Evaluating hypotheses for the origin of eukaryotes

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Summary

Numerous scenarios explain the origin of the eukaryote cell by fusion or endosymbiosis between an archaeon and a bacterium (and sometimes a third partner). We evaluate these hypotheses using the following three criteria. Can the data be explained by the null hypothesis that new features arise sequentially along a stem lineage? Second, hypotheses involving an archaeon and a bacterium should undergo standard phylogenetic tests of gene distribution. Third, accounting for past events by processes observed in modern cells is preferable to postulating unknown processes that have never been observed. For example, there are many eukaryote examples of bacteria as endosymbionts or endoparasites, but none known in archaea. Strictly post-hoc hypotheses that ignore this third criterion should be avoided. Applying these three criteria significantly narrows the number of plausible hypotheses. Given current knowledge, our conclusion is that the eukaryote lineage must have diverged from an ancestor of archaea well prior to the origin of the mitochondrion. Significantly, the absence of ancestrally amitochondriate eukaryotes (archezoa) among extant eukaryotes is neither evidence for an archaeal host for the ancestor of mitochondria, nor evidence against a eukaryotic host. ***BioEssays*** **29:74–84, 2007.**

Introduction

It is now accepted that all known modern eukaryotes evolved from a mitochondrion-bearing ancestor;[1–4] that is, there are no known living eukaryotes that never possessed a mitochondrion (archezoa). Consequently, it is unclear whether other eukaryote-specific features such as the nucleus, endomembrane system, mRNA splicing and linear chromosomes predate or postdate the origin of the mitochondrion. Genomic comparisons reveal that eukaryote genes can be divided into several 'classes': genes apparently specific to eukaryotes, genes that appear from gene trees to be most closely related to a specific bacterial group, genes with greatest sequence similarity to sequences in bacterial lineages, and genes most similar to sequences in archaeal lineages.[5–12] Numerous hypotheses attempt to account for these patterns but disagree about the nature of the host and the number of partners involved in the origin of the eukaryote cell. Here we consider four general models (Fig. 1). These are not exhaustive, but instead aim to generalise the main features of a large number of models (see Martin et al.,[13] and Embley and Martin[3] for detailed reviews of specific models).

(i) Fusion, where one partner is an archaeon, the other is a bacterium. Fusion implies physical fusion of two cells, creating a single new cellular compartment by the mixing of the cell contents of the two partners. This is separate to the origin of the mitochondrion (Fig. 1, panel *i*).
(ii) Endosymbiosis, where the nucleus evolves directly from an engulfed archaeal or other type of cell. The nature of the engulfing cell likewise varies, but is never a eukaryotic cell (Fig. 1, panel *ii*).
(iii) Endosymbiosis, where an archaeal cell takes up the ancestor to the mitochondrion (Fig. 1, panel *iii*).
(iv) Endosymbiosis, where a protoeukaryotic cell engulfs the ancestor of the mitochondrion (Fig. 1, panel *iv*).

There are several distinctions between the hypotheses. In contrast to model *i*, models *ii–iv* specify a known process (endosymbiosis) wherein one lineage incorporates a second cell type. Models *i and ii* require three partners: model *i* requires a fusion to explain the origin of the eukaryote cell and, subsequently, endosymbiosis for the origin of mitochondria; model *ii* requires two endosymbioses, one for the nucleus, one for the mitochondrion. Models *iii and iv*, however, require only two partners, a host cell which engulfs the ancestor of the mitochondrion. Model *iv*, in contrast to models *i–iii*, implies that the basic eukaryote cell had already evolved, and that the origin of the mitochondrion may have been one of the final steps in the evolution of modern eukaryotes.

Suggestions that the nucleus itself is an endosymbiont (*ii*)[7,14] have been firmly rejected[15,16] and, likewise, there is no strong genetic evidence for three-way fusions (i and

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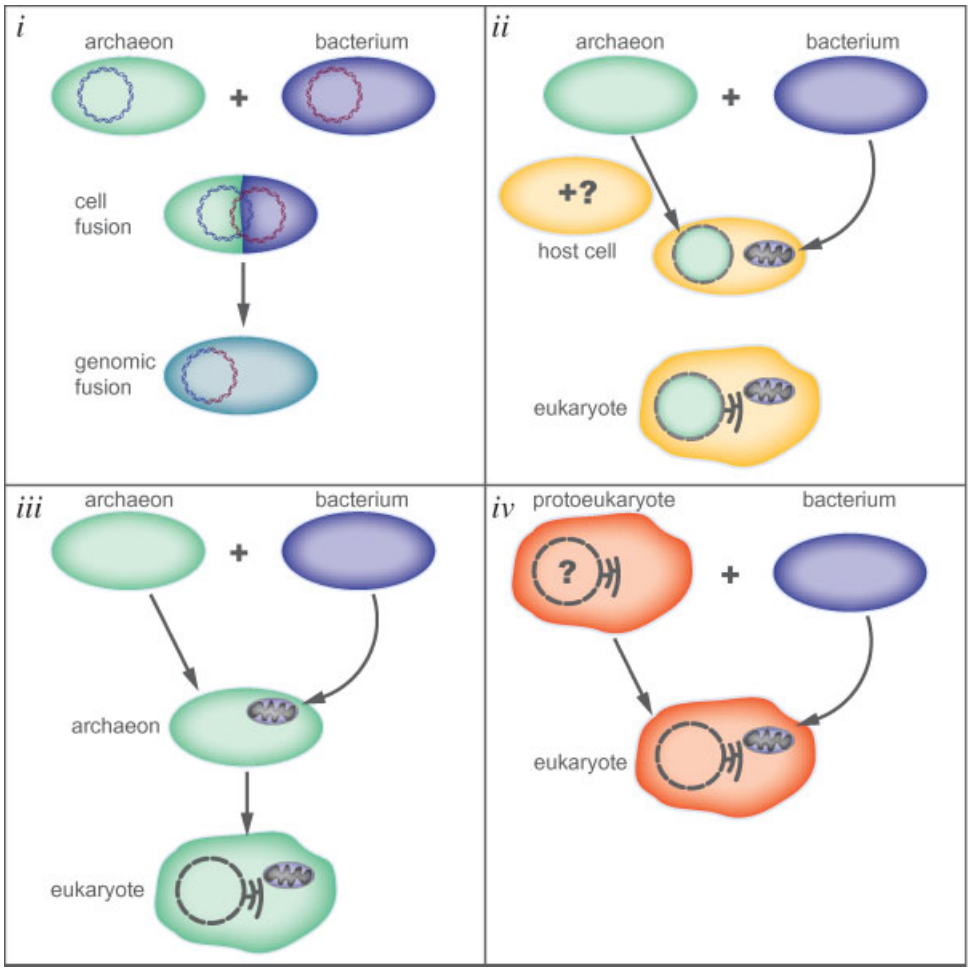

Figure 1. Four classes of models for the origin of eukaryotes. **Model *i*, top left panel:** fusion between an archaeon and a bacterium. As used here, fusion implies physical fusion of two cells leading to a single cellular compartment and a single integrated genome (whether or not the genome is separated by a membrane). This process is different to the origin of the mitochondrion, meaning that modern eukaryotes emerge from three cells, a fusion followed by endosymbiosis. This particular scenario was suggested by Zillig and colleagues,[57] though the term 'fusion' is now used widely but imprecisely. However, we restrict usage of the term to cell fusion, as indicated in model *i*. **Model *ii*, top right panel:** endosymbiosis with the nucleus evolving directly from an engulfed archaeal (or other cell). The nature of the engulfing cell varies, but it is never a eukaryotic cell. Thus models vary on both the nature of the host and the endosymbiont which evolves into the nucleus. Note that models of this type require three cells in order to include the mitochondrion, although some authors suggest the nucleus evolved directly from a virus.[6,7,14,84,85] **Model *iii*, bottom left panel:** endosymbiosis with an archaeal cell taking up the ancestor to the mitochondrion. This model implies that the first step in the evolution of eukaryotes was the evolution of the mitochondrion; all eukaryote-specific features thus evolved after this event.[32,45] **Model *iv*, bottom right panel:** endosymbiosis with a protoeukaryotic cell engulfing the ancestor of the mitochondrion. This model implies that eukaryotes were a separate lineage, distinct from archaea, and that the host cell possessed at least some eukaryote-specific features. In some formulations, it can be taken to imply that the final step in the evolution of the eukaryote lineage was engulfment of the mitochondrial ancestor.[69]

ii).[11,13,17] The discovery of eukaryote-specific genes has been used to argue that fusion must have involved three partners;[6] these authors prefer engulfment of an hypothetical RNA-based 'chronocyte' that evolved into the nucleus (variant of *ii*) rather than standard models of gene gain and loss. A simpler explanation is of course that the eukaryote domain had already split from the archaeal domain, as model *iv* suggests.[11,12] The confusion has arisen because two aspects of an 'archezoa' hypothesis were combined. These are (a) an early eukaryote cell engulfed the bacterial ancestor of mitochondria, and (b) some modern anaerobic eukaryotes, because they appeared to lack mitochondria, are examples of such early eukaryotes.

Point b is now disproved through the 'death' of the Archezoa hypothesis.[3,18–20] Unfortunately, many have mistakenly assumed that this also disproves the first part of the

hypothesis. Here we critically examine whether rejection of point b necessarily leads us to an archaeal origin for eukaryotes.

A major problem is that there is no agreement on what constitutes a viable hypothesis—unsurprisingly, there is no shortage of advocacy, but how should one evaluate these alternatives? We attempt to do this by considering the following points.

(1) The null hypothesis should be that the unique features of an extant group evolved along its stem lineage (that is, prior to the last common ancestor of the group—Fig. 2).
(2) Where possible, phylogenetic tests should be used to evaluate the various hypotheses (enabling rejection of the null hypothesis in certain cases).
(3) Hypotheses that require hitherto undemonstrated biological processes should be avoided when known mechanisms are available. This is to prefer 'known causes' to explain events in the past.[21]

We address each of these points in turn.

The null hypothesis: evolution along stem lineages

As there are no known extant archezoa, the nature of the host for the mitochondrial endosymbiont must obviously be reconsidered. This is a classic example of stem lineages and crown groups. Standard usage is that the last common ancestor of a group, plus all its descendents (living and extinct), is the crown group; the earlier groups form the stem lineage (Fig. 2a). For questions such as mammalian, bird, vertebrate or land plant evolution, we know from the fossil record that there has been successive acquisition of the complex characters seen in extant (crown group) species.

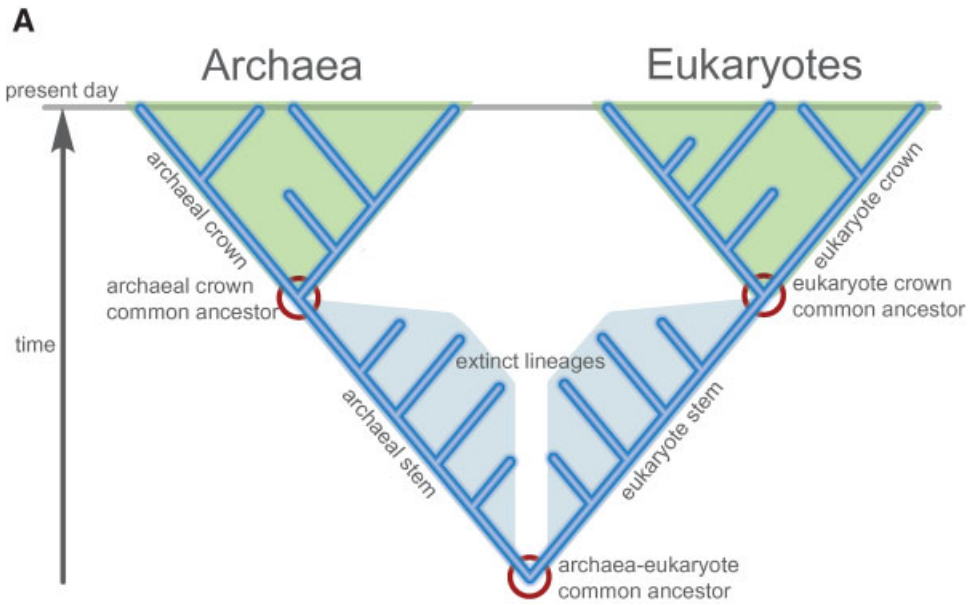

Figure 2. 'Stem' and 'crown' as applied to archaea and eukaryotes. **A:** If archaea and eukaryotes are each monophyletic and are sister groups then each can be defined as consisting of a stem group and a crown group. The crown group is the last common ancestor of a group, plus all its descendents both living and extinct. The stem group are all earlier diverging, but now extinct, taxa. Adapted from Donoghue.[86] **B:** Given the absence of any fossil forms, features in either the archaeal or eukaryote crown groups cannot be confidently placed in the common ancestor. Thus, crown-specific features present in either archaea and eukaryotes could have been present in the common ancestor, or have evolved in one stem group. Current data cannot shed light on these alternatives with the exception of the mitochondrion which, by definition, was incorporated into the eukaryote lineage along the stem. On current knowledge, phagocytosis preceded the origin of the mitochondrion, though, again, it is not possible to say if phagocytosis evolved along the eukaryote stem or was a feature of the last common ancestor of eukaryotes and archaea. **C:** Archaea and eukaryotes evolve from a fully formed archaeal ancestor. Assuming archaea and eukaryotes are sister groups, the implications in terms of stem and crown features, are as follows. All features that are universal among crown group archaea evolved at some very early stage before the common ancestor. On the other hand, *all* features universal among crown group eukaryotes evolved along the eukaryote stem, though the absence of any fossil data makes it impossible to divine the order of emergence. Key features are indicated schematically and include: phagocytosis, mitochondria, nucleus and endomembrane system, linear chromosomes, spliceosomal apparatus and introns. The postulated order of emergence differs depending on whether the archaeon is claimed to be an endosymbiont that became the nucleus (model *ii*), or whether it was the host that 'engulfed' the ancestor of the mitochondrion (model *iii*).

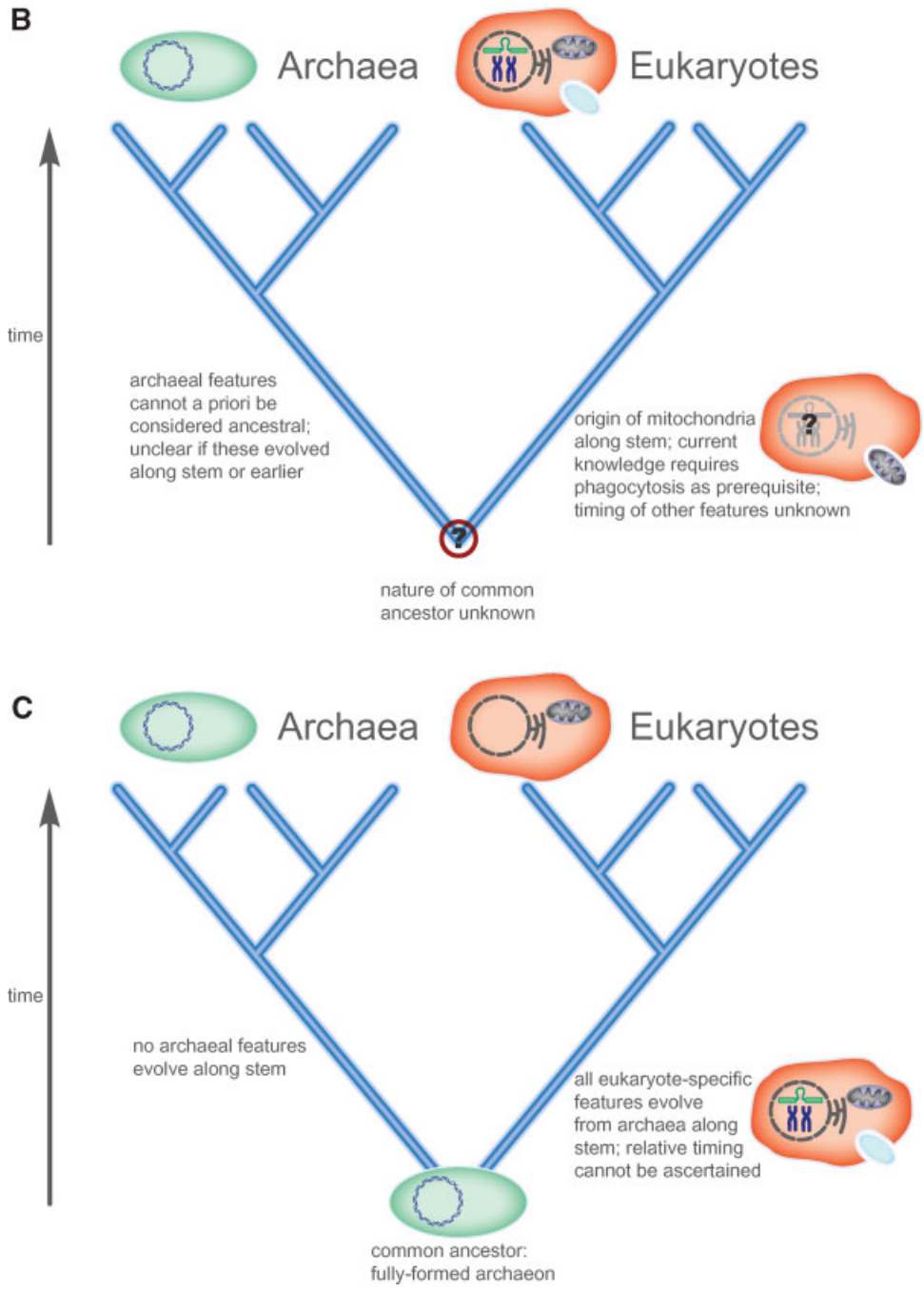

Figure 2. (*Continued*)

However, if data from fossils are ignored one could mistakenly conclude that numerous traits have appeared in evolutionary bursts.[22] This is exactly the problem that we face regarding the origin of eukaryotes: did eukaryote-specific features arise in successive stages, or in one burst? In one sense, the Archaea illustrate this point in that they are intermediate in 'informational genes' (such as those involved in replication and transcription) between Bacteria and Eukaryotes;[23–25] without their discovery the apparent gulf would be even larger. The central question is thus: had eukaryotes become a distinct lineage by the time of the origin of mitochondria by endosymbiosis?

Under point 1 above, the null hypothesis is that features specific to eukaryotes evolved in early eukaryotes after they

split from the lineage leading to modern archaea (assuming these are sister groups—this is discussed below). Likewise, the null hypothesis for the origin of archaeal-specific features is that these evolved in the archaeal stem. However, any given feature found in only one lineage (i.e. the crown) might of course have been present in the archaea–eukaryote common ancestor and been lost subsequently during evolution along the other stem. These two possibilities are indistinguishable based purely on presence in one lineage and absence in the other (Fig. 2b). Not surprisingly, there is little evidence for intermediate cellular forms in the evolution of either the crown eukaryotes or of the crown archaea, a point to which we shall return.

What is important is that models *i–iii* expect diversification of the archaeal crown group prior to the origin of eukaryotes, such that the common ancestor of eukaryotes and archaea was an archaeon (Fig. 2c). The exact mechanisms invoked by these three models for the origin of the eukaryote cell is not important for evaluating them on this criterion. In regard to the nature of the common ancestor of eukaryotes and archaea, all require a special case, shown in Fig. 2c, whereby the nature of the ancestor of the two lineages is already known.

To our knowledge, reasons for rejection of the null model of successive evolution of eukaryote features along a stem lineage have never been presented. Yet the stem group concept is used everywhere else in biology. If anything, invoking an archaeal ancestor under models *i–iii* is based on nothing more than the defunct tendency to view the progression of life from simple to complex.[26] This is grounded in orthogenesis[27] the theological organisation of life into a Great Chain of Being, and early representations of the relationships between organisms indeed marry the tree of life with this chain; Ernst Haeckel's *Stammbaum des Menschen* (Pedigree of Man) provides one such example.[28]

That said, the case shown in Fig. 2c need not be incorrect. On the contrary, it permits an explicit phylogenetic test that can distinguish between models *i–iii* and model *iv*.

A phylogenetic test of fusion

Our second point is that the models (*i–iv* above) can be tested phylogenetically. As demonstrated for the bacterial origin of mitochondria and chloroplasts[29,30] (see Fig. 3), genes from these organelles group them *within* modern bacteria; mitochondrial genes grouping within modern α-proteobacteria and plastids with cyanobacteria. A corollary of archaeal–bacterial fusion or archaeal–bacterial endosymbiosis (models *i–iii*) is that, excluding later additions to the eukaryote genome, eukaryote genes should group either *within* archaea or *within* bacteria (Fig. 3). For example, some hypotheses suggest a methanogenic archaeal host.[31,32] Evidence for these hypotheses would constitute the identification of numerous eukaryote genes that share a common ancestry with those in

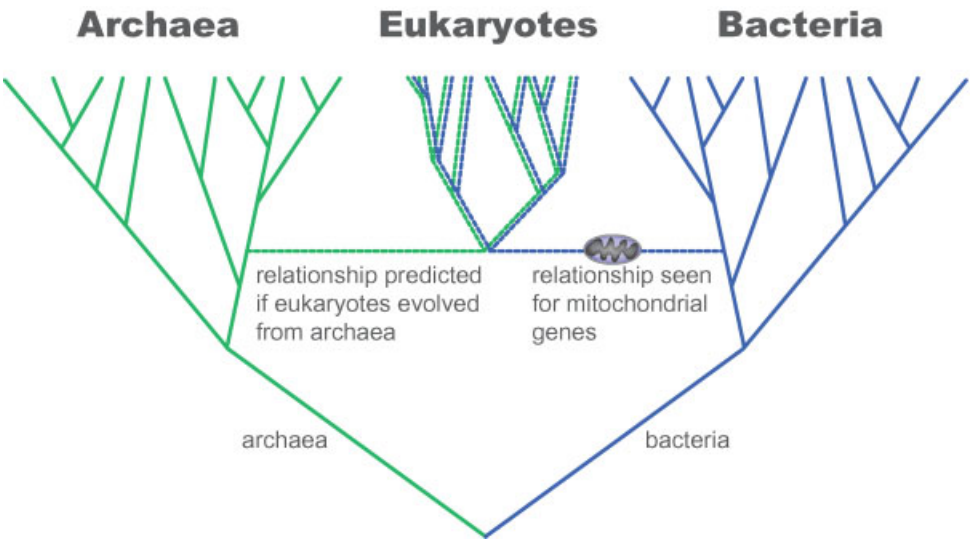

Figure 3. Phylogenetic test for an archaeal origin of eukaryote genes. The endosymbiotic origin of mitochondria has been tested phylogenetically, yielding the tree indicated schematically in blue. The bacterial origin of mitochondria explicitly predicts that mitochondrial genes should largely fall *within* the diversity of modern bacteria, because bacteria had already diversified prior to the origin of the mitochondrion. Using this same logic, hypotheses arguing for an archaeal origin of eukaryotes predict that a large proportion of eukaryote nuclear genes should fall *within* the diversity of crown group archaea because this latter group had already diversified prior to the origin of eukaryotes (green phylogeny). However, phylogenetic trees grouping archaea and eukaryotes do not appear to support an origin within archaea for eukaryotes. The conclusion that we draw at present is that archaea and eukaryotes share a common ancestor, and that the two lineages diverged in time prior to the divergence of each crown group (see Fig. 2b, lower panel).

extant methanogens, these eukaryote genes grouping *within* the euryarchaeal part of the archaeal tree. Methanogen-like genes in eukaryotes as judged by sequence similarity[17] are suggestive, but insufficient to demonstrate an intra-archaeal origin for these eukaryote genes.

Importantly, sisterhood between archaea and eukaryotes does not support a methanogenic archaeal host; methanogenesis cannot be unequivocally placed in either the archaeal crown group ancestor, or the archaea–eukaryote common ancestor (Fig. 2a). To our knowledge, the only proposed phylogenetic result suggesting eukaryotes group within archaea is the eocyte tree, which instead groups eukaryotes with crenarchaea,[5,33,34] not euryarchaeota. The eocyte tree is a source of ongoing debate[8,35–40] and, rightly or wrongly, the prevailing consensus is that archaea and eukaryotes are sister groups. Controversy over tree topology and possible tree-building artefacts aside, neither of these topologies support a methanogenic host in the emergence of modern eukaryotes, in spite of suggestive associations such as histone homologues in methanogens.[41]

These results indicate a difficulty with including an archaeal partner in a model for the origin of the eukaryote cell (models *i–iii*). Phylogenetic analyses strongly demonstrate bacterial origins for mitochondria and plastids,[8,9,11,17,42–44] so clearly at this phylogenetic depth, there is sufficient signal to establish the bacterial origin for the mitochondrion, and a later origin of the chloroplast. Why then would genes purported to be of archaeal origin not group *within* the diversity of modern archaea? Numerous *post hoc* explanations can be invoked (as typified below) but, in reality, the data as they stand do not support an archaeal origin for either eukaryote genes (as fusion partner, model *i*), or the nucleus (as endosymbiont, model *ii*) or as host (model *iii*, equivalent to fusion and the endosymbiotic origin for the nucleus in terms of a phylogenetic test for genes of archaeal origin, though only two cells are required). Furthermore, the mechanism of endocytosis resulting in the mitochondrion may be unspecified, as with fusion (model *i*), or unprecedented, as in endosymbiosis (models *ii* and *iii*—no extant archaeal species have thus far been shown to be either intracellular endosymbionts of bacteria, or capable of engulfment).

One post-hoc explanation that could be invoked to rescue these hypotheses is that the archaeal lineage that contributed genetically to eukaryotes (as well as any other more basal archaeal lineages) has since gone extinct. This would leave both archaea and eukaryotes appearing monophyletic. As the defining event in the origin of the eukaryote lineage is by definition the origin of the mitochondrion (under model *iii*), the additional requirement is that all eukaryote-specific features evolved after this event.[3,15,45] This requires that, at minimum, one feature *other than the mitochondrion* was sufficiently advantageous to the individual in which it first appeared that a selective sweep eliminated even distantly related lineages. (Excepting the unlikely possibility of rapid emergence of a selectively neutral complex feature becoming fixed by drift in a small otherwise undiversified population.[46]) Alternatively, one could argue post-hoc, that the reason eukaryotes do not group within archaea is that the former are fast-evolving, meaning the monophyly of archaea is a consequence of long-branch attraction (see comment by Martin in discussion on p85 in Ref. (47)). Long-branch attraction has been invoked in testing the eocyte hypothesis, as the slowest-evolving sites in several protein data sets recover the eocyte topology.[33] However, no evidence has been presented to indicate that this long-branch artefact accounts for the lack of a phylogenetic relationship between methanogens and eukaryotes.

Until evidence to the contrary is produced, the null hypothesis can only be that eukaryotes do not group within archaea, that is, archaea are strictly monophyletic. Thus current theories invoking an archaeal partner (of the general type *i–iii*) are backed at best by no more than circumstantial evidence. The clear phylogenetic result seen with mitochondria,[30] chloroplasts[29] and secondary endosymbioses[48] has been strengthened as additional data have become available (as cited above). Indeed all endosymbioses or endoparasitisms, where such a phylogenetic test of ancestry has been performed, provide a consistent picture. In contrast, phylogenetic data convincingly showing eukaryotes arising within archaea are simply not available. The evolution of features unique to each of these domains must therefore be considered to have occurred in their respective stems from an ancestor whose nature is not yet known (Fig. 2b).

Known mechanisms and endosymbioses

Science aims to explain events in the past by known mechanisms. There are countless examples of endosymbionts within eukaryote cells, and phagocytosis is a process widespread among eukaryotes. Chloroplasts provide a clear ancient example of endosymbiosis involving a eukaryotic host, but nitrogen-fixing spheroid bodies in freshwater diatoms,[49] the rhizobia of legumes,[50] bacterial endosymbionts of insects,[51] and red and green algal secondary and tertiary endosymbionts[52,53] are among numerous other examples. To these examples we shall return, but first we examine the evidence that can be brought to bear on models proposing eukaryotes emerging from fusion or symbiosis between a bacterium and an archaeon.

Model *i* predicts that genetic level fusion between archaea and bacteria should occur in nature. A possible example is that of *Thermotoga maritima*[54] where, on sequence similarity, as much as 24% of the genome is suggested to be of archaeal origin. For a subset of such genes several indicators of potential xenology (homology via horizontal gene transfer) were reported, including local GC content, synteny, codon usage and the presence of flanking repeat sequences.[54]

While this example suggests that a significant number of genes can be transferred between the two domains, several other points should be considered. Horizontal transfer could possibly produce a significant 'fusion' signal at the genomic level,[55] but this is not equivalent to fusion of archaeal and bacterial compartments (model *i*), and can be distinguished from fusion if there is continual transfer from a range of donors, as recently suggested.[55] In contrast to both horizontal gene transfer and endosymbiosis, no observations of fusion have thus far been reported. Second, the *T. maritima* genome result argued for horizontal gene transfer for 24% of the genome based on a blast analysis; the number of genes where additional data strongly support transfer are fewer and phylogenetic data, to our knowledge, exist only for one gene, reverse gyrase.[56] Third, a simple genomic fusion does not account for eukaryote cell structure, and all four models in Fig. 1 are in fact compatible with the identification of bacterial and 'archaeal' genes in eukaryotes, if the only measure is sequence similarity. Thus, model *i* is the least helpful in that it does not account for any eukaryotic cell architecture (not even the origin of the mitochondrion, which occurs subsequent to the fusion[57]). It does however predict the same phylogenetic relationship as models *ii* and *iii* for genes originating from the two fusion partners (Fig. 3), as discussed above.

It is a prediction of models *ii* and *iii* above that archaeal/bacterial endosymbioses would occur in nature. To our knowledge only one example of endosymbiosis between prokaryotes is known; that of γ-proteobacteria contained within β-proteobacteria, which are in turn contained within the cytoplasm of cells that make up the 'bacteriome', a specialised organ in mealybugs that hosts endosymbiont bacteria.[58] The γ-proteobacterial secondary endosymbionts appear to have taken up residence on four independent occasions and, subsequent to each infection event, have coevolved with the β-proteobacterial primary symbionts.[59]

Access to the bacterial cytoplasm has been documented previously, suggesting this is not a one-off observation. For instance, *Daptobacter*, a 'predatory' bacterium, appears to penetrate the cytoplasm of its bacterial prey,[60,61] though to our knowledge no endosymbioses resulting from invasion have been documented, and this genus is poorly studied. Several other examples suggest bacterial cells can take up residence or at least parasitize other bacteria. The 'predatory' bacterium, *Bdellovibrio bacteriovorus*,[60–62] invades the periplasm of its bacterial hosts, where it reproduces, ultimately bursting out of its host, which is lysed in the process. A similar mode of invasion is employed by α-proteobacterial symbionts of the tick, *Ixodes ricinus*; these bacteria invade and consume mitochondria of ovarian cells.[63,64]

Some authors have considered the mealybug example to indicate the plausibility of an archaeal–bacterial origin for the eukaryote cell.[47] However, this single example of a bacterial-bacterial endosymbiosis is not equivalent to an archaeal–bacterial endosymbiosis; no examples of the latter have so far been reported, and unless evidence appears that demonstrates this type of relationship, models *ii* and *iii* are not supported.

Furthermore, suggestions that the nucleus was an endosymbiont (model *ii*) do not explain the unique membrane structure of the nuclear envelope (or for that matter any other eukaryote-specific structure)[15,16,65] since this has no counterpart in either archaea or bacteria. The internal membranes of bacterial planctomycetes, in particular members of the genus *Gemmata*, are similar and possibly analogous to the nuclear envelope. However, current genomic data do not support an endosymbiotic or fusion scenario for planctomycete evolution. Nor do eukaryote cells appear to have received genes or organelles from this bacterial group.[66]

Endosymbiosis, where the 'engulfing' cell is archaeal in origin (model *iii*), requires, on current knowledge, that all extant archaea have lost the capacity to internalise bacterial symbionts. Modern examples of syntrophy between archaeal methanogens and hydrogen-producing bacteria are invoked as indicative of the first step in the hydrogen[32] and syntrophy[31,67] hypotheses for the origin of the eukaryote cell. While these hypotheses are persuasively argued from a metabolic viewpoint, neither explains the apparent absence of contemporary archaea harbouring endosymbionts, and the absence of phagocytosis in prokaryotic lineages, archaea in particular.

To recap, no archaea have been shown to carry bacterial endosymbionts (predicted by model *iii* and some variants of model *ii*), neither have any archaeal endosymbionts of bacteria been observed (predicted by some variants of model *ii*). Bacterial endosymbionts are extremely rare in bacteria, and phagocytosis has so far not been demonstrated to be an attribute of archaea. Thus, whichever way one looks at it, there is currently no known precedent for endosymbiosis involving an archaeon and a bacterium.

A 'eukaryotic' ancestor capable of engulfment (i.e. endosymbiosis of the mitochondrial ancestor into a protoeukaryotic cell (model *iv*), has been argued to present a common and known mechanism for incorporation of a bacterial cell into a eukaryotic cell.[68,69] In Fig. 2a, this ancestor would correspond to the 'eukaryote crown common ancestor'. Both engulfment and endosymbiosis are widespread in eukaryotes and phagocytosis is common. However, simple engulfment of prey seems to many to be too simplistic to account for the metabolic interdependence between the eukaryote cell and its fledgling mitochondrion (perhaps explaining the focus of previous models[31,32,70] on the possible metabolic nature of the interaction).

Having said that, as all subsequent endosymbioses leading to establishment of organelles must have occurred via phagocytosis,[71] it is hard to understand why the first endosymbiosis need be the exception. Indeed, the more

recent (and incontrovertible) endosymbioses demolish any suggestion that engulfment is too simplistic an explanation.

An important aspect of the origin of eukaryotic plastids (that is likewise relevant for the origin of eukaryotes and mitochondria) is establishing that phagotrophic cells can enter into symbiosis with their prey and evolve to become primary producers. A straightforward, though derived, example is given by mixotrophic eukaryotes—organisms capable of both phototrophy (on account of possessing photosynthetic plastids) and phagotrophy (engulfment of microbes as food). A diverse assemblage of eukaryotes, including dinoflagellates,[72] ciliates[73] and chlorarachniophytes,[74] are known to be mixotrophic. Consequently there is no controversy surrounding the statement that phagotrophy was central to the evolution of obligately photosynthetic eukaryotes via primary, secondary or tertiary endosymbioses.[52,71,75] Indeed, the spectrum of nutritional strategies, ranging from near-exclusive phagotrophy to near-exclusive phototrophy illustrates a feasible set of intermediates in the evolution of obligate phototrophs from phagotrophic ancestors.[71,73] It is beyond doubt that primary producers can and have evolved from phagotrophic ancestors, and the process of endosymbionts evolving into organelles in eukaryotes has clearly occurred multiple times.[52]

Likewise, very transient symbiotic interactions can arise from prey engulfment; a salient example is that of kleptochloroplasts. Some dinoflagellates, ciliates and sea slugs are known to engulf and digest photosynthetic algal cells, leaving only the chloroplast, which is transiently retained in a photosynthetically active state. After a short period, the kleptochloroplast is digested, further photosynthesis being only possible upon engulfment of additional photosynthetic eukaryote prey.[76–78] The recently characterised flagellate *Hatena*, which carries a plastid-bearing green algal symbiont, provides yet another example of this. Upon division, only one cell receives the engulfed symbiont; the cell without the symbiont develops a feeding apparatus, enabling engulfment of a new symbiont; this in turn leads to degeneration of the feeding apparatus.[79]

These examples of mixotrophic eukaryotes preying on other eukaryotes demonstrate the feasibility of many intermediate stages in the evolution of photosynthetic eukaryotes. However, this establishes the mechanistic feasibility of the phagotrophic origin of the chloroplast or of secondary or tertiary endosymbionts. Given such a firm basis for the endosymbiotic photosynthetic model, we can move ahead to consider a model for the origin of mitochondria.

A stepwise model for the origin of mitochondria

We will now look at whether a stepwise model for the origin of mitochondria by engulfment by the eukaryote crown common ancestor can be demonstrated by consideration of modern examples.

A simple phagotroph-prey scenario for the emergence of an obligate endosymbiosis would require the following steps:

(1) Phagotrophs engulfing prey cells via phagocytosis.
(2) Emergence of individuals within the prey population which are resistant to digestion, and which may escape from the interior of the phagotrophic cell.
(3) Emergence of a facultative symbiotic relationship between phagotroph and 'prey'; this could be mutualistic, commensal or a pathogenic interaction.
(4) Shift from a facultative to an obligate endosymbiotic association.
(5) The obligate endosymbiont evolves into an organelle.

There is no shortage of examples of phagotropic eukaryotes that engulf bacteria (step 1). Many types of amoebae engulf and digest bacteria, and Enterobacteria are one common food source.[80] Engulfment can have several outcomes: digestion, resistance or escape. The latter two are evolved traits amongst prey and often figure in strategies employed by pathogens, having developed resistance to destruction after engulfment (step 2). Amoebae have received particular attention, not least because they act as reservoirs for pathogenic bacteria; numerous bacterial pathogens survive and proliferate in both amoebae and human macrophages.[80]

Numerous examples of bacterial resistance to digestion are well known from studies of the immune response in multicellular organisms,[81] and a significant list has been collated of bacteria resistant to engulfment by amoebae,[80] all serving to illustrate the feasibility of point 2. For instance, pathogens from clinically important genera such as *Burkholderia*, *Legionella*, *Listeria*, *Mycobacterium* and *Salmonella* enter macrophage cells via the phagocytic pathway. Upon engulfment, these subsequently escape degradation by the phagosome through a variety of mechanisms, such as preventing fusion of the phagosome with the lysosome, or actively subverting the immune response.[81] Again, resistance to degradation is seen in amoebae, leading to intracellular persistence, proliferation and, in some cases, to lysis of the engulfing cell.[80]

Resistance to engulfment might well provide a mechanism from which the establishment of mutualistic or commensal endosymbioses occurs, with the initial contact being a phagotroph–prey interaction. As the above examples illustrate, this can frequently turn into a parasitic endosymbiosis. However, there are also cases where host cell lysis has been documented to be regulated by environmental cues, implying that a commensal relationship may emerge if favourable conditions prevail.

A spectrum of examples illustrating steps 3 and 4 are provided by α-proteobacterial endosymbionts of *Acanthamoeba*. In the case of *Candidatus Odysella thessalonicensis*, an increase in incubation temperature from 22°C to 30–37°C

results in a shift from stable intracellular occupation to lysis of its amoebal host, *A. polyphaga*.[82] Moreover, SSU rRNA trees of *Acanthamoeba* hosts and their α-proteobacterial endosymbionts (including *Candidatus O. thessalonicensis*) are congruent, suggesting that there has been coevolution and cospeciation between hosts and endosymbionts.[83]

Step 5 is well established; salient examples are green-algal-derived secondary endosymbionts that gave rise to Euglenids and Chlorarachniophytes, red-algal endosymbionts of Cryptomonads, Dinoflagellates and others. Perhaps the best-known example is given by *Buchnera*, vertically inherited intracellular bacterial endosymbionts of aphids. While this association is with a multicellular eukaryote, it illustrates a key intermediate stage in the evolution of organelles from free-living bacteria. *Buchnera* genomes are far less reduced than either plastids or mitochondria, and it is not yet clear whether any endosymbiont genes have been transferred to the host nuclear genome.[75]

In conclusion, given the massive number and diversity of endosymbiotic bacteria and organelles of bacterial-origin resident in modern eukaryote groups, there is overwhelming evidence for the establishment of endosymbioses between bacteria and eukaryotes. Again, there are currently no observations of archaea either as hosts of bacterial endosymbionts, no archaeal endosymbionts in bacteria, or for that matter archaea residing with other archaea. On the criterion of known processes, it is thus difficult to argue rationally for any other model than *iv* (Fig. 1).

Conclusions

The first part of the archezoa hypothesis, namely the idea that the eukaryote cell type had emerged before the incorporation of mitochondria into eukaryotes, was initially taken as given. However, at that time it was assumed that some eukaryotes that lacked obvious mitochondria were examples of eukaryotes that had never had mitochondria—that is, they were archezoa. With the realisation that extant 'archezoa' are secondarily amitochondriate (they are anaerobes and possess vestigial mitochondrial forms) the two independent elements of the archezoan hypothesis were lumped together. It is now recognised, correctly, that extant 'archezoa' have lost mitochondria, so the ancestor of all extant eukaryotes also possessed a mitochondrion.[3]

The first element of the archezoan hypothesis, the existence of an early protoeukaryote lineage, is what we have addressed here. Does rejection of the second point, whether there are any living archezoa, logically lead us to an archaeal origin for eukaryotes? Most definitely not. As we point out, a definitive phylogenetic test is lacking—modern eukaryotes should group within the diversity of modern archaea, in the same way as is seen for the relationship between mitochondria and α-proteobacteria. Second, no contemporary evidence exists for endosymbionts in archaea, nor are archaea known to be able to engulf cells. In contrast, there is abundant evidence for both these phenomena in eukaryotes. The third point is that, in addition to the mitochondrion, there are a number of eukaryote-specific features for which no counterpart exists in archaea.

Any theory that aims to explain the origin of mitochondria via engulfment by an archaeal ancestor must not only seek evidence for the first two points, but also explain why the acquisition of mitochondria was the first step. If Archaea and Eukaryotes are sister groups, as is widely held, similarity between these groups is expected on account of common ancestry, but such similarity does not allow us to deduce the nature of the ancestor (i.e. eukaryotic or archaeal). To argue an archaeal ancestry for eukaryotes, or a eukaryotic ancestry for archaea, requires that one group falls within the phylogenetic diversity of the other (Fig. 3); evidence for such a claim is basically non-existent. Hypotheses involving fusion of three groups seem superfluous; there is physical and genetic evidence for two cells, but no convincing genetic evidence for the inclusion of a third.[3,55]

On the basis of known mechanisms of engulfment and known examples of endosymbiosis, all data point to a mechanism of cell engulfment being a prerequisite for the origin of mitochondria. Again, on known mechanisms, engulfment can only have emerged during evolution along the eukaryote stem. The mealybug endosymbionts (a γ-proteobacterium within a β-proteobacterium within a eukaryote cell) are not evidence for an archaeal-bacterial endosymbiosis. How a bacterial cell can gain entry into another bacterium in this case is certainly an interesting and unsolved mystery, but this is a known phenomenon, as illustrated by *Bdellovibrio*. However, this does not bear a close resemblance to eukaryotic endosymbioses. To reiterate, no archaea are known to either host endosymbionts or to be hosted as endosymbionts of bacteria. Hence, it is reasonable to expect that there would be phagocytotic predators before the existence of modern eukaryotes. The simplest hypothesis, based on current knowledge, is that such cells were the stem lineage to modern eukaryotes.

To conclude, fusion and endosymbiotic hypotheses involving an archaeal and a bacterial partner require that eukaryote genes purported to be of archaeal origin have lost all signal of their origins, while this has not occurred for all eukaryotic genes of mitochondrial origin. Phylogenetic data fit best with the monophyly of the three domains, a common origin for eukaryotes and archaea, and acquisition of the endosymbiotic precursor of mitochondria early in eukaryote evolution by eukaryotic mechanisms of engulfment.[1] The alternative models require some series of untestable post hoc appeals to explain the data, such as *all* modern archaea losing their

[1]Note that this is not incompatible with interdomain transfer for specific genes—see Lester et al.[55] for a recent discussion.

abilities for endosymbiosis/engulfment, or genes evolving with a different mode and/or tempo on the archaeal side of the tree from the bacterial. Yes, archaea and eukaryotes appear to share a common ancestor, and yes, modern eukaryotes are a genetic chimera with many genes being transferred to the eukaryote nucleus from the mitochondrion. However, explaining the numerous differences between modern eukaryotes and archaea requires evolution of domain-specific features on both stems, archaeal and eukaryotic. Since this is a matter of the order of appearance of eukaryote-specific characters along the eukaryote stem lineage, and given the difficulties in accounting for the ultimate engulfment of a bacterium by an archaeon, the simplest explanation is that, by the time the mitochondrion was incorporated into ancestral eukaryotes, at minimum, phagocytosis must have evolved. The suggested specific link between eukaryotes and methanogens owing to the presence of histones the latter is further weakened by the discovery of histones in crenarchaea.[87]

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