Journal of Theoretical Biology 434 (2017) 2–10

Contents lists available at ScienceDirect

Journal of Theoretical Biology

journal homepage: www.elsevier.com/locate/jtbi

Physiology, anaerobes, and the origin of mitosing cells 50 years on

William F. Martin

Institute for Molecular Evolution, University of Düsseldorf, Universitätsstr. 1, 40225 Düsseldorf, Germany

ARTICLE INFO

Article history:
Received 3 November 2016
Revised 19 December 2016
Accepted 4 January 2017
Available online 11 January 2017

Keywords:
Endosymbiosis
Mitochondria
Chloroplasts
Hydrogenosomes
Eukaryote origin

ABSTRACT

Endosymbiotic theory posits that some organelles or structures of eukaryotic cells stem from free-living prokaryotes that became endosymbionts within a host cell. Endosymbiosis has a long and turbulent history of controversy and debate going back over 100 years. The 1967 paper by Lynn Sagan (later Lynn Margulis) forced a reluctant field to take endosymbiotic theory seriously and to incorporate it into the fabric of evolutionary thinking. Margulis envisaged three cellular partners associating in series at eukaryotic origin: the host (an engulfing bacterium), the mitochondrion (a respiring bacterium), and the flagellum (a spirochaete), with lineages descended from that flagellated eukaryote subsequently acquiring plastids from cyanobacteria, but on multiple different occasions in her 1967 account. Today, the endosymbiotic origin of mitochondria and plastids (each single events, the data now say) is uncontested textbook knowledge. The host has been more elusive, recent findings identifying it as a member of the archaea, not as a sister group of the archaea. Margulis's proposal for a spirochaete origin of flagellae was abandoned by everyone except her, because no data ever came around to support the idea. Her 1967 proposal that mitochondria and plastids arose from different endosymbionts was novel. The paper presented an appealing narrative that linked the origin of mitochondria with oxygen in Earth history: cyanobacteria make oxygen, oxygen starts accumulating in the atmosphere about 2.4 billion years ago, oxygen begets oxygen-respiring bacteria that become mitochondria via symbiosis, followed by later (numerous) multiple, independent symbioses involving cyanobacteria that brought photosynthesis to eukaryotes. With the focus on oxygen, Margulis's account of eukaryote origin was however unprepared to accommodate the discovery of mitochondria in eukaryotic anaerobes. Today's oxygen narrative has it that the oceans were anoxic up until about 580 million years ago, while the atmosphere attained modern oxygen levels only about 400 million years ago. Since eukaryotes are roughly 1.6 billion years old, much of eukaryotic evolution took place in low oxygen environments, readily explaining the persistence across eukaryotic supergroups of eukaryotic anaerobes and anaerobic mitochondria at the focus of endosymbiotic theories that came after the 1967 paper.

1. Introduction

Lynn Margulis (1938–2011) did a lot for biology. There is much to say about her 1967 paper "On the origin of mitosing cells", published under her name at the time, Lynn Sagan. She will be referred to here throughout as Lynn Margulis. It was a very important paper in the development of endosymbiotic theory. The main point of her proposal was that eukaryotes arose through symbiosis. The following passage aptly summarizes her 1967 version of symbiotic theory for eukaryote origin in her words:

> *"In keeping with the hypothesis, the following organisms should have evolved: a free-living complex flagellar counterpart; a free-living mitochondrion counterpart; and a heterotrophic prokaryote capable of ingesting cells. Free-living cells co-descendant with eukaryotic organelles might still contain cistrons homologous to those in (9+2) homologues, mitochondria, and plastids. For example, we may one day find different types of blue-green algae that are co-descendant with typical chrysophysean and rhodophysean plastids, which contain DNA with cistrons homologous to those in the plastids.*
>
> *If the theory is correct all eukaryotic cells must be seen as multi-genomed systems. This implies that a goal of cellular chemistry is understanding the way in which all biochemical reactions are coded off the nucleic acid of the nucleus and the subcellular organelles. All eukaryotes must contain at least three specific types of DNA: nuclear, mitochondrial, and (9+2) homologue. An additional DNA that is associated with the chloroplasts must be found in all eukaryotic plants."* (Sagan, 1967, p. 270).

The purpose of this contribution is to commemorate her 1967 paper in the broader context of endosymbiotic theories and to see

E-mail address: bill@hhu.de

http://dx.doi.org/10.1016/j.jtbi.2017.01.004
0022-5193/

where theories for the origin of mitosing cells are 50 years later. It is perhaps best to start by first recapping what Margulis's 1967 paper said. The introductory quote sums it up. Margulis envisaged three cellular partners at eukaryotic origin: the host (an engulfing bacterium), the mitochondrion (a respiring bacterium), the flagellum (a spirochaete), followed by a fourth partner, the plastid (a cyanobacterium) in the plant lineage. She very clearly says that new sets of genes entered the eukaryotic lineage via these symbioses and that, if her theory is correct, all eukaryotes are "multigenomed". Multigenomed is a 1967 way of saying that all eukaryotes arose through endosymbiosis, and that there should be no eukaryotes out there that arose autogenously, that is, solely by standard microevolutionary mechanisms (point mutation and gene duplication) operating in a prokaryote without endosymbiosis. "Multigenomed" is also a statement and prediction about a particular kind of genomic chimerism that, if endosymbiotic theory is correct, should exist in eukaryotes. In a different passage (p. 231) she wrote that "*some attraction between nucleic acid of the host and that of the symbiont*" is required for her theory to work, she was thus saying that genes should be found that were transferred from endosymbionts to the host, although one could also interpret her mention of "attraction" to relate to (presumed) basal body DNA origin. The gene transfer idea might seem to have been way ahead of its time in 1967, but endosymbiotic theory has a long history and the concept of gene transfers from endosymbionts was out there in the literature decades earlier, even before we knew for sure that genes are made of DNA, as a passage from Wallin attests:

> *"It appears logical, however, that under certain circumstances, in harmony with chemical and physical laws, bacterial organisms may develop an absolute symbiosis with a higher organism and in some way or another impress a new character on the factors of heredity. The simplest and most readily conceivable mechanism by which the alteration takes place would be the addition of new genes to the chromosomes from the bacterial symbiont."* (Wallin, 1925; p. 144).

Margulis's 1967 paper starts out by acknowledging prior contributions in the field of symbiotic theory. She mentions Mereschkowsky (by way of mention in Wilson's book), but not what he said: Mereschkowsky set forth powerful physiological arguments for a symbiotic origin of plastids from cyanobacteria (Mereschkowsky, 1905), but disfavoured a symbiotic origin of mitochondria, whereby the traits we today attribute to mitochondria he attributed to the nucleus, which he thought descended from an independent endosymbiosis that preceded the plastid (Mereschkowsky, 1910). She also mentions Wallin, but not what he said: He argued for a symbiotic origin of mitochondria, but not for plastids, as he thought the plastids of plants were transformed mitochondria (Wallin, 1927). Margulis's 1967 paper can probably be credited as having the first thorough formulation of endosymbiotic theory that has plastids and mitochondria both being derived from endosymbioses, but from different symbiotic associations involving different bacterial symbionts. There was a very curious paper by Goksøyr (1967) that also has mitochondria and chloroplasts descending from different endosymbionts, but the paper is extremely short and only has one reference (to work by Stanier that does not mention endosymbiosis) hence the literature and the reasoning underlying the conclusions in that paper remain somewhat of a puzzle. Margulis's 1967 paper also cites Ris and Plaut (1962), a rare example of papers that appeared during the time from 1928 to 1967 that discussed the old endosymbiotic ideas of Mereschkowsky and others in a positive light. The first author of that paper, Hans Ris, is important for endosymbiotic theory because he taught Margulis genetics at the University of Wisconsin, where she sat next to Jonathan Gressel (pers. comm.) and learned about endosymbiotic theory in Ris's lectures. In addition, Walter Plaut was Margulis's thesis advisor (Archibald, 2014) with whom she published on protists (Plaut and Sagan, 1958).

The 1967 paper moves at a very quick tempo over its first pages covering oxygen, aerobic bacteria, the origin of mitochondria, and larger cells. By page 229 she has reached the main course — mitosis and flagella — which continues through page 244. A half a page on plastid origin from cyanobacteria (an established but controversial concept at the time) is followed by several pages of crosschecking against available observations to see if the theory was internally consistent and consistent with geochemical evidence. After that come five pages on the accrual of oxygen on Earth history, a topic of general interest still today but with a very different narrative than in 1967 (Lyons et al., 2014; Fischer et al., 2016; Lenton et al., 2016; Reinhard et al., 2016), and the influence of oxygen on microbial metabolism followed by a discussion of mitochondrial division (organelle division was the point of departure for Mereschkowsky's theory for plastid origin), three pages on flagellar division and its relationship to the postulated symbiotic origin from spirochaetes, a terse but convincing summary of plastid division, and finally her section on predictions, from which stems the introductory quote.

She is uncompromising in her distinction between prokaryotes and eukaryotes. Mereschkowsky failed on that aspect by misinterpreting the nucleus of fungi, as reviewed elsewhere (Martin et al., 2001). She interprets the origin of mitochondria as corresponding to the appearance of oxygen in the Earth's atmosphere. The origin of photosynthesis in eukaryotes is interpreted as symbiotic acquisition from cyanobacteria, however she surmises that "*different photosynthetic prokaryotes (protoplastids) were ingested by heterotrophic protozoans at various times during the evolution of eumitosis*" (p. 247). If we look at her Figure 1 carefully, we can count 20 independent origins of plastids. Those 20 independent "protoplastid" endosymbioses are labelled by plastid colour (red, green, yellow, brown), much like Mereschkowsky's reasoning in his 1910 series, although he only had seven primary symbioses, all plastids of the same colour stemming from the same event. If we turn to her 1970 book (Margulis, 1970) for clarity on the number of plastid origins, we find that on three subsequent pages her Figure 2–6 shows 10 origins of plastids, her Figure 2–7 shows 15 origins of plastids, and her Figure 2–8 shows one origin of plastids.

On balance, Margulis's paper is as much about Earth history, oxygen, mitosis, flagella and the spirochaete endosymbiont as it is about mitochondria and plastids. Her 1967 Figure 2 spans 14 pages (some full page, some half page) summarizing variants of chromosome division during mitosis across different eukaryotic groups. The focus on mitosis is understandable, the paper was about the origin of mitosing cells. Overall it was quite a paper, for its time or otherwise.

2. Its impact

Why did the paper have such a big impact? Clearly the 1967 paper brought endosymbiosis back onto the evolutionary map, back into 'polite biological society' to use Wilson's famous 1925 words (quoted in Sagan (1967), p. 226 in the footnote), but that is not the whole story by far. The extremely broad appeal of the paper rests in Margulis's presentation of a more or less clearly articulated *narrative* that linked Earth history both to microbial evolution and to the prokaryote-eukaryote divide. Many people simply want to know more about early evolution, and her broad-brushed account of a possible series of evolutionary events linking endosymbiosis, oxygen, and the geological record connected a lot of dots. It probably had no precedent (the word 'probably' is used here deliberately because the literature is vast and nobody knows all of it). Virtually nothing was known about the workings of mitochondrial and plastid physiology when Mereschkowsky and Wallin were writing on

endosymbiosis and the very first insights into the course of oxygen accumulation during Earth's history were just coming to the fore in the 1960s. Margulis had the opportunity to put it all together, which she did. In a nutshell, her synthesis was this: cyanobacteria make oxygen, oxygen accumulates about 2.5 billion years ago, oxygen begets oxygen-respiring bacteria that become mitochondria via symbiosis, followed by later cyanobacterial symbioses (albeit in 20 different lineages independently) that bring photosynthesis to eukaryotes. That simple synthesis (if we disregard the 20 origins of plastids, which she did not mention explicitly in the text), with the link to Earth history, is perhaps what made the paper so exciting in its day and the reason why it was so widely embraced. Eukaryotic anaerobes did not fit into that narrative at all, however, we will return to that in later sections.

3. Endosymbiotic theory and the data

Her synthesis resonated well, but it resonated best without the spirochaete symbiont. In contrast to the mitochondrion (respiration of oxygen) and plastids (production of oxygen), the spirochaete flagellum had connections neither to physiology nor to Earth history. With the benefit of hindsight and many genomes worth of data, we can look back from today's standpoint and ask where the 1967 paper hit the mark and where it missed. She had the cyanobacterial origin of plastids right, though like Mereschkowsky she had too many cyanobacterial symbioses. She had the single bacterial origin of mitochondria right and she had mitochondria stemming from a different endosymbiont than plastids. That is possibly the only thing that Margulis maintained in her 1967 paper upon which everyone would still agree today. The spirochaete part of her theory, that had such a prominent place in the paper and in all of her subsequent works, never found any support in evidence.

What kind of evidence could have supported the spirochaete idea? The presence of DNA in chloroplasts and mitochondria played an important role in the formulation of her theory. Clearly, Margulis was also predicting the presence of DNA in basal bodies. There was one isolated report that seemed to provide evidence in favor of basal body DNA (Hall et al., 1989) but it turned out to be an irreproducible artefact (Johnson and Rosenbaum, 1990; Johnson and Dutcher, 1991).

Margulis did not anticipate an important development that would allow scientists to make genes from trees and to test the predictions of endosymbiotic theory: molecular phylogenetics. Fitch and Margoliash's paper on how to make trees from amino acid sequence comparisons also made its debut in 1967 (Fitch and Margoliash, 1967). Endosymbiotic theory turned out to be an important proving ground for molecular phylogenetics in that it made a clear prediction: Sequences in organelle DNA should be more similar to their prokaryotic counterparts than to DNA sequences in the nucleus. That proved to be true for plastids (Bonen and Doolittle, 1975) and for mitochondria (Schwartz and Dayhoff, 1978). Competing theories to explain the presence of DNA in organelles did not make that prediction (Raff and Mahler, 1972; Bogorad, 1975; Cavalier-Smith, 1975).

An incisive paper by John and Whatley (1975) made a strong case based on comparative physiology that the mitochondrion ancestor should be sought among the purple nonsulfur bacteria, perhaps in proximity to *Paracoccus denitrificans*, a member of the group that later came to be named α-proteobacteria (Stackebrandt et al., 1998) in rRNA-based systematics. That mitochondria and plastids branched with respiring bacteria and with cyanobacteria respectively in phylogenetic trees (Schwartz and Dayhoff, 1978) provided evidence in favor of endosymbiotic theory that was strong enough to eventually quiet its many critics. There was also a natural prediction that nuclear genes for organelle specific proteins might derive from the organelle genome, having been transferred to the nucleus, this kind of reasoning permeated Schwartz and Dayhoff's (1978) interpretations of trees for cytochrome *c*.

Thus, protein and gene sequence data supported the older theories of Mereschkowsky and Wallin for plastid and mitochondrial origin via endosymbiosis, while also supporting Margulis's incisive synthesis that the two organelles are descended from two different endosymbionts. There is still no consensus today on which lineages of proteobacteria and cyanobacteria are most closely related to the ancestors of mitochondria and plastids (Zimorski et al., 2014), the lack of consensus having to do with the number of genes investigated (Ku et al., 2015a), with sampling issues (Degli Esposti et al., 2016), and with lateral gene transfer among prokaryotes after the origin of organelles (Ku et al., 2015b). For plastids, genomic data point to heterocyst-forming cyanobacteria (Dagan et al., 2013) for mitochondria the genomic data point to facultatively anaerobic proteobacteria (Müller et al., 2012), respectively. However, Margulis's idea of a spirochaete origin of flagella, which she defended for over 40 years (Marguils et al., 2006) on the basis of morphological similarities, never found any real support in molecular, genetic or biochemical evidence and has been abandoned by everyone else.

4. The host

The host for the origin of mitochondria in Margulis's 1967 version of endosymbiotic theory was a non-descript fermenting cell, capable however of ingestion: *"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 (i.e. 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."* (Sagan, 1967, p. 228). Today we would call the "ingestion" property phagocytosis. Phagocytosis is also how de Duve (1969) imagined the mitochondrion entering the host, Stanier (1970) saw it the same way (although he had plastids arising before mitochondria). Of course, the discovery of archaebacteria (Woese and Fox, 1977), soon to be renamed archaea (Woese et al., 1990), changed thoughts on the phylogenetic nature of the host decisively. Archaeal RNA polymerases (Langer et al., 1995) and ribosomes (Fox et al., 1980) clearly linked the eukaryotic lineage to the archaea. The significance of that was immediately recognized (Woese, 1981). Woese and colleagues, however, had the origin of the host going right back to the days before there were individual free-living cells such that eukaryote complexity was more or less a direct inheritance from the earliest phases of cell evolution (Woese, 1981, 1998, 2002), with mitochondria tacked on to an otherwise prefabricated, ready made eukaryotic lineage. This "three domain" concept, traces eukaryotes basically back to the last universal common ancestor. The view that eukaryotes represent an ancient and independent lineage of descent encompassing complex cells that existed long before mitochondria entered the eukaryotic lineage still has many supporters today (Kurland and Harish, 2015; Forterre, 2013; Penny et al., 2014).

The rooted version of the three domain tree (Woese et al., 1990) had eukaryotes branching as sisters to the archaea and the three domains (eukaryotes and two kinds of prokaryotes, bacteria and archaea) at equal rank. Margulis adapted her views on the host to accommodate archaea by positing that the host was perhaps a relative of *Thermoplasma* (Margulis et al., 2000, 2006), as Dennis Searcy (1992) had been suggesting for some time. But in doing so, she modified the original formulation of the 1967 theory, which had mitochondria preceding the spirochaete flagellum, to have the spirochaete entering first into association with a *Thermoplasma* like host, giving rise to a nucleated cell that diversified into descendant eukaryotic (mitosing) lineages lacking mitochondria

(anaerobes like *Trichomonas* for example) and later arising aerobic lineages that acquired the mitochondrion.

Views on the host have changed in recent years. Thanks to improved phylogenetic methods (Cox et al., 2008; Williams et al., 2013) and environmental sequencing (Spang et al., 2015; Hug et al., 2016), microbiologists are currently finding new lineages of archaea whose ribosomal proteins branch more closely to the cytosolic ribosomes of eukaryotes than do those of other archaea. This new 'tree of life' result, which people are calling the two domain tree, has an archaeal host. With respect to Margulis's theory, that would mean either an archaeal host for the origin of flagella (Margulis et al., 2006) or an archaeal host for the origin of mitochondria (Sagan, 1967), depending on whether we take her older or newer formulations of her theory. With regard to eukaryote origins, an archaeal host is closer to what Margulis had in mind than to what Woese had in mind, and it is what I have been saying for 20 years (Martin and Müller, 1998; Sousa et al., 2016).

5. Endosymbiosis works best in the currency of physiology

In terms of physiology, Margulis got the plastid right, in terms of numbers she missed the mark. In 1967, she had photosynthesis as a symbiotic acquisition whose origin traced to cyanobacteria, albeit acquired by 20 different heterotrophic mitochondriate hosts, as suggested in her figure. Today, everybody would probably agree with the cyanobacterial part, but not with the multiple origins. The addition of the mitochondrion was a significant improvement upon Mereschkowsky's version of endosymbiotic theory, because he missed the mitochondrion altogether (just like Wallin missed the plastid). Similar to Mereschkowsky, who postulated seven independent plastid origins (Mereschkowsky, 1910), Margulis 1967 paper had many different endosymbiotic plastid acquisitions. Most of the independent plastid origins that both Margulis and Mereschkowsky postulated are today explained by the subsequent spread of plastids through secondary endosymbiosis (Lee, 1977; Gibbs, 1978; Archibald, 2015). Molecular data have indicated a single plastid origin from cyanobacteria for some time (Gould et al., 2008).

She also got it right concerning the symbiotic origin of mitochondrial energy metabolism as it was known at the time: *"oxidation of glucose using molecular oxygen via the Krebs cycle (H atoms from organic acids combine with DPN, FAD, and cytochromes; ATP is generated; and water is eliminated) occurred only in the symbiotic mitochondrion under the direction of its own genes."* (p. 229). It is difficult to name an earlier statement about the origin of eukaryotic respiration that was similarly explicit and that could be seen as similarly accurate from today's perspective.

The spirochaete had a structural role, not a physiological role, in the 1967 paper. In her later formulations it had a physiological role as well (sulfur metabolism), but in such a way as to generate the nucleus and, more problematically, primitively amitochondriate eukaryotes (Margulis et al., 2000, 2006). Today, the concept of primitively amitochondriate eukaryotes is no longer tenable, as all of the major clades (also called supergroups) of eukaryotes harbor mitochondriate forms, tracing the mitochondrion to the eukaryote common ancestor (Martin and Müller, 1998; Embley and Martin, 2006; Müller et al., 2012), which is much closer to that what Margulis had in mind in her 1967 paper than in her later writings.

6. Eukaryotic anaerobes, traditionally misplaced in endosymbiotic theory

To the same degree that she got the independent origin of chloroplasts and mitochondria right, she got the evolutionary status of eukaryotic anaerobes wrong. In 1967 she wrote (p. 228) that the origin of mitosis *"most likely occurred after the transition to the oxidizing atmosphere, since all eukaryotic organisms contain mitochondria and are fundamentally aerobic"*. Margulis, like everyone else, did not anticipate the discovery of hydrogenosomes (Lindmark and Müller, 1973) or how they would impact endosymbiotic theory. Hydrogenosomes are anaerobic, H_2-producing forms of mitochondria. The organelle of trichomonads harbors a short fermentative pathway that generates one ATP per pyruvate via substrate level phosphorylation yielding H_2, CO_2 and acetate as end products. From the standpoint of physiology, the organelles initially resembled clostridia more than mitochondria (Müller, 1980; Whatley et al., 1979) and for a short time in history were thought to represent independent endosymbionts that were distinct from the mitochondrion. Everyone now agrees by virtue of physiological and phylogenetic evidence that hydrogenosomes, and their even more reduced forms, mitosomes, are mitochondria (Tovar et al., 1999, 2003; Mai et al., 1999; Williams et al., 2002; Hrdy et al., 2004; Boxma et al., 2005; Embley and Martin, 2006; van der Giezen, 2009; Müller et al., 2012).

Until the mid 1990s the jury was still out on the evolutionary provenance of hydrogenosomes. The phylogeny of nuclear encoded hydrogenosomal proteins (Horner et al., 1996; Embley and Hirt, 1998) and the presence of a mitochondrial genome in ciliate hydrogenosomes (Boxma et al., 2005) resolved that issue, however. Margulis on the other hand, persistently denied that hydrogenosomes were mitochondria, maintaining that mitochondrial origin occurred after the diversification of eukaryotic lineages and corresponded to oxygen-dependent lifestyle, with eukaryotes that have aerobic mitochondria arising late, while (some) eukaryotic anaerobes were both primitively amitochondriate and basal branching in her view. That is to say, she placed eukaryotic anaerobes with hydrogenosomes (and mitosomes) basal in eukaryote phylogeny; eukaryotic anaerobes branched off before the acquisition of mitochondria in her schemes (Margulis et al., 2000, 2006). She classified organisms with hydrogenosomes as lacking mitochondria because she did not accept a common origin for hydrogenosomes and mitochondria. In that regard, Margulis's later view of mitochondrial origin (eukaryotic anaerobes being early-branching lineages, the mitochondrion being phagocytosed by a nucleated host) was similar to that of the archezoa hypothesis, which was refuted by all data that was ever obtained to test it (reviewed by Embley and Martin (2006)). In 1967, eukaryotic anaerobes were not salient to her theory, on p. 271 she wrote that it would be futile to search for "*eumitotic fossils dating from anaerobic times*". de Duve (2007) also was unwilling to accept a common origin for hydrogenosomes and mitochondria.

Eukaryotic anaerobes and anaerobic forms of mitochondria have been a problem for all versions of endosymbiotic theory where oxygen stands in the foreground, whether Sagan's (1967) version or subsequent formulations. There are basically three ways to explain the presence of mitochondria in eukaryotic anaerobes: we can label them for convenience as anaerobes amitochondriate, anaerobes late, and facultative anaerobes early.

The first possibility — anaerobes amitochondriate — is that eukaryotic anaerobes never possessed mitochondria (Vossbrinck et al., 1987; Cavalier-Smith, 1987; Margulis et al., 2006) and that mitochondria correspond to the origin of the aerobic lifestyle in eukaryotes (Sagan, 1967). That, however, does not mesh well with the observations that mitochondria and anaerobic forms of mitochondria are present in all eukaryotic supergroups studied to date and that the eukaryotic anaerobes, both with and without mitochondria, are spread out all across eukaryotic phylogeny (Müller et al., 2012; Stairs et al., 2015).

The second possibility — anaerobes late — is that the origin of mitochondria corresponds to the origin of an obligately aerobic lifestyle in eukaryotes, like Margulis said in 1967, but that all

eukaryotes are ancestrally obligate aerobes (taking the ubiquity of mitochondria across eukaryotes into account) such that all eukaryotes that inhabit anaerobic environments lacked the ability to colonize such habitats and therefore had to undergo some kind of lateral gene transfer (LGT) in order to be able to gain access anaerobic niches (Stairs et al., 2015). That view, though very popular these days (like eukaryote LGT narratives in general), does not however mesh well with the observation anaerobes from across the different eukaryotic supergroups always possess subsets of exactly the same small set of about a dozen key enzymes to synthesize ATP under anaerobic conditions (Müller et al., 2012). Of course, one could alternatively argue that anaerobic eukaryotes so diverse as fungi, animals, *Euglena*, *Trichomonas*, *Giardia* and *Entamoeba* always acquire exactly the same genes from prokaryotes in their independent anaerobic transitions.

In the case of 'anaerobes late', though, the phylogenetically diverse eukaryotes that inhabit anaerobic environments would have to be undergoing massively parallel LGT from the same donors in recently diverged evolutionary lineages. There is no evidence for the workings of such recent prokaryote to eukaryote LGT processes in eukaryotic genomes (Ku and Martin, 2016). As a fallback position, one could then alternatively argue that one lineage of eukaryotes acquired the genes required for anaerobic energy metabolism and then subsequently passed them around by some kind of eukaryote-to-eukaryote LGT. That kind of eukaryote-to-eukaryote LGT is, however, also not found at the comparative phylogenomic level (Ku et al., 2015a). The LGT claims for anaerobes late are founded in odd branching patterns observed in single gene trees, which are inherently prone to phylogenetic errors.

It is evident that there are two severe and general problems with the 'anaerobes late' theory: (i) The first problem is that if LGT is the way that eukaryotes gain access to anaerobic habitats, then genes for enzymes like cytochrome *bd* oxidase, a very common bacterial terminal oxidase that is not inhibited by sulfide (Forte et al., 2016; Korshunov et al., 2016), should be among the very first and most frequent genes that eukaryotes acquire via LGT when entering anaerobic environments. But in over 1.6 billion years (Parfrey et al., 2011), eukaryotes have never acquired *bd* oxidases (or sulfate reduction, or acetogenesis, or anaerobic photosynthesis, or methanogenesis or other pathways common among anaerobic prokaryotes). Instead they have the same handful of enzymes for anaerobic energy metabolism that trace, in the currency of gene presence, to the eukaryote common ancestor (Müller et al., 2012), any patchy distributions being readily attributable to differential loss (Ku et al., 2015a; Ku and Martin, 2016). (ii) In the bigger picture of Earth history, newer findings have it that oxygen arrived in the oceans much later than Margulis or anyone else thought; in fact geochemists have been telling us for two decades that the oceans had very low oxygen levels and were subject to widespread anoxia until about 580 million years ago (Lyons et al., 2014; Reinhard et al., 2016) with more recent evidence indicating that the rise to roughly our current atmospheric levels of O_2 were generated by early land plants only some 400 million years ago (Lenton et al., 2016). Thus, the Earth history narrative that was current in 1967 (oxygen early) has changed: oxygen is the latecomer in evolution, not anaerobes. Of course, the new view of oxygen in Earth history throws a monkey wrench into narratives that, like Margulis's initial version in 1967, either linked the origin of mitochondria, eukaryotes and atmospheric oxygen in a causal chain with temporal proximity to the advent of atmospheric oxygen roughly 2.4 billion years ago, or that assume a strictly aerobic ancestral state for eukaryotes from which anaerobic forms ostensibly arose recently (via LGT).

The third possibility — facultative anaerobes early — is that the common ancestor of mitochondria and hydrogenosomes (i) was a facultative anaerobic proteobacterium that was able to generate ATP with or without the help of O_2, whereby (ii) that endosymbiont was present in the eukaryote common ancestor, and (iii) as eukaryotes diversified into their descendant lineages (supergroups one would say nowadays), they underwent ecological specialization to oxic and anoxic environments (Martin and Müller, 1998; Tielens et al., 2002; Mentel and Martin, 2008; Müller et al., 2012), with differential loss accounting for the interspersed phylogeny of eukaryotes with typical mitochondria, anaerobic mitochondria, or hydrogenosomes. That view predicts the existence of intermediate states such as facultative anaerobic mitochondria, which are found for example in *Euglena* (Müller et al., 2012) and hydrogen producing mitochondria, which are found for example in some ciliates (Boxma et al., 2005). It furthermore directly accounts for the unexpected presence of highly reduced organelles of mitochondrial ancestry (now called mitosomes) in eukaryotes that were once thought to lack mitochondria altogether (Tovar et. al, 1999; Mai et al., 1999; Williams et al., 2002; Tovar et al., 2003) while also predicting the complete loss of mitochondria in some terminal lineages, which was recently reported. In the bigger picture of Earth history, 'facultative anaerobes early' fits seamlessly with the newer findings that oceans were oxygen poor and largely anoxic until about 580 million years ago (Poulton et al., 2004; Lyons et al., 2014; Planavsky et al., 2016). In the view of facultative anaerobes early, no convoluted LGT narratives are required to deliver the same genes for anaerobic survival to many different eukaryotic lineages, rather the genes required to underpin anaerobic growth were present in the eukaryote common ancestor, whereby most symbiont genes were transferred to the host's chromosomes (Martin and Müller, 1998; Timmis et al., 2004; Ku et al., 2015a), a select few for components of the electron transport chain were retained in organelles (Allen, 2015), and the symbiotic origin of mitochondria, not oxygen, was the decisive factor in eukaryote origin (Lane and Martin, 2010).

7. What do people think now: is there consensus?

The larger goal of Margulis's 1967 paper was to explain the origin of eukaryotes (mitosing cells) via endosymbiosis in an Earth history context. Fifty years later, there is still no consensus among evolutionary biologists regarding the origin of eukaryotes. Margulis believed that symbiosis was important as a mechanism of change (variation) in evolution. I completely agree (Lane and Martin, 2010; Martin et al., 2015), but she had a pinch too much symbiosis in her theory, the flagellum was unnecessary. Her opponents believed that endosymbiosis was altogether unnecessary in evolutionary theory, arguing that one could account for the differences between prokaryotes and eukaryotes with a long series of point mutations (Cavalier-Smith, 1975), and the DNA of chloroplasts and mitochondria could be explained by the budding off or sequestration of nuclear genes (Bogorad, 1975).

Arguably, the most radical aspect of Margulis's 1967 paper, and the aspect that was most difficult to accept for evolutionary biologists (as in the days of Mereschkowsky and Wallin), was her recourse to symbiosis as a *bona fide* evolutionary mechanism. For Margulis, that mechanism entailed the combination of physiological capabilties having to do with oxygen (and in her later papers, sulfur) and the combination of structures (the spirochaete flagellum) to produce novel lineages at the highest taxonomic rank via combination of existing cells. I could not agree more with Margulis's main argument that endosymbiosis is itself an evolutionary mechanism, but I think that she got its main mechanisms of action wrong in the cells whose origin she was striving to explain (eukaryotes). I maintain that the combination of anaerobic physiological capabilities involving hydrogen, namely anaerobic syntrophy with a facultative anaerobic mitochondrial ancestor (Martin and Müller, 1998), better accounts for observed eukaryote physiol-

ogy than does oxygen. I also maintain that the mere physical orientation of getting one cell inside another can itself lead to evolutionary novelty, via gene transfer from symbiont to host before the origin of organelle protein import machinery, and via host (archaeal) gene expression in the presence of bacterial lipid vesicles in the archaeal cytosol (Gould et al., 2016). Furthermore, I maintain that the physical, endosymbiosis-dependent transition from bioenergetics at the plasma membrane (in prokaryotes) to bioenergetics on the membranes of internal organelles in the eukaryote common ancestor was crucial to the origin of cell biological complexity in the eukaryote common ancestor, because major evolutionary transitions do not come from free, they come at an energetic price (Lane and Martin, 2010). Those are by no means mainstream views.

In 2016, many if not most authors publishing on the topic of eukaryogenesis still see no crucial role either for symbiosis or for mitochondria at eukaryote origin (Gray, 2014; Keeling et al., 2015; Booth and Doolittle, 2015; Forterre, 2011; Baum, 2015; Dacks et al., 2016; Lynch and Marinov, 2015, 2016; Koonin, 2015; Martijn and Ettema, 2013). For some, "luck" is preferable to endosymbiosis as an evolutionary mechanism (Keeling et al., 2015; Booth and Doolittle, 2015). Other authors see an important role for mitochondria at eukaryote origin, but also envisage other symbionts, typically the nucleus, preceding the mitochondrion (López-García and Moreira, 2006). Still others see a decisive and mechanistically pivotal role for mitochondria at eukaryote origin (Martin and Müller, 1998; Lane and Martin, 2010; Lane, 2015; McInerney et al., 2014; Speijer, 2015; Blackstone, 2013; Degli Esposti, 2014; Radzvilavicius and Blackstone, 2015; Sousa et al., 2016). Thus, 50 years later, the field is still debating the role and the significance of endosymbiosis in evolution.

8. On the origin of mitosing cells 50 years later

The origin of mitosis is a very challenging topic. Trying to bridge the evolutionary gap that separates prokaryotic and eukaryotic cell organization, chromosome division and cell division with the tools of logical inference is a daunting task. A look at the 14 pages of Margulis's 1967 Figure 2 readily explains why: there is a frightening breadth of observations for which to account. Margulis invoked processes related to the spirochaete origin of flagellae to help account for mitosis, but mitosis is only part of the story.

Mitosis is the cell division segment of the cell cycle. During mitosis, centromere-containing chromosomes are shut down in their activity, condensed, aligned and partitioned with the help of microtubules onto daughter cells. The formation of the nucleus that contains those eukaryotic chromosomes requires an endomembrane system for its formation. The eukaryotic endomembrane system in turn consists of membrane vesicles, which are in constant flux throughout the cytoplasm and are made out of bacterial lipids. All of the above, in addition to meiosis, the mechanism by which eukaryotes avoid extinction at the hands of Muller's ratchet, are missing in archaea, the lineage from which the host is descended.

In the 50 years since Margulis's 1967 paper, progress in understanding the origin of organelles has been substantial (Zimorski et al., 2014; Lane, 2015), while progress in understanding the origin of mitosis has been slow. More effort has been invested in trying to unravel the origin of the eukaryotic endomembrane system (a set of structures) (Dacks et al., 2016) than in trying to unravel the origin of mitosis itself (a process). The eukaryotic endomembrane system encompasses many functions, but the most widely touted in the context of the prokaryote-eukaryote transition has been phagocytosis: the ability to engulf, digest and oxidize other cells. Cavalier-Smith (1975, 2010) had argued for over 40 years that phagocytosis was the key invention at eukaryote origin. Many biologists follow that argument and view the matter similarly, interpreting mitochondria and the other traits that distinguish eukaryotes from prokaryotes as coming in the wake of a (primitive) phagocytotic feeding habit (Martijn and Ettema, 2013; Koonin, 2015; Dacks et al., 2016).

A problem with the phagocytosis theory is that it invariably starts from invaginations of the plasma membrane in a prokaryote, which no one has yet seen, even in reports that advertise evidence for the existence of "complex" archaeal cells supposedly capable of such invaginations (Spang et al., 2015). Endosymbioses of one prokaryote within another have been seen, though (von Dohlen et al., 2001), and such bacteria-within-prokaryote endosymbionts can even be repeatedly established during evolution (Husnik and McCutcheon, 2016), clearly indicating that a host cell need not be engulfing to acquire a bacterial endosymbiont. There are also many non-phagocytotic eukaryotes with endosymbionts (Bianciotto et al., 1996; Kneip et al., 2008). I have always doubted that the endomembrane system arose as a preadaptation for eating bacteria. I have also always doubted that the endomembrane system took root at the host's plasma membrane, because the plasma membrane is where prokaryotes make ATP via chemiosmosis; giving up that chemisomotic ATP synthesis before there were mitochondria for internal chemisomotic ATP synthesis has never seemed to me either likely or natural.

As a radically endosymbiotic alternative to phagocytosis first, perhaps the endomembrane system took root not at the *host*'s plasma membrane, but at the *mitochondrial endosymbiont*'s plasma membrane (Gould et al., 2016). Numerous bacteria and archaea do produce vesicles at their plasma membranes, but the vesicles are always excreted into the environment. They are called outer membrane vesicles, or OMVs (Deatherage and Cookson, 2012). If the mitochondrial ancestor came to reside within an archaeal host, OMVs produced by the endosymbiont would naturally generate an endomembrane system in the host's cytosol, forming from vesicles consisting of bacterial lipids with an ancestrally outward flux (Gould et al., 2016). In that view, the endomembrane system is the result of endosymbiosis, one cell living inside the other, and a consequence mitochondrial origin, not its prerequisite. The functional equivalent of OMVs are still pinched off into the cytosol by mitochondria today, they are called mitochondrial derived vesicles, or MDVs (Soubannier et al., 2012).

And mitosis? There is still no consensus on whence eukaryotic mitosis arose. A recent proposal has it that mitosis arose from meiosis (Garg and Martin, 2016). Indeed, sex traces to the eukaryote common ancestor (Ramesh et al., 2005; Speijer et al., 2015), just like mitochondria and the endomembrane system do. Eukarotes display more variation in mitosis (Parfrey and Katz, 2010; Raikov, 1994) as well-illustrated by her Figure 2 in Sagan (1967), than they do in meiosis (Ramesh et al., 2005; Speijer et al., 2015). This suggests that mitosis arose as a shortened form of meiosis, bypassing the recombination and reduction steps and that meiosis arose as a rescue from Muller's ratchet (Garg and Martin, 2016), but there is no need to summarize that proposal here. It has little to do with Sagan (1967) — aside from the circumstance that it is a radically symbiogenic model, even more radical than Sagan's (1967) proposals, because it first derives a process more complicated than mitosis, yet also more conserved, in the eukaryote common ancestor (meiosis) and operates with a bare minimum of symbiotic partners: the mitochondrion and its archaeal host. It also requires mitochondria to energetically finance the origin of evolutionary novelties required for basic cell division to evolve from binary fission in prokaryotes to mitosis embedded within a cell cycle in eukaryotes.

Finally, I point out that Margulis assumed in her 1967 paper that anaerobic fermenting cells were ancestral, both for the origin of life and as a host for the origin of mitochondria. 'Fermentation first' is an old but seldom inspected set of assumptions. It goes back to Haldane (1929) and stems from a time when we knew

very little either about how cells work in terms of conserving energy or about what substrates for microbial growth were available on the early Earth. From the standpoint of energetics and physiology, anaerobic autotrophs fit better as a starting point in evolution, both as it concerns the host for the origin of mitochondria (Martin et al., 2015; Sousa et al., 2016) and at the origin of life (Schönheit et al., 2016).

9. Conclusion

Did Margulis's 1967 paper change the views of a generation? In some ways yes, in some ways no. She was able to make her opponents (some grudgingly) admit that mitochondria and chloroplasts are descended from endosymbiotic bacteria. The importance of endosymbiosis as an evolutionary mechanism is however still debated, as many experts still prefer to bridge the prokaryote-eukaryote transition without resorting to any evolutionary mechanism that departs from point mutation (or gradualism, more generally) to the degree that endosymbiosis does. Other schools are quite comfortable with endosymbiosis as an evolutionary mechanism *per se*. When it comes to the origin of mitochondria and plastids, there are limits as to what point mutation can achieve. Respiration, photosynthesis, and organelle genomes in eukaryotes are the product of endosymbiosis (Allen, 2015), not point mutation. Endosymbiotic origins of mitochondria and chloroplasts were very rare events (Lane, 2015), but they provided the entire backbone of eukaryote physiology. If not for mitochondria we would not be here, and if not for plastids — primitive land plants, say newer data (Lenton et al., 2016) — we would not have oxygen to breathe. Eukaryotic anaerobes that do not need to breathe oxygen at all for a steady supply of ATP are spread out across the breadth of eukaryote diversity. Their origin and evolution is not an issue with which Margulis's 1967 paper dealt.

Acknowledgements

I thank the ERC (advanced Grant number 666053) for financial support, Sriram Garg, Verena Zimorski and Sven Gould for many productive discussions about endosymbiosis and Nick Lane for making sure I check the number of plastid symbioses in the 1967 paper.

References

Allen, J.F., 2015. Why chloroplasts and mitochondria retain their own genomes and genetic systems: colocation for redox regulation of gene expression. Proc. Natl. Acad. Sci. USA 112, 10231–10238. doi:10.1073/pnas.1500012112.

Archibald, J.M., 2014. One Plus One Equals One: Symbiosis and the Evolution of Complex Life. Oxford University Press, Oxford, UK.

Archibald, J.M., 2015. Genomic perspectives on the birth and spread of plastids. Proc. Natl. Acad. Sci. USA 112, 10147–10153.

Baum, D.A., 2015. A comparison of autogenous theories for the origin of eukaryotic cells. Am. J. Bot. 102, 1954–1965. doi:10.3732/ajb.1500196.

Bianciotto, V., Bandi, C., Minerdi, D., Sironi, M., Tichy, H.V., Bonfante, P., 1996. An obligately endosymbiotic mycorrhizal fungus itself harbors obligately intracellular bacteria. Appl. Environ. Microbiol. 62, 3005–3010.

Blackstone, N.W., 2013. Why did eukaryotes evolve only once? Genetic and energetic aspects of conflict and conflict mediation. Philos. Trans. R. Soc. B 368, 20120266. doi:10.1098/rstb.2012.0266.

Bogorad, L., 1975. Evolution of organelles and eukaryotic genomes. Science 188, 891–898. doi:10.1126/science.1138359.

Bonen, L., Doolittle, W.F., 1975. On the prokaryotic nature of red algal chloroplasts. Proc. Natl. Acad. Sci. USA 72, 2310–2314. doi:10.1073/pnas.72.6.2310.

Booth, A., Doolittle, W.F., 2015. Eukaryogenesis, how special really? Proc. Natl. Acad. Sci. USA 112, 10278–10285. doi:10.1073/pnas.1421376112.

Boxma, B., de Graaf, R.M., van der Staay, G.V.M., van Alen, T.A., Ricard, G., Gabaldon, T., van Hoek, A.H.A.M., Moon-van der Staay, S.Y., Koopman, W.J.H., van Hellemond, J.J., Tielens, A.G.M., Friedrich, T., Veenhuis, M., Huynen, M.A., Hackstein, J.H.P., 2005. An anaerobic mitochondrion that produces hydrogen. Nature 434, 74–79. doi:10.1038/nature03343.

Cavalier-Smith, T., 1975. The origin of nuclei and of eukaryotic cells. Nature 256, 463–468. doi:10.1038/256463a0.

Cavalier-Smith, T., 1987. Eukaryotes with no mitochondria. Nature 326, 332–333. doi:10.1038/326332a0.

Cavalier-Smith, T., 2010. Origin of the cell nucleus, mitosis and sex: roles of intracellular coevolution. Biol. Direct 5, 7. doi:10.1186/1745-6150-5-7.

Cox, C.J., Foster, P.G., Hirt, R.P., Harris, S.R., Embley, T.M., 2008. The archaebacterial origin of eukaryotes. Proc. Natl. Acad. Sci. USA 105, 20356–20361. doi:10.1073/pnas.0810647105.

Dacks, J.B., Field, M.C., Buick, R., Eme, L., Gribaldo, S., Roger, A.J., Brochier-Armanet, C., Devos, D.P., 2016. The changing view of eukaryogenesis – fossils, cells, lineages and how they all come together. J. Cell Sci. 129, 3695–3703. doi:10.1242/jcs.178566.

Dagan, T., Roettger, M., Stucken, K., Landan, G., Koch, R., Major, P., Gould, S.B., Goremykin, V.V., Rippka, R., Tandeau de Marsac, N., Gugger, M., Lockhart, P.J., Allen, J.F., Brune, I., Maus, I., Pühler, A., Martin, W.F., 2013. Genomes of Stigonematalean cyanobacteria (subsection V) and the evolution of oxygenic photosynthesis from prokaryotes to plastids. Genome Biol. Evol. 5, 31–44. doi:10.1093/gbe/evs117.

Deatherage, B.L., Cookson, B.T., 2012. Membrane vesicle release in bacteria, eukaryotes, and archaea: a conserved yet underappreciated aspect of microbial life. Infect. Immun. 80, 1948–1957.

Degli Esposti, M., 2014. Bioenergetic evolution in proteobacteria and mitochondria. Genome Biol. Evol. 6, 3238–3251. doi:10.1093/gbe/evu257.

Degli Esposti, M., Cortez, D., Lozano, L., Rasmussen, S., Nielsen, H.B., Romero, E.M., 2016. Alpha proteobacterial ancestry of the [Fe-Fe]-hydrogenases in anaerobic eukaryotes. Biol. Direct 11, 34. doi:10.1186/s13062-016-0136-3.

de Duve, C., 1969. Evolution of the peroxisome. Ann. N. Y. Acad. Sci. 168, 369–381. doi:10.1111/j.1749-6632.1969.tb43124.x.

de Duve, C., 2007. The origin of eukaryotes: a reappraisal. Nat. Rev. Genet. 8, 395–403. doi:10.1038/nrg2071.

Embley, T.M., Hirt, R.P., 1998. Early branching eukaryotes? Curr. Opin. Genet. Dev. 8, 655–661. doi:10.1016/S0959-437X(98)80029-4.

Embley, T.M., Martin, W., 2006. Eukaryotic evolution, changes and challenges. Nature 440, 623–630. doi:10.1038/nature04546.

Fischer, W.W., Hemp, J., Johnson, J.E., 2016. Evolution of oxygenic photosynthesis. Ann. Rev. Earth Planet Sci. 44, 647–683. doi:10.1146/annurev-earth-060313-054810.

Fitch, W.M., Margoliash, E., 1967. Construction of phylogenetic trees. Science 155, 279–284. doi:10.1126/science.155.3760.279.

Forte, E., Borisov, V.B., Falabella, M., Colaco, H.G., Tinajero-Trejo, M., Poole, R.K., Vicente, J.B., Sarti, P., Giuffre, A., 2016. The terminal oxidase cytochrome *bd* promotes sulfide-resistant bacterial respiration and growth. Sci. Rep. 6, 23788. doi:10.1038/srep23788.

Forterre, P., 2011. A new fusion hypothesis for the origin of Eukarya: better than previous ones, but probably also wrong. Res. Microbiol. 162, 77–91. doi:10.1016/j.resmic.2010.10.005.

Forterre, P., 2013. The common ancestor of archaea and Eukarya was not an archaeon. Archaea 2013, 1–18. doi:10.1155/2013/372396.

Fox, G., Stackebrandt, E., Hespell, R., Gibson, J., Maniloff, J., Dyer, T., Wolf, R., Balch, W., Tanner, R., Magrum, L., Zablen, L., Blekemore, R., Gupta, R., Bonen, L., Lewis, B., Stahl, D., Luehrsen, K., Chen, K., Woese, C., 1980. The phylogeny of prokaryotes. Science 209, 457–463. doi:10.1126/science.6771870.

Garg, S.G., Martin, W.F., 2016. Mitochondria, the cell cycle, and the origin of sex via a syncytial eukaryote common ancestor. Genome Biol. Evol. 8, 1950–1970. doi:10.1093/gbe/evw136.

Gibbs, S.P., 1978. The chloroplasts of *Euglena* may have evolved from symbiotic green algae. Can. J. Bot. 56, 2883–2889.

van der Giezen, M., 2009. Hydrogenosomes and mitosomes: conservation and evolution of functions. J. Eukaryot. Microbiol. 56, 221–231. doi:10.1111/j.1550-7408.2009.00407.x.

Goksøyr, J., 1967. Evolution of eucaryotic cells. Nature 214, 1161. doi:10.1038/2141161a0.

Gould, S.B., Waller, R.F., McFadden, G.I., 2008. Plastid evolution. Annu. Rev. Plant. Biol. 59, 491–517. doi:10.1146/annurev.arplant.59.032607.092915.

Gould, S.B., Garg, S.G., Martin, W.F., 2016. Bacterial vesicle secretion and the evolutionary origin of the eukaryotic endomembrane system. Trends Microbiol. 24, 525–534. doi:10.1016/j.tim.2016.03.005.

Gray, M.W., 2014. The pre-endosymbiont hypothesis: a new perspective on the origin and evolution of mitochondria. Cold Spring Harb. Perspect. Biol. 6, a016097. doi:10.1101/cshperspect.a016097.

Haldane, J.B.S., 1929. The origin of life. Ration. Annu. 3–10.

Hall, J.L., Ramanis, Z., Luck, D.J., 1989. Basal body/centriolar DNA: Molecular genetic studies in *Chlamydomonas*. Cell 59, 121–132. doi:10.1016/0092-8674(89)90875-1.

Horner, D.S., Hirt, R.P., Kilvington, S., Lloyd, D., Embley, T.M., 1996. Molecular data suggest an early acquisition of the mitochondrion endosymbiont. Proc. Biol. Sci. 263, 1053–1059. doi:10.1098/rspb.1996.0155.

Hrdy, I., Hirt, R.P., Dolezal, P., Bardonova, L., Foster, P.G., Tachezy, J., Embley, T.M., 2004. *Trichomonas* hydrogenosomes contain the NADH dehydrogenase module of mitochondrial complex I. Nature 432, 618–622. doi:10.1038/nature03149.

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., Thomas, B.C., Banfield, J.F., 2016. A new view tree life. Nat. Microbiol. 1, 16048. doi:10.1038/nmicrobiol.2016.48.

Husnik, F., McCutcheon, J.P., 2016. ↑Repeated replacement of an intrabacterial symbiont in the tripartite nested mealybug symbiosis. Proc. Natl. Acad. Sci. USA 113, E5416–E5424. doi:10.1073/pnas.1603910113.

John, P., Whatley, F.R., 1975. *Paracoccus denitrificans* and the evolutionary origin of the mitochondrion. Nature 254, 495–498. doi:10.1038/254495a0.
Johnson, D.E., Dutcher, S.K., 1991. Molecular studies of linkage group-XIX of *Chlamydomonas reinhardtii* – Evidence against a basal body location. J. Cell Biol. 113, 339–346. doi:10.1083/jcb.113.2.339.
Johnson, K.A., Rosenbaum, J.L., 1990. The basal bodies of *Chlamydomonas reinhardtii* do not contain immunologically detectable DNA. Cell 62, 615–619. doi:10.1016/0092-8674(90)90105-N.
Keeling, P.J., McCutcheon, J.P., Doolittle, W.F., 2015. Symbiosis is becoming permanent: survival of the luckiest? Proc. Natl. Acad. Sci. USA 112, 1010–10103. doi:10.1073/pnas.1513346112.
Kneip, C., Voβ, C., Lockhart, P.J., Maier, U.G., 2008. The cyanobacterial endosymbiont of the unicellular algae *Rhopalodia gibba* shows reductive genome evolution. BMC Evol. Biol. 8, 30. doi:10.1186/1471-2148-8-30.
Koonin, E.V., 2015. Origin of eukaryotes from within archaea, archaeal eukaryome and bursts of gene gain: eukaryogenesis just made easier? Philos. Trans. R. Soc. B. 370, 20140333. doi:10.1098/rstb.2014.0333.
Korshunov, S., Imlay, K.R.C., Imlay, J.A., 2016. The cytochrome *bd* oxidase of *Escherichia coli* prevents respiratory inhibition by endogenous and exogenous hydrogen sulfide. Mol. Microbiol. 101, 62–77. doi:10.1111/mmi.13372.
Ku, C., Martin, W.F., 2016. A natural barrier to lateral gene transfer from prokaryotes to eukaryotes revealed from genomes: the 70% rule. BMC Biol. doi:10.1186/s12915-016-0315-9.
Ku, C., Nelson-Sathi, S., Roettger, M., Garg, S., Hazkani-Covo, E., Martin, W.F., 2015. Endosymbiotic gene transfer from prokaryotic pangenomes: inherited chimerism in eukaryotes. Proc. Natl. Acad. Sci. USA 112, 10139–10146. doi:10.1073/pnas.1421385112.
Ku, C., Nelson-Sathi, S., Roettger, M., Sousa, F.L., Lockhart, P.J., Bryant, D., Hazkani-Covo, E., McInerney, J.O., Landan, G., Martin, W.F., 2015. Endosymbiotic origin and differential loss of eukaryotic genes. Nature 524, 427–432. doi:10.1038/nature14963.
Kurland, C.G., Harish, A., 2015. The phylogenomics of protein structures: the backstory. Biochimie 119, 284–302. doi:10.1016/j.biochi.2015.07.027.
Lane, N., 2015. The Vital Question: Why is Life the Way It Is?. Profile Books, London, UK.
Lane, N., Martin, W., 2010. The energetics of genome complexity. Nature 467, 929–934. doi:10.1038/nature09486.
Langer, D., Hain, J., Thuriaux, P., Zillig, W., 1995. Transcription in archaea: similarity to that in Eukarya. Proc. Natl. Acad. Sci. USA 92, 5768–5772. doi:10.1073/pnas.92.13.5768.
Lee, R.E., 1977. Evolution of algal flagellates with chloroplast endoplasmic reticulum from the ciliates. S. Afr. J. Sci. 73, 179–182.
Lenton, T.M., Dahl, T.W., Daines, S.J., Mills, B.J.W., Ozaki, K., Saltzman, M.R., Porada, P., 2016. Earliest land plants created modern levels of atmospheric oxygen. Proc. Natl. Acad. Sci. Usa. 113, 9704–9709. doi:10.1073/pnas.1604787113.
Lindmark, D.G., Müller, M., 1973. Hydrogenosome, a cytoplasmic organelle of the anaerobic flagellate *Tritrichomonas foetus*, and its role in pyruvate metabolism. J. Biol. Chem. 248, 7724–7728.
López-García, P., Moreira, D., 2006. Selective forces for the origin of the eukaryotic nucleus. Bioessays 28, 525–533. doi:10.1002/bies.20413.
Lynch, M., Marinov, G.K., 2015. The bioenergetic costs of a gene. Proc. Natl. Acad. Sci. USA. 112, 15690–15695. doi:10.1073/pnas.1514974112.
Lynch, M., Marinov, G.K., 2016. Mitochondria do not booth the bioenergetic capacity of eukaryotic cells. Proc. Natl. Acad. Sci. USA. 113, E667–E668. doi:10.1073/pnas.1523394113.
Lyons, T.W., Reinhard, C.T., Planavsky, N.J., 2014. The rise of oxygen in earth's early ocean and atmosphere. Nature 506, 307–315. doi:10.1038/nature13068.
Mai, Z., Ghosh, S., Frisardi, M., Rosenthal, B., Rogers, R., Samuelson, J., 1999. Hsp60 is targeted to a cryptic mitochondrion-derived organelle ("crypton") in the microaerophilic protozoan parasite *Entamoeba histolytica*. Mol. Cell. Biol. 19, 2198–2205.
Margulis, L., 1970. Origin of Eukaryotic Cells. Yale University Press, New Haven.
Margulis, L., Dolan, M.F., Guerro, R., 2000. The chimeric eukaryote: origin of the nucleus from the karyomastigont in amitochondriate protists. Proc. Natl. Acad. Sci. USA 97, 6954–6959. doi:10.1073/pnas.97.13.6954.
Margulis, L., Chapman, M., Guerrero, R., Hall, J., 2006. The last eukaryotic common ancestor (LECA): acquisition of cytoskeletal motility from aerotolerant spirochetes in the Proterozoic Eon. Proc. Natl. Acad. Sci. USA 103, 13080–13085. doi:10.1073/pnas.0604985103.
Martijn, J., Ettema, T.J.G., 2013. From archaeon to eukaryote: the evolutionary dark ages of the eukaryotic cell. Biochem. Soc. Trans. 41, 451–457. doi:10.1042/BST20120292.
Martin, W., Müller, M., 1998. The hydrogen hypothesis for the first eukaryote. Nature 392, 37–41. doi:10.1038/32096.
Martin, W., Garg, S., Zimorski, V., 2015. Endosymbiotic theories for eukaryote origin. Philos. Trans. R. Soc. Lond. B 370, 20140330. doi:10.1098/rstb.2014.0330.
Martin, W., Hoffmeister, M., Rotte, C., Henze, K., 2001. An overview of endosymbiotic models for the origins of eukaryotes, their ATP-producing organelles (mitochondria and hydrogenosomes) and their heterotrophic lifestyle. Biol. Chem. 382, 1521–1539. doi:10.1515/BC.2001.187.
McInerney, J.O., O'Connell, M., Pisani, D., 2014. The hybrid nature of the Eukaryota and a consilient view of life on earth. Nat. Rev. Microbiol 12, 449–455. doi:10.1038/nrmicro3271.
Mentel, M., Martin, W., 2008. Energy metabolism among eukaryotic anaerobes in light of Proterozoic ocean chemistry. Philos. Trans. R. Soc. Lond. B. 363, 2717–2729.
Mereschkowsky, C., 1905. Über natur und ursprung der chromatophoren im pflanzenreiche. Biol. Cent. 25, 593–604 (English translation in: Martin, W., Kowallik, K., 1999. Annotated English translation of Mereschkowsky's 1905 paper. Eur. J. Phycol. 34, 287–295).
Mereschkowsky, C., 1910. Theorie der zwei plasmaarten als grundlage der symbiogenesis, einer neuen lehre von der entstehung der organismen. Biol. Cent. 30, 278–288 (289-303, 321-347, 353-367).
Müller, M., 1980. The hydrogenosome. Symp. Soc. Gen. Microbiol. 30, 127–142. doi:10.1146/annurev.mi.42.100188.002341.
Müller, M., Mentel, M., van Hellemond, J.J., Henze, K., Woehle, C., Gould, S.B., Yu, R.-Y., van der Giezen, M., Tielens, A.G.M., Martin, W.F., 2012. Biochemistry and evolution of anaerobic energy metabolism in eukaryotes. Microbiol. Mol. Biol. Rev. 76, 444–495. doi:10.1128/MMBR.05024-11.
Parfrey, L.W., Katz, L.A., 2010. Dynamic genomes of eukaryotes and the maintenance of genomic integrity. Microbe 5, 156–163.
Parfrey, L.W., Lahr, D.J.G., Knoll, A.H., Katz, L.A., 2011. Estimating the timing of early eukaryotic diversification with multigene molecular clocks. Proc. Natl. Acad. Sci. USA 108, 13624–13629. doi:10.1073/pnas.1110633108.
Penny, D., Collins, L.J., Daly, T.K., Cox, S.J., 2014. The relative ages of eukaryotes and akaryotes. J. Mol. Evol. 79, 228–239. doi:10.1007/s00239-014-9643-y.
Planavsky, N.J., Cole, D.B., Reinhard, C.T., Diamond, C., Love, G.D., Luo, G.M., Zhang, S., Konhauser, K.O., Lyons, T.W., 2016. No evidence for high atmospheric oxygen levels 1,400 million years ago. Proc. Natl. Acad. Sci. USA 113, E2550–E2551. doi:10.1073/pnas.1601925113.
Plaut, W., Sagan, L.A., 1958. Incorporation of thymidine in the cytoplasm of Amoeba proteus. J. Biophys. Biochem Cytol. 4, 843–844.
Poulton, S.W., Fralick, P.W., Canfield, D.E., 2004. The transition to a sulphidic ocean ~1.84 billion years ago. Nature 431, 173–177. doi:10.1038/nature02912.
Radzvilavicius, A.L., Blackstone, N.W., 2015. Conflict and cooperation in eukaryogenesis: implications for the timing of endosymbiosis and the evolution of sex. J. R. Soc. Interface 12, 20150584. doi:10.1098/rsif.2015.0584.
Raff, R.A., Mahler, H.R., 1972. The non symbiotic origin of mitochondria. Science 177, 575–582. doi:10.1126/science.177.4049.575.
Raikov, I.B., 1994. The diversity of forms of mitosis in protozoa–a comparative review. Eur. J. Protistol. 30, 253–269.
Ramesh, M.A., Malik, S.B., Logsdon Jr., J.M., 2005. A phylogenomic inventory of meiotic genes, evidence for sex in *Giardia* and an early eukaryotic origin of meiosis. Curr. Biol. 15, 185–191. doi:10.1016/j.cub.2005.01.003.
Reinhard, C.T., Planavsky, N.J., Olson, S.L., Lyons, T.W., Erwin, D.H., 2016. Earth's oxygen cycle and the evolution of animal life. Proc. Natl. Acad. Sci. USA 113, 8933–8938. doi:10.1073/pnas.1521544113.
Ris, H., Plaut, W., 1962. Ultrastructure of DNA-containing areas in the chloroplast of *Chlamydomonas*. J. Cell Biol. 12, 383.
Sagan, L., 1967. On the origin of mitosing cells. J. Theor. Biol. 14, 225–274. doi:10.1016/0022-5193(67)90079-3.
Schönheit, P., Buckel, W., Martin, W.F., 2016. On the origin of heterotrophy. Trends Microbiol. 24, 12–24.
Schwartz, R.M., Dayhoff, M.O., 1978. Origins of prokaryotes, eukaryotes, mitochondria, and chloroplasts. Science 199, 395–403. doi:10.1126/science.202030.
Searcy, D.G., 1992. Origins of mitochondria and chloroplasts from sulphurbased symbioses. In: Hartman, H., Matsuno, K. (Eds.), The Origin and Evolution of the Cell. World Scientific, Singapore, pp. 47–78.
Soubannier, V., McLelland, G.L., Zunino, R., Braschi, E., Rippstein, P., Fon, E.A., McBride, H.M., 2012. A vesicular transport pathway shuttles cargo from mitochondria to lysosomes. Curr. Biol. 22, 135–141.
Sousa, F.L., Neukirchen, S., Allen, J.F., Lane, N., Martin, W.F., 2016. Lokiarchaeon is hydrogen dependent. Nat. Microbiol. 1, 16034. doi:10.1038/nmicrobiol.2016.34.
Spang, A., Saw, J.H., Jørgensen, S.L., Zaremba-Niedzwiedzka, K., Martijn, J., Lind, A.E., van Eijk, R., Schleper, C., Guy, L., Ettema, T.J.G., 2015. Complex archaea that bridge the gap between prokaryotes and eukaryotes. Nature 521, 173–179. doi:10.1038/nature14447.
Speijer, D., 2015. Birth of the eukaryotes by a set of reactive innovations: new insights force us to relinquish gradual models. Bioessays 37, 1268–1276. doi:10.1002/bies.201500107.
Speijer, D., Lukes, J., Eliáš, M., 2015. Sex is a ubiquitous, ancient, and inherent attribute of eukaryotic life. Proc. Natl. Acad. Sci. USA 112, 8827–8834. doi:10.1073/pnas.1501725112.
Stackebrandt, E., Murray, R.G.E., Trüper, H.G., 1998. *Proteobacteria* classis nov., a name for the phylogenetic taxon that includes the "purple bacteria and their relatives". Int. J. Syst. Bacteriol. 38, 321–325. doi:10.1099/00207713-38-3-321.
Stairs, C.W., Leger, M.M., Roger, A.J., 2015. Diversity and origins of anaerobic metabolism in mitochondria and related organelles Philos. Trans. R. Soc. Lond. B Biol. Sci. 370, 20140326. doi:10.1098/rstb.2014.0326.
Stanier, Y., 1970. Some aspects of the biology of cells and their possible evolutionary significance. Symp. Soc. Gen. Microbiol. 20, 1–38.
Tielens, A.G.M., Rotte, C., van Hellemond, J.J., Martin, W., 2002. Mitochondria as we don't know them. Trends Biochem. Sci. 27, 564–572. doi:10.1016/S0968-0004(02)02193-X.
Timmis, J.N., Ayliffe, M.A., Huang, C.Y., Martin, W., 2004. Endosymbiotic gene transfer: organelle genomes forge eukaryotic chromosomes. Nat. Rev. Genet. 5, 123–135. doi:10.1038/nrg1271.
Tovar, J., Fischer, A., Clark, C.G., 1999. The mitosome, a novel organelle related to mitochondria in the amitochondrial parasite *Entamoeba histolytica*. Mol. Microbiol. 32, 1013–1021. doi:10.1046/j.1365-2958.1999.01414.x.

Tovar, J., Leon-Avila, G., Sanchez, L.B., Sutak, R., Tachezy, J., van der Giezen, M., Hernandez, M., Müller, M., Lucoq, J.M., 2003. Mitochondrial remnant organelles of *Giardia* function in iron-sulphur protein maturation. Nature 426, 172–176. doi:10.1038/nature01945.

von Dohlen, C.D., Kohler, S., Alsop, S.T., McManus, W.R., 2001. Mealybug β-proteobacterial endosymbionts contain γ-proteobacterial symbionts. Nature 412, 433–436. doi:10.1038/35086563.

Vossbrinck, C.R., Maddox, J.V., Friedman, S., Debrunner-Vossbrinck, B.A., Woese, C.R., 1987. Ribosomal RNA sequence suggests microsporidia are extremely ancient eukaryotes. Nature 326, 411–414. doi:10.1038/326411a0.

Wallin, I.E., 1925. On the nature of mitochondria. IX. Demonstration of the bacterial nature of mitochondria. Am. J. Anat. 36, 131–139. doi:10.002/aja.1000360106.

Wallin, I.E., 1927. Symbionticism and the Origin of Species, 171. Bailliere, Tindall and Cox, London, UK.

Whatley, J.M., John, P., Whatley, F.R., 1979. From extracellular to intracellular: the establishment of mitochondria and chloroplasts. Proc. R. Soc. Lond. B 204, 165–187. doi:10.1098/rspb.1979.0020.

Williams, B.A., Hirt, R.P., Lucocq, J.M., Embley, T.M., 2002. A mitochondrial remnant in the microsporidian *Trachipleistophora hominis*. Nature 418, 865–869. doi:10.1038/nature00949.

Williams, T.A., Foster, P.G., Cox, C.J., Embley, T.M., 2013. An archaeal origin of eukaryotes supports only two primary domains of life. Nature 504, 231–236. doi:10.1038/nature12779.

Woese, C., 1998. The universal ancestor. Proc. Natl. Acad. Sci. USA 95, 6854–6859. doi:10.1073/pnas.95.12.6854.

Woese, C.R., 1981. Archaebacteria. Sci. Am. 98–122.

Woese, C.R., 2002. On the evolution of cells. Proc. Natl. Acad. Sci. USA 99, 8742–8747. doi:10.1073/pnas.132266999.

Woese, C.R., Fox, G.E., 1977. The concept of cellular evolution. J. Mol. Evol. 10, 1–6. doi:10.1007/BF01796132.

Woese, C.R., Kandler, O., Wheelis, M.L., 1990. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc. Natl. Acad. Sci. USA. 87, 4576–4579. doi:10.1073/pnas.87.12.4576.

Zimorski, V., Ku, C., Martin, W.F., Gould, S.B., 2014. Endosymbiotic theory for organelle origins. Curr. Opin. Microbiol. 22, 38–48. doi:10.1016/j.mib.2014.09.008.