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Eukaryogenesis and oxygen in Earth history

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The endosymbiotic origin of mitochondria during eukaryogenesis has long been viewed as an adaptive response to the oxygenation of Earth's surface environment, presuming a fundamentally aerobic lifestyle for the free-living bacterial ancestors of mitochondria. This oxygen-centric view has been robustly challenged by recent advances in the Earth and life sciences. While the permanent oxygenation of the atmosphere above trace concentrations is now thought to have occurred 2.2 billion years ago, large parts of the deep ocean remained anoxic until less than 0.5 billion years ago. Neither fossils nor molecular clocks correlate the origin of mitochondria, or eukaryogenesis more broadly, to either of these planetary redox transitions. Instead, mitochondria-bearing eukaryotes are consistently dated to between these two oxygenation events, during an interval of pervasive deep-sea anoxia and variable surface-water oxygenation. The discovery and cultivation of the Asgard archaea has reinforced metabolic evidence that eukaryogenesis was initially mediated by syntrophic H_2 exchange between an archaeal host and an α-proteobacterial symbiont living under anoxia. Together, these results temporally, spatially and metabolically decouple the earliest stages of eukaryogenesis from the oxygen content of the surface ocean and atmosphere. Rather than reflecting the ancestral metabolic state, obligate aerobiosis in eukaryotes is most probably derived, having only become globally widespread over the past 1 billion years as atmospheric oxygen approached modern levels.

In 1967, Lynn Margulis (then Lynn Sagan) published the first account[1] of what ultimately became known as the serial endosymbiotic theory (SET)[2]. According to SET, the origin of the eukaryotic cell (eukaryogenesis) occurred through a series of protracted endosymbiotic associations between an ancestrally anaerobic host and various bacterial symbionts, notably the cyanobacterial ancestors of chloroplasts and the α-proteobacterial ancestors of mitochondria[3] (Box 1). While the bacterial origins of chloroplasts[4] and mitochondria[5] had each been advocated separately, Margulis was the first to argue for the symbiotic origin of both organelles, each from a different bacterial endosymbiont[6]. Drawing upon the geologic and palaeontological literature, Margulis explicitly placed organelle physiology, as it was then understood, within the broader context of Earth's environmental evolution, particularly the evolutionary history of Earth's redox state (Box 2). Specifically, Margulis linked the origin of mitochondria to the oxygenation of the atmosphere, with the former serving as an adaptation to the latter (Fig. 1a). This cross-disciplinary synthesis resulted in an intuitively satisfying co-evolutionary narrative that remains influential to this day (reviewed by Martin[6]).

Despite the broad influence of SET on decades of eukaryogenesis research, its metabolic and biogeochemical context requires considerable updating. Foremost, the discovery of hydrogenosomes (H_2-generating organelles that synthesize ATP independently of O_2) and their common ancestry with aerobic mitochondria suggest a potential role for anaerobic energy metabolism during eukaryogenesis[7] (Box 1). The recent discovery[8] and cultivation[9] of the Asgard archaea reinforces predictions that eukaryogenesis began under anoxia (that is, with no O_2) between archaea and bacteria mutually dependent on one another via anaerobic syntrophy ('eating together' without O_2, the thermodynamic and obligately mutualistic[10] coupling of at least two different anaerobic energy metabolisms)[9,11–15]. These syntrophic models of eukaryogenesis spatially and mechanistically decouple the earliest stages of eukaryogenesis from the oxygen content of the atmosphere. Indeed, fossil and molecular-clock evidence consistently suggest that the last eukaryote common ancestor (LECA) emerged hundreds of millions of years after Earth's initial oxygenation[16–19]. Furthermore, recent advances in geochemistry and Earth system modelling suggest that the oceans at the time of eukaryogenesis were predominantly anoxic at depth, with only weakly oxygenated surface waters (probably no more than 1–10% of modern atmospheric saturation)[20–23]. These revisions have fostered major modifications[7,24–26] to the co-evolutionary narrative first synthesized by Sagan[1], inviting a reexamination of the redox conditions necessary for eukaryogenesis.

Oxygen and mitochondria

In the years immediately following SET[1], those advocating for a symbiotic origin of mitochondria maintained that the protomitochondrion (the bacterial ancestor of the mitochondrion) was fundamentally aerobic[2,27] (Box 2). Through this endosymbiosis, the protomitochondrion received metabolic substrates and physical protection from its host, while the host gained either the capacity for aerobic respiration (as in SET)[28], or a more efficient version of it[27,29], and thus more ATP than it was able to generate on its own[27,29]. As a later modification of this framework, the 'ox-tox' hypothesis argued that the initial functioning of the protomitochondrion was not providing ATP to the host (this was a secondary function), but rather lowering the host's intracellular O_2 concentration, allowing the host to survive in an oxygenated environment[30]. Numerous versions of these 'oxygen narratives' exist[31–33]. Generally, these reconstructions project the metabolism of modern aerobic mitochondria back to the ancestral mitochondrion and frame this symbiosis in terms of oxygen utilization (either aerobic respiration or detoxification) in the wake of atmospheric oxygenation. However, all such narratives either predate, ignore or minimize the recognition that

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Box 1 | Eukaryogenesis: model categories and terminology

In the decades following the initial publication of SET[1], the symbiotic origins of mitochondria and chloroplasts became widely accepted[153,154]. In the mid-1980s, an origin of mitochondria from within the α-proteobacteria was proposed based on mitochondrial 16S ribosomal RNA (rRNA) sequences[155,156], a view that quickly became the consensus[154]. Recently, however, a sister-group relationship between mitochondria and all other α-proteobacteria has also been proposed[157,158] (and debated[159]). While this topology is based on a rejection of the commonly accepted view of a close relationship between mitochondria and Rickettsiales, Martijn et al.[157] and Muñoz-Gómez et al.[158] exclude mitochondria from α-proteobacteria by redefining the clade to exclude Magnetococciales and the clade of 'marine α-proteobacteria' on the arbitrary basis that the branch length separating these two lineages from the remaining α-proteobacteria was too long. Following this same logic (using branch length to define clade membership), mitochondria should be included in α-proteobacteria. Setting aside these taxonomic deliberations, the ancestral mitochondrion would have been an α-proteobacterial-grade organism.

It is widely understood that the most recent common ancestor of all living eukaryotes, LECA, definitively possessed mitochondria[38,40], from which hydrogenosomes, mitosomes and anaerobic forms of mitochondria descend[7]. These anaerobic organelles (some of which are uninvolved in energy production, that is, mitosomes) descend from the same proteobacterial endosymbiont (the 'protomitochondrion' or the 'ancestral mitochondrion') as aerobic mitochondria[160] and have been variously called 'organelles of mitochondrial origin' or 'mitochondrion-related organelles' (MROs)[7,40]. Together, these organelle lineages (aerobic and anaerobic) form a clade (united by the last common ancestor of all aerobic mitochondrial lineages + MRO lineages), although no general term for members of this clade exists, to our knowledge, outside of 'mitochondria'. Importantly, aerobic mitochondria have no privileged phylogenetic position in the tree of eukaryotes. They interleave with anaerobic lineages in every major eukaryotic supergroup[7]. Rather, aerobic mitochondria simply represent the first organelle descendants of the α-proteobacterial endosymbiont to be described[161], as they are the most numerous in modern terrestrial (subaerial) and marine ecosystems where oxygen is continuously present[24]. Hereafter, all organelles descended from the α-proteobacterial endosymbiont (aerobic and anaerobic alike) are referred to as 'mitochondria'.

In framing scenarios for the relative timing of events within the process of eukaryogenesis, it is worth reflecting on the different stages in the ancestry of living eukaryotes (Fig. 2). It is widely appreciated that there is a distinction between a first eukaryote common ancestor (FECA) and LECA, but it is less widely appreciated that there are at least two FECAs and, therefore, at least two eukaryote stem-lineages. These are archaeal-FECA and mito-FECA. Archaeal-FECA is the first descendant, along the stem-lineage leading to the eukaryotes (Fig. 2, yellow branch), of the last common ancestor of eukaryotes and their archaeal sister lineage. Mito-FECA is the first descendant, along the stem-lineage leading to eukaryotes (Fig. 2, blue branch), arising from the last common ancestor of eukaryotes and their closest α-proteobacterial sister lineage. Extinct taxa more closely related to crown-eukaryotes comprise the mito- and archaeal stem-lineages (Fig. 2, blue and yellow, respectively), which coalesce at mitochondrial endosymbiosis (Fig. 2, node 3; hypotheses differ as to whether this was a single event or a protracted episode). After this endosymbiotic event occurred, we may consider eukaryote-grade organization to have been achieved (Fig. 2, green). However, views differ on the timing of origin of a nucleus, phagocytosis and so on, relative to the mitochondrion, leading to 'mitochondria-late' and 'mitochondria-early' hypotheses[40,55,162]. The amount of time between coalescence of the archaeal and mitochondrial lineages is not known and difficult to constrain because it is not identifiable in molecular phylogenies. Nevertheless, most molecular-clock studies and other interpretations of the fossil record predict that the time between the FECAs and LECA (Fig. 2, node 4) was on the order of hundreds of millions to more than a billion years (see, for example, Betts et al.[17]).

In general, mitochondria-early and mitochondria-late models make very different predictions about the nature and affinity of the host cell and how it acquired the ancestral mitochondrion. In mitochondria-late scenarios, the host cell is presumed to have been already capable of phagocytosis, which it used to internalize the protomitochondrion. Characters that are generally associated with the eukaryotic level of cellular organization (most relevantly, a cytoskeletal structure sufficiently dynamic to support some form of phagocytosis) are similarly assumed to have been acquired along the archaeal stem (Fig. 2, yellow branch) before the mitochondrial endosymbiosis. With respect to affinity, the hosts in mitochondria-late scenarios range from a primitively amitochondriate eukaryote (as in the Archaezoa hypothesis[163] and the most recent versions of SET[3]), a 'protoeukaryote' (a stem-eukaryote that was neither an archaeon nor a bacterium), or more recently, a phagocytosing archaeon[164]. As such, mitochondria-late models have also been called 'phagotrophic' models of eukaryogenesis. By contrast, mitochondria-early scenarios, the foundational example of which is the hydrogen hypothesis[11], predict that an archaeal host internalized the ancestral mitochondrion using nonphagocytic means. In this 'fusion' model, the origin of mitochondria and the onset of eukaryogenesis are synonymous, with all major eukaryotic traits, such as the nucleus and phagocytosis, evolving subsequently as a result of this endosymbiotic merger (Fig. 2). Accordingly, eukaryotic ancestors along the archaeal-stem and mito-stem (Fig. 2) were, respectively, typical archaea and α-proteobacteria with a prokaryotic cell organization. Because the hydrogen hypothesis emphasizes anaerobic syntrophy as the metabolic driver for this ancestral symbiosis, mitochondria-early models have also been called 'syntrophic' models of eukaryogenesis, although this general label should not be confused with the more specific syntrophic hypothesis[12], which is actually a mitochondria-late model of eukaryogenesis.

hydrogenosomes (H_2-producing eukaryotic organelles that are exclusively anaerobic) descend from the same endosymbiont as aerobic mitochondria[34] (Box 1).

First described in 1973 (ref. [35]), hydrogenosomes are double-membrane-bound organelles that oxidize pyruvate (or malate) to CO_2 while reducing protons to molecular hydrogen (H_2), generating ATP exclusively via substrate-level phosphorylation[36]. While a relationship to mitochondria or peroxisomes had initially been assumed, hydrogenosomes were primarily regarded as novel organelles, perhaps resulting from an independent symbiosis involving a *Clostridium*-like anaerobe, up until their shared ancestry with aerobic mitochondria became clear in the 1990s[36]. In 1998, Martin and Müller (co-discoverer of hydrogenosomes) published the first eukaryogenesis model to explicitly account for the evolutionary origins of hydrogenosomes[11]. In their model—the hydrogen hypothesis—the protomitochondrion was a facultative aerobe capable of

Box 2 | Serial endosymbiotic theory and oxygen

From Sagan[1]:

> "It is suggested that the first step in the origin of eukaryotes from prokaryotes was related to survival in the new oxygen-containing atmosphere: an aerobic prokaryotic microbe (that is the protomitochondrion) was ingested into the cytoplasm of a heterotrophic anaerobe. This endosymbiosis became obligate and resulted in the evolution of the first aerobic amitotic amoeboid organisms."

In Lynn Margulis' original SET narrative, the 'first step' of eukaryogenesis was seen as an adaptive response to the oxygenation of the atmosphere, ultimately driven by the evolution of oxygen-producing bacterial phototrophs (that is, Cyanobacteria and their ancestors). The free-living bacterial ancestors of mitochondria (Box 1), as well as mitochondria-bearing eukaryotes considered more broadly, were thus understood as 'fundamentally aerobic'[1]. The immediate free-living ancestor of the mitochondrion (the protomitochondrion; Box 1) was then 'ingested' (phagocytosed in later versions) into the cytosol of an anaerobic, heterotrophic bacterium[1] (later a primitively amitochondriate eukaryotic flagellate, resulting from the symbiotic merger between an archaeal host and a spirochaete)[3]. This endosymbiosis conveyed the capacity for aerobic respiration (and therefore survival in oxygenated settings) to the anaerobic host (Fig. 1a). In this scenario, both the origin of aerobic respiration in bacteria and the endosymbiotic transfer of this metabolism to anaerobic bacteria (that is, the origin of mitochondria) were understood as strategies for surviving planetary oxygenation. Afterwards, following the origin of mitochondria, chloroplasts originated independently and polyphyletically from photosynthetic bacteria ('protoplastids', homologous to modern cyanobacteria) perhaps up to 20 times[1,6].

In the 1960s, the timing of Earth's oxygenation, when free oxygen (O_2) first became a permanent feature of the atmosphere, was only beginning to be constrained[165]. In a pioneering effort, the geochemist Dick Holland placed this transition to between 1.8 and 0.5 billion years ago (Ga)[166]. Holland explicitly understood the oxygenation of the atmosphere as representing the point at which the rate of oxygen production by bacteria and the photodissociation of water surpassed the rate of oxygen consumption by volcanic gases and other surface reductants[166]. The palaeontologist Preston Cloud later estimated that oxygenic photosynthesis originated between 2.1 and 1.7 Ga, and that atmospheric oxygen levels sufficient for aerobic respiration (known as the 'Pasteur point,' equivalent to 1% of present atmospheric levels (PAL) of O_2) probably arose between approximately 1.2 and 0.6 Ga (ref. [167]). Margulis, citing Cloud, suggested that atmospheric oxygen accumulated somewhere between 2.1 and 0.6 Ga, and that aerobic bacteria, including the protomitochondrion, evolved in response (table 1 in Sagan 1967 more specifically dates the protomitochondrion to about 1.5 Ga)[1]. Importantly, Margulis was unable to independently date the oxygenation of the atmosphere and the origin of mitochondria to determine whether they correlated in time. Instead, Margulis predicted, on the basis of available evidence, that the evolution of aerobic respiration 'presumably occurred during the transition to the oxidizing atmosphere' and that the symbiosis between the protomitochondrion and the host was 'related to survival in the new oxygen-containing atmosphere'[1]. These predicted correlations continue to influence the most recent models of eukaryogenesis (see, for example, Imachi et al.[9]).

generating H_2 via fermentation in the absence of oxygen (Fig. 1b). In a major departure from SET and other eukaryogenesis models, the benefit of the symbiont to the host in the hydrogen hypothesis was not respiration-derived ATP in an oxic environment but interspecies H_2 transfer under anoxia (an example of anaerobic syntrophy). The host (an autotrophic archaeon dependent on H_2 for methanogenesis) became irreversibly dependent on the symbiont as its only source of H_2 as it decoupled itself from abiotic sources. The host, maximizing H_2 uptake, ultimately surrounded and encapsulated the symbiont (without phagocytosis), marking both the origin of mitochondria and the onset of the eukaryotic lineage (making the hydrogen hypothesis a 'fusion' model of eukaryogenesis, in the sense that eukaryotes arose from the fusion of an archaeal and a bacterial lineage[37]) (Box 1 and Fig. 2). Only after establishing this endosymbiosis did eukaryotes migrate out of anoxic niches and into oxic settings, within which the aerobic capacities ancestral to the symbiont became selectively advantageous. Aerobic respiration was therefore a secondary benefit to early eukaryotes, realized only after the symbiont had already been acquired. Syntrophic H_2 exchange drove the initial acquisition of the symbiont, serving as the obligate and proximate metabolic driver of eukaryogenesis by virtue of its irreversibility. The legacy of this anaerobic origin is reflected by the retention of these anaerobic energy pathways across the eukaryotic tree, as seen in hydrogenosomes[7].

In 1998, another eukaryogenesis model stressing anaerobic syntrophy between archaea and bacteria, that is, the syntrophic hypothesis, was independently published[12]. According to this hypothesis, eukaryogenesis commenced between a methanogenic archaeon and a fermentative ∂-proteobacterium engaging in interspecies H_2 transfer under anoxia (Fig. 1c). The archaeal partner ultimately gave rise to the nucleus as it became surrounded by multiple ∂-proteobacterial cells, whose membranes fused together to internalize the archaeal partner, forming the eukaryotic cytosol. The symbiosis with the protomitochondrion, imagined as a facultatively aerobic methanotrophic α-proteobacterium, developed as the symbiont consumed methane produced by this methanogenic host. Methanogenesis, however, was lost as the mitochondrion switched to aerobic respiration during the oxygenation of the atmosphere. Overall, while the syntrophic hypothesis and hydrogen hypothesis have considerable differences (Box 1 and Fig. 1b,c), both models predict that syntrophic H_2 exchange between archaea and bacteria served as the metabolic basis for eukaryogenesis and that the protomitochondrion was a facultative aerobe.

Today, the common ancestry of hydrogenosomes and aerobic mitochondria is undisputed[7,38,39] and extends to mitosomes (double-membrane-bound organelles uninvolved in ATP production) as well as anaerobic and H_2-producing forms of mitochondria[7] (Box 1). These anaerobic organelles are collectively labelled MROs[7,40] (Box 1). Given this common ancestry, the crucial question is whether the ancestral mitochondrion encompassed the total metabolic diversity of its organelle descendants[40–42]. An affirmative answer is realistic only if the ancestral mitochondrion was a facultative aerobe. This would imply that all modern 'mitochondria' (aerobic mitochondria and MROs alike) vertically inherited their respective metabolisms from the same bacterial symbiont[7]. Differential loss of anaerobic and aerobic energy metabolism pathways throughout the eukaryotic tree would therefore be explicable as the result of irreversible specializations by individual lineages to either the presence or absence of oxygen, respectively. This scenario contrasts with the traditional idea that anaerobic MROs descend from an obligately aerobic α-proteobacterium and acquired their anaerobic metabolisms secondarily from other bacterial lineages

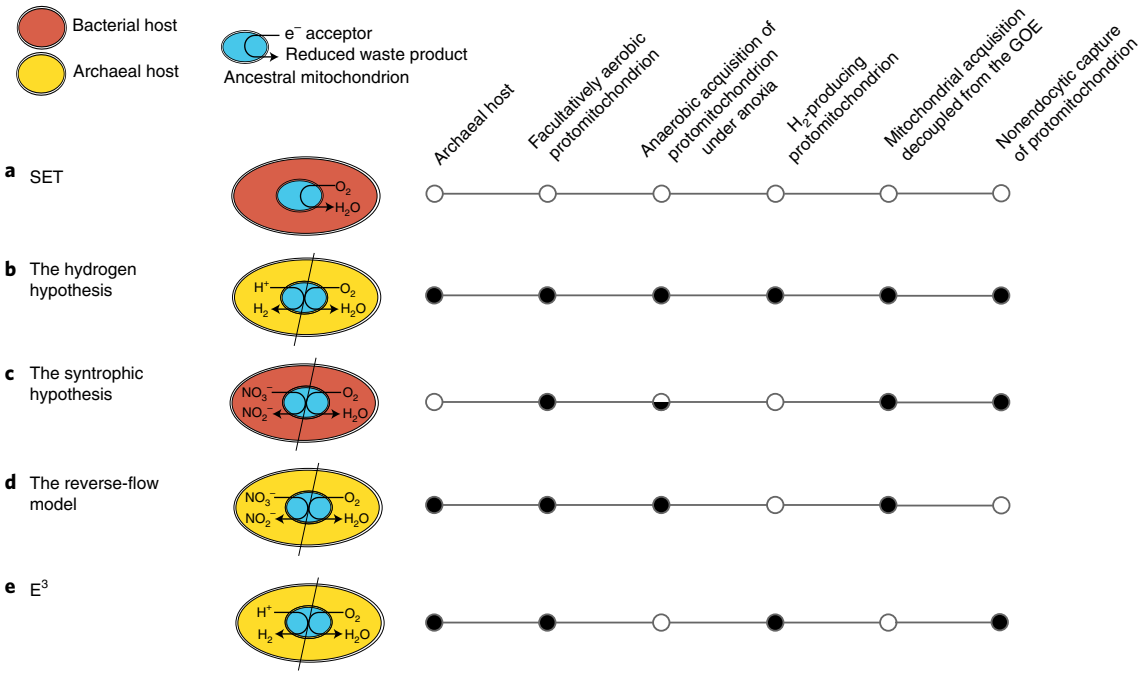

Fig. 1 | Models of eukaryogenesis. While the endosymbiotic origin of mitochondria from free-living bacteria is now undisputed, models of eukaryogenesis differ in terms of the affinity of the ancestral host cell, the metabolism of the free-living ancestor of the mitochondrion (the protomitochondrion) and the redox conditions under which mitochondria were acquired. **a**, SET originally predicted that an obligately anaerobic bacterial host 'ingested' (that is, phagocytosed) an obligately aerobic protomitochondrion in the presence of oxygen during the oxygenation of the global environment[1], what is now known as the GOE. **b**, The hydrogen hypothesis involves a H_2-dependent archaeal host (incapable of phagocytosis) engaging in anaerobic syntrophy with a H_2-producing, facultatively aerobic protomitochondrion under environmental anoxia[11,13]. **c**, The syntrophic hypothesis involves an archaeal origin of the eukaryotic nucleus (not depicted) and a bacterial origin of the eukaryotic cytoplasm and plasma membrane (thereby explaining the eukaryotic possession of bacteria-like phospholipids)[12,15,54]. This initial archaeal–bacterial fusion necessarily took place under anoxia, mediated by interspecies H_2 transfer, before the origin of mitochondria, which occurred without phagocytosis[15,54] in either the presence or absence of oxygen, depending on the hypothesized metabolism of the protomitochondrion[12,15]. **d**, The reverse-flow model is the hydrogen hypothesis with the flow of H_2 exchange reversed (that is, a H_2-consuming (not H_2-producing) protomitochondrion, capable of reducing nitrate, sulfate or fumarate under anoxia)[14] and a phagocytosing archaeal host[54]. **e**, The E^3 model, similar to the syntrophic hypothesis, predicts a syntrophic archaeal–bacterial consortium living under anoxia prior to the origin of mitochondria[9]. The H_2-producing archaeal host, originally syntrophically coupled to a sulfate-reducing bacterium (not pictured), abandoned this syntrophic partnership during the GOE as it encapsulated (without endocytosis) the protomitochondrion. This endosymbiosis was mediated by O_2 detoxification and aerobic respiration in the presence of O_2, even though the protomitochondrion was also capable of anaerobically generating H_2.

via multiple instances of lateral gene transfer (LGT), followed by additional LGT events within the eukaryotic tree[39,43].

Both the facultatively and obligately aerobic protomitochondrion scenarios are compatible with the phylogenetic intermingling of aerobic and anaerobic lineages within the eukaryotic clade[7]. To distinguish between these two scenarios, debate has primarily focused on the frequency of prokaryote-to-eukaryote LGT[39,44,45]. The relatively limited diversity of enzymes used to mediate anaerobiosis in eukaryotes has been used to invoke differential loss from a metabolically diverse ancestor, because a scenario involving polyphyletic acquisition of the relevant enzymes fails to explain why other enzymes common to anaerobic bacteria were never similarly transferred to eukaryotes[6]. On the other hand, homologues for the genes encoding enzymes used in anaerobic energy metabolism in eukaryotes, such as hydrogen-evolving [FeFe]-hydrogenases, lack a clear α-proteobacterial affinity, suggesting that these enzymes were sourced to eukaryotes from bacteria other than the protomitochondrion, thereby implying that the protomitochondrion was a strict aerobe[14,39,45]. Another interpretation of this pattern, however, is that these genes were indeed acquired by eukaryotes via the protomitochondrion but have since been laterally transferred throughout the bacterial tree to the extent that their previous α-proteobacterial affinity is no longer recovered[46,47].

Although it remains clear that the protomitochondrion was capable of aerobic respiration, debate continues as to whether the protomitochondrion was a facultative aerobe or an obligate aerobe (Box 3). This uncertainty has not prevented the development of a number of new eukaryogenesis models involving a facultatively aerobic protomitochondrion (Fig. 1). Central to many of these new eukaryogenesis models[9,13–15] is the discovery of novel archaeal lineages more closely related to eukaryotes than to other archaea.

The Asgard archaea

According to the hydrogen hypothesis, the host cell that acquired the ancestral mitochondrion was a bona fide archaeon (Fig. 1b)[11], a prediction bolstered by the increasing number of phylogenetic analyses recovering a two-domain tree of life[48–52]. In this topology, the eukaryotic nuclear lineage (that is, the lineage of the host cell that acquired the bacterial symbiont) branches among the archaea, even if the host cell was not a bona fide archaeon itself[49]. The recent metagenomic discovery of the proposed Asgard archaea superphylum (novel archaeal lineages more closely related to eukaryotes than to other known archaea) provides additional support for an archaeal origin of the eukaryotic host lineage[8,42,52,53]. The first published descriptions of cultivated representatives of the Asgard superphylum, which live syntrophically with bacterial symbionts in

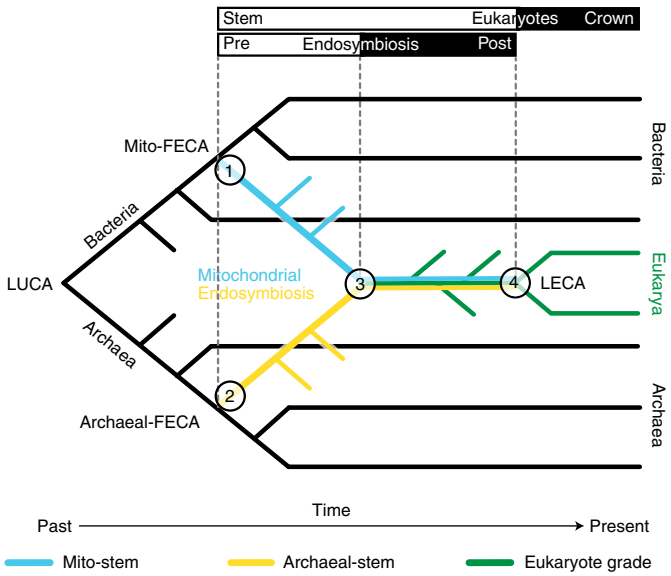

Fig. 2 | The many ancestors of eukaryotes. Eukaryotes can be determined to have possessed at least two FECAs, one reflecting divergence of the ancestral mitochondrion from its free-living α-proteobacterial relatives (mito-FECA; ①) and the other reflecting divergence of the eukaryotic host cell from its archaeal relatives (archaeal-FECA; ②). Eukaryotes, therefore, have at least two stem-lineages: the lineage belonging to the archaeal host cell (yellow) and the lineage belonging to the ancestral mitochondrion (blue). The coalescence of these two stem-lineages at mitochondrial endosymbiosis (③) may represent the achievement of a eukaryote-grade organization (green). Nevertheless, the eukaryote stem-lineage continues through to the LECA (④), which is the last common ancestor of all living eukaryotes. LUCA, last universal common ancestor.

anoxic marine sediments, reinforced these conclusions, as well as a syntrophic origin of eukaryotes[9,54]. These recent discoveries have inspired new eukaryogenesis models emphasizing anaerobic syntrophy between an archaeal host and different anaerobic bacterial symbionts[9,14], as well as corresponding reformulations of both the hydrogen hypothesis[13] and the syntrophic hypothesis[15].

In both the original hydrogen hypothesis and syntrophic hypothesis, the archaeal partner (that is, the host and future nucleus, respectively) was a methanogen[11,12]. However, the metagenome of *Lokiarchaeum* (the first described Asgard lineage[8]) lacks genes essential for methanogenesis but possesses a complete Wood–Ljungdahl pathway. These data inspired an updated hydrogen hypothesis in which the archaeal host was a strictly anaerobic H_2-dependent acetogen[13]. A survey of the metabolic repertoire of the Lokiarchaeota and the more recently described Asgard lineages (the Thor-, Odin- and Heimdallarchaeota[53]) concluded that the Asgard archaea are predominantly organoheterotrophs with lineage-specific capacities for both hydrogen production and consumption[14]. These conclusions inspired a new eukaryogenesis model, that is, the reverse-flow model, in which the archaeal host was a H_2-evolving organoheterotroph, with H_2 flowing from host to symbiont, the reverse direction compared with the hydrogen hypothesis[14] (Fig. 1d). Accordingly, the reverse-flow model involves a facultatively aerobic protomitochondrion capable of H_2-dependent growth.

The reverse-flow model's prediction of a H_2-producing archaeal host was reinforced by descriptions of the first cultured Asgard archaeon ('*Candidatus* Prometheoarchaeum syntrophicum' strain MK-D1), which performs H_2-evolving amino-acid degradation in syntrophic partnership with H_2-scavenging sulfate reducers and methanogens under anoxia[9]. Based on these observations, the entangle–engulf–endogenize (E^3) model was proposed, in which an H_2-producing archaeal host first developed a syntrophic relationship with a sulfate-reducing bacterium (never fated for endosymbiosis), before forming its symbiosis (without phagocytosis) with the ancestral mitochondrion, a facultative aerobe[9] (Fig. 1e). Unlike the hydrogen hypothesis and the reverse-flow model, the E^3 model proposes that the symbiosis with the ancestral mitochondrion was predicated on the energetic and detoxifying benefits of aerobic respiration in the wake of atmospheric oxygenation, in agreement with SET and the 'ox-tox' hypothesis. Interspecies H_2 transfer instead occurred between the H_2-producing archaeal host and the H_2-scavenging sulfate reducers. Thus, the E^3 model is a syntrophic model of eukaryogenesis, but one in which the ancestral relationship between the archaeal host cell and the ancestral mitochondrion was based on aerobic respiration rather than syntrophy. Lastly, following the published descriptions of the first cultured Asgard archaea, a revision of the syntrophic hypothesis was published, involving an H_2-producing organoheterotrophic archaeal symbiont (instead of the originally proposed methanogen) living syntrophically within a sulfate-reducing ∂-proteobacterial host in a cyanobacterial mat[15].

The hydrogen hypothesis explicitly predicted an archaeal host during eukaryogenesis[11] (and therefore invoked a two-domain tree of life[46]) almost two decades before the discovery of the Asgard archaea[8] and the increasing phylogenomic support for the two-domain scenario[42,52]. The first cultured representatives of the Asgard archaea are demonstrably nonphagotrophic and engage in syntrophic H_2 exchange with anaerobic bacteria under anoxia[9], observations similarly in agreement with the original predictions of the hydrogen hypothesis[11,13,55]. Speaking more broadly, all of the eukaryogenesis models inspired by the Asgard archaea involve syntrophic H_2 exchange between anaerobic archaea and bacteria, but make different predictions concerning the affinity and metabolism of the host, as well as the geologic timing and environmental location of the origin of mitochondria (Fig. 1).

Eukaryogenesis and environmental redox conditions

In SET, the E^3 model and other eukaryogenesis models emphasizing the energetic and detoxifying benefits of aerobic respiration, the symbiosis between the host cell and the ancestral mitochondrion began in environments sufficiently oxygenated to permit aerobic respiration, presumably marine surface waters, upper layers of some shallow marine sediments or oxygen-producing microbial mats. By contrast, eukaryogenesis models involving a syntrophic partnership between an archaeal host and a H_2-producing or H_2-consuming protomitochondrion necessarily place the onset of this symbiosis under anoxia, presumably in anoxic marine waters or sediments[13,14] (Fig. 1). However, even in these syntrophic models, the ancestral mitochondrion was a facultative aerobe (never an obligate anaerobe) and must have required some oxygen before engaging in syntrophic H_2 exchange with the archaeal host[40].

Historically, the Pasteur point, equivalent to 1% PAL O_2, represents the oxygen tension above which facultative aerobes begin respiring oxygen, approximating the limits of aerobic respiration[56]. More recently, cultures of *Escherichia coli* have been shown to grow (using glycerol as the sole carbon source, suggesting aerobic respiration rather than fermentation) at ≤3 nM O_2 (about 0.001% PAL), at least two orders of magnitude lower than the Pasteur point[57]. Likewise, cytochrome *c* oxidase, the terminal enzyme of the mitochondrial electron transport chain, has a K_m for molecular oxygen (O_2) between 0.1 and 10 μM (average of 1 μM), corresponding to 0.04–4% PAL (average of 0.4% PAL)[25,58]. Given these constraints, the free-living α-proteobacterial ancestor of the mitochondrion could have required only 3 nM to 1 μM O_2 (about 0.001–0.4% PAL O_2) to respire aerobically. Indeed, the microbial respiration of O_2 under nanomolar O_2 concentrations ('nanoxia') is becoming increasingly recognized in the modern ocean, including in taxa previously

Box 3 | Unresolved questions

Several key unknowns remain concerning eukaryogenesis. Below are five ongoing lines of investigation posed to guide future eukaryogenesis research:

1. **When did mitochondrial endosymbiosis occur?** While it remains controversial, constraining the timescale of eukaryogenesis is theoretically easy. It is the interval of time between archaeal- and mito-FECA, and LECA (Box 1 and Fig. 2). However, discerning the timing of mitochondrial endosymbiosis is more challenging, as the point of coalescence of the archaeal and free-living mitochondrial lineages is not, even in theory, an identifiable node within molecular phylogenies. Without some fundamental change in the nature of the constraints available, it may never be possible to determine whether the eukaryote grade was achieved early or late within the eukaryote stem-lineage.
2. **How many prokaryotes were involved in the origin of the eukaryotes?** The hydrogen hypothesis[11] and the ring of life hypothesis[168] suggest a simpler scenario in which the symbiosis of two prokaryotes (an archaeon and an α-proteobacterium) led to the origin of eukaryotes (Fig. 2). Other hypotheses, such as the syntrophic hypothesis[12], suggest a more complex process involving an initial symbiosis between an archaeon and a *∂*-proteobacterium, followed by a symbiosis with an α-proteobacterium. Additional confusion remains as eukaryotic genes of bacterial origin have multiple sources, even though they most strongly link eukaryotes with α-proteobacteria[169], and questions remain as to whether these genes entered the eukaryotic genome via multiple prokaryotic sources or because they had already been transferred to the genome of a single α-proteobacterial symbiont[170].
3. **What was the sequence of events within eukaryogenesis?** Debates surrounding mitochondria-early or mitochondria-late scenarios usually concern the relative timing of the acquisition of other eukaryote characters, such as the nucleus, Golgi apparatus and phagocytosis. Competing views are based on their perceived plausibility rather than on direct evidence of the sequence in which these characters were acquired. Practically, the required insights could only be arrived at using the fossil record. Recent taphonomy experiments provide some confidence in the achievability of resolving the timing of the origin of organelles based on fossil evidence[86].
4. **What does the fossil record tell us about eukaryote evolution?** Current interpretations of the eukaryote fossil record rely on the interpretation of proxy characteristics of eukaryote apomorphies, such as the presence of cell processes and excystment structures evidencing a cytoskeleton, or cell wall complexity that is perceived to be beyond prokaryote grade. These old keys to interpretation require a rethink since some of these characteristics evolved outside of Eukarya, or they are otherwise unsubstantiated. Furthermore, early stem-eukaryotes would have been morphologically indistinguishable from their archaeal forebears. A more probabilistic interpretation of the fossil record would greatly aid the calibration of molecular clocks, improving the accuracy (and probably also the precision) of evolutionary timescales.
5. **Was the ancestral mitochondrion an obligate or facultative aerobe?** While LECA definitively possessed mitochondria[38], it is unclear whether mitochondria are ancestrally obligate or facultative aerobes. In either case, the free-living ancestors of mitochondria could respire oxygen. Whether mitochondria could ancestrally metabolize anaerobically, like anaerobically functioning mitochondria and hydrogenosomes today, or whether these anaerobic capacities were laterally transferred to eukaryotes from other bacterial lineages, remains debated[7,39]. Both scenarios (differential loss of aerobic and anaerobic energy metabolism pathways versus acquisition of anaerobic energy metabolism pathways via LGT) predict the observed phylogenetic intermingling of anaerobic and aerobic lineages across the eukaryotic tree[7]. However, given that the oceans were predominantly anoxic at depth, with periodic upwelling leading to anoxic surface waters[21], during the time interval within which eukaryogenesis occurred, a facultative aerobic lifestyle is predicted to predate obligate aerobiosis[7,24], implying that the latter may only be as young as modern pO_2 levels, which in turn were probably reached well after eukaryogenesis concluded (Fig. 3).

considered to be strict anaerobes[59]. Therefore, in eukaryogenesis models emphasizing aerobic respiration (such as the E^3 model), nanomolar O_2 concentrations would have been sufficient to permit the aerobic symbiosis between the protomitochondrion and the host cell. For other syntrophic models such as the hydrogen hypothesis and the reverse-flow model, these are the minimum O_2 concentrations probably needed to make the transition to aerobic respiration following the anaerobic origin of mitochondria.

In addition to aerobic respiration, modern eukaryotes have an absolute O_2 requirement for steroid biosynthesis[60]. Sterols, a subgroup of steroids, are lipid components of the eukaryotic membrane essential for endocytosis[61]. In mitochondria-late models (Box 1), such as SET, where the ancestral mitochondrion was internalized by the host cell via endocytosis, sterol synthesis by the host can be seen as a prerequisite for the origin of mitochondria. This is not the case, however, in mitochondrial-early models (Box 1), such as the hydrogen hypothesis, involving nonendocytic means of symbiont acquisition[9,11]. Recently, a combination of ancestral sequence reconstruction and phylogenetic analyses of steroid biosynthesis genes suggested that the majority of these genes in eukaryotes were laterally transferred from myxobacteria prior to LECA, either before or after the origin of mitochondria[62]. This interpretation assumes that myxobacteria at that time in Earth history possessed the same genes as their modern representatives, whereas they may have only acquired these genes more recently via LGT[46,47]. Regardless of these uncertainties, sterol synthesis in yeast has been observed under oxygen concentrations as low as 7 nM O_2[63] (0.003% PAL O_2), constraining the oxygen levels needed to support sterol synthesis in both modern aerobic myxobacteria and the earliest sterol-synthesizing stem-eukaryotes (that is, extinct species more closely related to living eukaryotes than any other living group). Overall, considering the O_2 demands of both aerobic respiration and sterol biosynthesis, eukaryogenesis probably required only nanomolar O_2 concentrations to proceed[57,59,63], orders of magnitude lower than modern air-saturated conditions of about 250 μM O_2 (100% PAL O_2)[7].

Interspecies H_2 transfer (or H_2-based syntrophy) involves the metabolic coupling of H_2 production and consumption by multiple organisms, usually under anoxia where methanogenesis occurs (so-called methanic systems)[64,65]. In these low-energy environments, life operates at the thermodynamic limits of growth[10]. Extreme competition for electron donors keeps environmental H_2 concentrations low, driving the syntrophic exchange of H_2 between producers

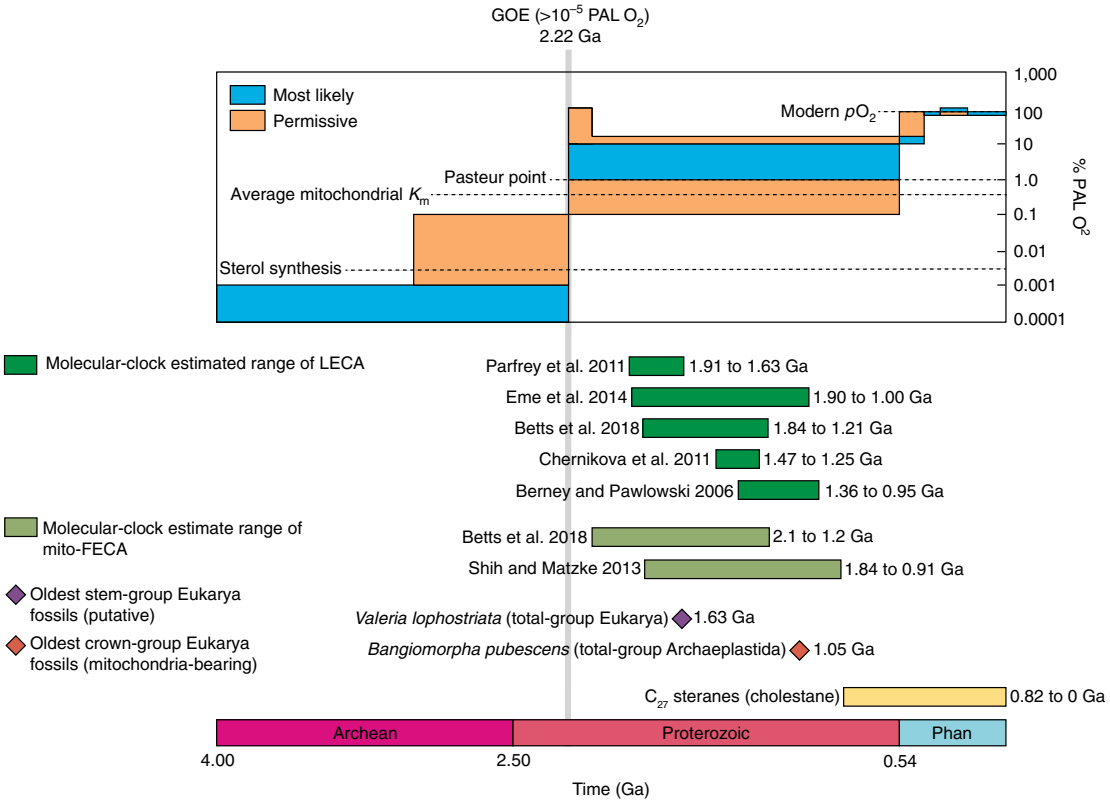

Fig. 3 | Correlated fossil, molecular and geochemical timeline. Molecular-clock estimates (horizontal green bars) and the fossil record (diamonds) both constrain the LECA to the middle of the Proterozoic Eon (2.5 to 0.541 Ga), hundreds of millions of years after the GOE (2.22 Ga (ref. [114])), when atmospheric oxygen (O_2) levels were still no more than 1–10% of PAL[21,23], approximating the Pasteur point (1% PAL)[26]. Atmospheric oxygen levels < 1.0% PAL, which have also been argued for the mid-Proterozoic Eon[134–136], fall below the Pasteur point, and even the average half-saturation constant (K_m) of mitochondrial cytochrome *c* oxidase for O_2 (0.4% PAL)[25]. These very low estimates would have therefore made an anaerobic origin of mitochondria even more likely. Regardless of these conflicting estimates, sterol synthesis, which is demonstrably operable under 7 nM O_2 (0.003% PAL)[63], was most probably permitted at this time. Modern pO_2 levels (100% PAL) were not met and sustained until the early Phanerozoic Eon (0.541 to 0 Ga)[129,130], well after (1.0 ± 0.5 billion years) the inferred origin of LECA. Data sources: the oldest total-group eukaryote fossils, such as *Valeria lophostriata*, date to around 1.625 Ga (ref. [88]), while the oldest unequivocal crown-eukaryote fossils (*Bangiomorpha pubescens*), which definitively possessed mitochondria, date to 1.05 Ga (ref. [101]). The oldest widely accepted steranes (horizontal yellow bar), the geologically stable form of eukaryotic sterols, only appear in the late Proterozoic Eon, roughly 0.8 billion years after the earliest total-group eukaryote fossils[74,75]. For the compiled molecular-clock estimates, the horizontal bars display the highest credibility intervals (95%) for the age of LECA (that is, the eukaryotic crown-group) and mito-FECA (Box 1). For Berney and Pawlowski[105], these values were obtained from fig. 1 (node 1). For Chernikova et al.[106], these values were obtained from the first set of values reported in table 2, where *B. pubescens* (then dated to 1.2 Ga) was used as a fossil calibration constraint. The values from Parfrey et al.[107] come from fig. 2, listed in table S1 under analysis 'a', while the values from Betts et al.[17] come from fig. 3. The range reported for Eme et al.[16] covers a variety of analyses exploring the impact of using different tree topologies, fossil calibration constraints and substitution models on estimating the age of LECA. The range from Shih and Matzke[108] comes from table 1. Atmospheric oxygen bounds were modified from Canfield[165]. Phan, Phanerozoic Eon.

and consumers[64]. Each partner in this relationship (the H_2 producer and consumer) is incapable of living alone under these geochemical conditions. The anaerobic production of H_2 via fermentation becomes thermodynamically self-limiting as H_2 concentrations increase in the local environment, ultimately rendering the reaction endergonic. Likewise, H_2-oxidation rates by methanogens, sulfate reducers and other H_2 consumers are constrained by the intense competition for H_2 under these energy-starved conditions, where H_2 is rapidly and efficiently scavenged[10,64]. The coupling of these two complementary metabolisms therefore solves both problems by keeping H_2 concentrations low enough to permit the continued reduction of protons to H_2 by producers (actively maintaining thermodynamic favourability) while securing a regular source of H_2 for consumers. The organisms in this syntrophic exchange ultimately become 'thermodynamically interdependent' on one another[66] as their metabolisms become obligately and mutualistically linked[10]. In other words, organisms in syntrophic relationships complete redox reactions together that would otherwise be thermodynamically unfavourable on their own under the same conditions[67,68]. Partners in syntrophic relationships therefore do much more than providing a nonessential benefit, such as extra ATP or detoxifying O_2. Indeed, they actively and obligately maintain one another's energy metabolism in an environment that would otherwise exclude them.

Many syntrophic partnerships observed in modern anoxic environments involve both bacterial and archaeal components[10,68]. In the hydrogen hypothesis, the α-proteobacterial ancestor of the mitochondrion was the 'syntrophic primary degrader' while the archaeal host was the consumer[10,13]. In the reverse-flow model, the inverse is proposed, that is, the ancestral mitochondrion was the H_2-dependent consumer while the archaeal host was the H_2-producing degrader[14] (Fig. 1d). However, several observations suggest that the ancestral mitochondrion was probably the H_2 producer while the archaeal host was the H_2 consumer. Modern mitochondria and MROs (and nonphotosynthetic eukaryotes more generally) do not consume

H_2[9,69]. While the Asgard archaea are empirically capable (at least in the case of '*Candidatus* Prometheoarchaeum syntrophicum' strain MK-D1) of H_2 production[9], metagenomic analyses also suggest that certain Asgard lineages such as the Lokiarchaeota are capable of H_2-dependent growth[13,14]. Indeed, the predicted flow of H_2 from the α-proteobacterial symbiont to the archaeal host agrees with the demonstrable H_2-producing nature of hydrogenosomes and H_2-evolving mitochondria[7].

To summarize, despite numerous competing eukaryogenesis models (Fig. 1), two general predictions regarding the environmental setting of eukaryogenesis can be made:

1. Eukaryogenesis could only have proceeded (or at least culminated) after the environmental O_2 concentrations necessary for aerobic respiration and sterol synthesis were reached, somewhere between 3 nM and 1 μM O_2 (about 0.001–0.4% PAL O_2).
2. H_2-based syntrophy under anoxia most probably supported the acquisition of the ancestral mitochondrion (as in the hydrogen hypothesis and the reverse-flow model) and/or the ecology of the archaeal host cell prior to mitochondrial acquisition (as in the syntrophic hypothesis and the E^3 model) (Fig. 1).

Together, these models suggest that eukaryogenesis occurred between anoxic and low-oxygen settings. Such conditions persist in marine sediments today but would have been met in the wider water column during the Proterozoic Eon (2.5 to 0.541 Ga) when pO_2 was probably no more than 1–10% of modern levels (discussed below). To place these predictions in the context of Earth history relative to atmospheric oxygenation, the onset of eukaryogenesis and the divergence of the major eukaryotic lineages first needs to be temporally constrained.

Dating eukaryogenesis and the origin of mitochondria

A timescale for eukaryogenesis must ultimately rest on the fossil record, which provides the only direct insight into evolutionary history. As with other controversial fossils, such as early animal fossils[70], claims for early eukaryotes must be carefully considered with respect to apomorphic characters and taphonomy, or how organisms are preserved in the rock record. The oldest claims for eukaryotes are Archaean (4.0 to 2.5 Ga) and early Palaeoproterozoic (2.5 to 1.6 Ga), including 2.7-billion-year-old sterol biomarkers[71] that have since been rejected as younger contaminants[72,73], along with all claims of eukaryote biomarkers prior to around 0.8 Ga[74,75]. Fungus-like remains have been recovered from the late Archaean[76] and early Palaeoproterozoic, such as *Tappania*[77], but lack indisputable fungal characters[78,79]. Nevertheless, *Tappania* along with *Shuiyousphaeridium* and *Valeria* (about 1.625 Ga (refs. [17,80])) preserve features that have conventionally been interpreted as evidence of eukaryote affinity[78,80–83]. Foremost among these traits are cell processes and excystment structures, which have been used as circumstantial evidence of a cytoskeleton[84]. However, evidence for some form of actin cytoskeleton has now been found in the Asgard archaeal relatives of eukaryotes[85]. Assuming vertical descent of these actin genes from archaea to eukaryotes, it therefore becomes difficult to discriminate between eukaryote and archaeal fossil remains on the basis of a cytoskeleton alone[19,86]. Eukaryote affinity has also been claimed for *Grypania*, which is known from records that extend to 1.87 Ga (refs. [87,88]). However, this organism is principally distinguished from cyanobacterial trichomes by its large size rather than any specific apomorphy[82]. Thus, while there are candidate eukaryotes known from deep in the Palaeoproterozoic[81], it is difficult to discriminate between fossil archaea or archaeal-grade stem-eukaryotes (Box 1 and Fig. 2). However, a case can be made for total-group eukaryote affinity for *Shuiyousphaeridium* and co-occurring *Dictyosphaera* about. 1.74 to 1.41 Ga (ref. [89]), which preserve intracellular structures that compare favourably in size and topology to nuclei[90], an interpretation that has recently been bolstered through experimental taphonomy[86].

The oldest-possible crown-eukaryotes are Mesoproterozoic, the earliest being *Rafatazmia chitrakootia* and *Ramathallus lobatus* (about 1.6 Ga, refs. [91,92]), which have been interpreted as red algae based principally on the presence of pit plugs and thalloid multicellular anatomy, respectively[92]. Taphonomy experiments, however, suggest that the pit-plug interpretation is unlikely[86]. The case for a chlorophyte affinity for the approximately 1-billion-year-old *Proterocladus* has been bolstered by new insights into its anatomy[93], although it is difficult to justify interpretations more refined than total-group Chlorophyta. Among a plethora of other candidate archaeplastids around the Mesoproterozoic–Neoproterozoic boundary about 1 Ga (refs. [94–97]), there are also claims of Amorphea/Unikonta, such as the putative holozoan *Bicellum*[98], although its purported holozoan affinity is not sufficiently robust to use as a fossil calibration point for eukaryote evolution. Claims of fungi at about 1 Ga (ref. [99]) and about 800 million years ago (Ma) (ref. [100]) are more convincing of the preservation of chitin than the affinity of their associated microfossils. Given these uncertainties, the oldest widely accepted record of crown-eukaryotes, constraining LECA to a minimum age of 1.047 Ga (ref. [101]), is *Bangiomorpha*, described as a bangiacean red alga based on its distinctive radially arranged cells, intercalary cell division, dimorphism, sporangial development and multicellular holdfast[102]. These characteristics are not exclusive to Bangiaceae, but they are nevertheless sufficient to justify membership in total-group Rhodophyta and, therefore, Archaeplastida and crown-Eukaryota[17].

Calibrating biological evolution to Earth history requires interpreting the fossil record of microbial evolution, which is biased by differential preservation of microbial lineages, life-history stages, environments and heterogeneities in the rock record itself[103]. Only molecular-clock methodologies are capable of integrating the biological record written in genomes and the geological record written in biomarkers, metabolically driven isotope fractionation and the fossil record. Molecular-clock methods are diverse, based on differing calibration information, lineage and genome locus sampling, evolutionary models and phylogenetic hypotheses[104]. Consequently, they yield equally disparate evolutionary timescales. Molecular-clock estimates for the age of LECA vary from about 1.91 to 0.95 Ga (refs. [16,17,105–107]) (Fig. 3). The only studies to sample the Asgard archaeal lineages estimate LECA to have emerged within the broad interval of 1.84 to 1.21 Ga, with archaeal-FECA (Box 1) emerging 3.0 to 2.3 Ga (ref. [17]). The estimates of Shih and Matzke[108] for mitochondrial endosymbiosis instead represent (at best) the divergence of mito-FECA (Box 1) from its α-proteobacterial relatives, and these range from 1.84 to 0.91 Ga. Despite the large uncertainties (a consequence of pooling the results from molecular-clock analyses that differ in analytic quality and taxonomic and genomic quantity, as well as a sparse fossil record that is difficult to interpret definitively), this timescale implies long eukaryotic stem-lineages (1.8 to 0.5 billion years long) during which eukaryogenesis occurred, and a comparatively late origin of mitochondria (irrespective of timing relative to other eukaryote apomorphies)[17]. To test the eukaryogenesis models that temporally correlate atmospheric oxygenation with the origin of mitochondria (for example, SET or the E^3 model), the estimated age of mitochondria needs to be compared with the latest evidence for Earth's long-term redox evolution.

Redox evolution of Earth's surface environment

In the decades following the initial publication of SET[1], geochemists have generally reconstructed a two-step increase in atmospheric oxygen over geologic time[109,110]. The first step, the so-called Great Oxidation Event (GOE)[111,112], represents the switch from a reducing to an oxidizing atmosphere, when pO_2 irreversibly accumulated to levels above trace concentrations (>10^{-5} PAL)[109,113] (Fig. 3).

Currently, this transition is constrained to about 2.22 Ga (ref. [114]), although it may extend slightly younger[115]. The evolutionary origin of oxygenic photosynthesis necessarily preceded the GOE, although how much earlier is debated[116,117]. Various molecular-clock estimates for the origin of crown-Cyanobacteria[118] and photosystem II[119], geochemical evidence for oxidative weathering[20] and organic carbon isotopic signatures consistent with aerobic cycling of methane[120,121] have all been used to argue for the presence of oxygenic photosynthesis hundreds of millions of years before the GOE, perhaps as early as 3.0 Ga (refs. [122,123]). Interpretation of geochemical redox proxies, however, remains debated[124,125]. Aerobic respiration may have evolved multiple times within crown-Cyanobacteria, perhaps in response to the GOE[126], but many oxygen-utilizing enzymes have also been dated to before the GOE[127], consistent with aerobic ecosystems by 2.72 Ga (ref. [120]). If both oxygenic photosynthesis and aerobic respiration substantially preceded the GOE, then SET, as originally articulated[1], could have conceivably occurred prior to Earth's oxygenation in localized 'oxygen oases' while the majority of Earth's surface environment remained anoxic. In this case, the evolutionary origin of oxygenic photosynthesis and aerobic respiration (not atmospheric oxygenation) would have been sufficient to permit SET[128].

While oxygen became a permanent feature of the atmosphere during the GOE, modern pO_2 levels were most probably only attained around 420–400 Ma, during the Palaeozoic Era[129,130] (Fig. 3). During the majority of the Proterozoic Eon, pO_2 was maintained at intermediate levels, probably around 10% PAL and possibly as low as around 1% PAL[20–22,131], leaving the deep oceans predominantly anoxic[132,133], with predominantly oxic surface waters except in (anoxic) upwelling regions[21]. Others argue for pO_2 <0.1% PAL[134] or <1% PAL[135,136] during the mid-Proterozoic, which would have left a mostly anoxic surface ocean containing 'oxygen oases' near primary producers (just as prior to the GOE)[21,137]. Thus, despite an oxidizing atmosphere after the GOE (about 2.22 Ga), the majority of the seafloor was in contact with anoxic bottom water, and much of the volume of the ocean was anoxic, until around 420 to 400 Ma (more than 1.5 billion years later). During this post-GOE interval of widespread marine anoxia, deep-water ferruginous (anoxic and nonsulfidic) conditions predominated[138]. Bottom-water euxinia (sulfide-rich anoxia) typically occurred in particular locations (for example, upwelling margins of the ocean) and sometimes transiently expanded over a greater volume of the ocean[139], but despite being more common than today, it was not as globally extensive in the mid-Proterozoic ocean as previously predicted[132,139,140]. A low-sulfate (or iron-reducing) ocean would have supported abundant sediment methanogenesis and methane accumulation in deep waters[141]. Redox conditions in benthic environments on ocean shelves were probably both spatially variable and dynamic, controlled by seasonal fluctuations in productivity and stratification, with transient episodes of oxygenation and anoxia.

A nascent co-evolutionary narrative

Synthesizing the above evidence produces an alternative, cross-disciplinary narrative linking eukaryogenesis to Earth's redox evolution. Crown-eukaryotes most probably emerged during the Mesoproterozoic Era (1.6 to 1.0 Ga) when pO_2 was perhaps no more than 1–10% PAL (Fig. 3) and key redox transition zones were located in the water column rather than the sediments[21,22]. There is no fossil, molecular or geochemical evidence suggesting that the GOE and mitochondrial acquisition were correlated in time, challenging the major co-evolutionary premise of SET, the E^3 model and every other eukaryogenesis model placing the origin of mitochondria at the GOE in the presence of O_2 (Fig. 1 and Box 2). Indeed, mitochondria were most probably acquired hundreds of millions of years after the GOE (in the late Palaeoproterozoic or Mesoproterozoic Era), during a period of pervasive deep-sea anoxia, with only weakly and dynamically oxygenated marine surface waters (Fig. 3). In contrast to SET, the oxygenation of the atmosphere (or at least the evolution of oxygenic photosynthesis and aerobic respiration) was more probably a basic prerequisite for, but not an evolutionary driver of, the origin of mitochondria.

The deep oceans during the late Palaeoproterozoic and Mesoproterozoic were predominantly anoxic and nonsulfidic, with air-saturated surface waters probably reaching the equivalent of 1–10% PAL (2.5–25 μM O_2). To modern oceanographers, these air-saturated environments would be characterized as 'hypoxic' or 'suboxic'[142]. Indeed, the Proterozoic Eon has also been labelled the 'Pasteurian', as atmospheric oxygen levels at this time largely hovered around the Pasteur point (1% PAL)[26] (Fig. 3). Such conditions are consistent with the ancestral mitochondrion only requiring 3 nM to 10 μM O_2 when living aerobically in contemporaneous air-saturated settings[25,58] and metabolizing anaerobically under anoxia, which consistently characterized the deep oceans and episodically characterized marine surface waters[137]. The mid-Proterozoic ocean probably hosted a variety of low-energy microbial ecosystems, analogous to modern microbial ecosystems from anoxic marine environments[10,68], and therefore was probably conducive to syntrophic couplings between bacteria and archaea and, by extension, a syntrophic origin of eukaryotes.

Obligate aerobiosis in eukaryotes most probably evolved after an initial period of anaerobic syntrophy and facultative aerobiosis, as originally predicted by the hydrogen hypothesis, and again by the syntrophic hypothesis, the reverse-flow model and the E^3 model (Fig. 1). After this initial stage of anaerobic syntrophy under anoxia, various eukaryotic lineages presumably migrated to shallower settings and adapted to the 1–10% PAL O_2 levels of the mid-Proterozoic surface ocean (dynamically oxygenated conditions conducive to facultative aerobiosis), and then again to more stably oxygenated settings over the course of the Phanerozoic as pO_2 reached 100% PAL (conditions conducive to obligate aerobiosis) (Fig. 3)[7]. The initial transition to aerobic respiration and the invasion of oxic settings by eukaryotes happened no later than the early Neoproterozoic Era (1.0 to 0.541 Ga) when eukaryotic microfossils definitively deposited in oxic settings first appear in the rock record (this dating may be pushed back, however, with additional geochemical analyses)[18,19]. If this date holds, then the apparent radiation of eukaryotes during the early to mid-Neoproterozoic[107,143] could represent the expansion of eukaryotes into more oxygenated settings, within which modern eukaryotic clades diversified.

Modern pO_2 levels and obligately aerobic eukaryotes adapted to 100% PAL O_2 are geologically recent phenomena that substantially post-date the origin of mitochondria (and eukaryogenesis more generally) by hundreds of millions of years (Fig. 3). The aerobic energy metabolism of extant eukaryotes adapted to life under modern pO_2 levels therefore cannot be projected back to the ancestral mitochondrion, which originated at a time in Earth history when pO_2 was no more than 1–10% of its present level[6,7,24]. Such metabolic projections are typically made in the absence of geologic context (as discussed by Martin[6]), or in the absence of discussions on hydrogenosomes, H_2-producing mitochondria and interspecies H_2 transfer[18,33,144]. When both geologic and metabolic lines of evidence are integrated, a consistent picture emerges: an anaerobic origin of mitochondria mediated by syntrophic H_2 exchange under anoxia[24].

Thus, while adaptation to oxic conditions may be fundamental to explaining the subsequent diversification of eukaryotic taxa, it is unlikely to have been critical in the origin of eukaryotes per se. This ordering (syntrophic origins under anoxia with a much later diversification under modern pO_2 levels) is ultimately an example of a simple and well-accepted principle, that is, that the selective pressures and environmental factors that initially drive a major evolutionary transition cannot be equated with the adaptive benefits that such a transition later produces[145]. Plausibly, the management

of a bacterial endosymbiont (and later an organelle) subsequently led to increased cell size and the evolution of the eukaryotic endomembrane system (allowed by mitochondrial genes and mitochondrial bioenergetic membranes), enabling access to additional food sources (that is, other cells) via phagocytosis[55,146].

The narrative broadly outlined above is the natural synthesis[24] of the hydrogen hypothesis[11] and the near-contemporaneous and independent recognition that the post-GOE Proterozoic ocean remained predominantly anoxic at depth[132]. Like SET, the hydrogen hypothesis has been explicitly set in Earth's historical context as a co-evolutionary narrative[7,24–26,58,128,147–149]. The hydrogen hypothesis has made successful and promising predictions concerning the universality of mitochondria in eukaryotes[7,38], the archaeal affinity of the eukaryotic host cell and the two-domain tree of life[46,52], and the syntrophic and nonphagotrophic lifestyle of the closest cultured archaeal relatives of eukaryotes[9,55]. The geologic record, specifically the temporal decoupling of Earth's oxygenation from the origin of mitochondria (discussed here), the earliest known fossil eukaryotes deposited in oxic settings post-dating the earliest known fossil eukaryotes by about 0.2–0.8 billion years[18] and the earliest known fossil evidence for phagocytosis post-dating the earliest known fossil eukaryotes by at least around 0.5 billion years[150], also agrees with the hydrogen hypothesis (Fig. 3). This demonstrable predictive and explanatory power, spanning both the molecular and geologic records, is a testament to the hydrogen hypothesis' internal consistency and compatibility with Earth's long-term redox evolution. Resolving the details of this emerging co-evolutionary framework requires continuing to integrate eukaryogenesis models into the field of historical geobiology[24], as well as generating and testing new predictions spanning the Earth and life sciences, as Lynn Margulis did in 1967 (ref. [1]).

Conclusions

The chronology (the origin of mitochondria substantially post-dated the GOE), environmental setting (methanic marine sediments or anoxic water column) and mechanism (interspecies H_2 transfer) for mitochondrial acquisition defended here all falsify the prediction that the oxygenation of the atmosphere provided the key selective pressure for this ancestral endosymbiosis. Instead, mitochondria most probably evolved in the mid-Proterozoic (late Palaeoproterozoic to Mesoproterozoic), under anoxia, driven by syntrophic H_2 exchange with an archaeal host, as originally predicted by the hydrogen hypothesis[11]. Eukaryotes are most probably ancestrally facultatively aerobic and may have only diversified within oxygenated settings (that were still 'hypoxic' by modern standards[142]) in the early Neoproterozoic Era[18,19]. Instead of representing a redox landscape 'challenging' or 'stifling' to eukaryotes[151,152], the primarily anoxic and low-energy ecosystems of the mid-Proterozoic Eon more reasonably served as the necessary cradle of eukaryotic life[7,24].

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Author contributions

D.B.M. conceived the study and wrote the paper with contributions from R.A.B., S.J.D., E.A.S., D.P., P.C.J.D. and T.M.L.

Competing interests

The authors declare no competing interests.

Additional information

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