of eukaryotic membranes. They are all produced by biosynthetic pathways that, while differing slightly, always involve the 14α-demethylation step (Desmond & Gribaldo, 2009). Inhibition of this activity usually results in cell death, and hence this enzyme is a popular target for anti-fungicides and antiparasitic drugs (Lamb *et al.*, 2001; Lepesheva *et al.*, 2007). The only trypanosomatid parasite shown to be resistant to these drugs was one

Leishmania species, which could incorporate lanosterol, the precursor of this enzymatic step, into its membranes (Rangel *et al.*, 1996). Similarly, studies on *Saccharomyces cerevisiae* showed that under certain circumstances, including lanosterol 14α-demethylase inhibition and decreased haem synthesis, lanosterol can function as the main membrane sterol (Gachotte *et al.*, 1997). In *P. serpens* cultured axenically without haem, lanosterol fully substitutes ergosterol in building the cellular membranes (Kořený *et al.*, 2012). Thus, for a eukaryote to uncouple its metabolism from haem a relatively complex series of adaptations are required including changes to energy dependencies, alternative enzymes, and ultimately a fundamental shift in the composition of its membranes.

VII. CONCLUSIONS

(1) The utility of haem and related tetrapyrroles has been instrumental in the development of cellular life. The need for haem for energy and redox metabolisms, as well as the importance of derivatives of its synthesis for ubiquitous coenzymes and photosynthetic pigments drove dynamic evolution of haem pathways in both prokaryotic and eukaryotic domains.

(2) The diversification of eukaryotes through multiple cellular symbioses, first between prokaryotes and later including mergers with each other, has seen ongoing reconfiguring of the originally prokaryotic pathways in an astonishing number of ways.

(3) The mitochondrion linked haem synthesis to carbon metabolism through the TCA cycle. The demands for haem in this organelle for energy metabolism drove the start, finish and regulation of its synthesis into this compartment.

(4) The gain of plastids as a second energetic compartment established a tension within the increasingly complex compartmentalisation of the eukaryotic cell with the plastid typically favoured as the new site for haem synthesis. Multiple independent gains of plastids have repeatedly tested the haem power-sharing arrangements of the cell, and a wide range of interim and stable solutions for haem control are found across eukaryotic diversity.

(5) The tensions of haem control remain evident as the secondary loss of photosynthesis often leads to the reversion to the mitochondrion-centric states.

(6) Serial endosymbioses also add to the genetic diversity of the pathway *via* gene replacement through EGT and/or HGT, further testimony to the adaptability of this pathway.

(7) Where eukaryotes can source haem or its intermediates from external sources they are frequently seen to lose most or all of its pathway for synthesis. In very rare examples a need for haem can even be nearly or completely eliminated.

(8) By broadly surveying taxonomic diversity it is clear that haem's history is highly convoluted and dynamic, and that negotiations for the control and influence of this key molecule have been at the centre of the evolution of cellular life.

VIII. ACKNOWLEDGEMENTS

This review is dedicated to the memory of Prof. Přemysl Po□ka (1941–2019), an expert in the field of iron metabolism and a friend. We thank Jan Perner (Institute of Parasitology) for comments. Czech Science Foundation grants 21-03224S, 20-07186S and 21-09283S, ERC CZ grant LL1601, and the ERD funds of the Czech Ministry of Education (CZ.02.1.01/0.0/0.0/16_019/0000759) supported this work. L.K. was supported by a Wellcome Investigator Award (214298/Z/18/Z) to R.F.W.

IX. REFERENCES

Akilov, O. E., Kosaka, S., O'Riordan, K. & Hassan, T. (2007). Parasiticidal effect of delta-aminolevulinic acid-based photodynamic therapy for cutaneous leishmaniasis is on direct and mediated through the killing of the host cells. *Experimental Dermatology* **16**, 651–660.

Allen, K. D., Wegener, G. & White, R. H. (2014). Discovery of multiple modified F (430) coenzymes in methanogens and anaerobic methanotrophic archaea suggests possible new roles for F(430) in nature. *Applied and Environmental Microbiology* **80**, 6403–6412.

Alves, J. M., Voegtly, L., Matveyev, A. V., Lara, A. M., Da Silva, F. M., Serrano, M. G., Buck, G. A., Teixeira, M. M. & Camargo, E. P. (2011). Identification and phylogenetic analysis of heme synthesis genes in trypanosomatids and their bacterial endosymbionts. *PLoS One* **6**, e23518.

Archibald, J. M. (2009). The puzzle of plastid evolution. *Current Biology* **19**, R81–R88.

Archibald, J. M. (2015). Endosymbiosis and eukaryotic cell evolution. *Current Biology* **25**, R911–R921.

Arnoult, D., Tatischeff, I., Estaquier, J., Girard, M., Sureau, F., Tissier, J. P., Grodet, A., Dellinger, M., Traincard, F., Kahn, A., Ameisen, J. C. & Petit, P. X. (2001). On the evolutionary conservation of the cell death pathway: mitochondrial release of an apoptosis-inducing factor during Dictyostelium discoideum cell death. *Molecular Biology of the Cell* **12**, 3016–3030.

Atteia, A., Van Lis, R. & Beale, S. I. (2005). Enzymes of the heme biosynthetic pathway in the nonphotosynthetic alga Polytomella sp. *Eukaryotic Cell* **12**, 2087–2097.

Bali, S., Lawrence, A. D., Lobo, S. A., Saraiva, L. M., Golding, B. T., Palmer, D. J., Howard, M. J., Ferguson, S. J. & Warren, M. J. (2011). Molecular hijacking of siroheme for the synthesis of heme and d1 heme. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 18260–18265.

Bali, S., Palmer, D. J., Schroeder, S., Ferguson, S. J. & Warren, M. J. (2014). Recent advances in the biosynthesis of modified tetrapyrroles: the discovery of an alternative pathway for the formation of heme and heme d 1. *Cellular and Molecular Life Sciences* **71**, 2837–2863.

Barbrook, A. C., Howe, C. J. & Purton, S. (2006). Why are plastid genomes retained in non-photosynthetic organisms? *Trends in Plant Science* **11**, 101–108.

Bayeva, M., Khechaduri, A., Wu, R., Burke, M. A., Wasserstrom, J. A., Singh, N., Liesa, M., Shirihai, O. S., Langer, N. B., Paw, B. H. & Ardehali, H. (2013). ATP-binding cassette B10 regulates early steps of heme synthesis. *Circulation Research* **113**, 279–287.

Bencze, K. Z., Yoon, T., Millán-Pacheco, C., Bradley, P. B., Pastor, N., Cowan, J. A. & Stemmler, T. L. (2007). Human frataxin: iron and ferrochelatase binding surface. *Chemical Communications (Cambridge, England)* **18**, 1798–1800.

Bonfio, C., Valer, L., Scintilla, S., Shah, S., Evans, D. J., Jin, L., Szostak, J. W., Sasselov, D. D., Sutherland, J. D. & Mansy, S. S. (2017). UV-light-driven prebiotic synthesis of iron-sulfur clusters. *Nature Chemistry* **9**, 1229–1234.

Bonifacio, A., Martins, M. O., Ribeiro, C. W., Fontenele, A. V., Carvalho, F. E., Margis-Pinheiro, M. & Silveira, J. A. (2011). Role of peroxidases in the compensation of cytosolic ascorbate peroxidase knockdown in rice plants under abiotic stress. *Plant, Cell & Environment* **34**, 1705–1722.

Borza, T., Popescu, C. E. & Lee, R. W. (2005). Multiple metabolic roles for the nonphotosynthetic plastid of the green alga *Prototheca wickerhamii*. *Eukaryotic Cell* **4**, 253–261.

Boyd, E. S., Thomas, K. M., Dai, Y., Boyd, J. M. & Outten, F. W. (2014). Interplay between oxygen and Fe-S cluster biogenesis: insights from the Suf pathway. *Biochemistry* **53**, 5834–5847.

Bryant, D. A., Hunter, C. N. & Warren, M. J. (2020). Biosynthesis of the modified tetrapyrroles-the pigments of life. *The Journal of Biological Chemistry* **295**, 6888–6925.

Brzezowski, P., Richter, A. S. & Grimm, B. (2015). Regulation and function of tetrapyrrole biosynthesis in plants and algae. *Biochimica et Biophysica Acta* **1847**, 968–985.

Cabello-Donayre, M., Orrego, L. M., Herráez, E., Vargas, P., Martínez-García, M., Campos-Salinas, J., Pérez-Victoria, I., Vicente, B., Marín, J. J. G. & Pérez-Victoria, J. M. (2020). Leishmania heme uptake involves LmFLVCRb, a novel porphyrin transporter essential for the parasite. *Cellular and Molecular Life Sciences: CMLS* **77**, 1827–1845.

Camadro, J. M., Chambon, H., Jolles, J. & Labbe, P. (1986). Purification and properties of coproporphyrinogen oxidase from the Yeast *Saccharomyces cerevisiae*. *European Journal of Biochemistry* **156**, 579–587.

Castelfranco, P. A. & Jones, O. T. G. (1975). Protoheme turnover and chlorophyll synthesis in greening barley tissue. *Plant Physiology* **55**, 485–490.

Cavallaro, G., Decaria, L. & Rosato, A. (2008). Genome-based analysis of heme biosynthesis and uptake in prokaryotic systems. *Journal of Proteome Research* **7**, 4946–4954.

Celis, A. I. & Dubois, J. L. (2015). Substrate, product, and cofactor: the extraordinarily flexible relationship between the CDE superfamily and heme. *Archives of Biochemistry and Biophysics* **574**, 3–17.

Cenci, U., Moog, D., Curtis, B. A., Tanifuji, G., Eme, L., Lukeš, J. & Archibald, J. M. (2016). Heme pathway evolution in kinetoplastid protists. *BMC Evolutionary Biology* **16**, 109.

Chelikani, P., Fita, I. & Loewen, P. C. (2004). Diversity of structures and properties among catalases. *Cellular and Molecular Life Sciences: CMLS* **61**, 192–208.

Chen, H., Ikeda-Saito, M. & Shaik, S. (2008). Nature of the Fe-O2 bonding in oxy-myoglobin: effect of the protein. *Journal of the American Chemical Society* **130**, 14778–14790.

Chen, W., Dailey, H. A. & Paw, B. H. (2010). Ferrochelatase forms an oligomeric complex with mitoferrin-1 and Abcb10 for erythroid heme biosynthesis. *Blood* **116**, 628–630.

Chen, W., Paradkar, P. N., Li, L., Pierce, E. L., Langer, N. B., Takahashi-Makise, N., Hyde, B. B., Shirihai, O. S., Ward, D. M., Kaplan, J. & Paw, B. H. (2009). Abcb10 physically interacts with mitoferrin-1 (Slc25a37) to enhance its stability and function in the erythroid mitochondria. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 16263–16268.

Choby, J. E. & Skaar, E. P. (2016). Heme synthesis and acquisition in bacterial pathogens. *Journal of Molecular Biology* **428**, 3408–3428.

Chow, K. S., Singh, D. P., Roper, J. M. & Smith, A. G. (1997). A single precursor protein for ferrochelatase-I from *Arabidopsis* is imported in vitro into both chloroplasts and mitochondria. *The Journal of Biological Chemistry* **272**, 27565–27571.

Chow, K. S., Singh, D. P., Walker, A. R. & Smith, A. G. (1998). Two different genes encode ferrochelatase in Arabidopsis: mapping, expression and subcellular targeting of the precursor proteins. *The Plant Journal* **15**, 531–541.

Cihlár, J., Füssy, Z., Horák, A. & Oborník, M. (2016). Evolution of the tetrapyrrole biosynthetic pathway in secondary algae: conservation, redundancy and replacement. *PLoS One* **11**, e0166338.

Cihlár, J., Füssy, Z. & Oborník, M. (2019). Evolution of tetrapyrrole pathway in eukaryotic phototrophs. *Advances in Botanical Research* **90**, 273–309.

Collin, F. (2019). Chemical basis of reactive oxygen species reactivity and involvement in neurodegenerative diseases. *International Journal of Molecular Sciences* **20**, E2407.

Cornah, J. E., Roper, J. M., Pal Singh, D. & Smith, A. G. (2002). Measurement of ferrochelatase activity using a novel assay suggests that plastids are the major site of haem biosynthesis in both photosynthetic and nonphotosynthetic cells of pea (*Pisum sativum* L.). *The Biochemical Journal* **362**, 423–432.

Corradi, N., Pombert, J. F., Farinelli, L., Didier, E. S. & Keeling, P. J. (2010). The complete sequence of the smallest known nuclear genome from the microsporidian *Encephalitozoon intestinalis*. *Nature Communications* **1**, 77.

Czarnecki, O. & Grimm, B. (2012). Post-translational control of tetrapyrrole biosynthesis in plants, algae, and cyanobacteria. *Journal of Experimental Botany* **63**, 1675–1687.

Dailey, H. A., Dailey, T. A., Gerdes, S., Jahn, D., Jahn, M., O'brian, M. R. & Warren, M. J. (2017). Prokaryotic heme biosynthesis: multiple pathways to a common essential product. *Microbiology and Molecular Biology Reviews* **81**, e00048–e00016.

Dailey, H. A., Dailey, T. A., Wu, C. K., Medlock, A. E., Wang, K. F., Rose, J. P. & Wang, B. C. (2000). Ferrochelatase at the millennium: structures, mechanisms and [2Fe-2S] clusters. *Cellular and Molecular Life Sciences: CMLS* **57**, 1909–1926.

Dailey, H. A. & Gerdes, S. (2015). HemQ: an iron-coproporphyrin oxidative decarboxylase for protoheme synthesis in Firmicutes and Actinobacteria. *Archives of Biochemistry and Biophysics* **574**, 27–35.

Dailey, H. A., Gerdes, S., Dailey, T. A., Burch, J. S. & Phillips, J. D. (2015). Noncanonical coproporphyrin-dependent bacterial heme biosynthesis pathway that does not use protoporphyrin. *Proceedings of the National Academy of Sciences of the United States of America* **112**, 2210–2215.

Dailey, T. A., Boynton, T. O., Albetel, A. N., Gerdes, S., Johnson, M. K. & Dailey, H. A. (2010). Discovery and characterization of HemQ: an essential heme biosynthetic pathway component. *The Journal of Biological Chemistry* **285**, 25978–25986.

Dailey, T. A., Woodruff, J. H. & Dailey, H. A. (2005). Examination of mitochondrial protein targeting of haem synthetic enzymes: in vivo identification of three functional haem-responsive motifs in 5-aminolevulinate synthase. *The Biochemical Journal* **386**, 381–386.

De Koning, A. P. & Keeling, P. J. (2004). Nucleus-encoded genes for plastid-targeted proteins in *Helicosporidium*: functional diversity of a cryptic plastid in a parasitic alga. *Eukaryotic Cell* **5**, 1198–1205.

De La Rosa, M. A., Navarro, J. A., Díaz-Quintana, A., De La Cerda, B., Molina-Heredia, F. P., Balme, A., Murdoch Pdel, S., Díaz-Moreno, I., Durán, R. V. & Hervás, M. (2002). An evolutionary analysis of the reaction mechanisms of photosystem I reduction by cytochrome c_6 and plastocyanin. *Bioelectrochemistry* **55**, 41–45.

De Souza, W. & Motta, M. C. (1999). Endosymbiosis in protozoa of the Trypanosomatidae family. *FEMS Microbiology Letters* **173**, 1–8.

Desmond, E. & Gribaldo, S. (2009). Phylogenomics of sterol synthesis: insights into the origin, evolution, and diversity of a key eukaryotic feature. *Genome Biology and Evolution* **1**, 364–381.

Domergue, F., Spiekermann, P., Lerchl, J., Beckmann, C., Kilian, O., Kroth, P. G., Boland, W., Zähringer, U. & Heinz, E. (2003). New insight into *Phaeodactylum tricornutum* fatty acid metabolism. Cloning and functional characterization of plastidial and microsomal delta12-fatty acid desaturases. *Plant Physiology* **131**, 1648–1660.

Ducluzeau, A. L. & Nitschke, W. (2016). When did hemes enter the scene of life? On the natural history of heme cofactors and heme-containing enzymes. In *Cytochrome Complexes: Evolution, Structures, Energy Transduction, and Signaling*. Advances in Photosynthesis and Respiration (Volume **41**, eds W. A. Cramer and T. Kallas), pp. 13–24. Springer, Dordrecht.

Elder, G. H. & Evans, J. O. (1978). Evidence that the coproporphyrinogen oxidase activity of rat liver is situated in the intermembrane space of mitochondria. *The Biochemical Journal* **172**, 345–347.

Espinas, N. A., Kobayashi, K., Sato, Y., Mochizuki, N., Takahashi, K., Tanaka, R. & Masuda, T. (2016). Allocation of heme is differentially regulated by ferrochelatase isoforms in *Arabidopsis* cells. *Frontiers in Plant Science* **7**, 1326.

Ettwig, K. F., Speth, D. R., Reimann, J., Wu, M. L., Jetten, M. S. & Keltjens, J. T. (2012). Bacterial oxygen production in the dark. *Frontiers in Microbiology* **3**, 273.

Ferreira, G. C., Andrew, T. L., Karr, S. W. & Dailey, H. A. (1988). Organization of the terminal two enzymes of the haem biosynthetic pathway: orientation of protoporphyrinogen oxidase and evidence for a membrane complex. *The Journal of Biological Chemistry* **263**, 3835–3839.

Flegontova, O., Flegontov, P., Malviya, S., Audic, S., Wincker, P., De Vargas, C., Bowler, C., Lukeš, J. & Horák, A. (2016). Extreme diversity of diplonemid eukaryotes in the ocean. *Current Biology* **26**, 3060–3065.

Foster, J., Ganatra, M., Kamal, I., Ware, J., Makarova, K., Ivanova, N., Bhattacharyya, A., Kapatral, V., Kumar, S., Posfai, J., Vincze, T., Ingram, J., Moran, L., Lapidus, A., Omelchenko, M., et al. (2005). The *Wolbachia* genome of *Brugia malayi*: endosymbiont evolution within a human pathogenic nematode. *PLoS Biology* **3**, e121.

Francklyn, C. S. & Minajigi, A. (2010). tRNA as an active chemical scaffold for diverse chemical transformations. *FEBS Letters* **584**, 366–375.

Fritz-Laylin, L. K., Prochnik, S. E., Ginger, M. L., Dacks, J. B., Carpenter, M. L., Field, M. C., Kuo, A., Paredez, A., Chapman, J., Pham, J., Shu, S., Neupane, R., Cipriano, M., Mancuso, J., Tu, H., et al. (2010). The genome of *Naegleria gruberi* illuminates early eukaryotic versatility. *Cell* **140**, 631–642.

Füssy, Z., Faitová, T. & Oborník, M. (2019). Subcellular compartments interplay for carbon and nitrogen allocation in *Chromera velia* and *Vitrella brassicaformis*. *Genome Biology and Evolution* **11**, 1765–1779.

Füssy, Z. & Oborník, M. (2017). Chromerids and their plastids. *Advances in Botanical Research* **84**, 187–218.

Füssy, Z. & Oborník, M. (2018). Complex endosymbioses I: from primary to complex plastids, multiple independent events. In *Methods in Molecular Biology* (Volume **1829**, ed. E. Maréchal), pp. 17–35. Humana Press, New York.

Füssy, Z., Záhonová, K., Tomčala, A., Krajčovič, J., Yurchenko, V., Oborník, M. & Eliáš, M. (2020). The cryptic plastid of *Euglena longa* defines a new type of nonphotosynthetic plastid organelle. *mSphere* **5**, e00675–e00620.

Gachotte, D., Pierson, C. A., Lees, N. D., Barbuch, R., Koegel, C. & Bard, M. (1997). A yeast sterol auxotroph (erg25) is rescued by addition of azole antifungals and reduced levels of heme. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 11173–11178.

Gawryluk, R. M. R., Tikhonenkov, D. V., Hehenberger, E., Husnik, F., Mylnikov, A. P. & Keeling, P. J. (2019). Non-photosynthetic predators are sister to red algae. *Nature* **572**, 240–243.

Gornik, S. G., Febrimarsa, Cassin, A. M., Macrae, J. I., Ramaprasad, A., Rchiad, Z., Mcconville, M. J., Bacic, A., Mcfadden, G. I., Pain, A. & Waller, R. F. (2015). Endosymbiosis undone by stepwise elimination of the plastid in a parasitic dinoflagellate. *Proceedings of the National Academy of Sciences of the United States of America* **112**, 5767–5772.

Gould, S. B., Waller, R. F. & Mcfadden, G. I. (2008). Plastid evolution. *Annual Review of Plant Biology* **59**, 491–517.

Gumsley, A. P., Chamberlain, K. R., Bleeker, W., Söderlund, U., De Kock, M. O., Larsson, E. R. & Bekker, A. (2017). Timing and tempo of the Great Oxidation Event. *Proceedings of the National Academy of Sciences of the United States of America* **114**, 1811–1816.

Guo, M. & Chen, Y. (2018). Coenzyme cobalamin: biosynthesis, overproduction and its application in dehalogenation—a review. *Reviews in Environmental Science and Biotechnology* **17**, 259–284.

Hadariová, L., Vesteg, M., Hampl, V. & Krajčovič, J. (2018). Reductive evolution of chloroplasts in non-photosynthetic plants, algae and protists. *Current Genetics* **64**, 365–387.

Hampl, V., Čepička, I. & Eliáš, M. (2019). Was the mitochondrion necessary to start eukaryogenesis? *Trends in Microbiology* **27**, 96–104.

Hamza, I. & Dailey, H. A. (2012). One ring to rule them all: trafficking of heme and heme synthesis intermediates in the metazoans. *Biochimica et Biophysica Acta* **1823**, 1617–1632.

Hansson, M. & Hederstedt, L. (1994). *Bacillus subtilis* HemY is a peripheral membrane protein essential for protoheme IX synthesis which can oxidize coproporphyrinogen III and protoporphyrinogen IX. *Journal of Bacteriology* **176**, 5962–5970.

Hardison, R. (1998). Hemoglobins from bacteria to man: evolution of different patterns of gene expression. *The Journal of Experimental Biology* **8**, 1099–1117.

Haskamp, V., Karrie, S., Mingers, T., Barthels, S., Alberge, F., Magalon, A., Müller, K., Bill, E., Lubitz, W., Kleeberg, K., Schweyen, P., Bröring, M., Jahn, M. & Jahn, D. (2018). The radical SAM protein HemW is a heme chaperone. *Journal of Biological Chemistry* **293**, 2558–2572.

He, Y., Alam, S. L., Proteasa, S. V., Zhang, Y., Lesuisse, E., Dancis, A. & Stemmler, T. L. (2004). Yeast frataxin solution structure, iron binding, and ferrochelatase interaction. *Biochemistry* **43**, 16254–16262.

Hehenberger, E., Imanian, B., Burki, F. & Keeling, P. J. (2014). Evidence for the retention of two evolutionary distinct plastids in dinoflagellates with diatom endosymbionts. *Genome Biology and Evolution* **6**, 2321–2334.

Heinemann, I. U., Jahn, M. & Jahn, D. (2008). The biochemistry of heme biosynthesis. *Archives of Biochemistry and Biophysics* **474**, 238–251.

Hey, D., Ortega-Rodes, P., Fan, T., Schnurrer, F., Brings, L., Hedtke, B. & Grimm, B. (2016). Transgenic tobacco lines expressing sense or antisense ferrochelatase 1 RNA show modified ferrochelatase activity in roots and provide experimental evidence for dual localization of ferrochelatase 1. *Plant & Cell Physiology* **57**, 2576–2585.

Hofbauer, S., Gysel, K., Bellei, M., Hagmüller, A., Schaffner, I., Mlynek, G., Kostan, J., Pirker, K. F., Daims, H., Furtmüller, P. G., Battistuzzi, G., Djinović-Carugo, K. & Obinger, C. (2014). Manipulating conserved heme cavity residues of chlorite dismutase: effect on structure, redox chemistry, and reactivity. *Biochemistry* **53**, 77–89.

Holliday, G. L., Akiva, E., Meng, E. C., Brown, S. D., Calhoun, S., Pieper, U., Sali, A., Booker, S. J. & Babbitt, P. C. (2018). Atlas of the radical SAM superfamily: divergent evolution of function using a "Plug and Play" domain. *Methods in Enzymology* **606**, 1–71.

Holliday, G. L., Thornton, J. M., Marquet, A., Smith, A. G., Rébeillé, F., Mendel, R., Schubert, H. L., Lawrence, A. D. & Warren, M. J. (2007). Evolution of enzymes and pathways for the biosynthesis of cofactors. *Natural Product Reports* **24**, 972–987.

Horáková, E., Changmai, P., Paris, Z., Salmon, D. & Lukeš, J. (2015). Simultaneous depletion of Atm and Mdl rebalances cytosolic Fe-S cluster assembly but not heme import into the mitochondrion of *Trypanosoma brucei*. *The FEBS Journal* **282**, 4157–4175.

Horáková, E., Changmai, P., Vancová, M., Sobotka, R., Van Den Abbeele, J., Vanhollebeke, B. & Lukeš, J. (2017). The *Trypanosoma brucei Tb*Hrg protein is a heme transporter involved in the regulation of stage-specific morphological transitions. *The Journal of Biological Chemistry* **292**, 6998–7010.

Hou, S., Reynolds, M. F., Horrigan, F. T., Heinemann, S. H. & Hoshi, T. (2006). Reversible binding of heme to proteins in cellular signal transduction. *Accounts of Chemical Research* **39**, 918–924.

Hug, L. A., Baker, B. J., Anantharaman, K., Brown, C. T., Probst, A. J., Castelle, C. J., Butterfield, C. N., Hernsdorf, A. W., Amano, Y., Ise, K., Suzuki, Y., Dudek, N., Relman, D. A., Finstad, K. M., Amundson, R., et al. (2016). A new view of the tree of life. *Nature Microbiology* **1**, 16048.

Hunt, R. D. (2006). *Radical S-adenosyl methionine domain containing-1 (rsad1): a novel gene essential for cell survival during vertebrate development*. Texas Medical Center Dissertations (via ProQuest). Paper AAI3328246.

Ilbert, M. & Bonnefoy, V. (2013). Insight into the evolution of the iron oxidation pathways. *Biochimica et Biophysica Acta* **1827**, 161–175.

Imlay, J. A. (2006). Iron-sulphur clusters and the problem with oxygen. *Molecular Microbiology* **59**, 1073–1082.

Iovieno, A., Ledee, D. R., Miller, D. & Alfonso, E. C. (2010). Detection of bacterial endosymbionts in clinical acanthamoeba isolates. *Ophthalmology* **117**, 445–452.

Jabłońska, J. & Tawfik, D. S. (2021). The evolution of oxygen-utilizing enzymes suggests early biosphere oxygenation. *Nature Ecology & Evolution* **5**, 442–448.

Jahn, D., Verkamp, E. & Söll, D. (1992). Glutamyl-transfer RNA: a precursor of heme and chlorophyll biosynthesis. *Trends in Biochemical Sciences* **17**, 215–218.

Janouškovec, J., Gavelis, G. S., Burki, F., Dinh, D., Bachvaroff, T. R., Gornik, S. G., Bright, K. J., Imanian, B., Strom, S. L., Delwiche, C. F., Waller, R. F., Fensome, R. A., Leander, B. S., Rohwer, F. L. & Saldarriaga, J. F. (2017). Major transitions in dinoflagellate evolution unveiled by phylotranscriptomics. *Proceedings of the National Academy of Sciences of the United States of America* **114**, E171–E180.

Janouškovec, J., Paskerova, G. G., Miroliubova, T. S., Mikhailov, K. V., Birley, T., Aleoshin, V. V. & Simdyanov, T. G. (2019). Apicomplexan-like parasites are polyphyletic and widely but selectively dependent on cryptic plastid organelles. *eLife* **8**, e49662.

Jun, S. R., Sims, G. E., Wu, G. A. & Kim, S. H. (2010). Whole-proteome phylogeny of prokaryotes by feature frequency profiles: an alignment-free method with optimal feature resolution. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 133–138.

Kalanon, M. & Mcfadden, G. I. (2010). Malaria, *Plasmodium falciparum* and its apicoplast. *Biochemical Society Transactions* **38**, 775–782.

Kachroo, A. H., Laurent, J. M., Akhmetov, A., Szilagyi-Jones, M., Mcwhite, C. D., Zhao, A. & Marcotte, E. M. (2017). Systematic bacterialization of yeast genes identifies a near-universally swappable pathway. *eLife* **6**, e25093.

Karnkowska, A., Vacek, V., Zubáčová, Z., Treitli, S. C., Petrželková, R., Eme, L., Novák, L., Žárský, V., Barlow, L. D., Herman, E. K., Soukal, P., Hroudová, M., Doležal, P., Stairs, C. W., Roger, A. J., et al. (2016). A eukaryote without a mitochondrial organelle. *Current Biology* **23**, 1274–1284.

Karunadharma, P. P., Basisty, N., Chiao, Y. A., Dai, D. F., Drake, R., Levy, N., Koh, W. J., Emond, M. J., Kruse, S., Marcinek, D., Maccoss, M. J. & Rabinovitch, P. S. (2015). Respiratory chain protein turnover rates in mice are highly heterogeneous but strikingly conserved across tissues, ages, and treatments. *FASEB Journal* **29**, 3582–3592.

Katinka, M. D., Duprat, S., Cornillot, E., Méténier, G., Thomarat, F., Prensier, G., Barbe, V., Peyretaillade, E., Brottier, P., Wincker, P., Delbac, F., El Alaoui, H., Peyret, P., Saurin, W., Gouy, M., et al. (2001). Genome sequence and gene compaction of the eukaryote parasite *Encephalitozoon cuniculi*. *Nature* **414**, 450–453.

Katz, A., Elgamal, S., Rajkovic, A. & Ibba, M. (2016). Non-canonical roles of tRNAs and tRNA mimics in bacterial cell biology. *Molecular Microbiology* **101**, 545–558.

Kayama, M., Maciszewski, K., Yabuki, A., Miyashita, H., Karnkowska, A. & Kamikawa, R. (2020). Highly reduced plastid genomes of the non-photosynthetic dictyochophyceans *Pteridomonas* spp. (Ochrophyta, SAR) are retained for tRNA-Glu-based organellar heme biosynthesis. *Frontiers in Plant Science* **11**, 602455.

Ke, H., Sigala, P. A., Miura, K., Morrisey, J. M., Mather, M. W., Crowley, J. R., Henderson, J. P., Goldberg, D. E., Long, C. A. & Vaidya, A. N. (2014). The heme biosynthesis pathway is essential for *Plasmodium falciparum* in mosquito stage but not in blood stages. *The Journal of Biological Chemistry* **289**, 34827–34837.

Keeling, P. J., Burki, F., Wilcox, H. M., Allam, B., Allen, E. E., Amaral-Zettler, L. A., Armbrust, E. V., Archibald, J. M., Bharti, A. K., Bell, C. J., Beszteri, B., Bidle, K. D., Cameron, C. T., Campbell, L., Caron, D. A., et al. (2014). The Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP): illuminating the functional diversity of eukaryotic life in the oceans through transcriptome sequencing. *PLoS Biology* **12**, e1001889.

Khan, A. A. & Quigley, J. G. (2011). Control of intracellular heme levels: heme transporters and heme oxygenases. *Biochimica et Biophysica Acta* **1813**, 668–682.

Kleine, T., Maier, U. G. & Leister, D. (2009). DNA transfer from organelles to the nucleus: the idiosyncratic genetics of endosymbiosis. *Annual Review of Plant Biology* **60**, 115–138.

Kobayashi, K., Masuda, T., Tajima, N., Wada, H. & Sato, N. (2014). Molecular phylogeny and intricate evolutionary history of the three isofunctional enzymes involved in the oxidation of protoporphyrinogen IX. *Genome Biology and Evolution* **6**, 2141–2155.

Kolluri, S., Sadlon, T. J., May, B. K. & Bonkovsky, H. L. (2005). Haem repression of the housekeeping 5-aminolaevulinic acid synthase gene in the hepatoma cell line LMH. *The Biochemical Journal* **392**, 173–180.

Kořený, L., Lukeš, J. & Oborník, M. (2010). Evolution of the haem synthetic pathway in kinetoplastid flagellates: an essential pathway that is not essential after all? *International Journal for Parasitology* **40**, 149–156.

Kořený, L. & Oborník, M. (2011). Sequence evidence for the presence of two tetrapyrrole pathways in *Euglena gracilis*. *Genome Biology and Evolution* **3**, 359–364.

Kořený, L., Oborník, M. & Lukeš, J. (2013). Make it, take it, or leave it: heme metabolism of parasites. *PLoS Pathogens* **9**, e1003088.

Kořený, L., Sobotka, R., Janouškovec, J., Keeling, P. J. & Oborník, M. (2011). Tetrapyrrole synthesis of photosynthetic chromerids is likely homologous to the unusual pathway of apicomplexan parasites. *The Plant Cell* **23**, 3454–3462.

Kořený, L., Sobotka, R., Kovárová, J., Gnipová, A., Flegontov, P., Horváth, A., Oborník, M., Ayala, F. J. & Lukeš, J. (2012). Aerobic kinetoplastid flagellate *Phytomonas* does not require heme for viability. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 3808–3813.

Kraeva, N., Horáková, E., Kostygov, A. Y., Kořený, L., Butenko, A., Yurchenko, V. & Lukeš, J. (2017). Catalase in Leishmaniinae: with me or against me? *Infection, Genetics and Evolution* **50**, 121–127.

Krishnamurthy, P. C., Du, G., Fukuda, Y., Sun, D., Sampath, J., Mercer, K. E., Wang, J., Sosa-Pineda, B., Murti, K. G. & Schuetz, J. D. (2006). Identification of a mammalian mitochondrial porphyrin transporter. *Nature* **443**, 586–589.

Kumar, S. & Bandyopadhyay, U. (2005). Free heme toxicity and its detoxification systems in human. *Toxicology Letters* **157**, 175–188.

Lakey, B. & Triemer, R. (2017). The tetrapyrrole synthesis pathway as a model of horizontal gene transfer in euglenoids. *Journal of Phycology* **53**, 198–217.

Lamb, D. C., Cannieux, M., Warrilow, A. G., Bak, S., Kahn, R. A., Manning, N. J., Kelly, D. E. & Kelly, S. L. (2001). Plant sterol 14 alpha-demethylase affinity for azole fungicides. *Biochemical and Biophysical Research Communications* **284**, 845–849.

Lane, N. (2014). Bioenergetic constraints on the evolution of complex life. *Cold Spring Harbor Perspectives in Biology* **6**, a015982.

Lane, N. & Martin, W. F. (2010). The energetics of genome complexity. *Nature* **467**, 929–934.

Lane, N. & Martin, W. F. (2012). The origin of membrane bioenergetics. *Cell* **151**, 1406–1416.

Lang, J. M., Darling, A. E. & Eisen, J. A. (2013). Phylogeny of bacterial and archaeal genomes using conserved genes: supertrees and supermatrices. *PLoS One* **8**, e62510.

Lara, F. A., Lins, U., Bechara, G. H. & Oliveira, P. L. (2005). Tracing heme in a living cell: hemoglobin degradation and heme traffic in digest cells of the cattle tick *Boophilus microplus*. *The Journal of Experimental Biology* **208**, 3093–3101.

Laranjeira-Silva, M. F., Hamza, I. & Pérez-Victoria, J. M. (2020). Iron and heme metabolism at the *Leishmania*-host interface. *Trends in Parasitology* **36**, 279–289.

Layer, G., Reichelt, J., Jahn, D. & Heinz, D. W. (2010). Structure and function of enzymes in heme biosynthesis. *Protein Science* **19**, 1137–1161.

Lepesheva, G. I., Ott, R. D., Hargrove, T. Y., Kleshchenko, Y. Y., Schuster, I., Nes, W. D., Hill, G. C., Villalta, F. & Waterman, M. R. (2007). Sterol 14alpha-demethylase as a potential target for antitrypanosomal therapy: enzyme inhibition and parasite cell growth. *Chemistry & Biology* **14**, 1283–1293.

Lepesheva, G. I. & Waterman, M. R. (2007). Sterol 14alpha-demethylase cytochrome P450 (CYP51), a P450 in all biological kingdoms. *Biochimica et Biophysica Acta* **1770**, 467–477.

Lermontova, I., Kruse, E., Mock, H. P. & Grimm, B. (1997). Cloning and characterization of a plastidal and a mitochondrial isoform of tobacco protoporphyrinogen IX oxidase. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 8895–8900.

Lhee, D., Ha, J. S., Kim, S., Park, M. G., Bhattacharya, D. & Yoon, H. S. (2019). Evolutionary dynamics of the chromatophore genome in three photosynthetic *Paulinella* species. *Scientific Reports* **9**, 2560.

Lill, R. (2009). Function and biogenesis of iron-sulphur proteins. *Nature* **460**, 831–838.

Lindsey, J. S., Ptaszek, M. & Taniguchi, M. (2009). Simple formation of an abiotic porphyrinogen in aqueous solution. *Origins of Life and Evolution of the Biosphere* **39**, 495–515.

Lister, R., Chew, O., Rudhe, C., Lee, M. N. & Whelan, J. (2001). *Arabidopsis thaliana* ferrochelatase-I and -II are not imported into *Arabidopsis* mitochondria. *FEBS Letters* **506**, 291–295.

Liu, J., Chakraborty, S., Hosseinzadeh, P., Yu, Y., Tian, S., Petrik, I., Bhagi, A. & Lu, Y. (2014). Metalloproteins containing cytochrome, iron-sulfur, or copper redox centers. *Chemical Reviews* **114**, 4366–4469.

Lunetti, P., Damiano, F., De Benedetto, G., Siculella, L., Pennetta, A., Muto, L., Paradies, E., Marobbio, C. M., Dolce, V. & Capobianco, L. (2016). Characterization of human and yeast mitochondrial glycine carriers with implications for heme biosynthesis and anemia. *The Journal of Biological Chemistry* **291**, 19746–19759.

Maio, N., Kim, K. S., Holmes-Hampton, G., Singh, A. & Rouault, T. A. (2019). Dimeric ferrochelatase bridges ABCB7 and ABCB10 homodimers in an architecturally defined molecular complex required for heme biosynthesis. *Haematologica* **104**, 1756–1767.

Maitra, D., Bragazzi Cunha, J., Elenbaas, J. S., Bonkovsky, H. L., Shavit, J. A. & Omary, M. B. (2019). Porphyrin-induced protein oxidation and aggregation as a mechanism of porphyria-associated cell injury. *Cellular and Molecular Gastroenterology and Hepatology* **8**, 535–548.

Marchetti, P., Hirsch, T., Zamzami, N., Castedo, M., Decaudin, D., Susin, S. A., Masse, B. & Kroemer, G. (1996). Mitochondrial permeability transition triggers lymphocyte apoptosis. *Journal of Immunology* **157**, 4830–4836.

Maréchal, E. (2018). Primary endosymbiosis: emergence of the primary chloroplast and the chromatophore, two independent events. In *Methods in Molecular Biology* (Volume **1829**, ed. E. Maréchal), pp. 3–16. Humana Press, New York.

Marin, B., Nowack, E. C. & Melkonian, M. (2005). A plastid in the making: evidence for a second primary endosymbiosis. *Protist* **156**, 425–432.

Martin, W. & Russell, M. J. (2003). On the origins of cells: a hypothesis for the evolutionary transitions from abiotic geochemistry to chemoautotrophic prokaryotes, and from prokaryotes to nucleated cells. *Philosophical transactions of the Royal Society of London. Series B, Biological Sciences* **358**, 59–83.

Martin, W. F., Sousa, F. L. & Lane, N. (2014). Energy at life's origin. *Science* **344**, 1092–1093.

Martinez, M., Fendley, G. A., Saxberg, A. D. & Zoghbi, M. E. (2020). Stimulation of the human mitochondrial transporter ABCB10 by zinc-mesoporphyrin. *PLoS One* **15**, e0238754.

Masuda, T., Suzuki, T., Shimada, H., Ohta, H. & Takamiya, K. (2003). Subcellular localization of two types of ferrochelatase in cucumber. *Planta* **217**, 602–609.

Matsumoto, T., Shinozaki, F., Chikuni, T., Yabuki, A., Takishita, K., Kawachi, M., Nakayama, T., Inouye, I., Hashimoto, T. & Inagaki, Y. (2011). Green-colored plastids in the dinoflagellate genus *Lepidodinium* are of core chlorophyte origin. *Protist* **162**, 268–276.

Mathur, V., Kolísko, M., Hehenberger, E., Irwin, N., Leander, B. S., Kristmundsson, Á., Freeman, M. A. & Keeling, P. J. (2019). Multiple independent origins of apicomplexan-like parasites. *Current Biology* **29**, 2936–2941.

Medlock, A. E., Shiferaw, M. T., Marcero, J. R., Vashisht, A. A., Wohlschlegel, J. A., Phillips, J. D. & Dailey, H. A. (2015). Identification of the mitochondrial heme metabolism complex. *PLoS One* **10**, e0135896.

Merli, M. L., Pagura, L., Hernández, J., Barisón, M. J., Pral, E. M., Silber, A. M. & Cricco, J. A. (2016). The *Trypanosoma cruzi* Protein TcHTE is critical for heme uptake. *PLoS Neglected Tropical Diseases* **10**, e0004359.

Michel, R., Müller, K. D., Haurõder, B. & Zöller, L. (2000). A coccoid bacterial parasite of *Naegleria* sp. (Schizopyrenida: Vahlkampfiidae) inhibits cyst formation of its host but not transformation to the flagellate stage. *Acta Protozoologica* **39**, 199–207.

Miyagishima, S., Kuroiwa, H. & Kuroiwa, T. (2001). The timing and manner of disassembly of the apparatuses for chloroplast and mitochondrial division in the red alga *Cyanidioschyzon merolae*. *Planta* **212**, 517–528.

Molina, J., Hazzouri, K. M., Nickrent, D., Geisler, M., Meyer, R. S., Pentony, M. M., Flowers, J. M., Pelser, P., Barcelona, J., Inovejas, S. A., Uy, I., Yuan, W., Wilkins, O., Michel, C. I., Locklear, S., et al. (2014). Possible loss of the chloroplast genome in the parasitic flowering plant *Rafflesia lagascae* (Rafflesiaceae). *Molecular Biology and Evolution* **31**, 793–803.

Montfort, W. R., Wales, J. A. & Weichsel, A. (2017). Structure and activation of soluble guanylyl cyclase, the nitric oxide sensor. *Antioxidants & Redox Signaling* **26**, 107–121.

Moore, S. J., Sowa, S. T., Schuchardt, C., Deery, E., Lawrence, A. D., Ramos, J. V., Billig, S., Birkemeyer, C., Chivers, P. T., Howard, M. J., Rigby, S. E., Layer, G. & Warren, M. J. (2017). Elucidation of the biosynthesis of the methane catalyst coenzyme F430. *Nature* **543**, 78–82.

Moulin, M. & Smith, A. G. (2005). Regulation of tetrapyrrole biosynthesis in higher plants. *Biochemical Society Transactions* **33**, 737–742.

Nagaraj, V. A., Arumugam, R., Chandra, N. R., Prasad, D., Rangarajan, P. N. & Padmanaban, G. (2009a). Localisation of *Plasmodium falciparum* uroporphyrinogen III decarboxylase of the heme-biosynthesis pathway in the apicoplast and characterisation of its catalytic properties. *International Journal for Parasitology* **39**, 559–568.

Nagaraj, V. A., Arumugam, R., Prasad, D., Rangarajan, P. N. & Padmanaban, G. (2010a). Protoporphyrinogen IX oxidase from *Plasmodium falciparum* is anaerobic and is localized to the mitochondrion. *Molecular and Biochemical Parasitology* **174**, 44–52.

Nagaraj, V. A., Prasad, D., Arumugam, R., Rangarajan, P. N. & Padmanaban, G. (2010b). Characterization of coproporphyrinogen III oxidase in *Plasmodium falciparum* cytosol. *Parasitology International* **59**, 121–127.

Nagaraj, V. A., Prasad, D., Rangarajan, P. N. & Padmanaban, G. (2009b). Mitochondrial localization of functional ferrochelatase from *Plasmodium falciparum*. *Molecular and Biochemical Parasitology* **168**, 109–112.

Narita, S., Tanaka, R., Ito, T., Okada, K., Taketani, S. & Inokuchi, H. (1996). Molecular cloning and characterization of a cDNA that encodes protoporphyrinogen oxidase of *Arabidopsis thaliana*. *Gene* **182**, 169–175.

Ng, S. M., Lee, X. W., Mat-Isa, M. N., Aizat-Juhari, M. A., Adam, J. H., Mohamed, R., Wan, K. L. & Firdaus-Raih, M. (2018). Comparative analysis of nucleus-encoded plastid-targeting proteins in *Rafflesia cantleyi* against photosynthetic and non-photosynthetic representatives reveals orthologous systems with potentially divergent functions. *Scientific Reports* **8**, 17258.

Nowack, E. C., Melkonian, M. & Glöckner, G. (2008). Chromatophore genome sequence of *Paulinella* sheds light on acquisition of photosynthesis by eukaryotes. *Current Biology* **18**, 410–418.

Nowack, E. C., Price, D. C., Bhattacharya, D., Singer, A., Melkonian, M. & Grossman, A. R. (2016). Gene transfers from diverse bacteria compensate for reductive genome evolution in the chromatophore of *Paulinella chromatophora*. *Proceedings of the National Academy of Sciences of the United States of America* **113**, 12214–12219.

Nowack, E. & Weber, A. (2018). Genomics-informed insights into endosymbiotic organelle evolution in photosynthetic eukaryotes. *Annual Review of Plant Biology* **69**, 51–84.

Oborník, M. (2018). The birth of red complex plastids: one, three or four times? *Trends in Parasitology* **34**, 923–925.

Oborník, M. (2019). Endosymbiotic evolution of algae, secondary heterotrophy and parasitism. *Biomolecules* **9**, 266.

Oborník, M. (2020). Photoparasitism as an intermediate state in the evolution of apicomplexan parasites. *Trends in Parasitology* **36**, 727–734.

Oborník, M. (2021). Enigmatic evolutionary history of porphobilinogen deaminase in eukaryotic phototrophs. *Biology* **10**, 386.

Oborník, M. & Green, B. R. (2005). Mosaic origin of the heme biosynthesis pathway in photosynthetic eukaryotes. *Molecular Biology and Evolution* **22**, 2343–2353.

Oliver, T., Sánchez-Baracaldo, P., Larkum, A. W., Rutherford, A. W. & Cardona, T. (2021). Time-resolved comparative molecular evolution of oxygenic photosynthesis. *Biochimica et Biophysica Acta – Bioenergetics* **1862**, 148400.

Orrego, L. M., Cabello-Donayre, M., Vargas, P., Martínez-García, M., Sánchez, C., Pineda-Molina, E., Jiménez, M., Molina, R. & Pérez-Victoria, J. M. (2019). Heme synthesis through the life cycle of the heme auxotrophic parasite Leishmania major. *FASEB Journal* **33**, 13367–13385.

Pánek, T., Eliáš, M., Vancová, M., Lukeš, J. & Hashimi, H. (2020). Returning to the fold for lessons in mitochondrial cristae diversity and evolution. *Current Biology* **30**, R575–R588.

Papenbrock, J., Mock, H. P., Kruse, E. & Grimm, B. (1999). Expression studies in tetrapyrrole biosynthesis: inverse maxima of magnesium chelatase and ferrochelatase activity during cyclic photoperiods. *Planta* **208**, 264–273.

Perally, S., Lacourse, E. J., Campbell, A. M. & Brophy, P. M. (2008). Heme transport and detoxification in nematodes: subproteomics evidence of differential role of glutathione transferases. *Journal of Proteome Research* **7**, 4557–4565.

Perner, J., Gasser, R. B., Oliveira, P. L. & Kopáček, P. (2019). Haem biology in metazoan parasites - 'The bright side of haem'. *Trends in Parasitology* **35**, 213–225.

Perner, J., Sobotka, R., Šíma, R., Konvičková, J., Sojka, D., Oliveira, P. L., Hajdušek, O. & Kopáček, P. (2016). Acquisition of exogenous haem is essential for tick reproduction. *eLife* **5**, e12318.

Pleyer, H. L., Strasdeit, H. & Fox, S. (2018). A possible prebiotic ancestry of porphyrin-type protein cofactors. *Origins of Life and Evolution of the Biosphere* **48**, 347–371.

Ponka, P. (1997). Tissue-specific regulation of iron metabolism and heme synthesis: distinct control mechanisms in erythroid cells. *Blood* **89**, 1–25.

Porcel, B. M., Denoeud, F., Opperdoes, F., Noel, B., Madoui, M. A., Hammarton, T. C., Field, M. C., Da Silva, C., Couloux, A., Poulain, J., Katinka, M., Jabbari, K., Aury, J. M., Campbell, D. A., Cintron, R., et al. (2014). The streamlined genome of *Phytomonas* spp. relative to human pathogenic kinetoplastids reveals a parasite tailored for plants. *PLoS Genetics* **10**, e1004007.

Pyrih, J., Harant, K., Martincová, E., Šuťák, R., Lesuisse, E., Hrdý, I. & Tachezy, J. (2014). *Giardia intestinalis* incorporates heme into cytosolic cytochrome b5. *Eukaryotic Cell* **13**, 231–239.

Rafferty, S. P. & Dayer, G. (2015). Heme proteins of *Giardia intestinalis*. *Experimental Parasitology* **159**, 13–23.

Rangel, H., Dagger, F., Hernandez, A., Liendo, A. & Urbina, J. A. (1996). Naturally azole-resistant *Leishmania braziliensis* promastigotes are rendered susceptible in the presence of terbinafine: comparative study with azole-susceptible *Leishmania mexicana* promastigotes. *Antimicrobial Agents and Chemotherapy* **40**, 2785–2791.

Rao, A. U., Carta, L. K., Lesuisse, E. & Hamza, I. (2005). Lack of heme synthesis in a free-living eukaryote. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 4270–4275.

Raux, E., Schubert, H. L. & Warren, M. J. (2000). Biosynthesis of cobalamin (vitamin B12): a bacterial conundrum. *Cellular and Molecular Life Sciences* **57**, 1880–1893.

Rogers, M. B., Gilson, P. R., Su, V., Mcfadden, G. I. & Keeling, P. J. (2007). The complete chloroplast genome of the chlorarachniophyte *Bigelowiella natans*: evidence for independent origins of chlorarachniophyte and euglenid secondary endosymbionts. *Molecular Biology and Evolution* **24**, 54–62.

Roper, J. M. & Smith, A. G. (1997). Molecular localisation of ferrochelatase in higher plant chloroplasts. *European Journal of Biochemistry* **246**, 32–37.

Sah, J. F., Ito, H., Kolli, B. K., Peterson, D. A., Sassa, S. & Chang, K. P. (2002). Genetic rescue of *Leishmania* deficiency in porphyrin biosynthesis creates mutants suitable for analysis of cellular events in uroporphyria and for photodynamic therapy. *The Journal of Biological Chemistry* **277**, 14902–14909.

Sanchez-Moreno, M., Lasztity, D., Coppens, I. & Opperdoes, F. R. (1992). Characterization of carbohydrate metabolism and demonstration of glycosomes in a *Phytomonas* sp. isolated from *Euphorbia characias*. *Molecular and Biochemical Parasitology* **54**, 185–199.

Sato, S., Clough, B., Coates, L. & Wilson, R. J. (2004). Enzymes for heme biosynthesis are found in both the mitochondrion and plastid of the malaria parasite *Plasmodium falciparum*. *Protist* **155**, 117–125.

Schenkman, J. B. & Jansson, I. (2003). The many roles of cytochrome b5. *Pharmacology & Therapeutics* **97**, 139–152.

Schulze, J. O., Schubert, W. D., Moser, J., Jahn, D. & Heinz, D. W. (2006). Evolutionary relationship between initial enzymes of tetrapyrrole biosynthesis. *Journal of Molecular Biology* **358**, 1212–1220.

Shanmugan, D., Wu, B., Ramirez, U., Jaffe, E. K. & Roos, D. S. (2010). Plastid-associated porphobilinogen synthase from *toxoplasma gondii*: kinetic and structural properties validate therapeutic potential. *The Journal of Biological Chemistry* **285**, 22122–22131.

Shirihai, O. S., Gregory, T., Yu, C., Orkin, S. H. & Weiss, M. J. (2000). ABC-me: a novel mitochondrial transporter induced by GATA-1 during erythroid differentiation. *The EMBO Journal* **19**, 2492–2502.

Sinclair, J. & Hamza, I. (2015). Lessons from bloodless worms: heme homeostasis in *C. elegans*. *Biometals* **28**, 481–489.

Smith, D. R. & Lee, R. W. (2014). A plastid without a genome: evidence from the nonphotosynthetic green algal genus *Polytomella*. *Plant Physiology* **164**, 1812–1819.

Sousa, F. L., Thiergart, T., Landan, G., Nelson-Sathi, S., Pereira, I. A., Allen, J. F., Lane, N. & Martin, W. F. (2013). Early bioenergetic evolution. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **368**, 20130088.

Stairs, C. W., Leger, M. M. & Roger, A. J. (2015). Diversity and origins of anaerobic metabolism in mitochondria and related organelles. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **370**, 20140326.

Suzuki, T., Masuda, T., Singh, D. P., Tan, F. C., Tsuchiya, T., Shimada, H., Ohta, H., Smith, A. G. & Takamiya, K. (2002). Two types of ferrochelatase in photosynthetic and nonphotosynthetic tissues of cucumber: their difference in phylogeny, gene expression, and localization. *The Journal of Biological Chemistry* **277**, 4731–4737.

Swenson, S. A., Moore, C. M., Marcero, J. R., Medlock, A. E., Reddi, A. R. & Khalimonchuk, O. (2020). From synthesis to utilization: the ins and outs of mitochondrial heme. *Cells* **9**, 579.

Tanaka, R. & Tanaka, A. (2007). Tetrapyrrole biosynthesis in higher plants. *Annual Review of Plant Biology* **58**, 321–346.

Timmis, J. N., Ayliffe, M. A., Huang, C. Y. & Martin, W. (2004). Endosymbiotic gene transfer: organelle genomes forge eukaryotic chromosomes. *Nature Reviews. Genetics* **5**, 123–135.

Tjhin, E. T., Hayward, J. A., Mcfadden, G. I. & Van Doren, G. G. (2020). Characterization of the apicoplast-localized enzyme TgUroD in *Toxoplasma gondii* reveals a key role of the apicoplast in heme biosynthesis. *The Journal of Biological Chemistry* **295**, 1539–1550.

Traut, T. (2008). Hemoglobin. In *Allosteric Regulatory Enzymes*, pp. 105–125. Springer, Boston.

Tripodi, K. E., Menendez Bravo, S. M. & Cricco, J. A. (2011). Role of heme and heme-proteins in trypanosomatid essential metabolic pathways. *Enzyme Research* **2011**, 873230.

Van Dooren, G. G., Kennedy, A. T. & Mcfadden, G. I. (2012). The use and abuse of heme in apicomplexan parasites. *Antioxidants & Redox Signaling* **17**, 634–656.

Vanhollebeke, B., De Muylder, G., Nielsen, M. J., Pays, A., Tebabi, P., Dieu, M., Raes, M., Moestrup, S. K. & Pays, E. (2008). A haptoglobin–hemoglobin receptor conveys innate immunity to *Trypanosoma brucei* in humans. *Science* **320**, 677–681.

Van Lis, R. V., Atteia, A., Nogaj, L. A. & Beale, S. I. (2005). Subcellular localization and light-regulated expression of protoporphyrinogen IX oxidase and ferrochelatase in *Chlamydomonas reinhardtii*. *Plant Physiology* **139**, 1946–1958.

Vavilin, D. V. & Vermaas, W. F. (2002). Regulation of the tetrapyrrole biosynthetic pathway leading to heme and chlorophyll in plants and cyanobacteria. *Physiologia Plantarum* **115**, 9–24.

Waller, R. F. & Koreny, L. (2017). Plastid complexity in dinoflagellates: a picture of gains, losses, replacements and revisions. *Advances in Botanical Research* **84**, 105–143.

Wang, Z. & Wu, M. (2017). Comparative genomic analysis of Acanthamoeba endosymbionts highlights the role of amoebae as a "melting pot" shaping the Rickettsiales evolution. *Genome Biology and Evolution* **9**, 3214–3224.

Watanabe, N., Che, F. S., Iwano, M., Takayama, S., Yoshida, S. & Isogai, A. (2001). Dual targeting of spinach protoporphyrinogen oxidase II to mitochondria and chloroplasts by alternative use of two in-frame initiation codons. *The Journal of Biological Chemistry* **276**, 20474–20481.

Watanabe, S., Hanaoka, M., Ohba, Y., Ono, T., Ohnuma, M., Yoshikawa, H., Tanekani, S. & Tanaka, K. (2013). Mitochondrial localization of ferrochelatase in a red alga *Cyanidioschyzon merolae*. *Plant & Cell Physiology* **54**, 1289–1295.

Williams, B. A., Elliot, C., Burri, L., Kido, Y., Kita, K., Moore, A. L. & Keeling, P. J. (2010). A broad distribution of the alternative oxidase in microsporidian parasites. *PLoS Pathogens* **6**, e1000761.

Williams, P., Hardeman, K., Fowler, J. & Rivin, C. (2006). Divergence of duplicated genes in maize: evolution of contrasting targeting information for enzymes in the porphyrin pathway. *The Plant Journal* **45**, 727–739.

Williams, T. A., Cox, C. J., Foster, P. G., Szöllősi, G. J. & Embley, T. M. (2020). Phylogenomics provides robust support for a two-domains tree of life. *Nature Ecology & Evolution* **4**, 138–147.

Woodson, J. D., Perez-Ruiz, J. M. & Chory, J. (2011). Heme synthesis by plastid ferrochelatase I regulates nuclear gene expression in plants. *Current Biology* **21**, 897–903.

Wu, B., Novelli, J., Jiang, D., Dailey, H. A., Landmann, F., Ford, L., Taylor, M. J., Carlow, C. K., Kumar, S., Foster, J. M. & Slatko, B. E. (2013). Interdomain lateral gene transfer of an essential ferrochelatase gene in human parasitic nematodes. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 7748–7753.

Wu, G. Z. & Bock, R. (2021). GUN control in retrograde signaling: how GENOMES UNCOUPLED proteins adjust nuclear gene expression to plastid biogenesis. *Plant Cell* **33**, 457–474.

Yamauchi, K., Hayashi, N. & Kikuchi, G. (1980). Translocation of delta-aminolevulinate synthase from the cytosol to the mitochondria and its regulation by hemin in the rat liver. *The Journal of Biological Chemistry* **255**, 1746–1751.

Yao, N., Eisfelder, B. J., Marvin, J. & Greenberg, J. T. (2004). The mitochondrion - an organelle commonly involved in programmed cell death in *Arabidopsis thaliana*. *The Plant Journal* **40**, 596–610.

Yao, N. & Greenberg, J. T. (2006). *Arabidopsis* accelerated cell death2 modulates programmed cell death. *The Plant Cell* **18**, 397–411.

Yeh, E. & DeRisi, J. L. (2011). Chemical rescue of malaria parasites lacking an apicoplast defines organelle function in blood-stage *Plasmodium falciparum*. *PLoS Biology* **9**, e1001138.

Yoon, T. & Cowan, J. A. (2004). Frataxin-mediated iron delivery to ferrochelatase in the final step of heme biosynthesis. *The Journal of Biological Chemistry* **279**, 25943–25946.

Zhang, Y., Ma, A., Liu, W., Bai, Z., Zhuang, X. & Zhuang, G. (2018). The occurrence of putative nitric oxide dismutase (NOD) in an alpine wetland with a new dominant subcluster and the potential ability for a methane sink. *Archaea* **2018**, 6201541.

Zheng, K., Ngo, P. D., Owens, V. L., Yang, X. P. & Mansoorabadi, S. O. (2016). The biosynthetic pathway of coenzyme F430 in methanogenic and methanotrophic archaea. *Science* **354**, 339–342.

Zíková, A., Verner, Z., Nenarokova, A., Michels, P. & Lukeš, J. (2017). A paradigm shift: the mitoproteomes of procyclic and bloodstream *Trypanosoma brucei* are comparably complex. *PLoS Pathogens* **13**, e1006679.

(*Received 16 December 2020; revised 12 August 2021; accepted 19 August 2021*)