Eukaryotic origins are heavily debated. The author as well as others have proposed that they are inextricably linked with the arrival of a pre-mitochondrion of alphaproteobacterial-like ancestry, in a so-called symbiogenic scenario. The ensuing mutual adaptation of archaeal host and endosymbiont seems to have been a defining influence during the processes leading to the last eukaryotic common ancestor. An unresolved question in this scenario deals with the means by which the bacterium ends up inside. Older hypotheses revolve around the application of known antagonistic interactions, the bacterium being prey or parasite. Here, in reviewing the field, the author argues that such models share flaws, hence making them less likely, and that a “pre-symbiotic stage” would have eased ongoing metabolic integration. Based on this the author will speculate about the nature of the (endo) symbiosis that started eukaryotic evolution—in the context of bacterial entry being a relatively “early” event—and stress the differences between this uptake and subsequent ones. He will also briefly discuss how the mutual adaptation following the merger progressed and how many eukaryotic hallmarks can be understood in light of coadaptation. Also see the video abstract here https://youtu.be/ekqtNleVJpU

1. Introduction

Most scientists, myself included, seem to live in a bubble when it comes to contentious issues in our field of study, engaging in too little real open-minded exchange of ideas. This is certainly true with regard to one of the great conundrums in biology: “How did eukaryotes come about?” I will try to set out what aspects of eukaryogenesis most researchers agree upon and what are the “bones of contention,” in a two-part contribution. The focus will be on a comparison of the two main models (“symbiogenic/chimeric” vs “autogenic/incremental,” see Table 1), which can be seen as opposite positions on a spectrum of hybrid views. I will try to show that a symbiotic relationship between prokaryotes, characterized by metabolic interdependency well before uptake (referred to as pre-uptake symbiosis or “pre-symbiosis”) seems essential for eukaryogenesis. The second article will attempt to highlight the many ways in which anachronistic reasoning (using examples from highly derived present-day eukaryotes to explain their origins) keep on clouding the debate.

I will try to carefully elucidate the reasoning behind my own, symbiogenic position. An extensive description of last eukaryotic common ancestor’s (LECA) complexity and a less biased overview of different ideas regarding its evolution can be found in a relatively up-to-date review by Koumandou et al.[2] Let me stress here that reconstructing interactions occurring more than 1.5 billion years ago, under unknown ecological conditions, involving organisms that have to be reconstructed from their present-day relatives is daunting. Thus all our statements should be cautious and conditional. When lacking such caution in what follows, please remember this proviso.

2. Symbiogenesis Versus Autogenesis: Did Eukaryogenesis Start with Bacterial Uptake or Not?

Many biologists agree that there were two (and only two) different cell types directly involved in the processes that gave rise to the evolution of the eukaryotes (one archaeon-related, the other a bacterium); see ref. [3] and references therein, though some alternatives have been proposed as well (e.g., Part III of ref. [1] and below). How and why the bacterium ended up in the archaeon is debated. An important aspect to point out is that most researchers that have wrestled with the mechanisms involved in eukaryogenesis either come from a metabolic/biochemical or a cell biology/protistology background. The first group tends to focus...
Table 1. Two basic models of eukaryogenesis.

| Characteristic                  | Symbiogenesis | Autogenesis |
|--------------------------------|---------------|-------------|
| Mitochondria                   | Early         | Late        |
| Phagocytosis                   | Late \(^{2}\) | Early       |
| Driving force(s)               | Integration of metabolism/ROS | ? |
| Intermediate states            | “Fleeting”    | ?           |
| Timespan                       | Short         | Long        |
| First uptake mechanism         | \( ^{2}\)     | Phagocytic  |
| Occurrence                     | Very rare     | Likely?     |
| Relation of organisms          | Merger (chimera) | Host acquisition |

\(^{2}\)Whether LECA was phagocytic is debated; \(^{2}\) Uptake in accordance with the inside-out model[8] Essential differences in the two competing models in the form of catchphrases.

... digestion in the absence of a “pre-mitochondrion.” The two main contending theories,

1) Autogenesis, in which small steps lead to a pre-eukaryote making a living by use of endo/phagocytic capabilities, allowing uptake of organisms, one of which will become the mitochondrion, the other,

2) Symbiogenesis, starting with the merger of two prokaryotes (archaeon and bacterium) by an unknown, “primitive” mechanism, postulating that most, if not all, eukaryotic characteristics arose as adaptations to challenges the resulting chimera faced, are compared in Figure 1 and Table 1.

Of course, several researchers have championed hybrid theories. Two extensive, well written, reviews give an overview of current thinking, one from researchers sharing the symbiogenic point of view, [9] the other tending toward “phagocytosis early.” [5]

2.1. The First Autogenic Hypothesis Had To Be Abandoned

As indicated, the older, more gradualist vision hypothesizes that eukaryotic features such as internal membrane structures, a nucleus, and phagocytosis developed early on. The phagocytic capability in turn allowed uptake of a bacterium related to present-day alphaproteobacteria that gave rise to mitochondria. Its oldest incarnation, the archean hypothesis, saw certain amitochondriate eukaryotes (“Archezoa”) as “living fossils,” reflecting the stage before endosymbiont uptake. [10] However, all amitochondriate groups are now known to have evolved from mitochondriate ancestors, so they are not seen as representing early branching clades anymore. Though the Archezoa have been abandoned, the idea that endo/phagocytic capabilities were a precondition for uptake of the endosymbiont is still thriving (see ref. [5] and references therein), because it is hard to envisage alternative mechanisms.

2.2. How Do Cells Merge in the Absence of Phagocytic Mechanisms?

Indeed, a weakness of a symbiogenic theory, with a form of phagocytosis developing after uptake, is the merger by an unknown mechanism. In this light, the very recent, first successful cultivation of an Asgard archaeon, Promethearchaeum, resembling the “hosts” involved in eukaryogenesis, but not belonging to the group most related to the “host”) shows it to have extensions, demonstrating increased curvature and possibilities for membrane–membrane interactions. Further research could show whether these archaea might occasionally take up their symbiotic partners. Even before this finding, some problems with the proposals based on full blown phagocytosis were obvious. The two main proposals for entry by established phagocytic mechanisms are entry as a pathogenic agent, for example, a facultative intracellular energy parasite, or engulfment by a predatory pre-eukaryote. Based on phylogenetic considerations, known non-phagocytic methods of entry by parasites (such as used by Bdellovibrio bacteria) are unlikely to have played a role. As mentioned, phylogenetic analyses have shown the “host”...
Figure 1. Alternative scenarios for eukaryogenesis. A) Phagocytotic engulfment or parasitic invasion. The further programmed development is interrupted and somehow endosymbiosis ensues. The “host” cell is a pre-eukaryote of archaeal origin (brown). The timing of many inventions as well as the driving forces responsible are uncertain. Only some further increase in complexity is seen to be dependent on bacterial uptake. B) Presymbiosis leading to symbiogenesis via many intermediate steps. Enhanced internal ROS formation is probably crucial in the symbiogenic process. Internal ROS leads to bacterial DNA (black circles) reduction and host DNA increase (thicker circles) plus possible membrane protection by bacterial OMVs (blue, forming a pre-ER?). Retargeting of FA oxidation enzymes to the pre-mitochondrion and (related) OMVs, combined with insertion of catalase, part of the prokaryotic secretome, gives rise to peroxisomes (olive). The archaeal outer membrane (brown) is replaced and a nuclear membrane (red) and full-blown ER (green) and Golgi (purple) evolve. The specific sequence of events here is again uncertain. For reasons of simplicity, mitochondria (blue with thin DNA circle) are depicted with a single membrane structure. The double nuclear membrane (derived from the stacking of OMVs?) is also depicted as a single structure (with ER and Golgi as connected “extensions”). Phago- or syntrophic modes refer to the use of these terms in ref. [86].

lineage leading to the eukaryotes to be most related to recently discovered groups of archaea, the Asgards, engendering speculations regarding their phagocytic capacities. However, work from Burns and co-workers based on the analysis of gene sets predictive of trophic mode seems to suggest that Asgard archaea are not phagocytic. Their findings highlight that eukaryotic phagocytosis is a capability based on a combination of archaeal and bacterial gene products. However, they cannot distinguish between a scenario in which the bacterial genes were picked up in slow progression before endosymbiont uptake or were (partly) derived from the pre-mitochondrion itself, after entry, instead. I will leave out further discussion of the highly contentious phylogenetic debates surrounding these uptake proposals (“swallowed/infected whom”) and just focus on some aspects making the whole model of an efficiently phagocytosing amitochondriate Asgard archaeon (relative) at the start of eukaryogenesis less likely. Here, it should be pointed out that there is an example of a phagocytosing amitochondriate prokaryote, though completely unrelated: a planctomycete bacterium. It demonstrates that the presence of a mitochondrion is not an absolute requirement for a form of phagocytosis to occur.

Indeed, in autogenous scenarios a more complex, larger, phagocytic cell is presupposed to have come about before efficient endogenous ATP generation (using O₂ as the final acceptor) became available from multitudes of (pre)mitochondria. Although precise reasoning vis-a-vis an energetic barrier to organismal complexity is difficult, there seems to be a correlation between increasing complexity levels and (mitochondrial) oxidation. Also, for the spoils of phagocytosis to be used efficiently, catabolic pathways linking proteins, carbohydrates, and fats to the oxidative electron transport chains (ETCs) of (pre)mitochondria seems necessary; the large majority of these came with the endosymbiont. The existence of anaerobic phagocytic eukaryotes (which are derived from aerobic, “normal” ancestors) does not invalidate these arguments; see, for example, ref. [3] and Part 2 of this paper (How anachronistic reasoning can lure us into inventing intermediates). Based on these inherent problems, and others opted for a non-phagocytic mechanism of entry, though inefficient phagocytosis, boosted by getting stuck with the endosymbiont, cannot be ruled out, especially in the light of the recent example of a phagocytosing prokaryote.

An extensive overview of the most persuasive arguments can be found in ref. [22]. The absence of all intermediate forms prior to LECA is a difficulty for both theories of eukaryogenesis, but especially for the more gradual one. Autogenesis also seems to lack credible driving forces for many of the eukaryotic “inventions.” Many of these do make sense in the context of symbiogenesis (see below).

3. Predator or Parasite Models of Eukaryogenesis Have Conceptual Problems

Let us leave aside the problem of the development of a larger and more complex cell capable of uptake of complete prokaryotes without efficient ATP generation from breakdown of its main components for now. Many of the eukaryotic traits also come from mergers of the capabilities present in either participant,
and eukaryotic endo/phagocytosis is no exception, highlighting a temporal difficulty.

Eörs Szathmáry and John Maynard Smith make an important observation in their illuminating review of “The major evolutionary transitions.”[23] They point out the real danger of selection at lower levels (in this case the individual prokaryotes) disrupting integration at the higher level (the eukaryote to be). To make such a difficult transition work (of course in the absence of foresight), starting with two essentially antagonistic cells does look less promising. A gradually developing symbiosis of groups of cells in a changing environment leading to mutual dependency seems a superior spot to look for the birthplace of the eukaryotes, with “pre-adapted” (consortia of) bacteria having better chances of surviving an “accidental” uptake,[20,24,25] because of a pre-existing metabolic integration. Here, it is worthwhile to be reminded that the successful primordial uptake of the mitochondrial forerunner depended on two aspects: entry and functional retention. Though the focus has been on the first, it might be that the second aspect represents the biggest hurdle. Retention is problematic because of the really challenging molecular and biochemical puzzle that arises upon entry and not because of “host defences”[26] as the host cells were not primed by evolution for relatively rare events (see also below). Still, one can imagine inadvertent uptake of a bacterium in symbiotic populations of archaea and bacteria, several of which have been shown to exist so far.[27]

Symbiotic interactions involving archaea and bacteria are probably very ancient. With the aid of secondary ion mass spectroscopy analysis, Schopf and co-workers found indications for interactions between CH₄ producing and CH₄ metabolizing prokaryotes already present ≈0.5 × 10⁹ years after the late heavy bombardment ended.[28] Rapid evolution of prokaryotic trade, starting with the use of the waste product of one participant, but ending with populations in which both partners secrete costly products, has even been observed in laboratory settings.[29–31] An important precondition for these stable symbioses to take hold is that they occur in spatially structured environments. There are different scenarios for the primordial symbiosis giving rise to euarkyogenesis, such as the “hydrogen hypothesis”[24] (named after the waste product involved) or alternating cellular electron-acceptors operating in the presence of varying amounts of oxygen (with intermediate metabolites as waste).[30] Both occur in highly structured environments.

Some archaea are capable of cell fusion; see ref. [32] and references therein. Bacteria can take up other bacteria (e.g., found in Mealybugs, in which insect cells contain betaproteobacteria, in turn harboring gammaproteobacteria). The last example shows such uptake is best understood as the end result of an increasingly close pre-symbiosis. However, we should be wary of modern examples, as they occur in the context of full-fledged modern eukaryotic phagocytosis.

4. Are Modern-Day Examples of Bacterial Entry Evolutionarily Misleading?

In a previous article I argued that the lessons modern-day uptake examples had to offer with regard to how the process played out early in life history, might be severely limited.[16,35] In the context of euarkyogenesis, many instances of interpreting current life forms or biological processes as representative of ancient stages have turned out to be misleading or might be so. The most notorious example was already mentioned: that the amitochondriate eukaryotes were living fossils, representing the complex phagocytosing stage before bacterium uptake. However, they are products of reductive evolution, having lost mitochondria; see, for example, ref. [36]. The current debate whether euarkyotic hydrogenosomes are reflective of ancient mitochondrial capabilities or late adaptations to new ecological niches following horizontal gene transfer (HGT; compare refs. [24, 36–40]) illustrates the difficulties involved; see also Part 2. But looking for the closest relative of the original endosymbiont among modern intracellular alphaproteobacterial parasites[26] might be misguided as well. The idea that mitochondria are derived from intracellular parasites, tricking the host into phagocytosis, got a boost from the presence of genes encoding proteins associated with the dynamic euarkyotic architecture (including phagocytosis) in archaeal “Asgard” metagenomes.[11,12] This induced Ball and co-workers to revisit the hypothesis that the parasite in question was “a Rickettsiales-like” organism.[26] Much is made of the fact that Rickettsia can multiply in the host cytosol without the use of inclusion vesicles, because this demonstrates an ability to cope with “host defences.” However, the scenario of rare, accidental, uptake, does not have to contend with such mechanisms being in place. These authors then suggest a “transition from endocytosis of nutrients to a full-fledged phagocytic lifestyle” powered by the mitochondrion. Here a conceptual flaw seems to present itself. The “endocytosis of nutrients” by what seems to be primitive/simple phagocytosis, resembling pinocytosis, functions as a construct to solve the paradox of the uptake and catabolic use of a full-size bacterium in the absence of mitochondrial power and metabolism. As pointed out by Sven Gould, all Rickettsia are obligate intracellular pathogens depending on eukaryotes for survival.[41,42] They represent highly derived adaptations to ecological niches that only became available after the flowering of eukaryotes, and co-evolved with them. Thus it becomes really difficult to deduce whether a free-living ancestor could have been involved. At this juncture it is appropriate to point out that many bacterial taxa contain organisms capable of intracellular parasitism of eukaryotes. Just looking at human pathogens: Mycobacterium tuberculosis (actinobacteria), Legionella pneumophila, Salmonella enterica, Yersinia pseudotuberculosis (all gammaproteobacteria), and Listeria monocytogenes (firmicutes) to name but a few. Even if alphaproteobacteria are overrepresented among the intracellular parasitizes of eukaryotes, this, first of all, reflects the fact that Wolbachia species can infect large amounts of arthropods (especially insects) and nematodes.[43] Crucially, the most recent phylogenetic evidence points to mitochondria evolving from a sister lineage splitting before the divergence of all sampled alphaproteobacteria, barring a meaningful choice between free-living or parasitic mitochondrial precursors (assuming such existed back then).[44] However, accidental uptake of an alphaproteobacterium-like organism that morphs into mitochondria would of course enable their modern-day relatives to escape euarkyotic host defences more easily. The recent cultivation of an Asgard representative without indications for endocytic mechanisms, but with membrane extensions makes the whole “parasite precursor for mitochondria” model less likely.[14]
5. Predator/Parasite Models of Eukaryogenesis Can Be Challenged by Physical Considerations

LECA, likely capable of phagocytosis, was a highly complex organism, using the specialized “new” functions enabled by repeated gene duplications to perform well-regulated endocytic processes.[2] Even the Asgard archaea do not come close to this level of complexity. The cultivated representatives, though encoding quite a few “eukaryotic specific proteins” remain archaea. Burns and co-workers, analyzing trophic mode predictive gene sets, conclude that these archaea are not phagocytic.[16] Dey et al. convincingly show that even claiming a “primitive” form is premature at best.[25] Such claims are based on the genomes encoding small GTPases, resembling those involved in vesicle transport, as well as COP (vesicle coat proteins) and TRAnsport protein particle (TRAPP) homologues. First of all: “homologous genes” do not automatically have “homologous functions” (see also Part 2). More importantly, for these GTPases to function in membrane traffic, proper localization is needed. In eukaryotes, most of them are anchored to membranes via hydrophobic groups. Interestingly, prenylated and palmitoylated GTPases are both involved in membrane/vesicle transport regulation, again stressing the hybrid nature of these processes, using both archaeal (isoprene building blocks) and bacterial (fatty acid building blocks) anchors. The Asgard GTPases miss canonical recognition sequences for the attachment of the hydrophobic groups and the metagenomes do not encode the necessary enzymes either.[25] Asgard metagenomes miss other endocytosis components, mediating inward membrane curvature as well as the enzymes capable of membrane scission: dynamins. These seem to be of (mitochondrial) endosymbiont origin as well.[45] One also has to consider that, though mixing of the two membrane types turns out to be easily feasible,[46] we do not know whether endocytosis with archaeal membranes is easy as well. In light of their analyses, Dey et al. stress the importance of an increasingly close pre-symbiosis.[25] This conclusion was born out upon the cultivation of an Asgard representative mentioned above.[14] Finally, as the “Asgard” metagenomes seem to predict operational ETCs, even pinocytosis could disturb their membrane potential. Thus, endo/phagocytic models need to explain adequate ATP generation in the absence of adequately functioning ETCs.

This brings us to the question of driving forces for eukaryotic inventions (e.g., the greatly increased eukaryotic gene set) beginning with the merger of two prokaryotes and why the “mitochondrial late” model[45] is less likely to be correct. Let us expand upon symbiogenic theories,[13,20,24,48] explaining their “revolutionary” character.[11,16]

6. How Symbiogenesis, the “Ultimate Coadaptation” Model, Gave Rise to the Last Eukaryotic Common Ancestor

Symbiogenic theories, explaining eukaryotic characteristics as mutual adaptations of two prokaryotes, started with the work of Martin and Muller[24] and Moreira and Lopez-Garcia.[48] Though Schopf and co-workers found indications for such interactions (possibly between archaea and γ-proteobacteria) dating back ≈3.5 × 10⁹ years,[28] eukaryotes appeared on the scene much later (between 2.1 × 10⁹ and 1.6 × 10⁹ years ago[49]). This time lag in eukaryotic evolution seems to correlate with the slow increase of O₂ due to cyanobacterial activity (and illustrated by the great oxygenation event or “GOE”; 2.3 × 10⁹ years ago). In light of such considerations, several alternative symbiotic scenarios have been proposed (see e.g. the reviews in refs. [5, 50] and references therein). I favour a primordial symbiosis based on intermediate carbohydrate metabolite exchange necessitated by recurring changes in oxygen availability alternatively hampering the different terminal electron chain complexes of the two prokaryotes involved.[20] This seems to be in line with observations in the cultivated Asgard archaeon that lives in symbioses and has metabolic pathways that could be enhanced by transferring metabolites to bacteria capable of using molecular oxygen as the final electron acceptor.[14] The essence of symbiogenesis is nicely described by Nick Lane: “The dominant selective forces driving eukaryotic evolution arose from within the cell, not the external environment.”[11] However, I disagree when he downplays the role of O₂ by stressing that even after the GOE many environments were still anoxic (which is hardly relevant). Also the existence of anaerobic eukaryotes does not mean that these in anyway reflect what might have happened during evolution towards LECA or that LECA might have had mitochondria with anaerobic capabilities. It is surprising that he correctly dismisses phagotrophic anaerobic eukaryotes as misleading later developments (stressing the difference between “origin” and “retention” of phagocytosis; see also Part 2, while he does not consider this possibility for anaerobic eukaryotes as such).[13] I stress this fact because eukaryotic evolution seems to me to only make sense in the light of an aerobic bacterium as one of the partners in the primordial merger (see below).

Whatever the specific model, symbiogenesis has turned out to be a fruitful concept, its many new insights making it a likely description of events (see ref. [6] and references therein). Let us try to reconstruct eukaryogenesis starting with our merger: we will see eukaryotic features make much more sense when seen through the prism of symbiogenic theory.

Starting out with a symbiosis between archaea and bacteria, the bacteria using oxygen when available, the archaea taking care of business when not, one can envisage cell/cell contacts arising for efficient exchange of intermediate metabolites.[28] A related model bases the symbiosis on “reverse flow” involving electron or hydrogen uptake by the bacterium.[11] Closer symbiosis led to more intimate interdependency. Rather early under these kind of circumstances “cheaters” will appear[30] and such archaeal cheaters might, for example, have lost their membrane potential. Such cells could have more easily survived increases in [O₂] and accidental uptake of the ever more interacting bacteria. Whatever the details, such a merger is not unlikely to have occurred in the Asgard communities described in.[14]
constellation in which the internal mitochondrial membranes generated copious ATP for eukaryotic inventions most likely requiring larger amounts of energy\[51\] and the driving force of highly damaging internal reactive oxygen species (ROS) formation; see, for example, refs. [52–55] and references therein. Why is internal ROS formation crucial?

1) ROS is an intrinsic by-product of oxidative phosphorylation (OXPHOS). The merger led to oxidation of many different substrates (reflecting modern-day eukaryotic metabolic versatility): carbohydrates (sugars), amino acids and fatty acids (FAs). Alternating between them leads to enhanced ROS formation.\[54,56\]

2) Eukaryotes often contain myriads of mitochondrial membranes, multiplying ROS effects. The endosymbionts used to produce ROS at their outer membranes, now these are produced smack in the middle of the pre-eukaryote.

3) ROS need metal ions (mostly Fe) for full reactive oxidative capacity, and these are concentrated inside as essential enzyme cofactors.

I will list the most important eukaryotic characteristics to be explained along these lines.

### 7.1. Endomembrane System, Nuclei, Peroxisomes, Autophagosomes, and Phagocytosis

The highly complex, diverse, eukaryotic endomembrane system possibly arose out of bacterial outer membrane vesicles (OMVs) released by the mitochondrial ancestor into the cytosol. However, some kind of internal membrane structure could have arisen prior to, or with, uptake in archaeal “hosts” by an inside-out mechanism.\[14,58\] Alternative scenarios focussing more on the evolution of the machinery involved can be found in refs. [2, 59]. An interesting aspect of the endosymbiont OMV theory is that diluting out archaeal plasma membranes would nicely explain eukaryotes ending up with bacterial ones. In this respect the theory is superior to the ad hoc explanation by the bacterium-archaeon fusion model of Forterre,\[60\] which was invoked to deal with phylogenetic challenges (see Section 8).

Nuclei, peroxisomes, autophagosomes, and phagocytosis are best understood as evolving in response to, or in the context of, pre-mitochondrial entry. The nucleus might have evolved from incremental OMV stacking around the host chromosomes, protecting against internal ROS formation of endosymbiont origin, and, after reaching a certain level of complexity, separating splicing from translation in the wake of migration of intron containing genes from mitochondrial ancestors to hosts.\[61\] This argument is sometimes countered with the observation that “the nuclear membrane would be a poor ROS defence as they can pass freely through nuclear pores.” However, a typical membrane has \(1000\) pores with a diameter of \(120\) nm, and an actual opening of \(5–10\) nm. Such openings are negligible compared to the complete surface surrounding the chromosomes, so the membrane would still be a formidable barrier indeed. Even small interposing vesicles would already reduce ROS reaching DNA behind it, so this could constitute a viable scenario for incremental evolution of the nucleus. OMVs enriched in sterols, another eukaryotic invention that makes sense in the light of endogenous ROS,\[62\] would be effective barriers. Descriptions of the use of membranes in ROS containment can be found in ref. [63]. Histone evolution also seems to have links to ROS protection.\[6\] Autophagy and autophagosomes, already “complete” in LECA,\[2\] could have evolved from mitophagy, the process of disposing of oxidatively damaged mitochondria. Phagocytosis might be seen as a variation on these mechanisms. Peroxisomes can be perfectly understood as organelles that evolved as a system to alleviate internal ROS formation associated with pre-mitochondrial FA oxidation and are indeed build from endosymbiont building blocks.[21,32,64,65] However, to illustrate the range of scenarios invoked to explain eukaryotic cell structures: Mans et al. speculated that nuclei emerged in an autogenic fashion in primitive eukaryotic ancestors, during compartmentalization triggered by archaeo-bacterial symbiosis.\[66\]

### 7.2. Meiotic Sex, Mitochondrial Genome Reduction, and Levels of Repair

The evolution of meiotic sex can be illuminated by internal ROS formation as well,[67–70] as can gradual mitochondrial genome reduction (see e.g. the effect of mitochondrial mutation rates\[71\]), bringing endosymbiont genes to places of greater safety. A revolutionary rapid period of adjustment characterized by a high mutation rate, as previously proposed, see, for example, refs. [6, 72] and references therein, is most easily understood in light of enhanced internal ROS formation. The combination of ROS and meiotic sex must have hugely sped up evolution rates, which should warn us against too easily using the normal phylogenetic algorithms (see below). Can we find traces of this “mutation bombardment”? Recently, it was found that large amounts of destabilizing mutations in mitoribosomal RNAs and the genes encoding hydrophobic core subunits of OXPHOS complexes were countered by the addition of extra (\(\approx \)75) protein subunits in the mature structures. This process was completed in LECA, while no such process was found to occur later on for chloroplast ribosomes.\[73\]

Thus, mitochondrial gene migration and targeting of mitochondrial proteins developed under natural selection, first distancing them from direct ROS generating complexes, second because behind the (beginnings of) protective nuclear membrane other beneficial effects of the selective force of “battling ROS” were evolving, such as chromosomes, extended anti-oxidant mechanisms, and superior DNA repair. A recent model looks at whether “mitochondrial complementation” could also have been a factor favoring the development of archaeal/early eukaryote cell fusion: a necessary ingredient of eukaryotic sex.\[74\]

### 7.3. Metabolic Restructuring and ROS Signaling

The long evolution of eukaryotes can be seen as a history of coping with internal ROS formation. It is reflected in, for example, the way in which low amounts of ROS are actually beneficial, as they invoke important antioxidant responses: mitohormesis. It is found in a number of examples of mitochondrial ROS acting...
as a cellular differentiation mechanism in complex multicellular organisms and the invention of uncoupling proteins (UCPs). Examples are given in refs. [54, 56] and further details can be found in references therein. Here I will only highlight one striking example. Myeloid cells confronted with pathogens and/or cellular stress can respond with NLRP3 inflammasome activation. The NLRP3 complex is highly versatile and can be activated by a range of diverse stimuli.[75] It has been suggested that at least three NLRP3 activating mechanisms (ROS increase, K⁺ efflux, and damage to lysosomes) all signal via the induction of oxidized mitochondrial DNA release, which then binds and activates NLRP3.[76] Recently it was demonstrated that this process depends on enhanced synthesis of mitochondrial DNA and that the newly made mitochondrial DNA is crucial. The authors of the study hypothesize that the new DNA, not packaged in condensed nucleoid structures yet, would be more susceptible to oxidation and subsequent fragmentation by nuclease(s).[77] These fragments are then released via membrane pores, opened by NLRP3 activators. This convergence of many signals on oxidative damage in mitochondria seems indicative of an old, evolutionarily basic, eukaryotic pathway.

8. Defining “Pre-Symbiosis” and Asking Whether the Endosymbiont Arrived Early or Late

The reconstruction of eukaryogenesis just presented seems rather convincing. Are there no major problems that have to be resolved? One difficulty resides in the fact that most phylogenetic analyses give unexpected results. Reconstructing LECA, we naturally find archaeal (Asgard archaea containing most) “host” and bacterial (“alphaproteobacterium-like”) genes. However, “eukaryotic inventions” (genes that probably evolved so quickly, they can only be classified as eukaryotic), together with bacterial genes seemingly coming from quite diverse bacteria, unrelated to the endosymbiont, form a very sizeable group. Looking at the mitochondrial proteome (i.e., the genes encoding the proteins that will end up in the mitochondrion) we encounter the same difficulty. Stating that at most 20% of the “mitoproteome” is of the “correct” bacterial clade, Gray proposed the pre-endosymbiont hypothesis, invoking an autologous organelle before uptake of the pre-mitochondrion.[78] Phylogenetic challenges like these recently led Gabaldón to valiantly propose another alternative, related theory, the “pre-mitochondrial symbioses” hypothesis.[79] He presumes eukaryogenesis to have involved multiple symbiotic interactions that predate the acquisition of mitochondria. This proposal seems nicely compatible with the latest models invoked upon studying a cultivated Asgard archaeon.[14] The multiple symbioses, invoked to explain the large diversity of organisms that seemed to have contributed to the mitoproteome, could have existed when the uptake of the “mitochondrion-to-be” took place, for example, in the context of a temporary cellular syncytium as proposed in ref. [80]. However, based on an earlier analysis[47] Gabaldón proposes the late arrival of the endosymbiont, that is, after many of the eukaryotic inventions occurred. Before we discuss these proposals, an important point has to be made. I stress the importance of pre-symbiosis because it explains metabolic routes crucial for the eukaryotes and makes the step toward a successful merger less unlikely, while Gray and Gabaldón come up with the idea to explain perceived phylogenetic difficulties (Gray with a pre-existing organelle, Gabaldón with a broader scenario of extensive gene uptake).

8.1. Phylogenetic Analysis of Eukaryogenesis Is Fraught with Difficulties

Phylogenetic analyses (especially with the availability of complete genomes and metagenomes) is a powerful technique to probe (even deep) evolution. However, it is clear that rampant HGT, the complexity of algorithms to choose from, and the degrees of freedom regarding dataset inclusion/exclusion as well as mutation rates easily lead to conflicting results. The analysis regarding the timing of endosymbiont entry is a case in point. Whether the “late arrival analysis” withstands subsequent critiques I leave up to the reader.[5,47,81,82] However, a few aspects should be pointed out. In the analysis of Degli Esposti the effects of differing grouping criteria are nicely illustrated: a well-reasoned grouping of “aerobic proteobacteria” gets rid of most of the bacterial outliers in the phylogenetic trees of 81 bioenergetic proteins; for details see ref. [81]. He also hints at distorting effects inherent to the mitochondrial clade, as they contain a lot of information derived from rapidly evolving eukaryotic parasite lineages. The recently proposed reordering of mitochondria as derived from a sister linage of the alphaproteobacteria further complicates matters.[44] as does the possibility of more “bacterial” genes in the archaeal contribution to eukaryotic ancestry than previously imagined.[83]

In his “pre-mitochondrial symbioses” hypothesis,[79] Gabaldón uses a nice metaphor to describe the phylogenetic problems. Observing that a long stem separates all the eukaryotes from their prokaryotic ancestors, he introduces the “palm tree of eukaryotes.” We encounter a massive radiation in the absence of surviving lineages reflecting intermediate stages (the trunk of the palm tree). This image can be used to illustrate some of the differences between his model and the strict symbiogenesis that is defended here.[3,6,84] If one uses a rather constant rate of change (a constant molecular clock), we are indeed missing branches. If, instead, one assumes a “revolutionary” period or bottleneck of rapid adaptations (with selection for new functions in many protein families, on populations of mitochondria inside a cell[43] with mutating ROS producing endosymbionts on their way to becoming mitochondria[52] clearly a situation never encountered before) the trunk grew so rapidly, there was no time for branching. Can I give some extra arguments for this scenario, which will also highlight some of the phylogenetic pitfalls? If we indeed start out with two different genomes, only partially integrated, still separate, metabolisms and a “mutator” in the form of strong internal ROS formation we can imagine a rapid acceleration in the rate of change. Also, separate, partly overlapping genomes allow large scale gene loss from mitochondria and make readjustment of enzymatic activities more easily feasible (using redundant copies[2]). Even HGT could have been more extensive due to oxidative membrane damage, though this is speculative.

To determine the relative influence of HGT on the findings with regard to levels of relatedness and timing of events, we should better distinguish between easily transferred genes and more hardwired ones. The latter ones encode proteins or RNAs
that are so central that they are involved in myriads of molecular interactions; these “hubs” are not easily swapped or lost. Such an “indexed HGT” algorithm might make the phylogenetic conclusions more reliable. Other problems have to do with the mitoproteome. The original genome being next to ROS forming sites of the readjusting ETC and the skewing effects of more rapidly evolving mitochondria (e.g., in parasites) have already been mentioned. But the novel circumstance of large-scale migration of genes to another genomic environment, and the combination of gene products from different genomes in molecular machines of an organelle in the making, encountering completely new stressors, might make the mitoproteome difficult to analyse with standard approaches.

Here we have been engaged in some speculation. Let us now instead use Occam’s razor. What are the things we really know? We know that the endosymbiont was present in LECA and we know that increased amounts of ATP[51] were needed to be able to make such a complicated organism as LECA,[2] I just referred to Lane and Martin,[5] who point out that eukaryotic gene complexity is expensive, and paid for by mitochondrial power. However, they explicitly state that oxygen is not essential for this power (referring to anaerobic eukaryotes), though they also mention that: “By enabling oxidative phosphorylation across a wide area of internal membranes, mitochondrial genes enabled a roughly 200000-fold rise in genome size compared with bacteria”[my italics]. This tension disappears when one recognizes that invoking anaerobic eukaryotes here might constitute an example of anachronistic reasoning, see Part 2. The role of oxygen (both as final electron acceptor in OXPHOS and as a motor driving change in its endogenous ROS form), can be considered essential.[6,20] We have explanations for many novel eukaryotic features that rely on the presence of the endosymbiont. It also seems clear that the entry of the first endosymbiont was different from all later ones (even of the cyanobacterium that gave rise to the chloroplast) because the later ones were uptakes by full-fledged eukaryotes with mitochondria (mostly?) using phagocytosis (as can be seen by extra membranes surrounding some of these entities). They also required less extensive adaptations in both host and endosymbiont.[6] Thus, strict symbiogenesis is our best bet to understand the evolution of the eukaryotes. The final picture of LECA: a completely new kind of organism based on a merger of an archaeon and a bacterium,[8] the evolution of which was the driving force for eukaryotic inventions. When? After the GOE, when more O2 became available. How can we study whether this relatively simple scenario is correct? Harcombe, Marx, and co-workers have been performing experiments to define parameters conductive to symbiosis, using different bacterial strains.[29,31]

What would happen during the co-culture of non-O2 using archaea and oxidizing bacteria under fluctuating O2? In other model systems, what will happen to membrane potentials and ROS formation in mitochondria missing UCP “safety valves”? Could archaenal membranes be more susceptible to oxidative damage than bacterial ones? I eagerly await further findings.

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9. Is This Indeed How Eukaryotes Evolved (Future Experiments and Conclusions)?

I argued against an autologous model of eukaryotic inventions in the absence of endosymbiont influences with “late” phagocytosis, because merging metabolic routes is more difficult in the absence of pre-symbiosis. The driving forces for eukaryotic inventions also seem less clear. However, the symbiogenic model seems to explain the how, why and when. How? Accidental entry of the (respiring) bacterium in the archaeon during presymbiosis, which could coevolve into an ever-increasing source of highly efficient ATP generation, more easily allowing eukaryotic complexity. Indeed, the curved extensions of Prometheoarchaeum as part of a syntrophic consortium with multiple bacterial species conjures up an image of accidental uptake in such consortia.[14,38,85]

Why? The inescapable by-product of mitochondrial OXPHOS, fluctuating internal ROS, both speeds up evolution because of higher mutation rates and represents acute selective forces for eukaryotic inventions. When? After the GOE, when more O2 became available. How can we study whether this relatively simple scenario is correct? Harcombe, Marx, and co-workers have been performing experiments to define parameters conductive to symbiosis, using different bacterial strains.[29,31]
