nature portfolio

Peer Review File

Oxygen metabolism in descendants of the archaeal-eukaryotic ancestor

Corresponding Author: Dr Brett Baker

Any redactions in this file are there to maintain patient confidentiality, the confidentiality of unpublished data, or to remove third-party material.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
Comments on Appler et al.

In this manuscript Appler and colleagues present a tour de force analysis of a huge data set of new Asgard archaeal MAGs (404 Asgard MAGs specifically and >10,000 new MAGs overall). This data set provides an order of magnitude more data to bear on the question of the archaeal ancestry of the eukaryotic nucleocytoplasmic lineage and eukaryogenesis. The authors combine phylogenetics, sophisticated microbial ecology analysis, in depth structural prediction and modeling and biochemical reconstructions in their analyses. They present a treasure trove of interesting and groundbreaking new findings that really challenge the current views of eukaryogenesis. The most interesting of these (in my view) is the finding that a very large fraction of the Heimdallarchaeia (the immediate asgard sister lineage to eukaryotes) are likely aerobic respirers with aerobic electron transport chains. This goes against many prominent hypotheses of eukaryogenesis (most notably the Hydrogen Hypothesis, but others as well) that have assumed that the eukaryotic host lineage came from a strictly anaerobic Asgard lineage. Indeed, the general assumption in the field is that Asgard archaea are 'all' anaerobes with only one or two exceptions. This paper turns that assumption on its head and convincingly, in my view, demonstrates that the last asgard/eukaryotic common ancestor was likely an aerobe possibly living in shallow coastal environments. This result will no doubt ruffle many feathers and lead to a re-evaluation of eukaryogenesis hypotheses. For this reason, and also because of the sheer number of other novel/exciting findings reported in this paper, I strongly support its publication in Nature.

Below I have made a number of comments on the manuscript/analyses that, if addressed, I believe could significantly improve the manuscript.

Major comments;

(1) The authors have done an amazing (dizzying!) number of analyses of different kinds in this manuscript and there is a huge amount of information summarized. I have concerns mainly about a 'corner' of those analyses that involve phylogenetic analyses of concatenated proteins (e.g. NM47, CoxABC) and individual proteins (e.g. Nuo subunits in supp. Figures 15-27). The methods used to infer these phylogenies are sometimes quite different depending on the data set and there is heterogeneity in the approaches described. I am fully aware that it can be difficult to coordinate many different collaborators who are experts in different biochemical systems to do analyses using the same approaches, so, in what follows, I do not expect everything to be 'redone' with a particular set of methods. That being said, 'deep time' phylogenetic analyses of proteins can be easily compromised by artefacts owing to large numbers of substitutions occurring on the branches. For such analyses the current gold standard is to use a profile mixture model with many components (e.g. LG+C60+Gamma or CAT+GTR or similar) to ameliorate potential problems with long branch attraction. These models also usually end up being selected by model selection criteria such as AIC or BIC when they are included amongst the models being tested.

For the main analyses to reconstruct the phylogeny of the new Asgard lineages with the NM47 data set shown in Figure 1b, the authors did use profile mixture models, although they only showed the analyses where the data had been recoded into 4

states (GTR+C60+G4 recoded into 4 states). For the other data sets it appears that the authors have opted for site homogeneous analyses with models such as LG+R10 (or other rate distributions selected by BIC). In the latter cases model selection was performed, but I'm almost certain that the authors did not include the profile mixture models in the set of models tested; this is a frequent oversight because IQ-TREE, by default, does not test these models (they must be specified using the '-madd' flag followed by the profile mixture models that you wish to test). I'm confident that had the authors tested the LG+CXX series of profile mixture models (XX= 10, 20…60), they would frequently would have found that the complex mixture models were preferred for both the concatenated analyses and the single-protein analyses.

How important is this and what should the authors do? I would suggest that, at least for the concatenated protein analyses shown in figures in the main text (except Fig. 1a which is just a 'rough' tree), that the authors try to fit site-heterogeneous mixture models using the above approach and estimate the trees to see if they see any differences in the resulting topologies. For Figure 1b, I think it's important that the authors present a non-recoded profile mixture model analysis of the data set (e.g. LG+C60+G4) and comment on what changes in the estimated topology were recovered with the recoding. As it currently stands the authors are arguing they recoded to avoid composition-related artefacts, but it would be nice to see if recoding changed anything because recoding is well known to have its own problems (see Foster et al. (2023) https://doi.org/10.1093/sysbio/syac042). It's important to have non-recoded analyses presented in addition to the recoded analyses and for the differences to be commented on (the authors could include these in the supplementary figures). For the single-protein analyses of the Nuo subunits (and others), there are a lot of analyses that would have to be redone using profile mixture models, so I wouldn't insist on the authors doing this. However, if it could be done, I think it would improve the quality of the analyses in the paper and assure readers that those trees are less likely to be influenced by long branch attraction.

(2) The authors have tried hard to make a detailed eukaryogenesis scenario in Figure 5 and an accompanying discussion in the last part of the paper, but several aspects of this scenario are, in my view, either poorly justified or unclear. I think the paper is already very interesting without the authors speculating to this degree so I have a general suggestion that the authors simplify their scheme, take out the more speculative claims and stick to the evidence they have presented. I think they could write a separate "scenario" paper where they develop their ideas more fully rather than in overly brief format here. Here are some specific concerns I have about this scheme and figure:

i) the authors discuss how many Heimdalls can't make vitamin B12 and they argue in various places that this could have been important in the mitochondrial symbiosis. But it is well-known that many prokaryotic and eukaryotic microbes in the ocean are B12 auxotrophs and they import B12 from the surrounding environment (produced by prototrophs). I don't think this is particularly convincing as part of this scenario.
ii) What specifically do the molecules hsp90, L28e, alphaMICOS have to do with this scenario. Is hsp90 only found in Heimdalls?
iii) What Cox subunit in modern day eukaryotes is homologous to CoxD found in these MAGs (I tried to find out but couldn't find the name of the homolog)? Are the authors suggesting that this subunit of Cox was 'host inherited' rather than symbiont inherited in eukaryotes? If so, they need to provide phylogenetic evidence for this claim. What about SCO1?
iv) the authors are showing 'contemporary' Hod and Kari lineages as having tentacle-like protrusions in the figure. There is no evidence for this cell morphology in these lineages is there?
v) there seems to be a 'quasi-timeline' at the top of Fig. 5, but it can't be drawn to scale given the numbers shown. Also I can't figure out which cell along the grey arrow diagram corresponds to the most recent common ancestor of Hod and eukaryotes. It can't be FECA because the cell depicted there has a mitochondrion and the authors did not present any evidence for any of their organisms having a mitochondrial symbiont. Yet FECA is, by definition, the immediate daughter cell of the most recent common ancestor of Hod and eukaryotes.
vi) I don't think the authors need to show "anaerobic eukaryotes" in this diagram or hydrogenosomes at all. They aren't relevant and they clutter up the diagram.
vii) the two arrows next to O2 and mtDNA are confusing. What does this mean?
viii) overall the diagram is too cluttered to be easily interpretable. I would suggest simplifying it and removing anything that isn't immediately relevant to the scheme.

Other comments:
- Line 31, Abstract: "These archaea encode hallmarks of aerobic eukaryotes". Consider rephrasing as "hallmark proteins" maybe?

- Line 34, "Heimdallarchaeia encodes" encode?

- Line 47, I suggest rephrasing as "some models of eukaryogenesis" (or "many models")

- Line 55-58, The authors are taking the molecular clock estimates from this one paper (ref. 12) very (too?) seriously. There is substantial variation in the age estimates of these nodes across different studies in the literature, so it might be prudent to word this more tentatively. "A recent study estimated that" and "If these estimates prove to be robust then these events would roughly coincide with the rise in the oxygen on the planet…"

- Line 61, "potentially some of its modern descendants could use oxygen." The phrase "use oxygen" here is a bit vague. Oxygen can be a substrate in a variety of biochemical reactions. Do they mean "use oxygen as a terminal electron acceptor in ATP generating pathways (i.e. aerobic respiration)" or do they mean this statement to include many other metabolic uses of oxygen. The sentences afterward suggest that perhaps they mean aerobic respiration. I would encourage the authors to be a bit more specific here.

- Line 82, “first eukaryotic cell.” The concept of the “first eukaryotic cell” can only be arbitrarily defined. What would be the first eukaryotic cell? The one with a proto-nucleus with only half the nuclear proteins that LECA had? Or maybe the one with the first fully developed nucleus? Or maybe it had only one nuclear pore protein and a rudimentary membranous structure? Since it’s not possible to uniquely define the “first eukaryote”, I think that this wording should be avoided completely. The authors could consider changing “bioenergetic advantages that the Hodarchaeales host may have conferred to the first eukaryotic cell” to “bioenergetic advantages of the Hodarchaeales lineage in this evolutionary transition” or something like that.

- Line 110, “the closest known ancestor to eukaryotes”. The Hodarchaeales representatives are not ancestors of eukaryotes, they are relatives (i.e. sister lineages) of eukaryotes. Consider rephrasing this.

- Line 129-130, The specific substitution model used with RAxML is not specified here or in the Methods section for Fig. 1a. I assume it is LG+Gamma or LG+F+Gamma? This should be specified in the Methods section.

- Figure 1a, It’s difficult to see the grey circles on the branches indicating >=90 bootstrap support. Perhaps the authors can increase the resolution of the figure and make these circles stand out a bit more (or maybe the figure resolution was compromised by the rendering of the PDF).

- Figure 1b in the Support value legend to the left of the tree you have “greater than or equal to 100” sign. Support values can’t be >100 so this should be just an equals sign (=). Same goes for Supplementary Fig. 6.

- Line 175-177, the authors should cite paper 47 at the end of this sentence (i.e. after the word “intolerant”) because this is the method used in that sentence. I don’t think it makes sense to cite 47 at the end of line 179.

- Line 180: This first sentence of the paragraph says: “The mitochondrial ancestor is predicted to have been a facultative aerobic Alphaproteobacteria-related lineage” and refs 48 and 49 are cited. It’s very clear that the mitochondrial ancestor had the capacity for aerobic respiration; the oxygen-dependent ETC in mitochondria is clearly phylogenetically related to the alphaproteobacterial system. Dozens of different papers in the literature have demonstrated this fact and no-one would dispute it. However, the claim that the mitochondrial ancestor had the ability to thrive in anoxic (or low oxygen) conditions (which would be required of a facultative aerobe) is based on very weak evidence. Reference 48 points to potential enzymes of anaerobic metabolism found within genomes of various alphaproteobacterial taxa, but there is no direct phylogenetic affinity with the eukaryotic homologs demonstrated. Reference 49 is a review paper and doesn’t present any specific evidence that shows the mitochondrial ancestor had the capacity for anaerobic metabolism. However, there are many published papers that show that enzymes of anaerobic metabolism in anaerobic eukaryotes (which have secondarily adapted to low oxygen) appear to have distinct phylogenetic origins by horizontal gene transfer from various kinds of bacteria (see Gawryluk, Vizitiu and Stairs BBA Bioenergetics 1863: 148627, Stairs et al. (2020) Sci. Adv. doi/full/10.1126/sciadv.abb7258 and reviewed in Roger et al. (2017) Curr. Biol. 27: R1177–R1192). I know this might be seen as a minor point, but I think it would be more balanced if the authors reworded this sentence along the lines of “The mitochondrial ancestor is predicted to have been an Alphaproteobacteria-related lineage capable of aerobic respiration [references], and some authors have argued it may have been facultatively aerobic [ref 48, 49].” I note that there isn’t any real need to discuss the potential for anaerobic metabolism in the mitochondrial symbiont since its aerobic respiratory functions are most relevant to the discussion in this paper.

- On lines 210-212 the authors indicate that none of their structural predictions suggest that the NuoEFG module formed a higher-order complex with the rest of the ‘complex I-like structure’ (or equivalently the [NiFe]-hydrogenase complex which apparently shares a similar architecture). I find it confusing, then, that in Figure 4b NuoEFG are shown in a complex with other complex I subunits and as separate soluble module. Furthermore, in the paragraph that starts on line 243 it is argued that the group 4 [NiFe]-hydrogenases of the entire group of Heimdallarchaeia are likely oxidizing ferredoxin and reducing protons to H2, using this redox reaction to pump protons out of the cell. This section is hard to square with Fig. 4b where no ferredoxin oxidation or H2 production is shown and instead NADH oxidation/MQ reduction is powering proton translocation by complex I. If I haven’t misunderstood what is being argued (and I easily could have), I think the authors need to justify why their Fig. 4b is showing something different than this section of the text (and in fact showing something different from that described in the figure legend itself).

- Line 243, “The group 4 [NiFe]-hydrogenases of Heimdallarchaeia are an evolutionarily missing link of Complex I” (Note: “evolutionarily” should be “evolutionary”). This is an intriguing hypothesis if I understand the author’s suggestion correctly. However, it would be helpful to know how this hypothesis relate to the phylogenies of the Nuo subunits shown in Suppl. Figs. 15 to 27? These trees show the actual inferred evolutionary relationships between subunits from Complex I of bacteria and the asgard and other archaeal subunits and therefore seem relevant to the evolutionary position of the group 4 lineages. I think it would be good if the authors made their reasoning more explicit here.

- Line 265 – “Moreover, detailed structural protein modeling using this expanded genomic catalog supports the reverse flow model of eukaryogenesis, with Heimdallarchaeia not having to rely on a symbiont for H2 production”. I think this is a bit of an ‘overreach’ and I would encourage the authors to tone it down a bit. If the authors are correct about their metabolic arguments on line 243 then it is likely these organisms produce oxidize ferredoxin and reduce protons to H2 with their [NiFe]-hydrogenase complex. The part of the ‘reverse flow’ model this supports is this metabolic capacity specifically (and the presence of [NiFe]-hydrogenase) in the eukaryotic/asgard last common ancestor. This does not say anything about whether or not the mitochondrial symbiont could harvest the H2 gas or whether mitochondrial symbiont came in at the same

time the ‘host’ lineage was doing this kind of metabolism. If mitochondria were relatively ‘late’, then there could be hundreds of millions of years between the LEAsCa and the initial mitochondrial symbiosis. I suggest the authors rephrase their comment here to be specific about what part of the reverse flow hypothesis specifically their data support.

- Line 363, I don’t understand this sentence. Perhaps the authors can clarify why oxygen tolerance in the Hodarchaeales would directly increase cell to cell contact? What does oxygen tolerance have to do with “providing” compartmentalization to the mitochondrial symbiont? The latter is a matter of the host cell surrounding the symbiont and doesn’t have anything directly to do with oxygen tolerance does it?

- Line 399, I suggest rephrasing it to make it clearer. For example, “…Lokiarcheaeles, which, in the latter, produce protrusions” (there isn’t any evidence they produce protrusions in Heimdalls yet).

- Line 402-408, these claims are too speculative and, without more detailed explanation, are very difficult to understand in this context. I think the paper would be much better if these sentences were just removed. There is no point in bringing up the “inside-out” model in conjunction with these speculations and it just seems a bit random (and needlessly distracting) to discuss this here.

Methods
- Line 583, what does “hyperthermal-sensitive marker genes” mean? Perhaps it means that proteins encoded by these genes have an extreme amino acid composition change relative to other archaea which results from adaptation to hyperthermophily. Consider rephrasing this to make it clearer.

Referee #2

(Remarks to the Author)
This manuscript presents a significant expansion of our understanding of Asgardarchaeota diversity and distribution, particularly focusing on the Hodarchaeales, the closest known archaeal relatives of eukaryotes. The authors have generated an impressive dataset of metagenome-assembled genomes (MAGs) and conducted extensive comparative genomic analyses.

The study's strengths lie in its comprehensive genomic dataset, which has led to the identification of a new class and numerous lower taxonomic levels within Asgardarchaeota. The expansion of key metabolic protein families, especially the doubling of [NiFe]-hydrogenase diversity, is a significant contribution to the field. The authors' focus on Hodarchaeales and their potential contributions to eukaryotic cellular complexity is particularly noteworthy. Overall, this is an interesting study with some promising results. Their findings provide valuable insights into the metabolic capabilities of these organisms and their potential role in eukaryogenesis.

However, the manuscript suffers from several inconsistencies and overstatements that need to be addressed as in detail comments. While this work represents a significant advance in our understanding of Asgardarchaeota and their potential role in eukaryogenesis, the manuscript would benefit from more rigorous analysis in some areas, clearer definitions of key concepts, and a more balanced discussion of alternative hypotheses. Addressing these issues would strengthen the impact and reliability of this important contribution to the field.

Major concerns：
1. The topology of Asgardarchaeota (also known as Promethearchaeota) and the branching position of Eukaryotes form the foundational basis for understanding eukaryogenesis and have been subjects of significant debate. A recent study by Eme et al. based on non-ribosomal marker proteins suggested Hodarchaeales as the closest relatives to Eukaryotes; however, the possibility of methodological artifacts cannot be completely dismissed. In the present study, the authors employ three marker gene sets to reconstruct the Asgard phylogeny, resulting in three distinct topologies with low bootstrap support. Considering the current phylogenetic analyses struggle to confidently reconstruct the Eukaryotes' branching position with Asgardarchaeota, the authors should revise their eukaryogenesis model in light of alternative phylogenetic scenarios, rather than definitively presenting Hodarchaeales as the closest relatives to Eukaryotes throughout the manuscript.

2. The current results do not provide sufficient evidence to support the aerobic lifestyle of LAECA, even if the phylogenetic issues are set aside. While the ancestor of Hodarchaeales and Kariarchaeaceae each developed aerobic metabolic capabilities, the ancestor of all Heimdallarchaeal lineages remained largely anaerobic. Hence, it remains an open question whether LAECA was aerobic or anaerobic, as its evolutionary stage was much earlier than the Hodarchaeal ancestors. The current results neither confirm nor refute the hypothesis of an aerobic LAECA. The undetermined position of LAECA further complicates the issue. Additional data is needed for further validation.

Detail comments

Lines 31-33: Replace "hallmarks of aerobic eukaryotes" with "hallmarks of aerobic lifestyle." Note that Complex III is only found in certain Hodarchaeales and should not be listed here.

Lines 54-56: Clarify that the 2.67–2.19 Ga reference (ref 12) relates to FECA, while the 1.84 Ga reference (ref 13) pertains to LECA.

Lines 59-61: The concept of LAECA is not clearly defined or explained. Is this referring to FECA (First Eukaryotic Common

Ancestor) or LECA (Last Eukaryotic Common Ancestor)? This distinction is crucial, given the vast temporal gap (on the order of billions of years) between FECA and LECA. The authors should provide a precise definition and clarify which ancestral state they are referring to, as this has significant implications for the interpretation of their findings and the evolutionary timeline under discussion.

Lines 61-64: The authors' assertion that "most contemporary Asgard archaea are inferred to be strict anaerobes" is an overstatement and lacks nuance. They have not given due credit to the work by Liu et al. (Nature, 593:553-557, 2021), which provides a more nuanced view of Asgard archaea metabolism. According to Liu et al., 66.67% of Asgard clades are predicted to be anaerobic heterotrophs, while 25% are predicted to be facultative aerobic heterotrophs. This distribution suggests a more diverse metabolic landscape than the authors imply. Furthermore, the recognition that Heimdall archaea are capable of an aerobic lifestyle is not a novel concept and does not warrant overemphasis. The authors should revise their statement to more accurately reflect the current understanding of Asgard archaea metabolism, incorporating the findings from at least Liu et al. and possibly other relevant literature. This would provide a more balanced and scientifically accurate representation of the metabolic diversity within the Asgard archaea, which is crucial for understanding their evolutionary significance and potential relationship to eukaryogenesis.

Line 70: The authors' statement that "Hodarchaeales, ..., just 23 MAGs available" appears to be inaccurate and understated. This claim contradicts the authors' own findings presented later in the manuscript, where they report an increased number of Hodarchaeales MAGs. Moreover, their distribution analysis indicates that Hodarchaeales prefer coastal and deep-sea sediment environments. It is worth noting that this conclusion could have been inferred even from the previously available 23 MAGs, which also originated from coastal and deep-sea sediment samples. The authors should revise this statement to accurately reflect the current state of knowledge, including their own contributions to the field. They should acknowledge that the environmental preferences of Hodarchaeales can already be suggested by the previously available MAGs, and Emphasize how their new data reinforces and expands upon these earlier data.

Lines 74-77: The phrase "the first detailed inferences of their metabolism" should be moderated.

Lines 94-96: The three phylogenetic analyses utilized different marker sets, software, substitution models, and outgroup genomes. While this is acceptable for preliminary analysis, these should be standardized in the final publication.

Lines 96-98: The bootstrap values are consistently low for Hodarchaeales and Heimdallarchaeia across all three topologies.

Lines 101-102: The phylogenetic position of Ranarchaeia is unstable across the three topologies; hence, the authors should not selectively display and discuss a seemingly more favorable result.

Line 114: The use of the term "pangenomic analyses" in this context is incorrect and misleading. Pangenomic analysis typically refers to a comparative genomic analysis of the entire set of genes from all closely related strains within a specific clade. Given the broad taxonomic scope of the Asgard archaea superphylum, the analysis conducted here cannot be accurately described as a pangenomic analysis. The authors should replace "pangenomic analyses" with "comparative genomic analyses".
Line 120, Fig 1a, L168 and Sup Fig 3: Hod and Kari are not phylogenetically most closely related to each other, but they are (1) more metabolically complex than other Heimdallarchaea or other Asgard archaea, and (2) enriched in epipelagic sediments. I wonder whether the observed (1) and (2) is cause or cog?

Line 127 Fig.1: The phylogenetic trees presented in Fig. 1a and Fig. 1b exhibit notable inconsistencies, particularly regarding the phylogenetic position of the Odin clade. This discrepancy raises concerns about the robustness and reliability of the phylogenetic analyses presented. The authors appear to have utilized their latest NM47 marker set (previously referred to as NM57 in their earlier publications, which was noted to have technical issues as reported by Eme et al., Nature 618, 992-999, 2023) for these analyses. The inconsistency between the trees, especially considering the use of this frequently updated marker set, requires thorough explanation and justification.

Line 155 Fig.2 and line 168: The use of the compound term "epipelagic/coastal" raises concerns about precision and accuracy in describing environmental niches. These two terms represent distinct ecological zones with potentially different physicochemical characteristics and biological communities. "Epipelagic" typically refers to the uppermost layer of the open ocean (0-200m depth), characterized by sufficient light penetration for photosynthesis. In contrast, "coastal" environments encompass a range of habitats near shorelines, including intertidal zones, estuaries, and shallow waters, which may or may not overlap with epipelagic zones. The conflation of these terms may lead to ambiguity in interpreting the ecological distribution and adaptations of the organisms under study.
The concept of "epipelagic sediments" is thus contradictory and scientifically inaccurate. Sediments are typically classified based on their location (e.g., coastal, continental shelf, abyssal plain) or their composition, but not by pelagic zones of the overlying water column.

Lines 169-171: The authors suggest that presence in "epipelagic sediments" implies oxic environments. However, sediments, even in shallow waters, can be anoxic just below the surface layer. This assumption lacks substantiation.

Line 176 modelling oxygen tolerant? In methods?

Line 182: The statement regarding the co-occurrence of Hodarchaeales and Alphaproteobacteria requires significant clarification and context. The authors need to specify whether this observation is based on contemporary samples or

includes historical data, as this temporal context is crucial for understanding potential evolutionary associations. Furthermore, the significance of this particular co-occurrence is not apparent, given that Alphaproteobacteria are widely distributed and likely co-occur with numerous other microbial groups. The authors should explain why this specific association is noteworthy and whether it is unique to Hodarchaeales or a general pattern observed with other Asgard archaea or archaea in general.

Line 199: I suggest the author should provide more structural details (e.g. primary structure and conserved amino acid residues) of these ETC subunits. Perhaps some biochemical analyses can be performed to advance our understanding of the new Asgard subunit.

Line 210-213, Line 247-248, Fig 3a, and Fig 4b: How do these stand-alone NuoEFG modules originate? NuoEFG is present in the Fig 4b, but Heimdallarchaea do not have NuoEFG in Fig 3a.

Line 270 fig3b: It is crucial for the authors to provide a clear and detailed explanation of the criteria used for selecting the reference sequences of CoxABC. This information is essential for readers to assess the robustness and reliability of the phylogenetic analysis presented.

Lines 298-299: Justify the choice of Complex IV over other aerobic hallmarks.

Lines 300-302: The results shown in Fig. 2b may be attributable to the limited number of genomes used, as coxABC subunits are present in many archaeal lineages in the KEGG database.

Lines 313-318: The statement "the presence of O2-dependent enzymes varies across Heimdallarchaeia" contradicts the oxygen tolerance modeling mentioned in Lines 175-179.

Lines 358-360: The authors should explain the absence of Complex III in most Asgard genomes.

Lines 363-365: The authors are linking "widespread oxygen tolerance" to "initial compartmentalization with the predicted aerobic mitochondrial ancestor" lacks a clear logical foundation and requires substantial clarification. This statement raises several critical issues that need to be addressed. The reference to the "predicted aerobic mitochondrial ancestor" needs elaboration, explaining how this prediction relates to the observed characteristics of extant Asgard archaea. The authors should also discuss alternative hypotheses that might explain the evolution of compartmentalization in eukaryotes and how their proposal fits within the broader context of eukaryogenesis theories.

Line 396-397 and Line 483-484: Considering Asgard archaea undergo billions of years evolution, today's coexistence may not be evidence of the same scenario billions of years ago.

Lines 399-402: The authors' implication in lines 399-402 that Heimdallarchaeia can produce protrusions, leading to an increased surface-to-volume ratio and consequent energy advantages, requires important caveats and clarification. While the presence of actin homologs in Heimdallarchaeia is intriguing, the authors should exercise caution in extrapolating from gene presence to complex cellular features. It is crucial to note that not all microorganisms possessing actin homologs necessarily develop protrusions or exhibit increased surface-to-volume ratios. A pertinent example that should be cited is Ca. Uab amorphum (Shiratori et al., Nature Communications 10, 5529, 2019), which possesses actin homologs but does not form protrusions. The authors should explicitly acknowledge this counter-example and discuss the implications for their hypothesis.

Fig 5: In a recent study conducted by Brocks et al. (PMID: 37286610), it was observed that modern sterols, which are characteristic biomarkers of crown eukaryotes, were notably absent in sediments dating from 1.6 to 1.0 Ga. Instead, the presence of protosterols, which are indicative of more primitive eukaryotic lineages, was detected. This finding suggests that eukaryotic organisms existing before 1.0 Ga belonged to the stem group rather than the crown group.

Lines 420-421: The authors raise a critically important question: "Why would the ancestral host partner with a bacterial symbiont for aerobic respiration and then lose that capability?" This query is fundamental to our understanding of eukaryogenesis and mitochondrial evolution. However, the authors' response to this pivotal question lacks the depth and rigor. Their proposed explanation that "the endosymbiont increased total respiratory capacity by expanding its membrane surface" is overly simplistic and fails to address several key issues. The authors should discuss the energetic trade-offs of maintaining two separate respiratory systems versus integrating them, address the concept of genomic streamlining in endosymbionts, explore the benefits of compartmentalized respiration in mitochondria, clarify the evolutionary timing of host respiratory capability loss and mitochondrial function acquisition, provide a more comprehensive comparative genomics analysis of respiratory genes in extant Asgard archaea and alphaproteobacteria, and consider alternative hypotheses, e.g., by using genome-scale metabolic modelling.

Lines 485-491: Elaborate functions other than phagocytosis is well-established in eukaryotic cells. The authors cannot convince me that the existence of respiratory function in the membrane of the host cell impedes the development of phagocytosis.

Figure 2: Define the meaning of the box and line in the figure.

Table S2: Correct the number of ASGARD genomes to 936 instead of the stated 937.

Table S4: Ensure that the 12 environmental categories match those presented in Fig. 2.

Referee #3

(Remarks to the Author)
The emergence of eukaryotes was a defining process that gave rise to almost all living beings that are visible to the naked human eye, as well as to the abundant, taxonomically and morphologically diverse, and ecologically and medically important eukaryotic microbes. Accordingly, the ancestral cells and events involved in this process are topics of fundamental biological importance. Spirited discussion has focused on the nature and timing of the mitochondrial endosymbiosis, with specific topics including: 1) the metabolism of the likely host and symbiont, and the metabolic basis of their relationship; 2) whether oxidative phosphorylation, presumably made possible by the symbiont, was a necessary prerequisite for the emergence of complex subcellular structures such as the endomembrane system and/or cytoskeleton in eukaryotes.

The discovery of the Asgard archaea, collectively comprising multiple sister lineages of eukaryotes, created an exciting opportunity to draw inferences about features that were likely present in the shared ancestors of Asgards and eukaryotes, with implications for our models of early eukaryotic evolution. Because of the diversity and patchily distributed genes found in this group, broad taxon sampling is essential to generate a well-resolved reconstruction of features in shared ancestors in the group, especially in the shared ancestors of eukaryotes with their immediate sister group, currently believed to be the Hodarchaeales.

Here, Appler and colleagues report the generation of Metagenome-assembled genomes (MAGs) from samples taken from waters off the coasts of Mexico and China. Combining these with publicly available data, they identified and analysed more than 400 new Asgard archaeal MAGs, nearly doubling the number of Asgard MAGs available, making this dataset a valuable resource for the community in and of itself. Beyond this, the paper represents an interesting and novel addition to the growing literature on Asgard archaea in several ways: the size of the dataset allows the authors to identify patterns of correlation between specific environments and specific Asgard archaeal groups, and to produce detailed reconstructions of gene content are possible on a much larger scale than previously possible.

The most interesting implications come from the authors' metabolic reconstructions, particularly those pertaining to Hodarchaeales (Heimdallarchaeia), the likely sister group to eukaryotes according to the most recent phylogenomic analyses. One of the leading eukaryogenesis models, the 'reverse flow' model, posits a hydrogen-producing shared ancestor of eukaryotes and Asgards. The authors of that paper also raised the possibility of this ancestor having been oxygen-tolerant, based on a small number of MAGs. The authors conclude that their metabolic reconstructions support these earlier, tentative conclusions, based on predictions of the oxygen tolerance of some Hodarchaeales, the presence of reactive oxygen species-detoxifying enzymes, and predictions of sequence and structural similarity between some of their protein complexes and elements of the eukaryotic mitochondrial respiratory chain (which are unlikely to be contaminants in individual MAGs, given that they form robustly supported clades).

This would have far-reaching repercussions for eukaryogenesis discussions: in the most conservative interpretation of the authors' findings, the proto-mitochondrial endosymbiont's ability to encounter and form a relationship with its host would be easier to understand and more likely if the host was already capable of tolerating oxygen. In the boldest interpretation, a host that already had the capacity for oxidative phosphorylation would call into question received ideas about the nature of the mitochondrial symbiosis, raising the possibility that oxidative phosphorylation was not a major factor and that nutritional symbioses, similar to those seen in modern prokaryotic symbioses, might have been more important. For those adhering to the idea that oxidative phosphorylation was necessary for the emergence of eukaryotic complexity, it would also raise the question of mitochondria having been dispensable for both processes. The possibility of this kind of oxygen tolerance in Asgards has been proposed before (that work is appropriately cited), but without the weight of evidence provided by Appler and colleagues' work.

The overall approach is appropriate, and the work is appropriately placed into context. The authors have generated and presented an impressive amount of data, and it's a pity that some of the paper's findings are a bit swamped as a result: some interesting and detailed discussion of metabolic inferences are relegated to 20 pages of Supplementary Discussion, and some of the methods are inconsistent and not always easy to follow between the main methods and the supplementary ones.

Overall, I would like to see greater clarity and consistent terminology throughout the text, and changes to some of the methods before I can fully evaluate the paper's claims. I've also highlighted specific places analyses that are mentioned in the text but for which no data are presented.

Major points and suggestions for improvement:

1) I have concerns about the ways in which the phylogenetic methods were carried out, including a lack of consistency between approaches and inappropriate model choice or interpretation:
• The same level of detail should be provided for all similar analyses, and I would find it helpful to have them described in the same place: currently the methods used to generate the NM47 phylogeny are described in detail in the supplementary methods, but the methods used to generate the Phylosift and arCOG phylogenies are only mentioned in the main text. More detail should also be provided on parameters used for alignments, trimming and (if applicable) single-gene tree construction

for the Phylosift and arCOG analyses (e.g., which MAFFT program was used for the arCOG analysis, which BMGE matrix, etc.)
• I would like to see the number of trimmed sites used in each final analysis mentioned either in the text or in each figure legend.
• Phylogenies should only be shown as rooted if the analyses used to generate them specified an outgroup; while outgroup taxa were included, the methods don't mention that an outgroup was specified for phylogenetic inferences, and so these roots should be removed from the figures.
• The developers of the ultrafast bootstrap feature have made it clear that the threshold for interpreting support as high is greater than that for nonparametric bootstrap support values: "95% support correspond roughly to a probability of 95% that a clade is true. […] For UFBoot, you should only start to rely on a branch if its support is >= 95%" (http://www.iqtree.org/doc/Frequently-Asked-Questions#how-do-i-interpret-ultrafast-bootstrap-ufboot-support-values); note that the authors also strongly suggest generating Shimodaira-Hasegawa approximate likelihood ratio test support values in addition to ultrafast bootstrap support values. Supplementary Figs. S3, S15-S27, S30, S37-S40, S42-S44, S53-S67 show ultrafast bootstrap support values >= 70%, which gives a misleading suggestion that these should all be considered high support. Similarly, support values >= 90% are shown for Fig. 3(c). Where individual values are legible, I would recommend removing some values and/or adding a note of caution regarding the interpretation of ultrafast bootstrap support to the figure legend, and where only circles for the highest support values are shown, these should be shown for values greater than or equal to 95%. This point is important because it affects the interpretation of the results by leading to the overestimation of support for some clades. (The legends for several Supplementary Figures also simply mention that these show 'bootstrap support', so I would specify that these are ultrafast bootstrap support values).
• For the majority of single-gene trees shown, and for the Phylosift phylogeny, the best-scoring model was chosen by ModelFinder in IQ-TREE according to the Bayesian information criterion. However, ModelFinder does not test mixture models approximating the CAT model (e.g. C20) by default, leading these to not be considered even though they would generally be the most appropriate choice (and more robust to artifacts, especially in the case of the Phylosift analysis). Instead, these models need to be explicitly included in ModelFinder analyses, as was done for NM47. I'd strongly recommend rerunning these phylogenies and including mixture models; this may also help to resolve some differences seen between the NM47 and Phylosift analyses.
• The authors mention that single-gene trees generated from trimmed alignments used for the concatenated NM47 alignment were screened for possible contamination or paralogs. I didn't see this mentioned for Phylosift or arCOG analyses, so if it was, the authors should mention it in the methods (and if it was not done, then they should do it).
• In the case of NM47, a C-series mixture model was (appropriately) included for ModelFinder model choice; however, I would expect C60 to be a more appropriate choice than C10 for a phylogenomic analysis, with a large dataset reconstructing relationships over deep evolutionary time. The authors did subsequently rerun their analysis with a larger number of mixture classes, but they did so on recoded data. I suspect this was done in the interests oof time, but I would suggest performing an analysis under conditions that incorporate C60 on the full, non-recoded dataset as well.
• In Fig. 1, I find the grey circles for support values almost impossible to see both on my screen and in print, so I would suggest using filled black circles instead.
• I understand the authors' desire to show their results cross-referencing as many types of data as possible, but the different concentric circles and many colours displayed in Fig. 1(a) make the figure very complex and difficult to read. I'd suggest removing some of these circles, maybe splitting the figure and moving one part to the supplementary materials if the authors would like to keep the information. (Elsewhere I've suggested the opposite, merging information, for a supplementary table; I feel that a large table is much easier to read and parse than a sub-figure).

2) I have concerns about some of the structural modeling predictions and their interpretation:
• The authors tried to overcome size limitations in Alphafold3 by modeling individual parts of the nuoEFG complex and producing a composite prediction from these predictions by structural superposition of the individual partial predictions in ChimeraX. The authors explain in the supplementary figure legends which individual parts were modeled, but this should be clearly laid out in the Supplementary methods as well. I also find the current wording unclear: the figure legends suggest that a full NuoEFG complex could be modeled by Alphafold3, but that a putative larger complex including NuoEFG, NiFe-hydrogenases and flavodoxins could not be modeled. However, the supplementary methods text ("AlphaFold3 (AF3) predicted a partial NuoEFG complex in this lineage, but a more complete model could not be obtained due to the 5000 amino acid input size limit") suggests that a complete NuoEFG could not be modeled either.
• I have concerns about this approach, namely that structural modeling of these partial sequences is less likely to be accurate given that interactions between more distantly located amino acids can't be modeled. Tools that generate structural predictions for large complexes based on predictions for smaller parts of the complex (such as CombFold) do exist, but these collate interaction predictions for more combinations of proteins or model protein complexes iteratively. The authors could explore the use of a tool designed for predicting the structure of large protein complexes, but I feel that the current approach lacks robustness.
• The authors state (Supplementary methods lines 297-298): "AF2 multimer indicated that annotated NuoEFG subunits in the Asgard lineages we tested do not form complexes with the other Nuo subunits (P and Q-modules) present in the same genome". These data should be shown.
• "However, AF2 multimer predictions of NuoEFG subunits in combination with the neighboring proteins predicted several multimers" – these data are also not shown, although they're skirted over in the main text: "*A subset* of structural predictions indicated *various* multimer structures, including…" (emphasis mine) – this really seems like cherry-picking, and needs to be explained further
• "Initial AF2 modeling indicated evidence of possible electron bifurcating complex with similarities to the Methanospirillum hungatei Fdh-Hdr-Fmd complex" – this should also be shown
• Moreover, Alphafold-multimer has a size limit of 4,000 amino acid residues; the authors explain how they tried to overcome the size limitation for Alphafold3, how was this done for Alphafold2?

• The authors model the nuoEFG complex using Alphafold3; Alphafold output files should also be made available with the supplementary materials on Figshare (I didn't see them there).

3) I'm unclear about some of the numbers and thresholds reported:
• the authors mention sometimes a threshold of >50% complete, <10% gene duplicates/contamination (e.g. lines 528-529), which would be consistent with their description of "medium-high quality" MAGs. However, in Supplementary Figure 1, a completeness cutoff of >=45% is mentioned, while in line 549, completeness > 45% (not also = 45%) is mentioned. Based on Table S1, it appears that only one Asgard MAG, D4993_C5_H3_Bin_385, is below 50% (despite having been generated from GE3 data; per the text, "The GE3 sequencing effort resulted in 5,606 archaeal and bacterial 529 MAGs (>50% complete, <10% multiple gene copies based on CheckM v1.2.196"). So I'd like to know why this was included in analyses (was it an oversight, and if so, how easy would it be to remove at this stage? Was this a deliberate choice, and if so why? Regardless, it would be good to check that consistent values are used throughout the manuscript, and making sure that only sequences with completeness >= 50% are being reported as 'medium-high quality' (and that if the authors then choose to exclude any MAGs for being below quality thresholds, they then ensure that these MAGs aren't included in phylogenies or other analyses)
• As a more minor but related point, the authors state that "From these, a total of 404 new MAGs were classified as Asgardarchaeota, expanding the known diversity of this group by 46.5%" (line 91), but in Figure 1 they seem to be showing 46.5% of the known diversity of Asgards coming from their new samples and state that "These MAGs constitute a 115.1% increase in the medium-high quality publicly available genomes" – I believe it's actually an 86.9% increase (115.1% would be 100 x the number of existing MAGs/the number of newly generated ones, as opposed to the reverse). The abstract correctly mentions 'nearly doubles'. See also my previous paragraph regarding the use of 'medium-high quality' here.
• Furthermore, Supplementary Table 1 is titled "Description of the 405 Asgard genomic dataset", not 404

4) The authors bring up co-occurrence of MAGs from the Heimdallarchaeia (especially Hodarchaeales) with alphaproteobacteria. This could point to an ability to form symbioses with alphaproteobacteria, which in turn would be consistent with archaeal ancestors of Hodarchaeales, with similar metabolism, being able to form symbioses with the alphaproteobacterial-like organisms that gave rise to mitochondria. However, this point is much weaker and based on conjecture than other parts of the manuscript. The authors can in any case only report co-occurrence, not confirmed symbiosis.
• While the authors compare co-occurrence with alphaproteobacteria between different groups of Asgard archaea, they do not compare them with co-occurrence with other types of prokaryotes, which could strengthen their case. I would like to see them do this; I think it should not be too onerous given that the data would already have been generated to identify the entire set of MAGs they assembled from their sampling data.
• The authors report the co-occurrence of Hodarchaeales and alphaproteobacteria as "demonstrating the co-occurrence between these lineages across sites and depths." 'across sites and depths' is doing some heavy lifting here, as co-occurrence was found in 2/5 GE3 cores, and none of the GE2 samples, so this sentence should be modified to clarify and avoid overstatement.

5) According to supplementary Table 6, the authors are finding 'Putative nuclear envelope organisation protein' in at least 10% of Lokiarchaeota (the same cutoff as that for cytochrome c oxidase subunit III and cytochrome c oxidase subunit IV in Heimdallarchaeota). That seems worth commenting on one way or the other.

6) Supplementary Table 8 seems to be missing currently.

7) I'm struck by the contrast between the paper's bolder conclusions and some of its coyer statements:
"Moreover, detailed structural protein modeling using this expanded genomic catalog supports the reverse flow model of eukaryogenesis, with Heimdallarchaeia not having to rely on a symbiont for H2 production." (lines 266-268)

"However, it cannot be definitively ruled out whether these Complex-I like complexes are instead H2-oxidizing, menaquinone-reducing proton pumps." [in which case the Heimdallarchaeia would in fact not be producing H2] (Fig. 4 legend)

"Our AF2 multimer models are primarily starting points for hypothesis generation and require future experimental validation" (Supplementary methods; a caveat worth mentioning in the main text)

• This sometimes leads to a lack of clarity around nomenclature and the solidity of the data: Fig. 4 shows Hodarchaeales having Complex I, the figure legend for Fig. 4 and the title of Fig. 3 describe 'Complex I-like' enzymes, and the figure legend for Fig. 3 describes 'Group 4 [NiFe]-hydrogenases'. I understand why this is the case, given the argument that these enzymes are 'missing links' with features of both NiFe-hydrogenases and Complex I and the fact that subunit organization varies between individual species. However, for the sake clarity, one term should be chosen, and I think that the least difficult to justify would be 'Group 4 [NiFe]-hydrogenases'.

Minor points

Typo in Fig. 4, 'chorsimate' should read 'chorismate'

Line 51, "acetate typically coupled with" should read "acetate, typically coupled with" for ease of reading

Line 55, "the bacterial ancestors of mitochondria" – or sister group, per Martijn et al. 2018 and Muñoz-Gómez et al. 2022

Lines 59-60, “Therefore, it is plausible that the last Asgard archaea and eukaryotes’ common ancestor” would be easier to read as “Therefore, it is plausible that the last common ancestor of Asgard archaea and eukaryotes”

Lines 68-69 “However, these lineages are very distinct from Hodarchaeales proposed to be the closest prokaryotic relatives of eukaryotes”

Lines 85-86, “we chose two ocean floor ecosystems rich in Asgard diversity” – I suggest including references here

Lines 138-139, “These MAGs constitute a 115.1% increase in the medium-high quality publicly available genomes” - could this sentence be made more specific? I assume ‘medium-high quality’ refers to MAGs with completeness >45% and contamination/redundancy below 10%?

Line 152, “hydrothermal-associated locations” is a bit vague - I think the casual reader might expect this to mean hydrothermal vents or maybe hydrothermal vents and hot springs, but looking at the locations in which Njordarchaeales and Odinarchaea were found in Fig. 2, these include bathypelagic samples and oilfieldsm as well.

Line 186, typo, “Rizobales” should be “Rizobiales”

Line 197, “Previous studies have suggested that the LAECA was hydrogen-dependent” – I agree with citing Sousa et al. here, but ref. 2 is the reverse flow model proposal, which I believe describes a hydrogen-dependent symbiont progenitor of mitochondria, rather than a hydrogen-dependent LAECA

Lines 266-267, “structural protein modeling using this expanded genomic catalog supports the reverse flow model of eukaryogenesis” – the reverse flow model paper states that “This model suggests that a metabolic syntrophy between *anaerobic* ancestral Asgard archaea and *facultative anaerobic* alphaproteobacteria has provided the selective force for the establishment of a stable symbiotic interaction that has subsequently led to the origin of the eukaryotic cell” (emphasis mine). So I think it would be helpful to reword this to mention which conclusions are being supported: 1) the conclusion that the may have produced hydrogen rather than consuming hydrogen from its endosymbiont, and 2) the conclusion (mentioned by Spang et al but not explicitly part of the model itself) that Heimdallarchaeota may be capable of aerobic respiration. This could be phrased as ‘supports the findings of Spang et al.’ for clarity.

Line 335, ‘Fig. 4, Increasing complexity’ - I’d suggest ‘Increased’ here since it refers to a single organism

Line 461, I’d suggest splitting the references here, moving those in support of mitochondria-early to the end of the previous sentence and keeping here only those in favour of mitochondria-late, to make it clearer to the reader which ones support which position

Line 514, “library prep” – writing out “library preparation” would be more appropriate

Line 535-536, “Resulting in a total of 5,233 BH MAGs and 3,000 GE2 MAGs >50% complete and <10% contamination based on CheckM v1.2.1” – I think this should have followed on from the last sentence with a comma, not a full stop

Line 544-546, “we compared several methods for accessing completeness and contamination (CheckM v1.2.1, miComplete v2, and MEBS v2)” - if these tools are being mentioned in the paper, I’d show the data on which the authors based their choice to go with CheckM, or otherwise explain briefly how CheckM was chosen

Line 551, “ASsemblage of Genomes from Asgard Archaea Resolving DIrection to our ANcestors (ASGARDIAN)” – cute!

Lines 559-561, “We also used SingleM/Sandpiper 0.3.0 to search the NCBI SRA public metagenome sets prior to 2021 for OTUs linked with GTDB R220 Hodarchaeales, Kariarchaeaceae, and Alphaproteobacteria” - should this sentence be placed earlier in the methods? Based on its current placement, it looks like these MAGs were not subjected to the same optimal oxygen tolerance, temperature, salinity and pH predictions as the others.

Lines 632-633, “T-tests to test if water depth significantly influenced genome size and if so in what direction.” – fragment, consider revising

The title of Supplementary Figure 6 is “Transitions between prokaryotic-eukaryotic life identified with functional pangenomics”, I don’t think this is a clear description of what it’s actually showing (the number of OGs uniquely shared between each Asgardarchaeota lineage with the single-cell eukaryote database)

It would be helpful to merge Supplementary Tables 4 and 9, so as to allow the reader to more easily compare predictions about the optimal environmental conditions of each sample with any available data on the environmental context of the sample

Supplementary Table 10, typo, “Alphaproteobactiera Total”

Supplementary methods line 146, “we obtained five cores split into 12 depth horizons”, reference Supplementary Figure S2 here

Supplementary methods lines 158-160, “Recent validation of the superphylum, Asgard archaea, as the phylum, Asgardarchaeota8 with all previously proposed phyla now listed as classes, orders, and families.” – sentence fragment, consider revising

Supplementary methods lines 181-182, “but the representatives in each of the 17 clades remain consistent (Fig. 1 and Supplementary Fig. 2” – I think the latter should be Supplementary Figure 3

Supplementary methods, structural modelling section, where several Alphafold structural predictions were compared to a solved structure, a table of individual RMSD results (for both pruned and unpruned atom pairs) should be provided, rather than a range of values

Supplementary methods line 1249, “Promethoarchaeum guaymasis”, should this be Prometheoarchaeum guaymasis?

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
Overall, I think the authors have done a good job of revising this exciting manuscript. I only have a few minor comments that I feel they need to address:

Minor comments:

Lines 47-48: The authors indicate that models of eukaryogenesis that imply hydrogen exchange between host and a ‘bacterial partner’ are “supported by the limited enrichment cultures refs 11-13”. These models of eukaryogenesis are not directly “supported” by the existence of cultures of living Asgard archaea that are involved in syntrophic interactions with other prokaryotes. At most what can be said is that is that they are “consistent with syntrophic interactions observed in limited enrichment cultures of Asgard archaea”. This may seem like a trivial change, but it is important to recognize that none of these cultures involve bacterial partners related to the mitochondrial symbiont or any other bacterial partner known to be involved in eukaryogenesis. So the evidence provided by the physiology of these living asgard archaea is circumstantial at best.

Lines 52-55: The authors state:
“A recent study estimated that the first eukaryotes (FECA, the first eukaryotic common ancestor) diverged from an interaction between Asgard archaeon and Alphaproteobacteria (the bacterial group related to the mitochondrial ancestor15,16) about 2.67-2.19 billion years ago (Ga) and 2.58-2.12 Ga, respectively17.”

This sentence needs to be revised for several reasons. Firstly, the “first eukaryotic common ancestor”, FECA, is incorrectly defined here. FECA was first defined in the literature by Makarova et al. (2005), https://pmc.ncbi.nlm.nih.gov/articles/PMC1187821/, to be the immediate descendant on the eukaryote line of the closest archaeal relative of eukaryotes. So it is basically the ‘first’ daughter cell on the eukaryote line descending from the most recent common ancestor of eukaryotes and Archaea. As such it must predate the mitochondrial symbiosis (= “interaction between Asgard archaeon and Alphaproteobacteria”). Note that FECA is also incorrectly specified to be the asgard/alphaproteobacterial holobiont in Fig. 5 as well; that should be fixed.

The second problem is the ambiguity regarding what the dates cited are referring to. The way it is worded, it looks like the interaction (mitochondrial symbiosis) is being dated but if so, then why are there two sets of dates and the use of the word “respectively”? The dates referred to from ref. 17 are for the last common ancestors of eukaryote/asgards and mitochondria/alphaproteobacteria respectively, which only imply that the mitochondrial symbiosis must be more recent than these dates. In reference 17, the age of LECA is estimated to be 1.84-1.93 Ga. So, if those dates are correct, this means that the mitochondrial symbiosis must have happened sometime between 2.58 Ga and 1.84 Ga; the symbiosis itself cannot be dated using molecular clocks because it doesn’t correspond to a node on a phylogenetic tree.
I think the authors should rephrase the sentence above to be more precise.

Line 87: “increase membrane potential” should be “increased membrane potential”

Line 453: I think the authors should be more cautious here and avoid making unqualified claims based on their metabolic predictions. I would suggest putting this as “Our findings suggest that the gain of ETC…by Heimdallarchaeia may have increased energy yield and…”

Line 455: This sentence is also making an assumption that increased energy was required for the evolution of cellular complexity. There is no evidence whatsoever that this claim (first made by Lane and Martin) is true. Complexification of protist cells over evolutionary time has occurred in anaerobic protist lineages that do not have aerobic respiration: an example is the origin of hypermastigids (a branch of the Parabasalia) with hundreds of flagella from much simpler biflagellated parabasalid ancestors. All parabasalids possess hydrogenosomes, not mitochondria and can only do substrate-level phosphorylation to produce ATP. I suggest removing this sentence and the following one and rewriting this

paragraph along the lines of:
“However, our analysis is consistent with an alphaproteobacterial origin of eukaryotic mitochondrial ETC components. This raises the question of why the mitochondrial symbiont containing protoeukaryote would ultimate lose the original ETC system of the host lineage. Perhaps the endosymbiont increased total respiratory….”
I don’t insist that the authors do this, but if they wish to keep in the original sentences, then they need to provide some evidence that excess energy over substrate-level phosphorylation is required to evolve cellular complexity, otherwise it’s just perpetuating a baseless claim.

Line 467: “while the redundant Asgard ETC subunits were likely lost in early eukaryotes.” I suggest rephrasing this because “early eukaryotes” might be interpreted by readers as “early crown eukaryotes” – i.e. post-LECA lineages of eukaryotes. I’m not sure the authors mean to suggest this or instead are suggesting that they were lost in the lineage leading (and prior to) LECA.

Line 531-532: “that LAECA conferred several bioenergetic advantages to the first eukaryotic cell…” As requested in the origin review, I think references to the “first eukaryotic cell” should be removed. Furthermore, how can an ancestor ‘confer’ advantages to a descendant? I suggest tightening up the language here. You could say something like “Our findings indicate that LAECA had aerobic bioenergetic capacities that may have played a role early in eukaryogenesis including genes for the ETC….”

Page 17, Fig. 5: The figure is much improved over the original version. I still think it’s a bit too cluttered and potentially confusing. As mentioned above the location of the label FECA on the above line needs to be changed. I would also perhaps suggest an arrow from the “Heimdall ancestor” cell goes to the “contemporary Hod and Kari lineages” cell so that it looks more like a tree where the latter are sister taxa to eukaryotes. I don’t know if the small thumbnail picture of Hod & kari cell and the “Alpha” thumbnail next to the top timeline needs to be there. It distracts from the overall picture…and is confusing given that Alpha appears to be in the future (relative to the “Today” dot in the timeline.

Referee #2

(Remarks to the Author)
I appreciate the authors' revisions to moderate claims about Hodarchaeales being the definitive closest relatives of eukaryotes and for incorporating language that acknowledges phylogenetic uncertainties and alternative scenarios. These revisions represent positive steps toward a more balanced presentation. However, additional modifications are needed to address the persistent overemphasis on Hodarchaeales. The text should more consistently reflect that Hodarchaeales represent one candidate lineage within Heimdallarchaeia, and that features observed in Hodarchaeales might be ancestral to Heimdallarchaeia (and thus relevant to eukaryogenesis) rather than directly or subtly implying special significance for Hodarchaeales specifically.
Besides, the manuscript should avoid using genomic inferences about metabolic capabilities (such as oxygen metabolism) as direct evidence or subtle support for conclusions about the much deeper LAECA node. The text needs to clearly distinguish between demonstrable aerobic capabilities in specific extant lineages (e.g., Kariarchaeaceae and Hodarchaeales) and the unresolved question of LAECA's physiology regarding oxygen metabolism.

Major comments 1
The hypothesis that Kariarchaeaceae and Hodarchaeales are adapted to oxygen is supported mainly by ecological distribution and by a few hallmark proteins of aerobic lifestyles. However, this evidence is not yet convincing, and the argumentation lacks logical continuity. While this adaptation is theoretically possible, it would be beneficial for the authors to provide more substantial evidence or revise the manuscript for a balanced presentation. Please consider the following points:
L172. Some Kariarchaeaceae and Hodarchaeales MAGs were recovered from aerobic water-column samples, yet no comparison is provided of their relative abundance in the water column versus anaerobic sediments. Adding these abundance data would substantiate the claim.
L182. Oxygen tolerance was predicted with GenomeSPOT, a tool known to misclassify taxa (e.g., Nitrososphaerota is labeled oxygen-intolerant). Please validate the GenomeSPOT results with additional methods (e.g., the XGBoost method used in 10.1126/science.adp1853).
L188. The co-occurrence analysis relies solely on a binary presence/absence model and fails to examine abundance dynamics. This is a critical limitation particularly for dominant taxa， which are often difficult to recover from metagenomic sequencing.
L194. Nearly all samples containing Hodarchaeales also harbor Alphaproteobacteria, yet sites lacking Hodarchaeales may likewise show high Alphaproteobacterial abundance. Provide comparative statistics to determine whether the association is significant.
L198. If some Kariarchaeaceae and Hodarchaeales are truly adapted to aerobic environments, indicate their positions on the phylogeny. Are these taxa dispersed across the tree or concentrated in particular clades (e.g., guild 12) or in other guilds? Mapping oxygen-tolerant lineages onto the phylogeny would clarify this point.
Table S10. I welcome the abundance information for Hodarchaeales, Alphaproteobacteria and other microbial lineages. However, the current data do not clearly support an interaction between Hodarchaeales and Alphaproteobacteria, nor do Alphaproteobacteria appear to be a reliable proxy for oxygen. For example, Kiloniellales are present in every Bohai sample but many of those samples lack Hodarchaeales or Kariarchaeaceae, whereas Hodarchaeales is present in most GE2 samples where Kiloniellales are absent. Additional evidence is needed, or the wording should be softened. More importantly, it is just a co-occurrence phenomenon, which does not provide evidence for symbiosis. I think it can be deleted

but depend on authors' choice.

Major comments 2
Metabolic reconstruction of Hodarchaeales, Kariarchaeaceae，and even LAECA rests largely on hydrogenases and on electron-transport chain complex IV.
Hydrogenases – Please label the phylogenetic tree with catalytic direction (hydrogen-evolving, hydrogen-oxidizing, bidirectional) and assess critically how confidently type can be inferred from phylogeny and structural models, especially for the novel Asgard-specific enzymes. Maybe it is better to explain why these hydrogenases were missed in earlier surveys; and further re-analyzed public archaeal genomes with the new HMMs to see whether they are genuinely novel. Check whether homologous group 4 hydrogenases occur outside Asgard and map their distribution among hydrogen-producing versus hydrogen-consuming lineages.
Complex IV – This complex may also occur in Aigarchaeota and Halobacteriota. Among these, Aigarchaeota, not Poseidoniia, may be the nearest sister lineage of Hodarchaeales, though this needs testing. A previous study linked the Hodarchaeales complex IV to nitrate respiration, whereas in Halobacteriota it is thought to function in copper metabolism. Revisit screening criteria, survey its archaeal distribution again, and discuss whether the Hodarchaeales complex uses oxygen directly.
Key functional genes should be mapped onto the species tree and examined for vertical versus horizontal inheritance. Only guild labels are shown at present, which is misleading: the basal lineage is Hodarchaeales, not Kariarchaeaceae (the main member of guild 12). The placement of GCA_022574115 is tricky; please show it clearly.
Finally, the manuscript focuses on the gain of aerobic traits but does not assess whether Hodarchaeales and Kariarchaeaceae have concurrently lost core anaerobic pathways. A genome-wide survey of canonical anaerobic markers would address this gap. To my knowledge, pyruvate formate-lyase—a hallmark gene of anaerobic metabolism—is widespread in Hodarchaeales.

Specific modifications needed
Line 24: "including 136 new Heimdallarchaeia (49 Hodarchaeales)" — please remove "(49 Hodarchaeales)"
Line 77: "both the Hodarchaeales and the Heimdallarchaeia class" appears redundant since the latter encompasses the former
L110. As shown in Fig. 1, the purported "expanded genomic diversity" does NOT "increases support in the phylogeny for the deep-rooted clades". Please verify this point and delete or revise the statement and any related claims.
L115. An increase in genome number does not automatically lead to an increase in phylogenetic diversity. Please quantify how much the additional genomes actually expand phylogenetic diversity, particularly for the Kariarchaeaceae and Hodarchaeales lineages.
L123. The current guild classification separates Kariarchaeaceae and Hodarchaeales into distinct branches. Please clarify the rationale for this split. Was it driven by horizontal gene transfer affecting particular genes, and if so, which ones?
L327. Please add a species phylogeny indicating the sampling location, predicted lifestyle (aerobic vs. anaerobic), and the presence or absence of hydrogenase, cox complex, narGHJ, and other key metabolic genes.
L376. The authors state that the first Hodarchaeales co-cultures "are unlikely to be representative of all Heimdallarchaeia lineages." In reality, these two strains exhibit remarkable genome expansion, evocative of the genomic complexification associated with eukaryogenesis. The manuscript should offer a more balanced appraisal of the relevant studies.
L424. The relationship between the coastal/deep-sea MAGs and guilds 11/12 should be clarified more explicitly.
L425. Please clarify how the structural models of Complex IV differ when configured for oxygen versus nitrate as the terminal electron acceptor.
Line 455: "The presence of ETC components in the Asgard ancestor of eukaryotes would have" should be modified to "The presence of ETC components in the Heimdallarchaeial ancestor would have"
Lines 472-474: "…especially in Hodarchaeales and Kariarchaeaceae, support that the Asgard archaeal ancestor of eukaryotes was likely…" should be modified to "…support that the Heimdallarchaeial ancestor was likely…"
L484. I wonder if it might be best to remove Ran and Asgardaechaeia from Fig. 5. The order of GOE, eukaryogenesis, and FECA appears to be problematic. Figure 5 is still to complex for clearly show your idea. The left panel with blue background is not so important, I think it can be removed. For example, what is relationship with the deep-sea hydrothermal vents? Does the interaction only happen in coastal water column?

Referee #3

(Remarks to the Author)
I would like to thank the authors; the revised version of the manuscript addresses my major concerns. My remaining suggestions would be to clarify some of the thinking, descriptions and figures regarding the evolutionary scenario presented, to investigate whether newly published information on Njordarchaeales MAGs might shed light on some of the phylogenetic analyses, and to ensure that methodological detail given is consistent between different parts of the manuscript (e.g., between the main text, figure legends, and supplementary materials).

Eukaryogenesis:

The abstract and the legend for Fig. 5 describe a 'Heimdallarchaeia-centric model of eukaryogenesis'; I find the use of '-centric' here confusing, it's unclear to me whether it's used because the model is developed based on inferences drawn from features in modern members of Heimdallarchaeia, or because it conflates eukaryogenesis with the mitochondrial symbiosis and focuses primarily on the evolution of the host. Either way, I feel that it would avoid confusion if either a more descriptive term were used, or if the evolutionary scenario were presented without the name.

(Less importantly, use of the word ‘updated’ would also be more appropriate when referring explicitly to a previously described and named model: for instance, ‘an updated version of the reverse-flow model of eukaryogenesis’.)
Probably to maintain the linguistic link with this model name, the Fig. 4 legend title mentions ‘Heimdallarchaeia”, but since the diagram only presents the ETC of Hodarchaeales, it would be more accurate to change the title to either reflect this specificity, e.g. ‘in Hodarchaeales (Heimdallarchaeia)’. The authors could also spell out the implications in the figure legend if they wished: that Hodarchaeales are the closest sister group to eukaryotes, and that the ETC of their shared ancestor with eukaryotes may have been similar.

The timeline shown in Fig. 5 is not to scale, but even beyond that, the combination of the timeline with some of the figure’s other elements is still confusing. The relative positioning of Hodarchaeales and Kariarchaeaceae (both members of Heimdallarchaeia) and ‘Heimdall ancestor’ makes it look as though the former pre-date the latter. It took a bit of squinting at the figure on my part to see that the authors probably mean the right-hand portion of the figure to be an inset from the small red cell positioned under the word ‘Aerobic’; but if so that’s not really clear given the timeline, and in any case I think that the relative positioning of the cell representing Hodarchaeales and Kariarchaeaceae below this cell is still confusing. The inclusion of ‘Heimdall’ near the top right is also confusing: does this represent the emergence of the clade (if so, why is it positioned after the emergence of eukaryotes), or the simple existence of modern members of Heimdallarchaea? (It’s also a confusing abbreviation given the existence of Heimdallarchaeaceae, see comment below.) Why show two different cells representing, respectively, ‘Hodarchaeales and Kariarchaeaceae’ and ‘Contemporary Hodarchaeales and Kariarchaeaceae lineages’? Basically, I feel that it would be much easier to understand this figure if it were more clearly split into several distinct sections (especially if the number of sections could be minimised).

The Fig. 5 legend suggests that ‘LAsCA diversification allowed widespread niche expansion’, and I think it’s worth clarifying what the authors mean by this. Firstly, the meaning of ‘diversification’ isn’t clear, and secondly, regardless of the meaning of this term, it’s unlikely to apply to the LAsCA itself. I think that ‘Genetic diversification in descendents of the LAsCA allowed their occupation of a variety of new niches’ could be what the authors meant, but I may be wrong.

Line 89, changing “the archaeal host was an anaerobe” to “the host was an anaerobic archaeon” or “the host was an archaeon and an anaerobe” would be more accurate

Line 99: The authors that FECA is the same thing as the first eukaryotes, but this is unlikely to be true: FECA is defined as the first descendant on the eukaryotic branch of the last common ancestor of eukaryotes and their closest archaeal relatives. It is therefore to be expected that many descendant lineages of the FECA went extinct in the interval between its existance, and the emergence of cells with key eukaryotic features. This is true whether one favours a ‘mitochondria-late’ scenario (in which the FECA might have had many descendents with increasing degrees of complexity in their endomembrane system and cytoskeletal organisation), or ‘mitochondria-early’ (in which the FECA would likely not have been a eukaryote at all, but rather a nucleus-lacking archaeon with an alphaproteobacterial endosymbiont).
Likewise, the position of the FECA should be drawn much earlier along the timeline shown in Fig. 5, immediately after the divergence of the Heimdall ancestor from the eukaryote ancestor

Line 104-105, “it is plausible that the archaeal host and potentially some of its modern descendants” - This is confusing, as eukaryotes themselves are descended from this host and tolerate oxygen. Maybe something like, “it is plausible that the archaeal host and potentially some of its close modern archaeal relatives”?

Njordarchaeales MAGs:

The authors mention (in supplementary methods and review responses) potential artifacts introduced by sequences from the Njordachaeales clade. Some MAGs from this clade have recently been reported to be chimeric (Zhang et al. 2025 https://doi.org/10.1038/s41586-025-08955-7), so it might be helpful to investigate whether this affects the authors’ dataset and might explain the apparent artifacts they report

Methodological detail:

The authors state in their response to one of my earlier comments (on the interpretation of ultrafast bootstrap values) that they are now showing only ultrafast bootstrap values equal to or higher than 95% for trees in Fig. 3c and supplementary figures, and that they are adding a note of caution regarding that ultrafast bootstrap values should ‘only be relied on when >=95%’. However, they have not done this for Fig. 1, which presents key findings on relationships between Asgard groups.

The legend for Fig. 1 states that the species phylogeny was reconstructed with the WAG+C10 model, but the supplementary methods instead state that WAG+C10+G4 was used

Fig. 1 legend, do these include only genomes with >50% (as written) or also >45%?

Line 1460, were publicly available MAGs with >45% completeness included here, or only >50%?

Minor points:

The authors mention which groups are included within Heimdallarchaeia, but for a general audience, it would really be helpful to show on Fig. 1b (not just on Suppl. Fig. 3) which groups are included within Heimdallarchaeia. Similarly, consistency should be used for the abbreviation ‘Heimdall’, which is used for Heimdallarchaeaceae in Fig. 1a but for

Heimdallarchaeia in Fig. 4.

Line 100, “between Asgard archaeon” should read “between an Asgard archaeon”

Line 308, “expanding the known diversity” should read “expanding their known diversity”

Line 314, “increase membrane potential” should read “increase in membrane potential”

Line 543, “public metagenomes prior to 2021” should read “public metagenomes published prior to 2021”

Line 552, “interest on” should read “interest in”

Line 554, the terms in brackets should be clarified, e.g. “that can break down carbohydrates (*using* CAZymes)”

Line 666, I would change “adopts a configuration like Complex I” to something like “adopts a configuration similar to that of Complex I”

Line 1707, “The output was filtered to remove those sequences >200 amino acids...” - I think this should be ‘extract’ or ‘retain’ rather than ‘remove’

Line 1737, ‘we chose a reference dataset’ - I think this should read ‘we constructed a reference dataset’ (unless the dataset was an existing one, in which case a citation should be given)

Fig. 1. I think my point about roots in my earlier review was misunderstood: if the phylogenies were generated without specifying a root, then the trees can be displayed rooted on any clade (as the authors have appropriately done), but the little line representing the root itself should be removed (this can be done easily in a figure editing program such as Inkscape or Illustrator)

Fig. 5 legend has a typo, “Heimall” instead of “Heimdall”

Supplementary materials line 377, the wording of “for each marker a total of 1000 ultrafast bootstraps and SH-like approximate likelihood ratio tests” should be clarified as “for each marker a total of 1000 ultrafast bootstraps and 1000 SH-like approximate likelihood ratio test replicates”

Supplementary materials line 640, a reference should be provided for CombFold.

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)
The authors have addressed all of my previous concerns in this revision. The manuscript represents an important step forward in our understanding of Asgard archaeal diversity and eukaryogenesis.

Referee #2

(Remarks to the Author)
The authors have done an outstanding job addressing the previous points of critique. I have no further substantive comments. Please remove any reference duplications that exist between the main article and the supplementary files.

Referee #3

(Remarks to the Author)
I would like to thank the authors for thoroughly addressing my concerns. I have no more major suggestions, and only a couple of minor ones:

- that the same support criteria (>=80% SH-aLRT as well as 95% ultrafast bootstrap support) be applied for Figure 3 as for Figure 1, and similarly that the lines representing the roots be removed from these figures (in line with an earlier suggestion; ditto supplementary figure 82)

- the legend for Supplemenary Figure 83 states that “In Heimdallarchaeia (red), we identify...” if the red cell shown is Heimdallarchaeia, then it’s not the FECA (as currently labelled)

- similarly, the same sentence appears in the figure legend for main Figure 5, where all of the descendants of the LAsCA, not just Heimdallarchaea, are shown in red

- some of the features of Fig. 5b are described in the figure legend for Fig. 5a

- both Fig. 5a and supplementary fig. 83a should show units (i.e. ‘Ga’ or similar) for the timeline

Referees' comments:

Referee #1 (Remarks to the Author):

Comments on Appler et al.

In this manuscript Appler and colleagues present a tour de force analysis of a huge data set of new Asgard archaeal MAGs (404 Asgard MAGs specifically and >10,000 new MAGs overall). This data set provides an order of magnitude more data to bear on the question of the archaeal ancestry of the eukaryotic nucleocytoplasmic lineage and eukaryogenesis. The authors combine phylogenetics, sophisticated microbial ecology analysis, in depth structural prediction and modeling and biochemical reconstructions in their analyses. They present a treasure trove of interesting and groundbreaking new findings that really challenge the current views of eukaryogenesis. The most interesting of these (in my view) is the finding that a very large fraction of the Heimdallarchaeia (the immediate asgard sister lineage to eukaryotes) are likely aerobic respirers with aerobic electron transport chains. This goes against many prominent hypotheses of eukaryogenesis (most notably the Hydrogen Hypothesis, but others as well) that have assumed that the eukaryotic host lineage came from a strictly anaerobic Asgard lineage. Indeed, the general assumption in the field is that Asgard archaea are 'all' anaerobes with only one or two exceptions. This paper turns that assumption on its head and convincingly, in my view, demonstrates that the last asgard/eukaryotic common ancestor was likely an aerobe possibly living in shallow coastal environments. This result will no doubt ruffle many feathers and lead to a re-evaluation of eukaryogenesis hypotheses. For this reason, and also because of the sheer number of other novel/exciting findings reported in this paper, I strongly support its publication in Nature. Below I have made a number of comments on the manuscript/analyses that, if addressed, I believe could significantly improve the manuscript.

We would like to thank the reviewer for evaluating our work. We sincerely appreciate the reviewer's positive feedback and support for the publication of this manuscript. We agree this work will be impactful in our understanding of the origin of eukaryotes.

In response to the reviewers' detailed and helpful comments, we have rerun 28 phylogenetic analyses incorporating complex mixture models, accounting for various rates of heterogeneity, and comparing species tree recoding versus non-recoding approaches. We now provide additional details in legends, main and supplementary methods and tables. These steps were taken to streamline our analysis and test the resulting evolutionary scenarios. Additionally, we have updated the comparative genomic analyses, providing 27 new AlphaFold models, offering more detailed descriptions of specific genes and proteins, and including several new analyses as requested, such as additional co-

occurrence identification. Lastly, we have revised our eukaryogenesis model to incorporate the reviewers' suggestions, updates, and added analyses. In total, these revisions included updating 66 figures and 6 tables while adding 10 additional requested figures.

Major comments;

(1) The authors have done an amazing (dizzying!) number of analyses of different kinds in this manuscript and there is a huge amount of information summarized.

We appreciate this positive view of the impact of this manuscript.

I have concerns mainly about a 'corner' of those analyses that involve phylogenetic analyses of concatenated proteins (e.g. NM47, CoxABC) and individual proteins (e.g. Nuo subunits in supp. Figures 15-27). The methods used to infer these phylogenies are sometimes quite different depending on the data set and there is heterogeneity in the approaches described. I am fully aware that it can be difficult to coordinate many different collaborators who are experts in different biochemical systems to do analyses using the same approaches, so, in what follows, I do not expect everything to be 'redone' with a particular set of methods.

We thank the reviewer for their insight and constructive feedback on the phylogenetic analyses. We greatly appreciate your acknowledgement of the amount of work that is required to do these phylogenies.

That being said, 'deep time' phylogenetic analyses of proteins can be easily compromised by artefacts owing to large numbers of substitutions occurring on the branches. For such analyses the current gold standard is to use a profile mixture model with many components (e.g. LG+C60+Gamma or CAT+GTR or similar) to ameliorate potential problems with long branch attraction. These models also usually end up being selected by model selection criteria such as AIC or BIC when they are included amongst the models being tested.

Thank you for reminding us of this, we have rerun all the NM47 (Fig. 1ab and Suppl Fig. 3), [NiFe]-hydrogenase (Fig 3a), CoxABC (Fig. 3bc, Suppl Fig. 72-74), and Nuo phylogenies (Suppl Fig. 15-27) with mixture models (C50 and C60) to reduce the impact of long branch attraction. We also reran the analyses of Complex II (Suppl Fig. 37-40), Complex III (Suppl Fig. 57-59), and Complex IV (Suppl Fig. 72-74) to streamline the approach. Detailed descriptions of the updated analyses are provided below.

These revised results strengthen the manuscript. However, these refined testing methods did not change the main outcomes, retaining the taxonomic classification of the named Asgardarchaeota orders (NM47). These results still

support the presence of the Asgardarchaeota electron transport chain subunits and the mitochondrial subunits are likely of bacterial origin.

For the main analyses to reconstruct the phylogeny of the new Asgard lineages with the NM47 data set shown in Figure 1b, the authors did use profile mixture models, although they only showed the analyses where the data had been recoded into 4 states (GTR+C60+G4 recoded into 4 states).

As suggested by the reviewer, we have updated the manuscript to include the species trees derived from the NM47 dataset with all the Asgardarchaeota MAGs with and without the use of a recoding scheme. The species tree has been reconstructed using the WAG+C10+R4 model, after applying Model Finder between the matrices LG, WAG and Q.pfam, and presented as Figure 1a. The SR4-recoded dataset is now presented as Figure 1b. In the un-recoded dataset, Heimdallarchaeia (Gerdarchaeales, Heimdallarchaeaceae, Kariarchaeaceae, Hodarchaeales, Njordarchaeales) appear paraphyletic and attracted toward the outgroup possibly indicating the presence of a long branch attraction artifact due to the long branch introduced by Njordarchaeales. The SR4-recoded dataset appears to mitigate both issues.

To ensure the recoding did not compromise the phylogenetic signal, we dereplicated our dataset to include species-level representatives of the Asgard ingroup. This allowed us to apply the more computationally intensive C60 mixture model. With this model, we observed increased support for the depicted topologies and importantly a monophyletic Heimdallarchaeia clade. We provide the subset un-recoded phylogeny in Supplementary Fig. 3b.

For the other data sets it appears that the authors have opted for site homogeneous analyses with models such as LG+R10 (or other rate distributions selected by BIC). In the latter cases model selection was performed, but I'm almost certain that the authors did not include the profile mixture models in the set of models tested; this is a frequent oversight because IQ-TREE, by default, does not test these models (they must be specified using the '-madd' flag followed by the profile mixture models that you wish to test). I'm confident that had the authors tested the LG+CXX series of profile mixture models (XX= 10, 20…60), they would frequently would have found that the complex mixture models were preferred for both the concatenated analyses and the single-protein analyses.

We chose to test the electron transport chain subunits with complex mixture models C50 and C60 in the CXX series. We now display the phylogeny with the model with the lowest Bayesian Information Criterion (BIC). Due to the number of phylogenies to rerun and the computational burden, we chose the CXX models with the most classes. Recent literature indicated simpler mixture models can impact tree estimation, but using class-rich models (C50-C60) does

not negatively impact tree topology, reducing the concern of overparameterization (Baños et al., 2023 Systematics Biology).

In addition to the main figures previously discussed, we have rerun and updated the NuoABCEFGHIJKLMN (Suppl Fig. 15-27), Complex II (Suppl Fig. 37-40), Complex III (Suppl Fig. 57-59), Complex IV (Suppl Fig. 60; 72-74), and NiFe Hydrogenases (Suppl Fig. 42-44) with mixture models (C50 and C60). Now accounting for site heterogeneity, we reduce the potential long branch attraction.

In comparing the updated analyses, we still find that only one Lokiarchaeales genome (Co_bin7) clusters with eukaryotic electron transport chain subunits (NuoAJ). Similarly, we note one Njordarchaeales hot spring genome that encodes NuoACFGHJKN that cluster with bacteria. Closer inspection indicates these single hits are either contamination or recent transfer events. Otherwise, most of our Asgardarchaeota sequences cluster by phyla or in most of the subunits by lower taxonomic levels.

Therefore, our updated analyses support previous claims that the electron transport chain subunits likely did not arise from the archaeal ancestor. As previously mentioned, instead our analysis continues to support bacterial origins of the electron transport chain, especially with the addition of the Alphaproteobacteria from the Bohai Sea and the Guaymas Basin.

Alphaproteobacteria were the only class that grouped consistently with mitochondrial sequences across all the analyzed Complex I subunits. More specifically, Ferrovibrionales, GCA-2731375, JAAEOK01, Kiloniellales, Rhodospirillales, Rhodospirillales_A, Rickettsiales, SMXQ01, Sphingomonadales, UBA1280, and UBA8366 representatives grouped with at least five of the analyzed mitochondrial subunits. This supports previous claims of the electron chain subunit's origin in bacteria, more specifically Alphaproteobacteria or their sister lineages. We now further discuss these evolutionary implications in Supplementary Discussion.

How important is this and what should the authors do? I would suggest that, at least for the concatenated protein analyses shown in figures in the main text (except Fig. 1a which is just a 'rough' tree), that the authors try to fit site-heterogeneous mixture models using the above approach and estimate the trees to see if they see any differences in the resulting topologies.

In addition to the phylogenies above, we have also rerun all the phylogenies and updated the figures included in the main text. The chosen models are as follows: Fig1a (WAG+C10+R4), Fig1b (WAG+C10+R4+SR4), Fig3a (Q.pfam+C50+R8), Fig3b (WAG+C50+R8), and Fig3c (LG+C50+R8). We did not

notice major differences when comparing the resulting topologies but again agree that addition of complex profile mixture models and rate heterogeneity helps to strengthen our analysis.

For Figure 1b, I think it's important that the authors present a non-recoded profile mixture model analysis of the data set (e.g. LG+C60+G4) and comment on what changes in the estimated topology were recovered with the recoding. As it currently stands the authors are arguing they recoded to avoid composition-related artefacts, but it would be nice to see if recoding changed anything because recoding is well known to have its own problems (see Foster et al. (2023) https://doi.org/10.1093/sysbio/syac042). It's important to have non-recoded analyses presented in addition to the recoded analyses and for the differences to be commented on (the authors could include these in the supplementary figures).

We have updated the main figures in the manuscript to include untreated (non-recoded) C10 species tree as Figure 1a and the recoded as Figure 1b for comparison. We add a WAG+C60+G4 analysis of a comprehensive taxonomic subsampling of the MAGs in Supplementary Figure 3. We chose a subset for the C60 analysis due to the computational requirement of the complex model. We have included all these updated analysis in the Figshare files, please see those for all the intermediate files.

Details on the reasoning for recoding and the differences in topology recovered after the recoding have been added to Supplementary Methods and Discussion. The updated phylogenies maximally support both already described and novel orders from this study across all performed phylogenetic analyses (recoded/non-recoded and all MAGs/subset). The complex mixture model C60 (Suppl. Fig. 3) and recoded analyses (Fig. 1b) conserve the monophyly of both Heimdallarchaeia and Lokiarchaeia.

For the single-protein analyses of the Nuo subunits (and others), there are a lot of analyses that would have to be redone using profile mixture models, so I wouldn't insist on the authors doing this. However, if it could be done, I think it would improve the quality of the analyses in the paper and assure readers that those trees are less likely to be influenced by long branch attraction.

We agree with the reviewer's insights and have reanalyzed all the Nuo subunits (Complex I), SdhABCD (Complex II), QcrABC (Complex III), and Complex IV (CoxABCD) data as suggested. Briefly, we re-analyzed the phylogenies using three substitution models (LG, Q.pfam, and WAG) along with multiple rate heterogeneity models (G4, R4, R6, and R8). The models were selected using the Model Finder Plus (MFP) algorithm based on Bayesian Information Criterion (BIC). Additionally, we incorporated complex mixture models (C50 and C60) to account for more sophisticated patterns of sequence evolution.

This updated analysis increases the bootstrap support for our original results and helps to mitigate long branch attraction in deep-evolutionary time (Supplementary Figures 15-27; 37-40; 42-44; 57-59; 60; 72-74).

(2) The authors have tried hard to make a detailed eukaryogenesis scenario in Figure 5 and an accompanying discussion in the last part of the paper, but several aspects of this scenario are, in my view, either poorly justified or unclear. I think the paper is already very interesting without the authors speculating to this degree so I have a general suggestion that the authors simplify their scheme, take out the more speculative claims and stick to the evidence they have presented. I think they could write a separate "scenario" paper where they develop their ideas more fully rather than in overly brief format here. Here are some specific concerns I have about this scheme and figure:

Thank you for the detailed examination of the model. We agree there is a lot here, and considered not including it, but given all the new findings from this expanded database we feel it is important to frame them with respect to eukaryogenesis. However, we have modified specific parts of the model as suggested and detail these changes below.

i) the authors discuss how many Heimdalls can't make vitamin B12 and they argue in various places that this could have been important in the mitochondrial symbiosis. But it is well-known that many prokaryotic and eukaryotic microbes in the ocean are B12 auxotrophs and they import B12 from the surrounding environment (produced by prototrophs). I don't think this is particularly convincing as part of this scenario.

We have removed vitamin B12 from Figure 5 and discussion of the exchange in the legend "and vitamin B12 (vB12) synthesis" and "Furthermore, vitamin B12, necessary for coenzyme synthesis, is absent in all coastal Hodarchaeales genomes. This suggests that it requires a partner, sharing of this coenzyme has been linked to other host-symbiont metabolic interactions" from the main text to avoid confusion with potential for import of vitamin B12.

We have moved part of the discussion to the supplementary to highlight that our analysis indicates a switch of vitamin B12 prototrophy to auxotrophy between deep-sea and coastal representatives (see below), which remains ecologically relevant in adaptations to distinct environmental conditions.

Vitamin B12 (vB12), necessary for coenzyme synthesis, is absent in all coastal Hodarchaeales genomes. Indicating a potential metabolic shift from vB12 prototrophy to auxotrophy between deep-sea and coastal environments. This adaptation could be influenced by nutrient availability or microbial interactions. vB12 has been suggested as linked in other host-symbiont

metabolic interactions (Jerlström-Hultqvist et al., 2024 Nature Communications; Croft et al., 2005 Nature; Grant et al., 2014 ISME).

ii) What specifically do the molecules hsp90, L28e, alphaMICOS have to do with this scenario. Is hsp90 only found in Heimdalls?

These proteins were highlighted in the model because they are eukaryotic signature proteins (ESPs) (hsp90 and L28e) or proteins necessary for transition to endosymbiotic lifestyle (alphaMICOS). L28e is only within Hodarchaeales (27 genomes) and Hsp90 in Hodarchaeales, Gerdarchaeales, Hodarchaeales, Kariarchaeaceae, and Thorarchaeia (in the Supplementary Discussion).

The inclusion of ESPs in the model was to highlight potential *"increased cellular regulation and chaperoning through L28e and hsp90"*. However, we recognize the reviewer's concerns and have exchanged specific proteins in Figure 5 for "ESPs" and removed the statement in the legend.

iii) What Cox subunit in modern day eukaryotes is homologous to CoxD found in these MAGs (I tried to find out but couldn't find the name of the homolog)?

CoxABC (*coxABC; ctaDCE*) are homologous to mitochondrial cytochrome c oxidase subunits 1, 2, and 3 (I, II, and III) in eukaryotes. We have clarified this in the main text changing the previous statement *"Phylogenetic analyses of individual CoxABC proteins yielded consistent branching orders.*" to "*Phylogenetic analyses of individual CoxABC proteins (homologous to eukaryotic Cox123) yielded consistent branching patterns."* We also now cite Ludwig, 1987 FEMS Microbiology Reviews.

In prokaryotes, *coxD* is also called cytochrome c oxidase subunit IV or *ctaF*. As far as we know, this has not been recently tested, but earlier papers indicated no known homolog with mitochondrial subunits (Saiki et al., 1996 The Journal of Biological Chemistry; Iwata et al., 1995 Nature; Haltia, 1994 Biochemistry). We briefly looked into both sequence and structural homology. Due to the short length of this sequence, we have added the structural results in Supplementary Table 11 and Supplementary Fig. 75. We compared the predicted Asgardarchaeota CoxD to bacterial oxidase, bacterial cytochrome bo_3 ubiquinol oxidase, eukaryotic Cox3 and eukaryotic Cox4. We found the lowest root-mean square deviation (RMSD) in alignment of the Hodarchaeales CoxD and a bacterial cytochrome bo_3 ubiquinol oxidase. In bacteria, cytochrome bo_3 ubiquinol oxidase is a subunit of Complex IV in the electron transport chain, transferring electrons to reduce oxygen to water (Guo et al., 2023 Protein Science). This further indicates this subunit is part of Complex IV with the greatest structural similarity to a bacterial sequence.

Are the authors suggesting that this subunit of Cox was 'host inherited' rather than symbiont inherited in eukaryotes? If so, they need to provide phylogenetic evidence for this claim.

No, based on our analysis we are not suggesting Cox was host inherited. Instead, we are suggesting translating CoxABC and proteins to reduce the toxicity of reactive oxygen species (ROS) by the host enabled interaction with the suggested aerobic mitochondrial ancestor.

Our phylogenetic analyses of Complex I (Supplementary Figures 15-27), Complex II (Supplementary Figures 37-40), Complex III (Supplementary Figures 57-59), and Complex IV (Supplementary Figures 72-74) support eukaryotes likely obtained their capacity for aerobic respiration from the bacterial symbiont. We have added "Our analysis is consistent with an alphaproteobacterial ancestry of ETC components" to our model description for clarity.

What about SCO1?

Sco proteins have been linked to various functions, including COX regulation and assembly, protection against oxidative stress, and redox regulation through Cu-binding signaling molecules or Cu homeostasis (Banci et al., 2011 FEB Journal). We now cite this literature and provide a description of the potential function in eukaryotes. In response to requests to streamline the model, this section is now found in the Supplementary Discussion.

Primarily, SCO1 (Sco1) has been implicated in copper transfer (ion loading) to cytochrome c oxidase, likely involved in the posttranslational steps of the complex's formation (Balatri et al., 2003 Structure; Tan et al., 2014 BMC Structural Biology) across bacteria, archaea (Banci et al., 2007 Journal of Proteome Research), and eukaryotes. Analysis of pathogenic *Neisseria gonorrhoeae* indicate Sco is related to its prevention of oxidative damage instead of formation of Cu-containing proteins (Seib et al., 2003 FEBS letters).

iv) the authors are showing 'contemporary' Hod and Kari lineages as having tentacle-like protrusions in the figure. There is no evidence for this cell morphology in these lineages is there?

Our analysis of eukaryotic signature proteins identified Lokiactin (PF00022) in 60 Hodarchaeales (~83.3%) and 17 Kariarchaeaceae (68%) genomes. We provide this information in Supplementary Table 12, which is now cited after the initial statement in the Supplementary Discussion. Lokiactin have been previously postulated (Stairs and Ettema, 2020 Current Biology; Akıl et al., 2021

Current Opinion in Cell Biology; Nobs et al., 2022 Trends in Microbiology) and more recently visualized in *Lokiarchaeum ossiferum* to produce protrusions (Rodrigues-Oliveira et al., 2023 Nature). Rodrigues-Oliveira et al. cryo-ET and immunofluorescence analyses concluded widespread lokiactin provides scaffolding for hypothesized architecture of Asgardarchaeota cells.

We agree that without any published cultures or images of representatives of each of the Heimdallarchaeia lineages morphology cannot be confirmed. So, in response to the reviewer, we have clarified the current state of analysis in the Supplementary Discussion, adding "Heimdallarchaeia contain actin homologs like those in Lokiarchaeales, which in Rodrigues-Oliveira et al., 2023, produce protrusions (Supplementary Table 12). Yet in a cultured bacterium (*Candidatus* Uab amorphum), actin does not produce this morphology, necessitating image verification" and "Metabolite exchanges and coordinated response to rising oxygen levels may have increased cell-to-cell contact between partners, enabling initial compartmentalization via predicted lokiactin protrusions." in the legend of Figure 5.

v) there seems to be a 'quasi-timeline' at the top of Fig. 5, but it can't be drawn to scale given the numbers shown.

We appreciate the reviewer's feedback and have clarified this in the legend of Figure 5 "Timeline not drawn to scale".

Also I can't figure out which cell along the grey arrow diagram corresponds to the most recent common ancestor of Hod and eukaryotes. It can't be FECA because the cell depicted there has a mitochondrion and the authors did not present any evidence for any of their organisms having a mitochondrial symbiont. Yet FECA is, by definition, the immediate daughter cell of the most recent common ancestor of Hod and eukaryotes.

It is the first cell labeled as Heimdall Ancestor. We have moved the labels and reduced the clutter to hopefully clarify this concern. Specifically, we removed FECA and LECA cell labels to avoid confusion in progression of the updated scenario.

vi) I don't think the authors need to show "anaerobic eukaryotes" in this diagram or hydrogenosomes at all. They aren't relevant and they clutter up the diagram.

We have removed anaerobic eukaryotes and hydrogenosomes from the figure.

vii) the two arrows next to O2 and mtDNA are confusing. What does this mean?

The arrows were to represent the transition to anaerobic eukaryotes. Particularly, evolution in decreasing oxygen conditions and a reduction in mitochondrial DNA. However, as suggested by previous comment we have removed these from Figure 5, as well as transition to anaerobic eukaryotes.

viii) overall the diagram is too cluttered to be easily interpretable. I would suggest simplifying it and removing anything that isn't immediately relevant to the scheme.

Given the reviewer's detailed and helpful suggestions, we have removed all requested portions of the diagram to reduce clutter and provide an updated scenario in Figure 5.

Other comments:
- Line 31, Abstract: "These archaea encode hallmarks of aerobic eukaryotes". Consider rephrasing as "hallmark proteins" maybe?

We have revised this sentence as suggested by the reviewer.

- Line 34, "Heimdallarchaeia encodes" encode?

We have revised this sentence as suggested by the reviewer.

- Line 47, I suggest rephrasing as "some models of eukaryogenesis" (or "many models")

We have revised this sentence to "Many models of eukaryogenesis…".

- Line 55-58, The authors are taking the molecular clock estimates from this one paper (ref. 12) very (too?) seriously. There is substantial variation in the age estimates of these nodes across different studies in the literature, so it might be prudent to word this more tentatively. "A recent study estimated that" and "If these estimates prove to be robust then these events would roughly coincide with the rise in the oxygen on the planet…"

We agree and have updated the sentence to reflect the variance in approximated ages. See updated text "A recent study estimated that the first eukaryotes (FECA, the first eukaryotic common ancestor) diverged from an interaction between Asgard archaeon and Alphaproteobacteria (the bacterial group related to the mitochondrial ancestor) about 2.67-2.19 billion years ago (Ga) and 2.58-2.12 Ga, respectively. If these estimates prove robust, then eukaryogenesis would roughly coincide with the rise in oxygen on the planet ~2.5-2.1 Ga, referred to as the Great Oxidation Event (GOE)."

- Line 61, "potentially some of its modern descendants could use oxygen." The phrase "use oxygen" here is a bit vague. Oxygen can be a substrate in a variety of biochemical reactions. Do they mean "use oxygen as a terminal electron acceptor in ATP generating pathways (i.e. aerobic respiration)" or do they mean this statement to include many other metabolic uses of oxygen. The sentences afterward suggest that perhaps they mean aerobic respiration. I would encourage the authors to be a bit more specific here.

We have updated the statement to ''Given our new knowledge of these events, it is plausible that the archaeal host and potentially some of its modern descendants tolerated oxygen or even benefited from it as a terminal electron acceptor'', which more clearly reflects the ability to tolerate oxygen via ROS protection (Supplementary Figures 62-66) and use it for aerobic respiration.

- Line 82, "first eukaryotic cell." The concept of the "first eukaryotic cell" can only be arbitrarily defined. What would be the first eukaryotic cell? The one with a proto-nucleus with only half the nuclear proteins that LECA had? Or maybe the one with the first fully developed nucleus? Or maybe it had only one nuclear pore protein and a rudimentary membranous structure? Since it's not possible to uniquely define the "first eukaryote", I think that this wording should be avoided completely. The authors could consider changing "bioenergetic advantages that the Hodarchaeales host may have conferred to the first eukaryotic cell" to "bioenergetic advantages of the Hodarchaeales lineage in this evolutionary transition" or something like that.

We agree the definition of the first eukaryotic cell is arbitrary and not defined in this context. We have changed the statement as suggested to "Our analysis revealed that several Asgard archaeal lineages most closely related to eukaryotes can generate a membrane potential through both H_2 production (not obligatory consumption as previously proposed) and aerobic respiration with multiple lines of evidence suggesting they are adapted to oxygen exposure. This leads us to propose an updated model of eukaryogenesis that incorporates the increase membrane potential and oxygen tolerance within Heimdallarchaeia during this evolutionary transition", which appropriately reflects our analysis.

- Line 110, "the closest known ancestor to eukaryotes". The Hodarchaeales representatives are not ancestors of eukaryotes, they are relatives (i.e. sister lineages) of eukaryotes. Consider rephrasing this.

We have modified the text "sister lineage of eukaryotes".

- Line 129-130, The specific substitution model used with RAxML is not specified here or in the Methods section for Fig. 1a. I assume it is LG+Gamma or LG+F+Gamma? This should be specified in the Methods section.

Thank you for catching this, we have replaced Fig. 1a according to previous suggestions and the model for the updated Figure 1a-b is specified in both the legend and methods.

- Figure 1a, It's difficult to see the grey circles on the branches indicating >=90 bootstrap support. Perhaps the authors can increase the resolution of the figure and make these circles stand out a bit more (or maybe the figure resolution was compromised by the rendering of the PDF).

We updated Figure 1, including providing darker circles for the bootstrap values.

- Figure 1b in the Support value legend to the left of the tree you have "greater than or equal to 100" sign. Support values can't be >100 so this should be just an equals sign (=). Same goes for Supplementary Fig. 6.

Thank you, this has been updated in Figure 1.

- Line 175-177, the authors should cite paper 47 at the end of this sentence (i.e. after the word "intolerant") because this is the method used in that sentence. I don't think it makes sense to cite 47 at the end of line 179.

We have revised the position of the citation as suggested by the reviewer.

- Line 180: This first sentence of the paragraph says: "The mitochondrial ancestor is predicted to have been a facultative aerobic Alphaproteobacteria-related lineage" and refs 48 and 49 are cited. It's very clear that the mitochondrial ancestor had the capacity for aerobic respiration; the oxygen-dependent ETC in mitochondria is clearly phylogenetically related to the alphaproteobacterial system. Dozens of different papers in the literature have demonstrated this fact and no-one would dispute it. However, the claim that the mitochondrial ancestor had the ability to thrive in anoxic (or low oxygen) conditions (which would be required of a facultative aerobe) is based on very weak evidence. Reference 48 points to potential enzymes of anaerobic metabolism found within genomes of various alphaproteobacterial taxa, but there is no direct phylogenetic affinity with the eukaryotic homologs demonstrated. Reference 49 is a review paper and doesn't present any specific evidence that shows the mitochondrial ancestor had the capacity for anaerobic metabolism. However, there are many published papers that show that enzymes of anaerobic metabolism in anaerobic eukaryotes (which have secondarily adapted to low oxygen) appear to have distinct phylogenetic origins by horizontal gene transfer from various kinds of bacteria (see Gawryluk, Vizitiu and Stairs BBA Bioenergetics 1863: 148627, Stairs et al. (2020) Sci. Adv. doi/full/10.1126/sciadv.abb7258 and reviewed in Roger et al. (2017) Curr. Biol. 27: R1177–R1192). I know this might be seen as a minor point, but

I think it would be more balanced if the authors reworded this sentence along the lines of "The mitochondrial ancestor is predicted to have been an Alphaproteobacteria-related lineage capable of aerobic respiration [references], and some authors have argued it may have been facultatively aerobic [ref 48, 49]." I note that there isn't any real need to discuss the potential for anaerobic metabolism in the mitochondrial symbiont since its aerobic respiratory functions are most relevant to the discussion in this paper.

We appreciate the reviewer's insight in this area and have altered the sentence to "The mitochondrial ancestor is predicted to have been a Alphaproteobacteria-related lineage capable of aerobic respiration, and some authors have argued it may have been facultatively aerobic" and included Gawryluk and Stairs, 2021 and Stairs et al., 2020 as citations, indicating the evolution of anaerobic eukaryotes outside of Alphaproteobacteria to more fairly highlight both viewpoints.

- On lines 210-212 the authors indicate that none of their structural predictions suggest that the NuoEFG module formed a higher-order complex with the rest of the 'complex I-like structure' (or equivalently the [NiFe]-hydrogenase complex which apparently shares a similar architecture). I find it confusing, then, that in Figure 4b NuoEFG are shown in a complex with other complex I subunits and as separate soluble module.

We see the confusion and have modified Figure 4b to remove the NuoEFG subunits from the representation of the Complex I-like complex. These are now more thoroughly discussed in the supplementary discussion.

Furthermore, in the paragraph that starts on line 243 it is argued that the group 4 [NiFe]-hydrogenases of the entire group of Heimdallarchaeia are likely oxidizing ferredoxin and reducing protons to H2, using this redox reaction to pump protons out of the cell. This section is hard to square with Fig. 4b where no ferredoxin oxidation or H2 production is shown and instead NADH oxidation/MQ reduction is powering proton translocation by complex I. If I haven't misunderstood what is being argued (and I easily could have), I think the authors need to justify why their Fig. 4b is showing something different than this section of the text (and in fact showing something different from that described in the figure legend itself).

Thank you for catching this oversight. We have updated Figure 4b, to reflect the main text and legend, which is supported by our phylogenetic and structural analyses. Specifically, we have updated the figure to show ferredoxin oxidation and H_2 production of the Nuo complexes.

- Line 243, "The group 4 [NiFe]-hydrogenases of Heimdallarchaeia are an evolutionarily missing link of Complex I" (Note: "evolutionarily" should be "evolutionary").

Fixed.

This is an intriguing hypothesis if I understand the author's suggestion correctly. However, it would be helpful to know how this hypothesis relate to the phylogenies of the Nuo subunits shown in Suppl. Figs. 15 to 27? These trees show the actual inferred evolutionary relationships between subunits from Complex I of bacteria and the asgard and other archaeal subunits and therefore seem relevant to the evolutionary position of the group 4 lineages. I think it would be good if the authors made their reasoning more explicit here.

Using the updated Nuo subunit phylogenies with mixture models, we have updated the text discussing the modularity of these respiratory complexes, connecting our phylogenetic and structural analyses.

We also discuss the evolutionary implications in the Supplementary Discussion sections "Evolutionary Implications" and "Complex I-like NADH:ubiquinone oxidoreductase subunits". Briefly, we discuss the evolution of the modularity of these complexes, loss and duplication of membrane subunits, and use of alternatives to NuoEFG to obtain electrons. We also tie this to previous descriptions of archaea and the implication for evolution of eukaryotic Complex I.

- Line 265 – "Moreover, detailed structural protein modeling using this expanded genomic catalog supports the reverse flow model of eukaryogenesis, with Heimdallarchaeia not having to rely on a symbiont for H2 production". I think this is a bit of an 'overreach' and I would encourage the authors to tone it down a bit. If the authors are correct about their metabolic arguments on line 243 then it is likely these organisms produce oxidize ferredoxin and reduce protons to H2 with their [NiFe]-hydrogenase complex. The part of the 'reverse flow' model this supports is this metabolic capacity specifically (and the presence of [NiFe]-hydrogenase) in the eukaryotic/asgard last common ancestor. This does not say anything about whether or not the mitochondrial symbiont could harvest the H2 gas or whether mitochondrial symbiont came in at the same time the 'host' lineage was doing this kind of metabolism. If mitochondria were relatively 'late', then there could be hundreds of millions of years between the LEAsCa and the initial mitochondrial symbiosis. I suggest the authors rephrase their comment here to be specific about what part of the reverse flow hypothesis specifically their data support.

We agree with the reviewer's point and have revised to clarify which portion of the reverse flow model our data supports. It now states "Moreover, detailed

structural protein modeling using this expanded genomic catalog supports the capacity for H_2 production in Heimdallarchaeia, as proposed in the reverse flow model of eukaryogenesis, potentially reducing reliance on a symbiont for H_2."

- Line 363, I don't understand this sentence. Perhaps the authors can clarify why oxygen tolerance in the Hodarchaeales would directly increase cell to cell contact?

We have rewritten this section to highlight that it is the suite of oxygen tolerance genes/proteins, now listed with supplementary figures that provide the capacity for oxygen tolerance. We now also link this increased ability to tolerate ROS and byproducts of aerobic respiration to enable close knit spatial interaction with the aerobic/facultative aerobic mitochondrial ancestor.

What does oxygen tolerance have to do with "providing" compartmentalization to the mitochondrial symbiont? The latter is a matter of the host cell surrounding the symbiont and doesn't have anything directly to do with oxygen tolerance does it?

Here we were describing the advantage afforded by oxygen tolerance to a potential increase in close interactions with an aerobic/facultative aerobic mitochondrial ancestor. So, we clarify this second part of the statement to "potentially enabling close knit spatial interaction with the predicted facultative anaerobic mitochondrial ancestor".

- Line 399, I suggest rephrasing it to make it clearer. For example, "…Lokiarcheaeles, which, in the latter, produce protrusions" (there isn't any evidence they produce protrusions in Heimdalls yet).

We have updated the statement in the supplementary discussion as suggested "Heimdallarchaeia contain actin homologs like those in Lokiarchaeales, which in Rodrigues-Oliveira et al., 2023, produce protrusions (Supplementary Table 12). Yet in a cultured bacterium (*Candidatus* Uab amorphum), actin does not produce this morphology, necessitating image verification."

- Line 402-408, these claims are too speculative and, without more detailed explanation, are very difficult to understand in this context. I think the paper would be much better if these sentences were just removed. There is no point in bringing up the "inside-out" model in conjunction with these speculations and it just seems a bit random (and needlessly distracting) to discuss this here.

We have removed these statements from the main text. To meet the requests by the other reviewer's, this section has been modified for clarity and to avoid overspeculation. The modified text was relocated to the supplementary to avoid distraction.

Methods

- Line 583, what does "hyperthermal-sensitive marker genes" mean? Perhaps it means that proteins encoded by these genes have an extreme amino acid composition change relative to other archaea which results from adaptation to hyperthermophily. Consider rephrasing this to make it clearer.

Yes, the statement reflects the decision to exclude Korarchaeia from the outgroup to prevent a potential grouping of Njordarchaeales with Korarchaeia. This precaution was taken as their proteomes have adapted to a hyperthermophilic lifestyle and thus exhibit high compositional similarity (Eme et al., 2023 Nature). We have clarified the above statement in the Methods section.

Referee #2 (Remarks to the Author):

This manuscript presents a significant expansion of our understanding of Asgardarchaeota diversity and distribution, particularly focusing on the Hodarchaeales, the closest known archaeal relatives of eukaryotes. The authors have generated an impressive dataset of metagenome-assembled genomes (MAGs) and conducted extensive comparative genomic analyses.

The study's strengths lie in its comprehensive genomic dataset, which has led to the identification of a new class and numerous lower taxonomic levels within Asgardarchaeota. The expansion of key metabolic protein families, especially the doubling of [NiFe]-hydrogenase diversity, is a significant contribution to the field. The authors' focus on Hodarchaeales and their potential contributions to eukaryotic cellular complexity is particularly noteworthy. Overall, this is an interesting study with some promising results. Their findings provide valuable insights into the metabolic capabilities of these organisms and their potential role in eukaryogenesis.

However, the manuscript suffers from several inconsistencies and overstatements that need to be addressed as in detail comments. While this work represents a significant advance in our understanding of Asgardarchaeota and their potential role in eukaryogenesis, the manuscript would benefit from more rigorous analysis in some areas, clearer definitions of key concepts, and a more balanced discussion of alternative hypotheses. Addressing these issues would strengthen the impact and reliability of this important contribution to the field.

We appreciate the reviewer's acknowledgment of the advancements provided by this study and specific feedback on how to strengthen its contribution.

Major concerns :

1. The topology of Asgardarchaeota (also known as Promethearchaeota) and the branching position of Eukaryotes form the foundational basis for understanding

eukaryogenesis and have been subjects of significant debate. A recent study by Eme et al. based on non-ribosomal marker proteins suggested Hodarchaeales as the closest relatives to Eukaryotes; however, the possibility of methodological artifacts cannot be completely dismissed. In the present study, the authors employ three marker gene sets to reconstruct the Asgard phylogeny, resulting in three distinct topologies with low bootstrap support.

We have updated our phylogenomic analyses to rely on a single set of markers, NM47 (subset of the non-ribosomal archaeal markers reported in Eme et al., 2023 Nature and Petitjean et al., 2015 Molecular Biology and Evolution). As you suggest below, this standardizes our analysis with the most reliable set of markers. To better understand the branching order of the different Asgardarchaeota lineages, but also identify potential systematic errors incorporated in our analyses, we infer species trees using two datasets: the entire dataset (WAG+C10+R4 & SR4-recoded[GTR+C60+G4]) and the dereplicated—on species-level representatives—dataset (WAG+C60+R4). Furthermore, by using the NM47 marker set, potential phylogenetic artifacts caused by compositional bias of thermophilic lineages are reduced. As was show in Eme et al., Nature 2023, ribosomal markers are more compositionally biased compared to non-ribosomal markers, and hence more prone to cause phylogenetic artifacts.

We argue that the incongruence observed between the two species trees of the expanded dataset (un-recoded; Fig1a & recoded; Fig1b) likely results from the application of an inadequate evolutionary model (C10) that fails to address artifacts such as long branch attraction on the un-recoded dataset (Supplementary Discussion). Such artifacts are mitigated when applying recoding but indeed the bootstrap support of inner nodes remains low possibly because of decreased phylogenetic signal. Despite the above, well-defined clades like Loki-/Helarchaeales and the Heimdallarchaeia orders remain monophyletic.

In the species tree inferred from the dereplicated dataset, deeper nodes show increased bootstrap support when applying a more complex mixture model (C60). The use of C60 is now computationally feasible due to the reduced size of the dataset, which still captures the representative diversity of the Asgard clades. In our updated analyses, the use of more sophisticated models and/or treatments consistently recovers Heimdallarchaeia, the class in which eukaryotes are shown to be well-nested, as monophyletic.

In response to the reviewer's suggestion, we have replaced the 47 arCOG and 37 Phylosift marker sets with these phylogenomic analyses described above. Both of those marker sets (47 arCOG and 37 Phylosift) contain primarily ribosomal proteins (RP). In the previous submission, we provided these

analyses to show the conservation of the named clades (order level and lower) with different marker sets but agree this introduces confusion in the overall topology of the species tree.

Considering the current phylogenetic analyses struggle to confidently reconstruct the Eukaryotes' branching position with Asgardarchaeota, the authors should revise their eukaryogenesis model in light of alternative phylogenetic scenarios, rather than definitively presenting Hodarchaeales as the closest relatives to Eukaryotes throughout the manuscript.

We have updated the text to reflect that Hodarchaeales are inferred but not proven to be the closest relatives to eukaryotes and allow for alternative phylogenetic scenarios. Specifically, we highlight the widespread distribution of ROS tolerance genes across Asgardarchaeota, suggesting at the very least the ability to increase interaction with the mitochondrial ancestor in nano-microoxic conditions, "A suite of ROS protection is provided by encoded superoxide dismutases (Supplementary Fig. 62), catalases (Supplementary Fig. 63), and peroxidases (Supplementary Fig. 64-66 and Supplementary Discussion), potentially enabling close-knit spatial interactions with the predicted facultative anaerobic mitochondrial ancestor." and "...oxygen tolerance mediated by ROS defenses would enable close-knit spatial interactions with potential aerobic or facultatively anerobic partners in the microbial consortium" in the supplementary.

In several areas, we have broadened the description to the Heimdallarchaeia class, which has consistently been shown as a sister-lineage of eukaryotes (Spang et al., 2019 Nature; Eme et al., 2023 Nature). Despite the addition of numerous genomes, Hodarchaeales (formerly Heimdall LC-3) has remained closest to the last common ancestor of eukaryotes since the second Asgard archaea paper in 2016 (Spang et al., 2016 Nature). In response we revise the results, "It should be noted that inferences regarding the closest relative of eukaryotes remain debated and may shift with improved taxonomic sampling (including several undersampled groups) and improvement of phylogenetic methods and algorithms. Yet since its discovery, Heimdallarchaeia has remained the closest Asgardarchaeota clade to the eukaryotes in robust phylogenetic analyses. The widespread presence of complexes involved in oxygen respiration, oxygen-dependent enzymes, and ROS defense systems, especially in Hodarchaeales and Kariarchaeaceae, support that the Asgard archaeal ancestor of eukaryotes was likely exposed to elevated oxygen levels, highlighting the importance of oxygen metabolism in early eukaryotic evolution" and "Based on available data, an alternative scenario in which the capacity for aerobic respiration was not present in FECA, but more recently acquired via HGT from bacteria cannot be excluded. In that case, the acquisition of aerobic respiration genes via HGT and via the mitochondrial

endosymbiosis should be regarded as two independent energetic adaptions towards the increasing oxygen levels on Earth."

2. The current results do not provide sufficient evidence to support the aerobic lifestyle of LAECA, even if the phylogenetic issues are set aside. While the ancestor of Hodarchaeales and Kariarchaeaceae each developed aerobic metabolic capabilities, the ancestor of all Heimdallarchaeal lineages remained largely anaerobic. Hence, it remains an open question whether LAECA was aerobic or anaerobic, as its evolutionary stage was much earlier than the Hodarchaeal ancestors. The current results neither confirm nor refute the hypothesis of an aerobic LAECA. The undetermined position of LAECA further complicates the issue. Additional data is needed for further validation.

We have revised our document to discuss alternative scenarios.

> **"It should be noted that inferences regarding the closest relative of eukaryotes remain debated and may shift with improved taxonomic sampling (including several undersampled groups) and improvement of phylogenetic methods and algorithms. Yet since its discovery, Heimdallarchaeia has remained the closest Asgardarchaeota clade to the eukaryotes in robust phylogenetic analyses. The widespread presence of complexes involved in oxygen respiration, oxygen-dependent enzymes, and ROS defense systems, especially in Hodarchaeales and Kariarchaeaceae, support that the Asgard archaeal ancestor of eukaryotes was likely exposed to elevated oxygen levels, highlighting the importance of oxygen metabolism in early eukaryotic evolution.**
>
> **Based on available data, an alternative scenario in which the capacity for aerobic respiration was not present in FECA, but more recently acquired via HGT from bacteria cannot be excluded. In that case, the acquisition of aerobic respiration genes via HGT and via the mitochondrial endosymbiosis should be regarded as two independent energetic adaptions towards the increasing oxygen levels on Earth."**

Throughout the main text, we also now more clearly highlight phylogenetic distribution of ROS protection genes (see Supplementary Figs. 63-66) in other Heimdallarchaeia and Asgardarchaeota. Further, we discuss a few cytochrome *c* oxidases proteins in Gerdarchaeales, agreeing with the reviewer that oxygen metabolism may have been more widespread, but lost in evolution or as suggested in the revised main text gained later via HGT.

Detail comments

Lines 31-33: Replace "hallmarks of aerobic eukaryotes" with "hallmarks of aerobic lifestyle." Note that Complex III is only found in certain Hodarchaeales and should not be listed here.

We have updated the text to "hallmark proteins of an aerobic lifestyle" and removed Complex III.

Lines 54-56: Clarify that the 2.67–2.19 Ga reference (ref 12) relates to FECA, while the 1.84 Ga reference (ref 13) pertains to LECA.

The statement has been revised to focus on FECA, which more directly relates to our analysis.

Lines 59-61: The concept of LAECA is not clearly defined or explained. Is this referring to FECA (First Eukaryotic Common Ancestor) or LECA (Last Eukaryotic Common Ancestor)? This distinction is crucial, given the vast temporal gap (on the order of billions of years) between FECA and LECA. The authors should provide a precise definition and clarify which ancestral state they are referring to, as this has significant implications for the interpretation of their findings and the evolutionary timeline under discussion.

We have revised the text accordingly to more clearly define our use of LAECA (Last Asgard Eukaryotic Common Ancestor), which we use to discuss the archaeal partner (host cell). However, given the timing LAECA and FECA are equivalent, and we now more clearly show this in our revisions.

Lines 61-64: The authors' assertion that "most contemporary Asgard archaea are inferred to be strict anaerobes" is an overstatement and lacks nuance. They have not given due credit to the work by Liu et al. (Nature, 593:553-557, 2021), which provides a more nuanced view of Asgard archaea metabolism. According to Liu et al., 66.67% of Asgard clades are predicted to be anaerobic heterotrophs, while 25% are predicted to be facultative aerobic heterotrophs. This distribution suggests a more diverse metabolic landscape than the authors imply.

We removed the term strict and updated the statement to provide more nuance represented by the literature. We have also fixed the citation of the statement to include the Liu et al. 2021 Nature manuscript, which was previously miscited. Our statement reflects the analysis from Liu et al., which suggests only a subsection of the Heimdallarchaeia class (previously Hodarchaeota, Gerdarchaeota, Kariarchaeota, Heimdallarchaeota, and Wukongarchaeota) as potential facultative aerobic heterotrophs.

Furthermore, the recognition that Heimdall archaea are capable of an aerobic lifestyle is not a novel concept and does not warrant overemphasis. The authors should revise

their statement to more accurately reflect the current understanding of Asgard archaea metabolism, incorporating the findings from at least Liu et al. and possibly other relevant literature. This would provide a more balanced and scientifically accurate representation of the metabolic diversity within the Asgard archaea, which is crucial for understanding their evolutionary significance and potential relationship to eukaryogenesis.

We have corrected the citation of the Liu et al., paper as previously suggested, verified the other indications of oxygen tolerance are appropriately cited, and have revised the manuscript to avoid overstating these claims. However, we agree with the other reviews that this study provides the genomic expansion, and the substantial support needed for claims of oxygen tolerance across Asgardarchaeota lineages.

As suggested by Reviewer 3 *"For those adhering to the idea that oxidative phosphorylation was necessary for the emergence of eukaryotic complexity, it would also raise the question of mitochondria having been dispensable for both processes. The possibility of this kind of oxygen tolerance in Asgards has been proposed before (that work is appropriately cited), but without the weight of evidence provided by Appler and colleagues' work."*

Line 70: The authors' statement that "Hodarchaeales, ..., just 23 MAGs available" appears to be inaccurate and understated. This claim contradicts the authors' own findings presented later in the manuscript, where they report an increased number of Hodarchaeales MAGs.

We have clarified in the introduction the statement refers to the "public" MAGs (previously 23 now updated to 51, including the recent Zhang et. al, study). In the results, we present our study that increases the diversity of Hodarchaeales representatives by 49% (from 51 to 100 MAGs).

Moreover, their distribution analysis indicates that Hodarchaeales prefer coastal and deep-sea sediment environments. It is worth noting that this conclusion could have been inferred even from the previously available 23 MAGs, which also originated from coastal and deep-sea sediment samples. The authors should revise this statement to accurately reflect the current state of knowledge, including their own contributions to the field. They should acknowledge that the environmental preferences of Hodarchaeales can already be suggested by the previously available MAGs, and Emphasize how their new data reinforces and expands upon these earlier data.

We have updated the text to indicate there were previously Hodarchaeales MAGs from both environmental sites, but this study increases the number of

genomes and provides the first detailed metabolic description of the transitions between these environments.

Lines 74-77: The phrase "the first detailed inferences of their metabolism" should be moderated.

We have moderated the statement removing "first" to "…expanding the known diversity and enabling the detailed inferences of their metabolism across environments".

Lines 94-96: The three phylogenetic analyses utilized different marker sets, software, substitution models, and outgroup genomes. While this is acceptable for preliminary analysis, these should be standardized in the final publication.

We have now standardized our phylogenomic efforts that are based on the 47 non-ribosomal marker set (NM47). As mentioned above we expanded our analyses to employ additional models and treatments to assess incongruence and explain our efforts in a clear fashion within the Supplementary Discussion.

Lines 96-98: The bootstrap values are consistently low for Hodarchaeales and Heimdallarchaeia across all three topologies.

With the updated phylogenies (Fig 1ab and Supplementary Fig. 3), Hodarchaeales is maximally supported across all species trees. Regarding support for the Heimdallarchaeia, the clade is moderately supported (>90% standard bootstrap support; SBS) in the species tree of the dereplicated dataset where we now apply a more complex C60 mixture model. If it was computationally feasible, we believe additional support (i.e., >95% SBS) could be achieved with the use of even more realistic mixture models (e.g, CAT) and/or application of treatments to decrease bias (e.g, saturation, compositional bias) as has been showcased before (Eme et al., 2023 Nature). Thus, future studies with expanded taxon sampling and a broader exploration of different phylogenomic methods and treatments may fully support the monophyly of Heimdallarchaeia.

Lines 101-102: The phylogenetic position of Ranarchaeia is unstable across the three topologies; hence, the authors should not selectively display and discuss a seemingly more favorable result.

We have updated the text to state Ranarchaeia is a deep-branching lineage within the NM47 SR4 recoded phylogenies. We also now reference the following supplementary methods statement "Additional high-quality genomes will continue to increase the phylogenomic support, especially in the predicted deep-branching lineages."

Line 114: The use of the term "pangenomic analyses" in this context is incorrect and misleading. Pangenomic analysis typically refers to a comparative genomic analysis of the entire set of genes from all closely related strains within a specific clade. Given the broad taxonomic scope of the Asgard archaea superphylum, the analysis conducted here cannot be accurately described as a pangenomic analysis. The authors should replace "pangenomic analyses" with "comparative genomic analyses".

We have changed the terminology to "comparative genomic".

Line 120, Fig 1a, L168 and Sup Fig 3: Hod and Kari are not phylogenetically most closely related to each other, but they are (1) more metabolically complex than other Heimdallarchaea or other Asgard archaea, and (2) enriched in epipelagic sediments. I wonder whether the observed (1) and (2) is cause or cog?

We appreciate this insightful question, which highlights a central challenge in interpreting evolutionary and ecological patterns: whether expansion into a new ecological niche facilitated the independent acquisition of similar genes, or whether a more complex ancestral metabolism enable such ecological expansion.

To explore this, we used a combination of taxonomic, environmental, and protein content similarity ("guilds") as filters. These include the evolutionary adaptations enabling niche expansion, the impact of increasing environmental distribution, and the selective pressures arising from environmental variability (e.g., oxygenation events such as the GOE).

As part of this analysis, we describe several proteins and genes that are uniquely present in specific environments or lineages. For instance, we found a fatty acid amide hydrolase [EC:3.5.1.99] across three Asgardarchaeota lineages, but found exclusively in the coastal regions—suggesting an environmental advantage that may have driven HGT. Similarly, within Hodarchaeales we find malate dehydrogenase (*mdh*) in the TCA cycle—likely inherited from the archaeal partner—is present only in coastal zones. In contrast, cytochrome c oxidase subunits CoxABC are found in both deep-sea and coastal zones, but only within Heimdallarchaeia.

In the supplementary discussion, we present several instances of proteins encoded in specific environments or lineages. These finds offer valuable targets for future investigation in additional environments and genomes. Ultimately, fully resolving the directionality—whether complexity preceded or followed ecological diversification—would require broader sampling of

Heimdallarchaeia, resulting in high-quality genomes and additional in-depth analyses that are beyond the scope of this paper.

Line 127 Fig.1: The phylogenetic trees presented in Fig. 1a and Fig. 1b exhibit notable inconsistencies, particularly regarding the phylogenetic position of the Odin clade. This discrepancy raises concerns about the robustness and reliability of the phylogenetic analyses presented.

The phylogenetic position of Odinarchaeia remains incongruent between the updated species trees (WAG+C10+R4 & SR4-recoded[GTR+C60+G4]) based on the full dataset. However, the topology placing Odin- as the sister group to Frey(Jord)-/Atabeyarchaeia is better supported (i.e., WAG+C10+R4). When applying the mixture model C60 on the subset of the dataset, Odinarchaeia is positioned with maximal support as the sister to the Frey(Jord)-/Atabeyarchaeia. We argue that using a more complex and realistic model of evolution is essential to mitigate potential compositional bias, particularly given that Odin members are predicted thermophiles (Eme et al., 2023 Nature). This is further discussed in the updated supplementary discussion.

The authors appear to have utilized their latest NM47 marker set (previously referred to as NM57 in their earlier publications, which was noted to have technical issues as reported by Eme et al., Nature 618, 992-999, 2023) for these analyses. The inconsistency between the trees, especially considering the use of this frequently updated marker set, requires thorough explanation and justification.

We chose a subset of the archaeal core machinery presented in Petitjean et al., 2015 as phylogenetic markers. This subset greatly overlaps with the marker set used by Eme et al., Nature 618, 992-999, 2023 and hence we retain the connotation of "NM". Our phylogenetic inquiry, however, remains independent and includes manual checks to exclude paralogs, contaminants or HGT transfers from gene trees before proceeding with inferring species trees.

The prior phylogenomic analyses with 47 arCOG and 37 Phylosift marker sets were to show that each of the named groups (especially the new clades) remained consistent between phylogenetic markers. Since several predicted (hyper-)thermophilic lineages are included in the dataset we have removed these marker sets to avoid including bias that would ultimately affect the perceived evolutionary relationships of the studied lineages (Eme et al., 2023 Nature). We describe the inconsistency of these markers in more detail above. We now implement complex mixture models to increase the robustness of the NM47 phylogenomic analysis and describe the differences between the presented phylogenies in the Supplementary Discussion for further clarity.

Line 155 Fig.2 and line 168: The use of the compound term "epipelagic/coastal" raises concerns about precision and accuracy in describing environmental niches. These two terms represent distinct ecological zones with potentially different physicochemical characteristics and biological communities. "Epipelagic" typically refers to the uppermost layer of the open ocean (0-200m depth), characterized by sufficient light penetration for photosynthesis. In contrast, "coastal" environments encompass a range of habitats near shorelines, including intertidal zones, estuaries, and shallow waters, which may or may not overlap with epipelagic zones. The conflation of these terms may lead to ambiguity in interpreting the ecological distribution and adaptations of the organisms under study.

We have changed the compound term in Fig. 2 to Shallow Coastal. In the main text, we have changed the name to coastal to avoid confusion as our analysis does include shorelines, estuaries, etc. within this set of genomes.

The concept of "epipelagic sediments" is thus contradictory and scientifically inaccurate. Sediments are typically classified based on their location (e.g., coastal, continental shelf, abyssal plain) or their composition, but not by pelagic zones of the overlying water column.

Similarly, we have clarified this in the main text by describing the environmental location at shallow coastal sediments (<200 mbsl).

Lines 169-171: The authors suggest that presence in "epipelagic sediments" implies oxic environments. However, sediments, even in shallow waters, can be anoxic just below the surface layer. This assumption lacks substantiation.

We have changed this statement to "To determine environmental transitions, we investigated signatures of potential aerobic adaptation within this group." As we acknowledge in the manuscript, while oxygen level was not reported for most sediments for which Heimdallarchaeia MAGs were obtained, some are clearly oxygenated, including the MAG from the Persian Gulf (DO: 7.2 mg/L).

Our analysis is also supported by previous work indicating frequent changes in oxygen availability in coastal sediments (Kessler et al., 2019 Nature Microbiology and Hutchinson et al., 2024 ISME). Kessler et al., 2019 Nature Microbiology showed these shallow coastal environments select for metabolically flexible microbes and exclude strict anaerobes.

Line 176 modelling oxygen tolerant? In methods?

In response, we have moved the citation from the next sentence to the end of this statement and added (see methods). In the methods, we describe how we

predicted microbial growth conditions from amino acid composition in the Curation of Asgard archaea database section.

Line 182: The statement regarding the co-occurrence of Hodarchaeales and Alphaproteobacteria requires significant clarification and context. The authors need to specify whether this observation is based on contemporary samples or includes historical data, as this temporal context is crucial for understanding potential evolutionary associations.

Separately, we analyze both the new samples from the Guaymas Basin and the Bohai Sea described in this study, as well as samples from publicly available databases. For the public database, we searched the NCBI SRA public metagenome sets prior to 2021 using Sandpiper (v0.3.0) and provide the sample numbers from NCBI. We now clarify the results of the separate analyses in the main text and the public dataset used. The analyses are also split within Supplementary Table 10 between the sheets "This_Study" and "Public" to make this delineation clear.

Furthermore, the significance of this particular co-occurrence is not apparent, given that Alphaproteobacteria are widely distributed and likely co-occur with numerous other microbial groups. The authors should explain why this specific association is noteworthy and whether it is unique to Hodarchaeales or a general pattern observed with other Asgard archaea or archaea in general.

The significance is the samples released in this study and previous metagenomic data both support that at least some Hodarchaeales and Kariarchaeaceae are adapted to aerobic environments, where they co-occur with various Alphaproteobacteria. Specifically aerobic lineages of Alphaproteobacteria, which has not been previously identified.

We have updated our analysis of public databases to evaluate the co-occurrence patterns between all available Asgardarchaeota lineages and Alphaproteobacterial orders (see Supplementary Table 10). This has been updated in the methods with a note that the analysis is based on GTDB-tk taxonomic classification and includes only metagenome datasets prior to 2021. We find all Asgardarchaeota lineages co-occur with at least one aerobic Alphaproteobacteria lineage (e.g., Kiloniellales) in at least one sample, further supporting their potential to tolerate conditions in the same samples as aerobic Alphaproteobacteria. This information has been updated in the main text.

We also expanded our analysis of MAGs from the Bohai Sea and Guaymas Basin, using dataset from the three expeditions (BH, GE2, and GE3). For each site, we identified the number of Asgardarchaeota MAGs at the order level (GTDB v2.4.0) to assess co-occurrence patterns across all binned genomes.

These results are now reported in the updated Supplementary Table 10 and briefly discussed below.

We used pairwise co-occurrence analysis to determine probability of co-occurrence at both sampling sites. At the Bohai Sea site, Hodarchaeales MAGs co-occur with Acidobacteriota, Chloroflexota, Desulfobacterota_E, Desulfobacterota, Gemmatimonadota, Myxococcota_A, Nitrospirota, Planctomycetota, and Pseudomonadota phyla. Within Pseudomonadota, there are representatives from Alphaproteobacteria (orders Rhizobiales and UBA8366) and Gammaproteobacteria classes (orders GCA-272945, HK1, and Woeseiales). At the same site, Lokiarchaeia Thorarchaeia occurs with both archaea and bacteria. Thorarchaeia co-occurs with the same bacterial phyla and an additional six phyla (Bacteroidota, BMS3Abin14, Calditrichota, Desulfobacterota_B, Myxococcota, and Krumholzibacteriota). Lokiarchaeales co-occurs all the same phyla as Thorarchaeia with additional Desulfobacterota_B and Myxococcota_A. Thorarchaeia also co-occurs with Alphaproteobacterial orders Rhizobiales, UBA8366, Kiloniellales and Gammaproteobacterial orders GCA-272945, HK1, and Woeseiales, Burkholderiales, GCA-001735895, Pseudomonadales, and UBA9214. We also note archaeal (Aenigmatarchaeota, Hydrothermarchaeota, Nanoarchaeota, and Thermoplasmatota) and bacterial Acidobacteriota, Armatimonadota, Caldritrichota, CG2-30-53-67, Chloroflexota, Dependentiae, Desulfobacerota, Gemmatimonadota, JADFOP01, JAUYMQ01, Marinisomatota, Myxococcota, Nitrospirota, Omnitrophota, Patescibacteria, Planctomycetota, Pseudomonadota, Spirochaetota, and Zixibacteria) lineages that have potential positive co-occurrence (p_gt <0.05) with both Hodarchaeales and Kariarchaeaceae and bacterial phyla. This more accurately represents co-occurrence at this shallow coastal site.

In analyzing the Guaymas Basin samples we included both sequencing expeditions. In these samples, only three Asgardarchaeota lineages had a probability of co-occurrence above 0.5. Heimdallarchaeaceae co-occurred with bacterial phyla Acidobacteriota, Bipolaricaulota, and Planctomycetota. Gerdarchaeales co-occurred with those lineages and Chloroflexota and Desulfobacterota, as well as the archaeal phylum Thermoproteota. Lokiarchaeales co-occurred with all the same lineages as Heimdallarchaeaceae plus Bacteroidota, Caldisericota, and Zixibacteria. Hodarchaeales had potential positive co-occurrence (p_gt <0.05) with archaeal (Nanoarchaeota and Thermoplasmatota, and Lokiarchaeales) and bacterial phyla (Abyssobacteria, Acidobacteriota, Bacillota, CG02, Chlamydiota, Chloroflexota, Desulfobacterota, JAGLTZ01, Myxococcota, Omnitrophota, Patescibacteria, Pseduomonadota, and Zixibacteira).

Together, the updated results from both the publicly available metagenomes and the binned MAGs in this study provide additional evidence of co-occurrence between Heimdallarchaeia lineages and aerobic Alphaproteobacteria. More broadly, the results expand potential for oxygen tolerance across a wider range Asgardarchaeota lineages. We appreciate the reviewer's suggestion and have incorporated this revised analysis into the manuscript.

Line 199: I suggest the author should provide more structural details (e.g. primary structure and conserved amino acid residues) of these ETC subunits. Perhaps some biochemical analyses can be performed to advance our understanding of the new Asgard subunit.

We appreciate the reviewer's suggestion and have moved some of the more detailed discussion of structural details from the supplementary to the main text. We have added more details of the conservation of known key amino acid residue positions for Complex I in the Supplementary Discussion, and we have updated several supplementary figures to show sequence alignment information (Supplementary Figs. 48, 79, and 80). Further, we now include the key multiple sequence alignments in added supplementary figures and additional in Figshare.

We agree experimental biochemical analyses would be informative to clarify interaction and substrate but is beyond the scope of this paper. This suggestion has multiple challenges and limitations, including 1) most Asgardarchaeota lineages are uncultivated, which prohibits natively purifying these enzymes, and 2) typically membrane-bound multi-subunit metalloenzymes typically have very complex maturation pathways, which makes heterologous expression in *E. coli* or other model organisms difficult and prone to failure. In lieu of likely unsuccessful protein purification attempts, the provided structural predictions enable us to bridge the gaps between sequence and function. Given the reviewer's feedback, we now more clearly provide future protein targets for purification and structural characterization by cryogenic electron microscopy.

Line 210-213, Line 247-248, Fig 3a, and Fig 4b: How do these stand-alone NuoEFG modules originate?

The NuoEFG subunits in Complex I likely originate from an ancestral NAD^+-reducing hydrogenase complex, which over the course of evolution was incorporated into the structure of Complex I as a prebuilt module. Other researchers prior to our work have proposed this evolutionary origin for NuoEFG in Complex I (for reviews on the topic, see Efremov & Sazanov, 2012 Biochimica et Biophysica Acta-Bioenergetics; Friedrich & Weiss, 1997 Journal

of Theoretical Biology; Marreiros et al., 2013 Biochimica et Biophysica Acta-Bioenergetics). This hypothesis is supported by sequence homology and structural similarity between Complex I NuoEFG and NAD^+-reducing hydrogenase complexes (Shomura et al., 2017 Science). Based on sequence and structural homology, these putative stand-alone Asgardarchaeota NuoEFG complexes also potentially originate from an ancestral NAD^+-reducing hydrogenase. We have added a brief discussion of the evolutionary origins of Complex I NuoEFG to the Supplementary Discussion and clarified with added relevant citations.

NuoEFG is present in the Fig 4b, but Heimdallarchaea do not have NuoEFG in Fig 3a.

Thank you for catching this oversight. We have updated Fig. 4b to remove the NuoEFG subunits from the Complex I cartoon.

Line 270 fig3b: It is crucial for the authors to provide a clear and detailed explanation of the criteria used for selecting the reference sequences of CoxABC. This information is essential for readers to assess the robustness and reliability of the phylogenetic analysis presented.

We have updated the methods to provide for detailed description of the reference selection. We included four reference sets in addition to the 869 Asgardarchaeota MAGs to determine if Asgard archaeal sequences were clustering directly with mitochondrial sequences.

1) <u>Bohai Sea and Guaymas Basin Alphaproteobacterial MAGs:</u> 310 MAGs (>50% complete, <10% contamination), binned by this study from the Bohai Sea and Guaymas Basin. These were included to rule out recent horizontal gene transfer events or contamination during the binning from the same expeditions binning the Asgardarchaeota.

2) <u>Publicly available Alphaproteobacterial reference MAGs:</u> We included 20 high-quality public reference genomes from GTDB-tk. These supplemented the Alphaproteobacterial MAGs released by our study, providing more coverage across the phyla. Alphaproteobacterial MAGs were selected due to their evolutionary significance as the likely lineage or sister lineage of the mitochondrial ancestor.

3) <u>Set of archaeal and bacterial references:</u> Chosen to include representatives from all NCBI and GTDB taxonomic orders. This dataset included 664 bacterial representatives and 136 non-Asgardarchaeota archaeal sequences.

4) <u>Set of mitochondrial genomes:</u> 15,980 mitochondrial genomes from RefSeq were included to place the eukaryotic subunits within the prokaryotic diversity. This dataset was later subset in our analysis to 10 representatives due to oversaturation. Subsetting enabled us to increase complexity of the mixture models in the concatenated analyses.

After removing any duplicates in generating each of the dataset, our final reference dataset included 1,679 genomes. This can be broken down to 664 bacteria, 1005 archaea (including 869 Asgardarchaeota), and 10 mitochondrial genomes. This is now more clearly described in the methods.

Lines 298-299: Justify the choice of Complex IV over other aerobic hallmarks.

Our analysis was holistic and considered a wide diversity of enzymes for aerobic respiration and ROS detoxification. We focus particularly on Complex IV as (i) this enzyme enables not just O_2 reduction but also proton-motive force generation through proton pumping, (ii) it is a well-studied marker often used in metabarcoding (Hebert et al., 2003 R. Soc. Biology Letters; Hebert et al., 2003 Proc. R. Soc. Long. B.; Balech et al., 2018 Bioinformatics and Genomics; Dopheide et al., 2019 Ecological Applications; etc.) and phylogenetic analyses due to its perceived strong phylogenetic signal (Gjerde, 2013 International Journal for Parasitology; Bertini et al., 2007 Journal of Inorganic Biochemistry), and (iii) it is most relevant to the discussion of the expansion of the ETC. In the main text, however, we also discuss O_2-dependent enzymes involved in heme *o* biosynthesis, aromatic degradation (quercetin 2,3-dioxygenase), and amino acid metabolic (4-hydroxyphenylpyruvate dioxygenase).

Lines 300-302: The results shown in Fig. 2b may be attributable to the limited number of genomes used, as coxABC subunits are present in many archaeal lineages in the KEGG database.

We believe the reviewer is referring to Fig. 3b as there is no Fig. 2b and respond accordingly. In Fig. 3b, we use a database of 1,005 archaea (26 TACK; 30 DPANN; 869 Asgard archaea; 2 Methanopyrales; 4 Methanococcales; 4 Methanobacteriales; 4 Hydrothermarchaea; 22 Diaphorachaea; 26 Methanotecta; 10 Acherontia; 8 Stygia) archaeal genomes (see Supplementary Discussion). This is now more accurately described in the methods section.

Lines 313-318: The statement "the presence of O2-dependent enzymes varies across Heimdallarchaeia" contradicts the oxygen tolerance modeling mentioned in Lines 175-179.

In this approach, we are combining multiple lines of evidence to assess the potential for oxygen tolerance in Heimdallarchaeia. As suggested, the O_2-dependent enzyme analysis indicates a broader range of oxygen tolerance than the predicted by amino acid composition alone. While the presence of O_2-dependent enzymes does not definitely confirm aerobic respiration or oxygen tolerance, it may suggest a pattern of environmental variation or even the loss of previously acquired oxygen tolerance.

Lines 358-360: The authors should explain the absence of Complex III in most Asgard genomes.

We were strict in the identification of Complex III (see Supplementary Discussion). Briefly, we only included sequences that had the three flanking proteins and checked across multiple annotations to delineate the three subunits. This was done because previous ancestral reconstruction and comparative analysis indicated the complexes' absence in the Heimdallarchaeial ancestor (Eme et al., Nature 2023). Instead, this study suggested the Hodarcheales ancestor either used nitrate to nitrite reduction or combination of NarI within the Rieske center to transport elections.

Here, we identified loss of nitrate reduction subunits (NarGHJ) in all coastal Hodarchaeales, reducing the likelihood of the first hypothesis. Yet, we note the presence of NarI in guild 11 coastal Hodarchaeales and guild 9 Heimdallarchaeaceae. This protein had patchy distribution across Hodarchaeales and Heimdallarchaeia. The presence of NarI despite the absence of the NarGHJ in coastal genomes likely suggests an environmental metabolic transition in the evolution of these Hodarchaeales.

Considering both the presence and absence of Complex III and NarI we identify two potential evolutionary scenarios to explain the distribution: 1) Hodarchaeales' ancestor encoded Complex III, which was later lost in a portion of the genomes. 2) Hodarchaeales' ancestor encoded a Complex III-NarI complex like the previously proposed hypothesis (Eme et al., 2023 Nature) and has more recently gain additional Complex III subunits. In both cases, the complex provides the capacity to shuttle electrons. This is now included in the supplementary to provide a more comprehensive view of the results. We also have noted the importance of biochemical confirmation of these complexes.

Eme et al., 2023, Nature indicated ancestral reconstruction of scenario 2 is not computationally feasible with ALE, requiring improved techniques beyond the scope of this study. Both cases provide capacity to shuttle electrons, which is the main take away from the updated model.

Lines 363-365: The authors are linking "widespread oxygen tolerance" to "initial compartmentalization with the predicted aerobic mitochondrial ancestor" lacks a clear logical foundation and requires substantial clarification. This statement raises several critical issues that need to be addressed.

Here we were describing the advantage afforded by oxygen tolerance to increase close interactions with an aerobic/facultative aerobic mitochondrial ancestor. Thus, we clarify this second part of the statement to "…potentially enabling close-knit spatial interactions with the predicted facultative anaerobic mitochondrial ancestor".

The reference to the "predicted aerobic mitochondrial ancestor" needs elaboration, explaining how this prediction relates to the observed characteristics of extant Asgard archaea.

We discuss the prediction of an aerobic mitochondrial ancestor as it is relevant to the oxygen tolerance of its potential partner.

The authors should also discuss alternative hypotheses that might explain the evolution of compartmentalization in eukaryotes and how their proposal fits within the broader context of eukaryogenesis theories.

Within the manuscript, we now more clearly discuss which portions of alternative hypotheses our results support. Briefly, our model supports the reverse flow model, while refining and updating it with additional genomes and robust analyses. Most of the other models are based on Lokiarchaeia, which has a very different metabolism, which is highlighted throughout the manuscript and supporting supplementary materials.

Based on sequence and structure-based analyses, we indicate potential evolution of compartmentalization through close-knit interaction with oxygen tolerant Asgard archaea and aerobic/facultatively aerobic bacterial partners in coastal regions. This is broadly discussed throughout the manuscript.

The four most popular published eukaryogenesis models after the discovery of Asgardarchaeota include:
1) Revised Hydrogen Hypothesis (Sousa et al., 2016)
2) Reverse Flow model (Spang et al., 2019)
3) Revised Syntropy model (López-García and Moreira, 2020)
4) Entangle-Engulf-Endogenize model (Imachi et al., 2020)

Many of the initial models discussed hydrogen use as central to the model. Our analysis continues to explore hydrogen metabolism in Asgardarchaeota. Specifically, we find novel [NiFe] and [FeFe]-hydrogenases. The [NiFe]-

hydrogenases form structures linked to increased production of proton motive force and electron bifurcation/confurcation, specifically novel complexes in Hodarchaeales (Suppl. Fig. 28-29). However, our analysis does not indicate Heimdallarchaeia rely on a hydrogen-producing symbiont. As such, our results do not support the previously proposed hydrogen hypotheses (initial or revised) based on Lokiarchaeales (previously Lokiarchaeota) with an anaerobic autotrophic metabolism. Our analysis instead provides for flexibility in production of hydrogen by the host and indicates the archaeal partner was heterotrophic.

The Reverse Flow model based on Heimdallarchaeia (Heimdallarchaeota) indicates an organoheterotrophic archaeal host, which is supported and refined by the expansion of genomes in our analysis along with description between environments. We provide robust support for oxygen tolerance and potential to use oxygen as a terminal electron acceptor within Heimdallarchaeia lineages. We further determine the loss of nitrate reduction in the coastal Hodarchaeales genomes and provide a more complete picture of the metabolism of these lineages closest to eukaryotes. The specific components of this model supported by our analysis are discussed within the main text.

Like the hydrogen hypothesis, the initial syntrophic hypothesis indicated an autotrophic methanogenic archaeon partner that required hydrogen, acetate, and carbon dioxide from the partner. The revised syntrophy model includes interactions between three lineages and the transfer of hydrogen and sulfur. We support this revised model that the Asgardarchaeota partner has the capacity to produce hydrogen. However, we refine the discussion of the environmental parameters, including the environmental distribution of the descendants of these organisms. Further, we provide additional evidence for potential close-knit spatial interaction through oxygen tolerance genes between Alphaproteobacteria and Asgardarchaeota, including co-occurrence. Without additional evidence to support the tripartite interaction with sulfate reducer (based on the Lokiarchaeales culture) it is not included in our updated model. Similarly, this model indicated the first eukaryogenetic steps were anaerobic, which again we refine.

The Entangle-Engulf-Endogenize model suggests interactions between three partners with hydrogen-producing Asgardarchaeota. We have included in our model the protrusion imaged and discussed within this model, expanding on why mistargeting could be an issue and how these could eventually develop into the ER as discussed in the Inside-Out Model of eukaryogenesis (Baum & Baum, 2014; see Supplementary Discussion).

Line 396-397 and Line 483-484: Considering Asgard archaea undergo billions of years evolution, today's coexistence may not be evidence of the same scenario billions of years ago.

We agree with the reviewer and only reference this coexistence as the first indication of this coexistence across depths and samples and as potentially interesting regarding current proposed scenarios. Further, we have added analysis suggested by the reviewer to determine coexistence of Asgardarchaeota representatives from all Heimdallarchaeia lineages.

Lines 399-402: The authors' implication in lines 399-402 that Heimdallarchaeia can produce protrusions, leading to an increased surface-to-volume ratio and consequent energy advantages, requires important caveats and clarification.

We have altered the statement, to include these caveats and clarifications to "Heimdallarchaeia contain actin homologs like those in Lokiarchaeales, which in Rodrigues-Oliveira et al., 2023, produce protrusions (Supplementary Table 12). Yet in a cultured bacterium (*Candidatus* Uab amorphum), actin does not produce this morphology, necessitating image verification." This discussion was relocated to the supplementary to streamline the model and provide enough space to discuss the following suggestion to add a note about *Ca. Uab amorphum*.

While the presence of actin homologs in Heimdallarchaeia is intriguing, the authors should exercise caution in extrapolating from gene presence to complex cellular features. It is crucial to note that not all microorganisms possessing actin homologs necessarily develop protrusions or exhibit increased surface-to-volume ratios. A pertinent example that should be cited is Ca. Uab amorphum (Shiratori et al., Nature Communications 10, 5529, 2019), which possesses actin homologs but does not form protrusions. The authors should explicitly acknowledge this counter-example and discuss the implications for their hypothesis.

We have revised the statement as follows and added the citation as an important counterexample. See revised "Heimdallarchaeia contain actin homologs like those in Lokiarchaeales, which in Rodrigues-Oliveira et al., 2023, produce protrusions (Supplementary Table 12). Yet in a cultured bacterium (*Candidatus* Uab amorphum), actin does not produce this morphology, necessitating image verification."

We note in Shiratori et al. *Candidatus* Uab amorphum actin was shown to group within the diverse lokiactin. However, this branching point has a bootstrap support value of 38, which indicate poor support for this branching point. The Bayesian posterior probability indicates moderate support but does not definitively indicate this sequence belongs to Lokiactin. More recently in Stairs

et al., 2020 Current Biology the planctomycetes bacterium *Candidatus* Uab amorphum sequences were included in a phylogenetic analysis of actin and Arps (actin-related proteins). In this phylogenetic analysis, the divergent *Candidatus* Uab amorphum sequences appeared related to Asgard archaeal Arps between clade 1 and 2 instead of Lokiactin with bootstrap values below 80. It is also important to denote there are differences between the archaeal membrane that could impact this process.

Fig 5: In a recent study conducted by Brocks et al. (PMID: 37286610), it was observed that modern sterols, which are characteristic biomarkers of crown eukaryotes, were notably absent in sediments dating from 1.6 to 1.0 Ga. Instead, the presence of protosterols, which are indicative of more primitive eukaryotic lineages, was detected. This finding suggests that eukaryotic organisms existing before 1.0 Ga belonged to the stem group rather than the crown group.

We agree, the Brocks et al., 2023 paper provides an intriguing hypothesis for the sterane gap between the fossil and biomarker record. They provide evidence for sterane biomarkers up to 800 million years ago, suggesting that prior to this stem group eukaryotes were producing protosterols. Further, that "crown" sterols enabled crown eukaryotes to inhibit shallow water, oxygenated environments prior to that pelagic/deeper. Yet, shallow water, oxygenated settings are poorly suited for preserving biomarkers. It is possible crown eukaryotes emerged earlier but could have been inhabiting settings with poor indigenous preservation of these biomarkers. Instead, the appearance of crown sterols highlights not the beginning of crown eukaryotes, but instead their transition to offshore or pelagic environments, which are more suitable for preservation of these markers.

Lines 420-421: The authors raise a critically important question: "Why would the ancestral host partner with a bacterial symbiont for aerobic respiration and then lose that capability?" This query is fundamental to our understanding of eukaryogenesis and mitochondrial evolution. However, the authors' response to this pivotal question lacks the depth and rigor. Their proposed explanation that "the endosymbiont increased total respiratory capacity by expanding its membrane surface" is overly simplistic and fails to address several key issues.

We agree this is a key question that reshapes our understanding of eukaryogenesis and the development of this interaction.

In revising the manuscript, we have expanded on this question, while avoiding unnecessary speculation. This discussion can be broken down into more digestible questions:

1) **What instigated and sustained the interaction between the organisms? Our analysis provides some of the most robust support, suggesting that oxygen tolerance and potential for aerobic respiration is more widespread within Asgardarchaeota than previously appreciated. Oxygen tolerance would have enabled potential interactions in close spatial proximity in the *same environment* (ecological compatibility) with the aerobic or facultative aerobic mitochondrial ancestor. We also provide examples of potential metabolic sharing and environmentally or evolutionarily triggered metabolic transitions that could have promoted a sustained interaction.**
2) **Why does the eukaryotic aerobic respiration likely arise from a bacterial partner rather than the archaeal? We now address this more fully within the main text. Phylogenetic analyses continue to support a bacterial origin of ETC subunits. Additionally, we further discuss the role endosymbiotic gene transfer (EGT) and potential vulnerability of the host genome to degradation by reactive oxygen species. The asymmetry between nuclear and symbiont genome enabled by EGT and genomic streamlining, reduced redundancy and further integrated the potential partners.**
3) **Why did the archaea lose the capacity for aerobic respiration? In our model, we consider the potential degradation of the host genome prior to the formation of a nucleus. This has been suggested previously to indicate the similarity between bacteria and eukaryotic energetic pathways. Loss of the host's respiratory capability would have reinforced the dependency on the endosymbiont.**

The authors should discuss the energetic trade-offs of maintaining two separate respiratory systems versus integrating them, address the concept of genomic streamlining in endosymbionts, explore the benefits of compartmentalized respiration in mitochondria, clarify the evolutionary timing of host respiratory capability loss and mitochondrial function acquisition, provide a more comprehensive comparative genomics analysis of respiratory genes in extant Asgard archaea and alphaproteobacteria, and consider alternative hypotheses, e.g., by using genome-scale metabolic modelling.

We appreciate the reviewer's suggestion. However, we would like to acknowledge there are extensive requests here for additional analyses, considering the amount of data/analysis provided in this study, previous suggestion by reviewers to split into separate papers, and numerous added and updated analyses in the revision.

Below, we comment on how our analysis impacts the following concepts as suggested by the reviewer.

1) **<u>Address genomic reduction in endosymbionts:</u> In the updated model, we discuss endosymbiont gene transfer (EGT) relocated mitochondrial-encoded**

ETC subunits to the nuclear genome (nDNA). Then the Asgard ETC subunits may have been lost due to redundancy in early eukaryotes. We have added a statement in this section indicating this is likely due to genomic reduction, "Endosymbiotic gene transfer (EGT) from the organelle to the host would have relocated symbiont subunits (e.g., NuoA-M) to the nuclear DNA. Symbiont genome reduction would have created asymmetry with the host genome further integrating the partners, while the redundant Asgard ETC subunits were likely lost in early eukaryotes."

2) **<u>Benefits of compartmentalized respiration in mitochondria:</u> In describing the model we mention "Considering our findings, perhaps the endosymbiont increased total respiratory capacity by expanding its membrane surface and allowed the host outer membrane to facilitate other processes, such as endo/phago/pinocytosis." Further, we add in the main text "Compartmentalization provided by the endosymbiont would have enabled increased regulation and spatial distribution of bioenergetic efficiency (e.g., membrane potential), redox conditions, and toxic byproducts." We feel this will provide a clearer understanding of the role of compartmentalization in eukaryogenesis and today's eukaryotic cells.**

3) **<u>Clarify evolutionary timing of host respiratory loss and mitochondrial function acquisition:</u> We have updated Fig. 5 to reduce visual clutter and provide a clearer representation of the timing of key events in the proposed models. This revised figure is supported by the corresponding model description in the main text, as well as an expanded discussion in the Supplementary Information on the presence and absence of specific respiratory genes across Asgardarchaeota.**

   However, we do not attempt to extrapolate the precise timing of host respiratory loss and the acquisition of mitochondrial function. Such inferences are difficult to make because of the long evolutionary distances confounded by ghost lineages (extinct or unsampled), limiting our knowledge about key transitional forms after FECA. To determine specific timing would require time-calibrated markers for molecular clock models, which comes with its own limitations and error margins.

4) **<u>Comprehensive comparative genomic analysis of respiratory genes in extant Asgard archaea and Alphaproteobacteria:</u> We now provide additional detailed descriptions of the presence of respiratory genes across Asgardarchaeota, environments, and guilds. Further, we discuss the Alphaproteobacterial lineages most commonly clustering with mitochondrial ETC subunits in supplementary. However, we primarily focus on the implication of ETC in Asgardarchaeota. We support our comparative analysis with phylogenetic**

analyses of these subunits all provided in Figshare and the supplementary data.

5) **<u>Consider alternative hypotheses:</u> We now provide alternative hypotheses statement**: *"***Based on available data, an alternative scenario in which the capacity for aerobic respiration was not present in FECA, but more recently acquired via HGT from bacteria cannot be excluded. In that case, the acquisition of aerobic respiration genes via HGT and via the mitochondrial endosymbiosis should be regarded as two independent energetic adaptions towards the increasing oxygen levels on Earth." Based on previous reviewer comments, we also more clearly detail which portion of previously published models our updated model expands, supports, and refines.**

Lines 485-491: Elaborate functions other than phagocytosis is well-established in eukaryotic cells. The authors cannot convince me that the existence of respiratory function in the membrane of the host cell impedes the development of phagocytosis.

We agree with the reviewer and have modified this section not to imply that respiratory function impedes phagocytosis but instead provides a contained respiratory system. We have rephrased the last sentence "This reorganization could have allowed the archaeal membrane to evolve more complex endomembrane systems, including the capacity to perform phagocytosis, while the integrated endosymbiont provided a more centrally coordinated respiratory system." Thereby discussing the potential dual impact of removing the respiratory system from the outer membrane and containing it within the endosymbiont. Clarifying this shift likely did not impede phagocytosis but instead could have provided more membrane capacity to support various processes.

Figure 2: Define the meaning of the box and line in the figure.

We have clarified this in the sentence, "The bar chart shows the number of MAGs from each lineage by environment (shown in the connected box below), indicating lineages found only in a single environment…"

Table S2: Correct the number of ASGARD genomes to 936 instead of the stated 937.

Supplementary Table 2 has been updated to match the redone analyses.

Table S4: Ensure that the 12 environmental categories match those presented in Fig. 2.

We have updated Table S4, so all category names match Fig. 2. There is only one genome GCA_002728275_1_ASM272827v1, which we list as Unknown

(Mesopelagic) in Table S4 since it was binned from samples 5-1000 mbsl. We now add clarification of this in the method section, "One published MAG (GCA_002728275.1) was binned from pelagic waters (5-1000 mbsl) and is included in our analysis as mesopelagic."

Referee #3 (Remarks to the Author):

The emergence of eukaryotes was a defining process that gave rise to almost all living beings that are visible to the naked human eye, as well as to the abundant, taxonomically and morphologically diverse, and ecologically and medically important eukaryotic microbes. Accordingly, the ancestral cells and events involved in this process are topics of fundamental biological importance. Spirited discussion has focused on the nature and timing of the mitochondrial endosymbiosis, with specific topics including: 1) the metabolism of the likely host and symbiont, and the metabolic basis of their relationship; 2) whether oxidative phosphorylation, presumably made possible by the symbiont, was a necessary prerequisite for the emergence of complex subcellular structures such as the endomembrane system and/or cytoskeleton in eukaryotes.

The discovery of the Asgard archaea, collectively comprising multiple sister lineages of eukaryotes, created an exciting opportunity to draw inferences about features that were likely present in the shared ancestors of Asgards and eukaryotes, with implications for our models of early eukaryotic evolution. Because of the diversity and patchily distributed genes found in this group, broad taxon sampling is essential to generate a well-resolved reconstruction of features in shared ancestors in the group, especially in the shared ancestors of eukaryotes with their immediate sister group, currently believed to be the Hodarchaeales.

Here, Appler and colleagues report the generation of Metagenome-assembled genomes (MAGs) from samples taken from waters off the coasts of Mexico and China. Combining these with publicly available data, they identified and analysed more than 400 new Asgard archaeal MAGs, nearly doubling the number of Asgard MAGs available, making this dataset a valuable resource for the community in and of itself. Beyond this, the paper represents an interesting and novel addition to the growing literature on Asgard archaea in several ways: the size of the dataset allows the authors to identify patterns of correlation between specific environments and specific Asgard archaeal groups, and to produce detailed reconstructions of gene content are possible on a much larger scale than previously possible.

We thank the reviewer for their time in assessing our work and their appreciation for the effort and impact of producing this expanded dataset.

The most interesting implications come from the authors' metabolic reconstructions, particularly those pertaining to Hodarchaeales (Heimdallarchaeia), the likely sister

group to eukaryotes according to the most recent phylogenomic analyses. One of the leading eukaryogenesis models, the 'reverse flow' model, posits a hydrogen-producing shared ancestor of eukaryotes and Asgards. The authors of that paper also raised the possibility of this ancestor having been oxygen-tolerant, based on a small number of MAGs. The authors conclude that their metabolic reconstructions support these earlier, tentative conclusions, based on predictions of the oxygen tolerance of some Hodarchaeales, the presence of reactive oxygen species-detoxifying enzymes, and predictions of sequence and structural similarity between some of their protein complexes and elements of the eukaryotic mitochondrial respiratory chain (which are unlikely to be contaminants in individual MAGs, given that they form robustly supported clades).

This would have far-reaching repercussions for eukaryogenesis discussions: in the most conservative interpretation of the authors' findings, the proto-mitochondrial endosymbiont's ability to encounter and form a relationship with its host would be easier to understand and more likely if the host was already capable of tolerating oxygen. In the boldest interpretation, a host that already had the capacity for oxidative phosphorylation would call into question received ideas about the nature of the mitochondrial symbiosis, raising the possibility that oxidative phosphorylation was not a major factor and that nutritional symbioses, similar to those seen in modern prokaryotic symbioses, might have been more important. For those adhering to the idea that oxidative phosphorylation was necessary for the emergence of eukaryotic complexity, it would also raise the question of mitochondria having been dispensable for both processes. The possibility of this kind of oxygen tolerance in Asgards has been proposed before (that work is appropriately cited), but without the weight of evidence provided by Appler and colleagues' work.

We thank the reviewer for their positive feedback on our findings and agree this analysis will be impactful and expand our understanding of these evolutionarily pivotal interactions.

The overall approach is appropriate, and the work is appropriately placed into context. The authors have generated and presented an impressive amount of data, and it's a pity that some of the paper's findings are a bit swamped as a result: some interesting and detailed discussion of metabolic inferences are relegated to 20 pages of Supplementary Discussion, and some of the methods are inconsistent and not always easy to follow between the main methods and the supplementary ones.

We appreciate the reviewer's feedback and have used their suggestions, as well as the other reviewers, to help highlight more of the Supplementary Discussion within the main text. Further, we thank the reviewer for their detailed suggestions on methods and have not made the appropriate clarification to streamline the methods and discussion between the two documents.

Overall, I would like to see greater clarity and consistent terminology throughout the text, and changes to some of the methods before I can fully evaluate the paper's claims. I've also highlighted specific places analyses that are mentioned in the text but for which no data are presented.

We have used the suggested methods indicated by the reviewer and have provided additional data files, specifically those requested by the reviewer multiple sequence alignments and RMSD for the structural analysis. We value the reviewer's expertise and have included their suggestions to improve the manuscript.

Major points and suggestions for improvement:

1) I have concerns about the ways in which the phylogenetic methods were carried out, including a lack of consistency between approaches and inappropriate model choice or interpretation:

• The same level of detail should be provided for all similar analyses, and I would find it helpful to have them described in the same place: currently the methods used to generate the NM47 phylogeny are described in detail in the supplementary methods, but the methods used to generate the Phylosift and arCOG phylogenies are only mentioned in the main text.

We now describe all the details for species-phylogeny in the methods of the main text. The arCOG and Phylosift phylogenies were removed as suggested by other reviewers in place of additional complex mixture model testing and recoding to mitigate long-branch attraction.

More detail should also be provided on parameters used for alignments, trimming and (if applicable) single-gene tree construction for the Phylosift and arCOG analyses (e.g., which MAFFT program was used for the arCOG analysis, which BMGE matrix, etc.)

Thank you for catching this oversight. We revised the analysis in response to the other reviewers and no longer include the arCOG or the Phylosift analysis. We do include this information for all the remaining species-trees.

• I would like to see the number of trimmed sites used in each final analysis mentioned either in the text or in each figure legend.

We agree this is a great suggestion and now add the number of positions for the untrimmed and trimmed alignments for Fig. 1ab and Fig. 3abc in the methods of the main text. The untrimmed and trimmed positions for the

supplemental are included in the legend of each figure (Supplementary Figs. 3; 15-27; 30; 37-40; 42-44; 53-59; 61-67; 69-74).

• Phylogenies should only be shown as rooted if the analyses used to generate them specified an outgroup; while outgroup taxa were included, the methods don't mention that an outgroup was specified for phylogenetic inferences, and so these roots should be removed from the figures.

We agree with the reviewer that this should be clarified. Species tree phylogenies outgroups are defined. To display essential lineage and environmental context of the ETC subunits we rooted these trees at the midpoint. To clarify this, it is now clearly described in the legend of each figure. We provide all phylogeny files to view unrooted.

• The developers of the ultrafast bootstrap feature have made it clear that the threshold for interpreting support as high is greater than that for nonparametric bootstrap support values: "95% support correspond roughly to a probability of 95% that a clade is true. […] For UFBoot, you should only start to rely on a branch if its support is >= 95%" (http://www.iqtree.org/doc/Frequently-Asked-Questions#how-do-i-interpret-ultrafast-bootstrap-ufboot-support-values); note that the authors also strongly suggest generating Shimodaira-Hasegawa approximate likelihood ratio test support values in addition to ultrafast bootstrap support values. Supplementary Figs. S3, S15-S27, S30, S37-S40, S42-S44, S53-S67 show ultrafast bootstrap support values >= 70%, which gives a misleading suggestion that these should all be considered high support. Similarly, support values >= 90% are shown for Fig. 3(c). Where individual values are legible, I would recommend removing some values and/or adding a note of caution regarding the interpretation of ultrafast bootstrap support to the figure legend, and where only circles for the highest support values are shown, these should be shown for values greater than or equal to 95%. This point is important because it affects the interpretation of the results by leading to the overestimation of support for some clades. (The legends for several Supplementary Figures also simply mention that these show 'bootstrap support', so I would specify that these are ultrafast bootstrap support values).

We appreciate the reviewer's request. We have updated Fig. 3c and all the redone phylogenies in the supplementary to show only ultrafast bootstrap values ≥95.

We now also specify in the legend the specific type of analysis either non-parametric (Suppl Fig. 3) or ultrafast bootstrap replicates (Suppl Fig. 15-27; 30; 37-40; 42-44; 53-59; 61-67; 69-74).

For any analyses display UFBoot values < 95 we now add a note of caution in the legend, "Ultrafast bootstrap support should only be relied on when ≥ 95" or "but should only be relied on when ≥ 95".

• *For the majority of single-gene trees shown, and for the Phylosift phylogeny, the best-scoring model was chosen by ModelFinder in IQ-TREE according to the Bayesian information criterion. However, ModelFinder does not test mixture models approximating the CAT model (e.g. C20) by default, leading these to not be considered even though they would generally be the most appropriate choice (and more robust to artifacts, especially in the case of the Phylosift analysis). Instead, these models need to be explicitly included in ModelFinder analyses, as was done for NM47. I'd strongly recommend rerunning these phylogenies and including mixture models; this may also help to resolve some differences seen between the NM47 and Phylosift analyses.*

We agree and have rerun and revisualized several of the phylogenies (Fig. 1ab; Fig. 3abc; Fig. Suppl Figs. 3; 15-27; 30; 37-40; 42-44; 57-59; 72-74), testing complex mixture models. The Phylosift analysis was removed due to the concerns of the other reviewers, especially in relation to the impact of thermophily on some of the marker genes included within this set. We provided detailed descriptions of the decision in our response to the reviewers.

• *The authors mention that single-gene trees generated from trimmed alignments used for the concatenated NM47 alignment were screened for possible contamination or paralogs. I didn't see this mentioned for Phylosift or arCOG analyses, so if it was, the authors should mention it in the methods (and if it was not done, then they should do it).*

We have removed the Phylosift and arCOG analyses due to the concerns of the other reviewers. We include the methods for screening possible contamination or paralogs in the NM47 analyses.

• *In the case of NM47, a C-series mixture model was (appropriately) included for ModelFinder model choice; however, I would expect C60 to be a more appropriate choice than C10 for a phylogenomic analysis, with a large dataset reconstructing relationships over deep evolutionary time. The authors did subsequently rerun their analysis with a larger number of mixture classes, but they did so on recoded data. I suspect this was done in the interests oof time, but I would suggest performing an analysis under conditions that incorporate C60 on the full, non-recoded dataset as well.*

Indeed, our choice of phylogenetic models for the full dataset was constrained by computational demands and prohibitively long run times. To address these limitations, we dereplicated the dataset to include species-level

representatives of the Asgard ingroup. This allowed us to apply the more computationally intensive C60 mixture model. With this model, we observed increased support for the depicted topologies and importantly a monophyletic Heimdallarchaeia clade. We now present this phylogeny in Supplementary Fig. 3a and discuss the above within the supplementary discussion and updated methods.

• In Fig. 1, I find the grey circles for support values almost impossible to see both on my screen and in print, so I would suggest using filled black circles instead.

We agree and have darkened the circles to make it easier to read.

• I understand the authors' desire to show their results cross-referencing as many types of data as possible, but the different concentric circles and many colours displayed in Fig. 1(a) make the figure very complex and difficult to read. I'd suggest removing some of these circles, maybe splitting the figure and moving one part to the supplementary materials if the authors would like to keep the information. (Elsewhere I've suggested the opposite, merging information, for a supplementary table; I feel that a large table is much easier to read and parse than a sub-figure).

We agree with the reviewer and in revising the figure have removed several outer rings that are better displayed in the other main figures.

2) I have concerns about some of the structural modeling predictions and their interpretation:
• The authors tried to overcome size limitations in Alphafold3 by modeling individual parts of the nuoEFG complex and producing a composite prediction from these predictions by structural superposition of the individual partial predictions in ChimeraX. The authors explain in the supplementary figure legends which individual parts were modeled, but this should be clearly laid out in the Supplementary methods as well. I also find the current wording unclear: the figure legends suggest that a full NuoEFG complex could be modeled by Alphafold3, but that a putative larger complex including NuoEFG, NiFe-hydrogenases and flavodoxins could not be modeled. However, the supplementary methods text ("AlphaFold3 (AF3) predicted a partial NuoEFG complex in this lineage, but a more complete model could not be obtained due to the 5000 amino acid input size limit") suggests that a complete NuoEFG could not be modeled either.

We have updated our structural modelling method using CombFold as the reviewer suggests (see next point below). As a result, this section of the supplementary methods has been rewritten, and the new methodological details are clearly laid out.

• *I have concerns about this approach, namely that structural modeling of these partial sequences is less likely to be accurate given that interactions between more distantly located amino acids can't be modeled. Tools that generate structural predictions for large complexes based on predictions for smaller parts of the complex (such as CombFold) do exist, but these collate interaction predictions for more combinations of proteins or model protein complexes iteratively. The authors could explore the use of a tool designed for predicting the structure of large protein complexes, but I feel that the current approach lacks robustness.*

We now clarify this in the methods and greatly appreciate the reviewer's expertise and suggestion to take advantage of CombFold. Briefly, we ran CombFold's three-to-four step pipeline for the combinatorial assembly of AlphaFold multimer models (https://github.com/dina-lab3D/CombFold). The putative Nuo(EFG)$_2$-Mvh(ADG)$_2$-Etf(AB)$_2$-Hdr$_2$ complex was remodeled with the default CombFold pipeline (steps 1, 2, and 4 according to the documentation). The CombFold assembled model has an identical structural configuration to our previous model. Details about how we ran CombFold are included in the Supplementary methods, and we also include the CombFold running parameters and model inputs and outputs in the Figshare data repository.

• *The authors state (Supplementary methods lines 297-298): "AF2 multimer indicated that annotated NuoEFG subunits in the Asgard lineages we tested do not form complexes with the other Nuo subunits (P and Q-modules) present in the same genome". These data should be shown.*

We now provide this data in Supplementary methods, Supplementary discussion, and Figshare repository. In brief, we ran AlphaFold multimer to predict if group 4 [NiFe]-hydrogenase Complex I-like structures could form larger complexes with NuoEFG modules. We first searched for *nuoEFG* and *nuoLMNJKAHBDCI* genes within the same MAG to ensure we did not run AlphaFold multimer between complexes from different species. Two MAGs satisfied this requirement (see Supplementary methods and discussion). The AlphaFold multimer predictions were then ran with AlphaFold3 due to the large amino acid input size of *nuoEFG* and *nuoLMNJKAHBDCI* sequences together. No larger multimeric complex was predicted between NuoEFG modules and other Nuo subunits in these two cases with Hodarchaeales genomes. We now provide the low confidence scores (specifically the PAE plots showing maximal PAE scores between the NuoEFG subunits and the rest of the complex) in Supplementary Fig. 78.

• *"However, AF2 multimer predictions of NuoEFG subunits in combination with the neighboring proteins predicted several multimers" – these data are also not shown, although they're skirted over in the main text: "*A subset* of structural predictions*

*indicated *various* multimer structures, including…" (emphasis mine) – this really seems like cherry-picking, and needs to be explained further*

We agree and have analyzed the synteny of the annotations provided in Supplementary Table 8 to identify several candidates for further multimer testing, spanning multiple lineages and other archaeal representatives. We searched our MAG dataset for gene clusters harboring *nuoEFG* homologs, and focused our downstream analysis on gene clusters from larger contigs and diverse lineages. Our expanded structural analysis now includes 26 NuoEFG homologs (includes one previously predicted structure in Suppl Fig. 28-29). We have added 24 additional AlphaFold2 (AF2) multimer representatives of these NuoEFG complexes which originate from 7 Asgardarchaeota lineages (Asgardarchaeia, Gerdarchaeales, Hodarchaeales, Kariarchaeaceae, Lokiarchaeales, Njordarchaeales, Ranarchaeia) and 3 non-Asgard TACK and Euryarchaeota classes (Bathyarchaeia, Methanobacteria, Thermococci/Theionarchaea). Additionally, we have added two CombFold assembled NuoEFG complexes originating from Hodarchaeales. One of these models, the Nuo$(EFG)_2$-Mvh$(ADG)_2$-Etf$(AB)_2$-Hdr$_2$ complex, was included in our previous submission, but it has been re-modelled with the CombFold software.

We compared the structures of the new AF2 multimer models to experimental structures in the PDB (using FoldSeek-multimer and UCSF ChimeraX) to assign putative functions to them. To help with discussing their different putative functions, we grouped these models into five broad classes based on the similarity of their multimeric structures. We have classified the NuoEFG complexes as: (i) Fdh-BfuABC-like, (ii) Nfn-BfuABC-like, (iii) Nfn-BfuABCLS-like, (iv) [NiFe]-BfuABCLS-like (v) small NuoEFG-like complexes. We note that while we used FoldSeek-multimer and structural similarity comparisons to help group the NuoEFG complexes, this classification scheme is arbitrary and is only intended to help visualize and discuss their putative functions and structures. Most predicted NuoEFG complexes appear to have an electron bifurcating function based on their structural similarity with experimentally characterized electron bifurcating complexes in the PDB (hence the BfuABC nomenclature). Exceptions to the electron bifurcating function are the "NuoEFG-like with small NuoG" complexes, which have unknown functions (see Supplementary discussion).

Interestingly, the predicted Nfn-BfuABCLS-like complexes and the megacomplexes in the Hodarchaeales lineage appear to be previously undescribed electron-bifurcating complexes. Nfn-BfuABCLS-like complexes combine both previously described FBEB (flavin-based electron bifurcation) complexes: StnABC (Kremp et al., 2020 Scientific Reports; Kumar et al., 2023 Nature Communications) and NfnLS (Demmer et al., 2015 Journal of Biological

Chemistry), typically present in separate species (Kremp et al., 2020 Scientific Reports).

During our structural prediction workflow, we found a NuoEFG complex in Hodarchaeales where AF2 multimer hinted at it forming a large (>5000 amino acid) symmetrical multimeric complex. We decided to pursue the modelling of this complex further with CombFold, which predicted a large putatively electron bifurcating complex: Nuo(EFG)$_4$-Frh(AG)$_4$. We present this model alongside the previously mentioned Nuo(EFG)$_2$-Mvh(ADG)$_2$-Etf(AB)$_2$-Hdr$_2$ complex to highlight the interesting structural diversity of electron bifurcating complexes in coastal Hodarchaeales. Both predicted NuoEFG complexes have unique structural configurations that have little resemblance to other complexes in the PDB to the best of our knowledge. As a result, we were unable to group their structures into the previously described classes of NuoEFG complexes. We instead refer to these two CombFold predicted models as the Hodarchaeales electron bifurcating "megacomplexes" at points throughout the study.

• *"Initial AF2 modeling indicated evidence of possible electron bifurcating complex with similarities to the Methanospirillum hungatei Fdh-Hdr-Fmd complex" – this should also be shown*

We have removed this sentence since it may confuse readers. Our original intention was to highlight that both complexes are composed of heterodisulfide reductases. However, this is where the structural similarities end. Furthermore at the quaternary structure level, the Nuo(EFG)2-Mvh(ADG)2-Etf(AB)2-Hdr2 complex is structurally distinct from *M. hungatei* Fdh-Hdr-Fmd. As we now state in the Supplementary Discussion, the Nuo(EFG)2-Mvh(ADG)2-Etf(AB)2-Hdr2 complex is structurally unique and does not resemble other comples it to the best of our knowledge. Therefore, we have opted to remove this sentence about Fdh-Hdr-Fmd. We apologize for this confusion and oversight.

• *Moreover, Alphafold-multimer has a size limit of 4,000 amino acid residues; the authors explain how they tried to overcome the size limitation for Alphafold3, how was this done for Alphafold2?*

To clarify, all the AlphaFold2-multimer predictions shown in this study had an input size of less than 3000 amino acids, hence there was no input size limitation necessary to overcome in most cases. The few cases where AF2-multimer input size limitations did become an issue (and which we resorted to AF3 for) are listed in the methods.

• The authors model the nuoEFG complex using Alphafold3; Alphafold output files should also be made available with the supplementary materials on Figshare (I didn't see them there).

We thank the reviewer for catching this and now provide all the output files for the structural analysis in Figshare.

3) I'm unclear about some of the numbers and thresholds reported:
• the authors mention sometimes a threshold of >50% complete, <10% gene duplicates/contamination (e.g. lines 528-529), which would be consistent with their description of "medium-high quality" MAGs. However, in Supplementary Figure 1, a completeness cutoff of >=45% is mentioned, while in line 549, completeness > 45% (not also = 45%) is mentioned. Based on Table S1, it appears that only one Asgard MAG, D4993_C5_H3_Bin_385, is below 50% (despite having been generated from GE3 data; per the text, "The GE3 sequencing effort resulted in 5,606 archaeal and bacterial 529 MAGs (>50% complete, <10% multiple gene copies based on CheckM v1.2.196"). So I'd like to know why this was included in analyses (was it an oversight, and if so, how easy would it be to remove at this stage? Was this a deliberate choice, and if so why? Regardless, it would be good to check that consistent values are used throughout the manuscript, and making sure that only sequences with completeness >= 50% are being reported as 'medium-high quality' (and that if the authors then choose to exclude any MAGs for being below quality thresholds, they then ensure that these MAGs aren't included in phylogenies or other analyses)

We appreciate the reviewer pointing out this concern. The analysis of this MAG (D4993_C5_H3_Bin_385) was reported in Supplementary Table 4 for marker set p__Euryarchaeota (UID4), which reduced the completion estimation to 46.5%. However, when run with the correct marker set k__Archaea (UID2) completeness was 51.87% and Contamination 0.93%. This information has been updated in Supplementary Table 4 with all MAGs >50% complete and <10% contamination based on CheckM v.1.2.1.

• As a more minor but related point, the authors state that "From these, a total of 404 new MAGs were classified as Asgardarchaeota, expanding the known diversity of this group by 46.5%" (line 91), but in Figure 1 they seem to be showing 46.5% of the known diversity of Asgards coming from their new samples and state that "These MAGs constitute a 115.1% increase in the medium-high quality publicly available genomes" – I believe it's actually an 86.9% increase (115.1% would be 100 x the number of existing MAGs/the number of newly generated ones, as opposed to the reverse). The abstract correctly mentions 'nearly doubles'. See also my previous paragraph regarding the use of 'medium-high quality' here.

Thank you for catching this oversight! We have now corrected the increase throughout the manuscript.

• *Furthermore, Supplementary Table 1 is titled "Description of the 405 Asgard genomic dataset", not 404*

This has also been corrected to 404.

4) The authors bring up co-occurrence of MAGs from the Heimdallarchaeia (especially Hodarchaeales) with alphaproteobacteria. This could point to an ability to form symbioses with alphaproteobacteria, which in turn would be consistent with archaeal ancestors of Hodarchaeales, with similar metabolism, being able to form symbioses with the alphaproteobacterial-like organisms that gave rise to mitochondria. However, this point is much weaker and based on conjecture than other parts of the manuscript. The authors can in any case only report co-occurrence, not confirmed symbiosis.

Yes, currently we are only reporting co-occurrence, not evidence of symbiosis. To our knowledge this is the first time this co-occurrence has explicitly been noted between Hodarchaeales and Alphaproteobacteria. However, to avoid confusion we now add the statement behind the result, "It should be noted however that the observed co-occurrence does not confirm symbiotic interaction".

• *While the authors compare co-occurrence with alphaproteobacteria between different groups of Asgard archaea, they do not compare them with co-occurrence with other types of prokaryotes, which could strengthen their case. I would like to see them do this; I think it should not be too onerous given that the data would already have been generated to identify the entire set of MAGs they assembled from their sampling data.*

Thank you for this suggestion. We now expand this analysis to include all prokaryotic groups, identifying co-occurrence between Asgardarchaeota lineages and GTDB-tk identified groups by site and depth in Guaymas Basin and Bohai Sea. This is provided in updated Supplementary Table 10.

• *The authors report the co-occurrence of Hodarchaeales and alphaproteobacteria as "demonstrating the co-occurrence between these lineages across sites and depths." 'across sites and depths' is doing some heavy lifting here, as co-occurrence was found in 2/5 GE3 cores, and none of the GE2 samples, so this sentence should be modified to clarify and avoid overstatement.*

We have removed "demonstrating the co-occurrence between these lineages across sites and depth" to avoid an overstatement of our findings.

5) According to supplementary Table 6, the authors are finding 'Putative nuclear envelope organisation protein' in at least 10% of Lokiarchaeota (the same cutoff as that for cytochrome c oxidase subunit III and cytochrome c oxidase subunit IV in Heimdallarchaeota). That seems worth commenting on one way or the other.

We appreciate the reviewer's thorough analysis of our data and have added a discussion of this finding to the supplementary discussion as a potential eukaryotic characteristic. We annotated PF10281 in 43 Lokiarchaeales genomes from guilds 1 and 2 (excluding guild 3), spanning environments and sampling expeditions. Taxonomic distribution appears to be limited to viruses, eukaryotes, bacteria, and Lokiarchaeales. In fungi, this Pfam is a repeat that is used to identify the protein lsh1, which is linked to stress-response in the nuclear envelope (Asawaka et al., 2022 Genes to Cells: Devoted to Molecular & Cellular Mechanisms; Dey et al., 2020 Nature). These proteins also share sequence similarity with other smaller regulating cytoskeletal, cytoplasmic, and nuclear proteins, including gelsolin repeat and SAP domain. We have added this information into the supplemental for future targeted analysis, especially given the Lokiarchaeales cultures.

6) Supplementary Table 8 seems to be missing currently.

It showed that it was uploaded on our end, but we have now double-checked all supplementary tables including the updates are now provided.

7) I'm struck by the contrast between the paper's bolder conclusions and some of its coyer statements:
"Moreover, detailed structural protein modeling using this expanded genomic catalog supports the reverse flow model of eukaryogenesis, with Heimdallarchaeia not having to rely on a symbiont for H2 production." (lines 266-268)

"However, it cannot be definitively ruled out whether these Complex-I like complexes are instead H2-oxidizing, menaquinone-reducing proton pumps." [in which case the Heimdallarchaeia would in fact not be producing H2] (Fig. 4 legend)

"Our AF2 multimer models are primarily starting points for hypothesis generation and require future experimental validation" (Supplementary methods; a caveat worth mentioning in the main text)

• *This sometimes leads to a lack of clarity around nomenclature and the solidity of the data: Fig. 4 shows Hodarchaeales having Complex I, the figure legend for Fig. 4 and the title of Fig. 3 describe 'Complex I-like' enzymes, and the figure legend for Fig. 3 describes 'Group 4 [NiFe]-hydrogenases'. I understand why this is the case, given the argument that these enzymes are 'missing links' with features of both NiFe-hydrogenases and Complex I and the fact that subunit organization varies between*

individual species. However, for the sake clarity, one term should be chosen, and I think that the least difficult to justify would be 'Group 4 [NiFe]-hydrogenases'.

We agree that this needs clarification within the manuscript and figures. We have updated Figure 4 to show Complex I-like to match Figure 3. We have also updated the nomenclature and renamed "Complex I-like complexes" to "group 4 [NiFe]-hydrogenase membrane-bound complexes" for clarity. This nomenclature has the benefit of describing the putative function of the complex as opposed to their structural resemblance to Complex I. We note that the term "Complex I-like" still appears in the manuscript, but it is now used specifically in contexts when we need to describe the structural configuration of these complexes, such as when we distinguish them from the "MBH-like" structures, or when we need to specifically refer to the subset of group 4 [NiFe]-hydrogenase membrane-bound complexes that have "Complex I-like" structure.

We agree with the reviewer that the statement regarding the reverse flow model of eukaryogenesis in contrast with the statement in the Fig. 4 legend could confuse readers, and we thank the reviewer for catching this. We have updated the first statement with more appropriate (less bold) language to "detailed structural protein modeling using this expanded genomic catalog supports the capacity for H_2 production in Heimdallarchaeia, as proposed in the reverse flow model of eukaryogenesis", where the emphasis is on how the data supports capacity for H_2 production first and the reverse flow model second. Regarding the statement in the legend of Fig. 4, we were originally intending to highlight that (under certain cellular conditions) Complex I can reverse pump to oxidize quinone and reduce NAD^+ to NADH, and that a similar reverse pumping action may be possible in the group 4 [NiFe]-hydrogenase membrane-bound complexes. However, this may confuse readers without the proper context and longer discussion. Furthermore, while our structural modelling cannot definitively rule out any hypothesis, it does strongly suggest that menaquinone cannot enter or bind within the group 4 [NiFe]-hydrogenase complexes. Therefore, for the sake of clarity we have removed this sentence in the Fig. 4 legend.

Regarding the caveat about AF2 being a starting point for hypothesis generation and requiring experimental validation, we have now added this caveat/limitation to the main text's description of the AF2 modelling: "...though experimental studies are required to distinguish the functions of these unique complexes".

Minor points

Typo in Fig. 4, 'chorsimate' should read 'chorismate'

The figure has been changed.

Line 51, "acetate typically coupled with" should read "acetate, typically coupled with" for ease of reading

Thank you, we have updated the text accordingly.

Line 55, "the bacterial ancestors of mitochondria" – or sister group, per Martijn et al. 2018 and Muñoz-Gómez et al. 2022

We have revised the statement "the bacterial group related to the mitochondrial ancestor" and cited the two suggested papers.

Lines 59-60, "Therefore, it is plausible that the last Asgard archaea and eukaryotes' common ancestor" would be easier to read as "Therefore, it is plausible that the last common ancestor of Asgard archaea and eukaryotes"

We agree and have updated this statement in accordance with other reviewers' suggestions to "Given our new knowledge of these events, it is plausible that the archaeal host and potentially some of its modern descendants …".

Lines 68-69 "However, these lineages are very distinct from Hodarchaeales proposed to be the closest prokaryotic relatives of eukaryotes"

This statement has been updated in the text.

Lines 85-86, "we chose two ocean floor ecosystems rich in Asgard diversity" – I suggest including references here

We have added references of Asgardarchaeota diversity in Guaymas Basin (Eme et al., 2023 Nature; Dombrowski et al., 2018 Nature Communications) and Bohai Sea (Li et al., 2021 Science of The Total Environment and Guo et al., 2025 Ecology and Evolution).

Lines 138-139, "These MAGs constitute a 115.1% increase in the medium-high quality publicly available genomes" - could this sentence be made more specific? I assume 'medium-high quality' refers to MAGs with completeness >45% and contamination/redundancy below 10%?

We have added our cut-off for medium-high quality MAGs as >50% completeness and <10% contamination/redundancy of single-copy marker genes. This is also now corrected throughout the manuscript.

Line 152, "hydrothermal-associated locations" is a bit vague - I think the casual reader might expect this to mean hydrothermal vents or maybe hydrothermal vents and hot springs, but looking at the locations in which Njordarchaeales and Odinarchaea were found in Fig. 2, these include bathypelagic samples and oilfieldsm as well.

Thank you for catching this, we have modified the statement to primarily associated with hydrothermal vent sediments and hot springs.

Line 186, typo, "Rizobales" should be "Rizobiales"

We have corrected this to Rhizobiales.

Line 197, "Previous studies have suggested that the LAECA was hydrogen-dependent" – I agree with citing Sousa et al. here, but ref. 2 is the reverse flow model proposal, which I believe describes a hydrogen-dependent symbiont progenitor of mitochondria, rather than a hydrogen-dependent LAECA

We thank the reviewer for catching this and move the references to the discussion of the reverse flow model later in this section.

*Lines 266-267, "structural protein modeling using this expanded genomic catalog supports the reverse flow model of eukaryogenesis" – the reverse flow model paper states that "This model suggests that a metabolic syntrophy between *anaerobic* ancestral Asgard archaea and *facultative anaerobic* alphaproteobacteria has provided the selective force for the establishment of a stable symbiotic interaction that has subsequently led to the origin of the eukaryotic cell" (emphasis mine). So I think it would be helpful to reword this to mention which conclusions are being supported: 1) the conclusion that the may have produced hydrogen rather than consuming hydrogen from its endosymbiont, and 2) the conclusion (mentioned by Spang et al but not explicitly part of the model itself) that Heimdallarchaeota may be capable of aerobic respiration. This could be phrased as 'supports the findings of Spang et al.' for clarity.*

We have revised the statement to "Moreover, detailed structural protein modeling using this expanded genomic catalog supports the capacity for H_2 production in Heimdallarchaeia, as proposed in the reverse flow model of eukaryogenesis (Spang et al.), potentially reducing reliance of Heimdallarchaeia on a symbiont for H_2."

We have added the following a statement "These results substantiate and broaden previous findings that hinted at the potential for aerobic respiration in Heimdallarchaeia" in the section "Potential for aerobic respiration in the

archaeal eukaryotic ancestor", indicating our analysis support the findings in Spang et al. although not included in their model.

Line 335, 'Fig. 4, Increasing complexity' - I'd suggest 'Increased' here since it refers to a single organism

We have updated the figure legend.

Line 461, I'd suggest splitting the references here, moving those in support of mitochondria-early to the end of the previous sentence and keeping here only those in favour of mitochondria-late, to make it clearer to the reader which ones support which position

Thank you, this is an excellent point. We have split the two primary references between the sentences to more clearly indicate which timeline for acquisition is being supported.

Line 514, "library prep" – writing out "library preparation" would be more appropriate

We have updated the text.

Line 535-536, "Resulting in a total of 5,233 BH MAGs and 3,000 GE2 MAGs >50% complete and <10% contamination based on CheckM v1.2.1" – I think this should have followed on from the last sentence with a comma, not a full stop

We have updated the text.

Line 544-546, "we compared several methods for accessing completeness and contamination (CheckM v1.2.1, miComplete v2, and MEBS v2)" - if these tools are being mentioned in the paper, I'd show the data on which the authors based their choice to go with CheckM, or otherwise explain briefly how CheckM was chosen

We have included the results from all three sets of single-copy markers in Supplementary Table 4. This provides continuity with previous work Zaremba-Niedzwiedzka et al., 2017, Nature, which reported both miComplete and CheckM quality metrics. Added here is a more recent set of marker genes used by the MEBs to produce a completeness score (Gutiérrez-Preciado et al., 2018, Nature Ecology and Evolution). The three sets of markers are comparable, and we felt important to include for future analyses.

However, we chose to use CheckM as the standard for quality metrics. We used high-quality circularized complete genomes to assess the three software marker sets (2 Atabeyarchaeia, 1 Frey/Jordarchaeia, 1 Lokiarchaeia, 1 Odinarchaeia, and 2 Heimdallarchaeia). CheckM more accurately captures

completeness across the highest quality genomes with the highest completeness score for 4/7 genomes. For example, the complete Atabeyarchaeia and Freyarchaeia genomes (verified with short and long read sequencing data) had completeness scores of 97.2-98.13 in comparison to miComplete and MEBs with scores of 93.85-95.36 and 94.9-96.9, respectively. We only compared redundancy scores between CheckM and miComplete as MEBs does not currently output this data. CheckM had higher redundancy scores for 5 out the seven genomes, which is likely due to duplications in marker genes, but also provides a higher threshold to meet quality standards.

Line 551, "ASsemblage of Genomes from Asgard Archaea Resolving DIrection to our ANcestors (ASGARDIAN)" – cute!

Thank you! We think so too!

Lines 559-561, "We also used SingleM/Sandpiper 0.3.0 to search the NCBI SRA public metagenome sets prior to 2021 for OTUs linked with GTDB R220 Hodarchaeales, Kariarchaeaceae, and Alphaproteobacteria" - should this sentence be placed earlier in the methods? Based on its current placement, it looks like these MAGs were not subjected to the same optimal oxygen tolerance, temperature, salinity and pH predictions as the others.

Both SingleM and Sandpiper search through shotgun metagenomic datasets deposited in the public domain prior to 2021. Specifically, Sandpiper searches through the NCBI Sequencing Read Archive (SRA), which includes both binned and unbinned data. If the MAGs were binned and published with the metagenomic datasets they would be included in the previous growth condition analysis. The analysis of optimal growth conditions is for only the binned representatives (genomic references). However, SingleM/Sandpiper also captures unbinned portions by taxonomic identification through OTUs, expanding our analysis. Therefore, these represent separate analyses of medium-high quality MAGs (growth condition predictions) and co-occurrence (SingleM/Sandpiper 0.3.0). We now add "Sequencing Read Archive (SRA)" to clarify the database and "including binned and unbinned data".

Lines 632-633, "T-tests to test if water depth significantly influenced genome size and if so in what direction." – fragment, consider revising

We have modified the sentence to "T-tests were conducted to determine whether water depth significantly influenced genome size, and if so, to identify the direction of the effect."

The title of Supplementary Figure 6 is "Transitions between prokaryotic-eukaryotic life identified with functional pangenomics", I don't think this is a clear description of

what it's actually showing (the number of OGs uniquely shared between each Asgardarchaeota lineage with the single-cell eukaryote database)

We agree and have modified the legend to "Conserved functional OGs unique to Asgardarchaeota lineages and single-cell eukaryotes across evolutionary branching points".

It would be helpful to merge Supplementary Tables 4 and 9, so as to allow the reader to more easily compare predictions about the optimal environmental conditions of each sample with any available data on the environmental context of the sample

We appreciate the suggestion and now provide the combined tables as the updated Supplementary Table 4 for easier comparison.

Supplementary Table 10, typo, "Alphaproteobactiera Total"

Thanks, this has been corrected.

Supplementary methods line 146, "we obtained five cores split into 12 depth horizons", reference Supplementary Figure S2 here

Thank you, we have added this reference to the supplementary figure.

Supplementary methods lines 158-160, "Recent validation of the superphylum, Asgard archaea, as the phylum, Asgardarchaeota8 with all previously proposed phyla now listed as classes, orders, and families." – sentence fragment, consider revising

We have revised this sentence to "Recent validation of the superphylum, Asgard archaea, as the phylum, Asgardarchaeota reclassified all previously proposed phyla as classes, orders, and families."

Supplementary methods lines 181-182, "but the representatives in each of the 17 clades remain consistent (Fig. 1 and Supplementary Fig. 2" – I think the latter should be Supplementary Figure 3

Due to the suggestions by the reviewers this statement has been removed.

Supplementary methods, structural modelling section, where several Alphafold structural predictions were compared to a solved structure, a table of individual RMSD results (for both pruned and unpruned atom pairs) should be provided, rather than a range of values

We now reference an added table (Supplementary Table 9), which provides details on the structural model and specifically the individual RMSD results for both pruned and unpruned atom pairs, which were calculated with UCSF ChimeraX during our cofactor modelling steps. We have also included tables with information from our FoldSeek search results, which provides additional metrics on the structural similarity between the AF2 models we present and PDB models chosen for structural comparison and cofactor modelling.

Supplementary methods line 1249, "Promethoarchaeum guaymasis", should this be Prometheoarchaeum guaymasis?

Yes, thank you that has now been corrected.

Referees' comments:

Referee #1 (Remarks to the Author):

Overall, I think the authors have done a good job of revising this exciting manuscript. I only have a few minor comments that I feel they need to address:

We appreciate the reviewer's continued positive view of this manuscript.

Minor comments:

Lines 47-48: The authors indicate that models of eukaryogenesis that imply hydrogen exchange between host and a 'bacterial partner' are "supported by the limited enrichment cultures refs 11-13". These models of eukaryogenesis are not directly "supported" by the existence of cultures of living Asgard archaea that are involved in syntrophic interactions with other prokaryotes. At most what can be said is that is that they are "consistent with syntrophic interactions observed in limited enrichment cultures of Asgard archaea". This may seem like a trivial change, but it is important to recognize that none of these cultures involve bacterial partners related to the mitochondrial symbiont or any other bacterial partner known to be involved in eukaryogenesis. So the evidence provided by the physiology of these living asgard archaea is circumstantial at best.

We agree with the reviewer and have made the suggested revision.

Lines 52-55: The authors state:
"A recent study estimated that the first eukaryotes (FECA, the first eukaryotic common ancestor) diverged from an interaction between Asgard archaeon and Alphaproteobacteria (the bacterial group related to the mitochondrial ancestor15,16) about 2.67-2.19 billion years ago (Ga) and 2.58-2.12 Ga, respectively17."

This sentence needs to be revised for several reasons. Firstly, the "first eukaryotic common ancestor", FECA, is incorrectly defined here. FECA was first defined in the literature by Makarova et al. (2005), https://pmc.ncbi.nlm.nih.gov/articles/PMC1187821/, to be the immediate descendant on the eukaryote line of the closest archaeal relative of eukaryotes. So it is basically the 'first' daughter cell on the eukaryote line descending from the most recent common ancestor of eukaryotes and Archaea. As such it must predate the mitochondrial symbiosis (= "interaction between Asgard archaeon and Alphaproteobacteria"). Note that FECA is also incorrectly specified to be the asgard/alphaproteobacterial holobiont in Fig. 5 as well; that should be fixed.

We agree this was an oversight and has now been corrected when described in the introduction and Fig. 5.

The second problem is the ambiguity regarding what the dates cited are referring to. The way it is worded, it looks like the interaction (mitochondrial symbiosis) is being dated but if so, then why are there two sets of dates and the use of the word "respectively"? The dates referred to from ref. 17 are for the last common ancestors of eukaryote/asgards and mitochondria/alphaproteobacteria respectively, which only imply that the mitochondrial symbiosis must be more recent than these dates. In reference 17, the age of LECA is estimated to be 1.84-1.93 Ga. So, if those dates are correct, this means that the mitochondrial symbiosis must have happened sometime between 2.58 Ga and 1.84 Ga; the symbiosis itself cannot be dated using molecular clocks because it doesn't correspond to a node on a phylogenetic tree. I think the authors should rephrase the sentence above to be more precise.

We have updated the statement to "A recent study estimated the age of the last eukaryotic common ancestor (LECA) to be between 1.84-1.93 billion years ago (Ga)[16]. This finding suggests the timing of mitochondrial symbiosis, likely resulting from an interaction between an Asgard archaeon and Alphaproteobacteria (the bacterial group related to the mitochondrial ancestor[17,18]), occurred after the divergence of eukaryotes from their archaeal relatives and prior to LECA, between 2.58-1.84 Ga[16]."

Line 87: "increase membrane potential" should be "increased membrane potential"

We have modified the statement.

Line 453: I think the authors should be more cautious here and avoid making unqualified claims based on their metabolic predictions. I would suggest putting this as "Our findings suggest that the gain of ETC…by Heimdallarchaeia may have increased energy yield and…"

We agree and have revised the sentence as suggested.

Line 455: This sentence is also making an assumption that increased energy was required for the evolution of cellular complexity. There is no evidence whatsoever that this claim (first made by Lane and Martin) is true. Complexification of protist cells over evolutionary time has occurred in anaerobic protist lineages that do not have aerobic respiration: an example is the origin of hypermastigids (a branch of the Parabasalia) with hundreds of flagella from much simpler biflagellated parabasalid ancestors. All parabasalids possess hydrogenosomes, not mitochondria and can only do substrate-level phosphorylation to produce ATP. I suggest removing this sentence and the following one and rewriting this paragraph along the lines of: "However, our analysis is consistent with an alphaproteobacterial origin of eukaryotic mitochondrial ETC components. This raises the question of why the mitochondrial symbiont containing protoeukaryote would ultimate lose the original ETC system of the host lineage. Perhaps the endosymbiont increased total respiratory…."
I don't insist that the authors do this, but if they wish to keep in the original sentences,

then they need to provide some evidence that excess energy over substrate-level phosphorylation is required to evolve cellular complexity, otherwise it's just perpetuating a baseless claim.

We agree this was not the intended purpose of this sentence. Therefore, we have revised as suggested.

Line 467: "while the redundant Asgard ETC subunits were likely lost in early eukaryotes." I suggest rephrasing this because "early eukaryotes" might be interpreted by readers as "early crown eukaryotes" – i.e. post-LECA lineages of eukaryotes. I'm not sure the authors mean to suggest this or instead are suggesting that they were lost in the lineage leading (and prior to) LECA.

Thank you for helping us to clarify this statement. We have now revised the statement to "prior to the LECA".

Line 531-532: "that LAECA conferred several bioenergetic advantages to the first eukaryotic cell…" As requested in the origin review, I think references to the "first eukaryotic cell" should be removed. Furthermore, how can an ancestor 'confer' advantages to a descendant? I suggest tightening up the language here. You could say something like "Our findings indicate that LAECA had aerobic bioenergetic capacities that may have played a role early in eukaryogenesis including genes for the ETC…."

We have removed the phrasing first eukaryotic cell from the manuscript to minimize confusion with the timing we are discussing.

Page 17, Fig. 5: The figure is much improved over the original version. I still think it's a bit too cluttered and potentially confusing. As mentioned above the location of the label FECA on the above line needs to be changed. I would also perhaps suggest an arrow from the "Heimdall ancestor" cell goes to the "contemporary Hod and Kari lineages" cell so that it looks more like a tree where the latter are sister taxa to eukaryotes. I don't know if the small thumbnail picture of Hod & kari cell and the "Alpha" thumbnail next to the top timeline needs to be there. It distracts from the overall picture…and is confusing given that Alpha appears to be in the future (relative to the "Today" dot in the timeline.

We appreciate the reviewer's input on the model figure and have made the suggested modifications.

Referee #2 (Remarks to the Author):

I appreciate the authors' revisions to moderate claims about Hodarchaeales being the definitive closest relatives of eukaryotes and for incorporating language that acknowledges phylogenetic uncertainties and alternative scenarios. These revisions represent positive steps toward a more balanced presentation. However, additional modifications are needed to address the persistent overemphasis on Hodarchaeales. The text should more consistently reflect that Hodarchaeales represent one candidate lineage within Heimdallarchaeia, and that features observed in Hodarchaeales might be ancestral to Heimdallarchaeia (and thus relevant to eukaryogenesis) rather than directly or subtly implying special significance for Hodarchaeales specifically.

We appreciate the reviewer's input and have further modified the main text to show Hodarchaeales as one of the lineages within Heimdallarchaeia. In addition, we removed the specific mention of the number of Hodarchaeales genomes from the abstract to deemphasize them. We have gone through the document again and did not identify any other place where this change is necessary.

In particular during the previous round of revisions we added "Recent studies have suggested the Hodarchaeales (formerly Heimdall LC-3), a newly proposed order within the class Heimdallarchaeia, is the Asgard archaeal lineage most closely related to eukaryotes[2]. However, another recent analysis suggested a deeper-rooted origin of eukaryotes, indicating they share a common ancestor with the class Heimdallarchaeia (previously Heimdallarchaeota)[15]." to the introduction. We have provided balance representation while also acknowledging the studies that have indicated Hodarchaeales (Heimdall LC-3) as the sister group to eukaryotes. We also have added analysis of Kariarchaeaceae metabolism.

Besides, the manuscript should avoid using genomic inferences about metabolic capabilities (such as oxygen metabolism) as direct evidence or subtle support for conclusions about the much deeper LAECA node. The text needs to clearly distinguish between demonstrable aerobic capabilities in specific extant lineages (e.g., Kariarchaeaceae and Hodarchaeales) and the unresolved question of LAECA's physiology regarding oxygen metabolism.

We understand the reviewer's concern and have modified the text to ensure this is more clearly illustrated. For example, we clarified extant lineages in the following statement "Based on available data from extant lineages, an alternative scenario in which the capacity for aerobic respiration was not present in FECA, but more recently acquired via HGT from bacteria cannot be excluded. In that case, the acquisition of aerobic respiration genes via HGT and via the mitochondrial endosymbiosis should be regarded as two independent energetic adaptations towards the increasing oxygen levels on Earth."

Major comments 1
The hypothesis that Kariarchaeaceae and Hodarchaeales are adapted to oxygen is supported mainly by ecological distribution and by a few hallmark proteins of aerobic lifestyles. However, this evidence is not yet convincing, and the argumentation lacks logical continuity. While this adaptation is theoretically possible, it would be beneficial for the authors to provide more substantial evidence or revise the manuscript for a balanced presentation. Please consider the following points:

We appreciate the reviewers detailed feedback and suggestions for improvement. In response, we have both revised the manuscript text for a more balanced presentation and added several of the analyses requested by the reviewer. Our revisions are detailed under each point below. We believe this two-fold approach along with all the previous revisions will now satisfy the reviewer's concerns.

L172. Some Kariarchaeaceae and Hodarchaeales MAGs were recovered from aerobic water-column samples, yet no comparison is provided of their relative abundance in the water column versus anaerobic sediments. Adding these abundance data would substantiate the claim.

This previously written statement on line 172 "Hodarchaeales and Kariarchaeaceae were most associated with shallow coastal sediments (<200 mbsl), including 78% and 56% of the MAGs from each lineage") described the distribution of the MAGs in sediments. Almost all Asgardarchaeota MAGs have been recovered in the absence of biogeochemical measurements (e.g., oxygen, etc.) and through thorough investigation of the literature we report when oxygen concentrations were provided (i.e., 7.2 mg/L in the Persian Gulf). Without oxygen measurement relative abundance in the water column versus sediment does not fully address your concern.

However, we used Sandpiper v0.3.0 to determine abundance estimates in water columns versus sediment estimates for Heimdallarchaeia lineages. We manually checked the 1687 SRA accessions to obtain the correct sampling location since in our initial tests of this added analysis, we found sampling was often listed as metagenomic data or marine system without specifying the actual source. We found higher mean relative abundance for Kariarchaeaceae within the water column and higher mean relative abundance of Hodarchaeales in sediments and soils. However, we note the marine system is dynamic with the water column (e.g., dead zones) and sediments (e.g., with high turbation) have varying oxygen conditions.

Therefore, we scanned all the available metadata for the SRAs with Heimdallarchaeia sequences based on our Sandpiper results. Then, we searched for any information on oxygen with the keywords "oxygen", "diss_oxy", "oxy_sat", "anaerobe", "aerobe", "microaerophile", "oxygen_requirement", etc. Based on this, we found 4 samples containing Hodarchaeales with aerobic conditions (listed as aerobic, oxygen_mass > 6.5, DO > 2.0 mg/L, DO > 60.98

µmol/kg, DO > 1.4 mL/L, DO > 62.5 µmol/L, Oxygen saturation > 90 pct), 2 with microaerobic conditions (listed as microaerophilic, oxygen_mass 0.8<=x<6.5 mg/L, DO 0.3<=x<2.0 mg/L, DO 9.15<=x<60.98 µmol/kg, DO 0.21<=x<1.4 mg/L), and 26 samples with anaerobic conditions (listed as anaerobic, oxygen_mass < 0.8 mg/L, DO < 0.3 mg/L, DO < 9.15 µmol/kg, DO < 0.21 mg/L). In cases where data points differed, we took the author's provided relationship to oxygen. Unfortunately, oxygen concentrations were only gathered for sediment or microbial mat samples. No oxygen concentration was reported for the 44 water samples containing Hodarchaeales.

We found 114 samples containing Kariarchaeaceae with aerobic conditions, 8 with microaerobic conditions, 10 samples with anaerobic conditions, and 5 in oxygen minimum zone (no reported oxygen concentration). Further, supporting metabolic flexibility of Hodarchaeales as a facultative anaerobe or at the very least containing the capacity to tolerate oxygen. However, this additional data further supports that Kariarchaeaceae likely have an aerobic lifestyle.

We acknowledge results from both these added analyses are reliant on correct information being submitted to NCBI. This information is now provided in Supplementary Table 10 with this caveat noted.

L182. Oxygen tolerance was predicted with GenomeSPOT, a tool known to misclassify taxa (e.g., Nitrososphaerota is labeled oxygen-intolerant). Please validate the GenomeSPOT results with additional methods (e.g., the XGBoost method used in 10.1126/science.adp1853).

We appreciate the author's feedback on our methodology and have run the XGBoost method on our Asgardarchaeota genome catalog presented in Davín et al. This method identified 32% of Kariarchaeaceae (8 MAGs) as aerobes and predicted the rest of the Asgardarchaeota MAGs as anaerobes. We add these results to Supplementary Table 4 with the GenomeSPOT results.

Due to the binary classification (aerobe:anaerobe) of XGBoost this method is more likely to label aerobes and facultative anaerobes as an anaerobe and less likely to categorize an anaerobe as an aerobe. As discussed in the supplemental of the cited paper "In no case was an anaerobic organism incorrectly predicted as being aerobic." Yet, the model was designed for dating atmospheric oxygenation and bacterial aerobic metabolism, but does not characterize the spectrum for microaerophilic, facultative anaerobes, aerotolerant anaerobes. Therefore, our catalog includes Kariarchaeaceae MAGs that are likely aerobes and Hodarchaeales that are more likely to be facultative or aerotolerant anaerobes. Separately, GenomeSPOT analyzes oxygen tolerance and includes microaerophilic and facultative anaerobes in classification as oxygen tolerant.

For example, in the paper cited by the reviewer, Table S2 (Unified Human Gastrointestinal Genome (UHGG) database), presents 10 randomly selected

bacteria predicted as aerobes and 10 predicted as anaerobes by XGBoost. Of the 20 bacteria compared to the literature-derived phenotypes there were 7 mismatches (3 facultative anaerobes were predicted to be anaerobes, 2 facultative anaerobes were predicted to be aerobes, 1 facultative aerobe was found to be aerobic, and 1 microaerophile was found to be an anaerobe). Similarly in Table S3, when applied to OceanDNA MAG database 2 aerobes and one facultative anaerobe were all classified as anaerobes.

Although we value the insight given by this software published since our initial submission, it is difficult to determine accuracy in archaea and due to the strict binary cutoff for numerous uncultivated lineages within Asgardarchaeota. Based on the available supplemental information it appears GenomeSPOT was trained on archaeal and bacterial genomes. Yet, despite the method description of testing XGBoost on archaea this information is not currently clear. GenomeSPOT uses locations and amino acid proteins whereas XGBoost uses nucleotides and duplications of bacterial aerobic/anaerobic markers. We believe it elicits the discrepancy as duplications are common within the Asgardarchaeota phylum (Eme et al., 2023). XGBoost is published but still new with the github stating "TODO" on one of the steps and when installing required modifications in the code to run accurately.

In response to the reviewer's comments, we have been unable to find any published documents or comments that GenomeSPOT misclassifies Nitrososphaerota as oxygen-intolerant, but again these models are more likely to classify as oxygen intolerant than tolerant. We ran GenomeSPOT on 154 Nitrososphaerota (Nitrososphaeria) previously classified as aerobic, microaerobic, or anaerobic by Luo et al., 2024 ISME. GenomeSPOT classified all Nitrososphaeria MAGs as not oxygen tolerant. Again, the software does not classify any of the anaerobes or microaerobic microbes as aerobes. We ran the same genomes using XGBoost, which similarly classified the microaerobe and 2 aerobes as anaerobic, but also surprisingly misclassified 23 anaerobes (79%) as aerobic and 2 aerobes as anaerobic based on Luo et al., 2024 (See Table below). Both software are great resources for identifying physiochemical conditions of uncultured genomes, but neither is without error. By reporting the results of both programs and our metagenomic analyses we provide a more complete view of the aerobicity of these lineages.

As previously stated, we have added the results of XGBoost method to the supplemental but acknowledge the limitations of the software given our questions are outside its initial software's scope and may not provide the most complete picture of our results. Therefore, we provide data from both methods GenomeSpot and XGBoost (Supplementary Table 4), mapped the requested proteins onto the species tree (Supplementary Figs. 81 and 82), and have gone through the manuscript again to make sure we clearly state our findings. We hope this multi-prong approach alleviates the reviewer's concerns and will present the most complete picture of the data at this timepoint.

Accession	Respiration type	GenomeSPOT	Aerobicity
GCA_000018465.1	Aerobic	not tolerant	Aerobe
GCA_000200715.1	Aerobic	not tolerant	Aerobe
GCA_000204585.1	Aerobic	not tolerant	Aerobe
GCA_000220175.2	Aerobic	not tolerant	Aerobe
GCA_000299395.1	Aerobic	not tolerant	Aerobe
GCA_000303155.1	Aerobic	not tolerant	Aerobe
GCA_000328945.1	Aerobic	not tolerant	Aerobe
GCA_000402075.1	Aerobic	not tolerant	Aerobe
GCA_000685395.1	Aerobic	not tolerant	Aerobe
GCA_000698785.1	Aerobic	not tolerant	Aerobe
GCA_000722915.1	Anaerobic	not tolerant	Aerobe
GCA_000723185.1	Aerobic	not tolerant	Aerobe
GCA_000730285.1	Aerobic	not tolerant	Aerobe
GCA_000802205.2	Aerobic	not tolerant	Aerobe
GCA_000812185.1	Aerobic	not tolerant	Aerobe
GCA_000875775.1	Aerobic	not tolerant	Aerobe
GCA_000955905.3	Aerobic	not tolerant	Aerobe
GCA_000956175.1	Aerobic	not tolerant	Aerobe
GCA_001437625.1	Aerobic	not tolerant	Aerobe
GCA_001443365.1	Aerobic	not tolerant	Aerobe
GCA_001510275.1	Aerobic	not tolerant	Aerobe
GCA_001541925.1	Aerobic	not tolerant	Aerobe
GCA_001543015.1	Aerobic	not tolerant	Aerobe
GCA_001627235.1	Aerobic	not tolerant	Aerobe
GCA_001674955.1	Anaerobic	not tolerant	Aerobe
GCA_002156965.1	Aerobic	not tolerant	Aerobe
GCA_002494485.1	Aerobic	not tolerant	Aerobe
GCA_002495315.1	Anaerobic	not tolerant	Aerobe
GCA_002495905.1	Aerobic	not tolerant	Aerobe
GCA_002499005.1	Microaerobic	not tolerant	Anaerobe
GCA_002499525.1	Anaerobic	not tolerant	Aerobe
GCA_002506665.1	Anaerobic	not tolerant	Aerobe
GCA_002713205.1	Aerobic	not tolerant	Aerobe
GCA_002730325.1	Aerobic	not tolerant	Aerobe
GCA_002737455.1	Aerobic	not tolerant	Aerobe
GCA_002787055.1	Aerobic	not tolerant	Aerobe
GCA_002788515.1	Aerobic	not tolerant	Aerobe
GCA_002789395.1	Aerobic	not tolerant	Aerobe
GCA_002906215.1	Aerobic	not tolerant	Aerobe
GCA_003135575.1	Aerobic	not tolerant	Aerobe
GCA_003164815.1	Aerobic	not tolerant	Aerobe
GCA_003175215.1	Aerobic	not tolerant	Aerobe
GCA_003331425.1	Aerobic	not tolerant	Aerobe
GCA_003695185.1	Anaerobic	not tolerant	Aerobe
GCA_003702465.1	Anaerobic	not tolerant	Aerobe
GCA_003702525.1	Aerobic	not tolerant	Aerobe
GCA_003702545.1	Anaerobic	not tolerant	Aerobe
GCA_003724275.1	Aerobic	not tolerant	Aerobe
GCA_003724285.1	Anaerobic	not tolerant	Aerobe
GCA_003724325.1	Aerobic	not tolerant	Aerobe
GCA_004322465.1	Aerobic	not tolerant	Aerobe

GCA_005798405.1	Aerobic	not tolerant	Aerobe
GCA_005877205.1	Aerobic	not tolerant	Aerobe
GCA_005877225.1	Aerobic	not tolerant	Aerobe
GCA_005877305.1	Aerobic	not tolerant	Aerobe
GCA_005877345.1	Anaerobic	not tolerant	Aerobe
GCA_005877385.1	Aerobic	not tolerant	Aerobe
GCA_005877425.1	Anaerobic	not tolerant	Aerobe
GCA_005877475.1	Aerobic	not tolerant	Aerobe
GCA_006740685.1	Aerobic	not tolerant	Aerobe
GCA_007280335.1	Aerobic	not tolerant	Aerobe
GCA_007571135.1	Aerobic	not tolerant	Aerobe
GCA_007826885.1	Aerobic	not tolerant	Aerobe
GCA_008080815.1	Aerobic	not tolerant	Aerobe
GCA_008389435.1	Aerobic	not tolerant	Aerobe
GCA_009377575.1	Aerobic	not tolerant	Aerobe
GCA_009379865.1	Anaerobic	not tolerant	Aerobe
GCA_009665115.1	Aerobic	not tolerant	Aerobe
GCA_009839185.1	Aerobic	not tolerant	Aerobe
GCA_009840065.1	Aerobic	not tolerant	Aerobe
GCA_009898475.1	Anaerobic	not tolerant	Aerobe
GCA_009903775.1	Anaerobic	not tolerant	Anaerobe
GCA_009903795.1	Anaerobic	not tolerant	Anaerobe
GCA_010028495.1	Aerobic	not tolerant	Aerobe
GCA_011319465.1	Aerobic	not tolerant	Aerobe
GCA_011319475.1	Aerobic	not tolerant	Aerobe
GCA_011523285.1	Aerobic	not tolerant	Aerobe
GCA_011605775.1	Anaerobic	not tolerant	Anaerobe
GCA_011772925.1	Anaerobic	not tolerant	Aerobe
GCA_012271275.1	Aerobic	not tolerant	Aerobe
GCA_012959225.1	Aerobic	not tolerant	Aerobe
GCA_013043295.1	Anaerobic	not tolerant	Aerobe
GCA_013114715.1	Aerobic	not tolerant	Aerobe
GCA_013114725.1	Aerobic	not tolerant	Aerobe
GCA_013215285.1	Aerobic	not tolerant	Aerobe
GCA_013288545.1	Aerobic	not tolerant	Aerobe
GCA_013306035.1	Aerobic	not tolerant	Aerobe
GCA_013330055.1	Aerobic	not tolerant	Aerobe
GCA_013340765.1	Anaerobic	not tolerant	Anaerobe
GCA_013378375.1	Aerobic	not tolerant	Aerobe
GCA_013388925.1	Anaerobic	not tolerant	Anaerobe
GCA_013388945.1	Aerobic	not tolerant	Aerobe
GCA_013390375.1	Aerobic	not tolerant	Aerobe
GCA_013390485.1	Aerobic	not tolerant	Aerobe
GCA_013390745.1	Anaerobic	not tolerant	Aerobe
GCA_013390785.1	Aerobic	not tolerant	Aerobe
GCA_013407145.1	Aerobic	not tolerant	Aerobe
GCA_013407165.1	Aerobic	not tolerant	Aerobe
GCA_013407185.1	Aerobic	not tolerant	Aerobe
GCA_013407275.1	Aerobic	not tolerant	Aerobe
GCA_013407385.1	Aerobic	not tolerant	Aerobe
GCA_013521055.1	Aerobic	not tolerant	Aerobe
GCA_013694585.1	Aerobic	not tolerant	Aerobe
GCA_013821245.1	Anaerobic	not tolerant	Aerobe

GCA_013867335.1	Aerobic	not tolerant	Aerobe
GCA_013911135.1	Aerobic	not tolerant	Aerobe
GCA_014075315.1	Aerobic	not tolerant	Aerobe
GCA_014078525.1	Aerobic	not tolerant	Aerobe
GCA_014193235.1	Aerobic	not tolerant	Aerobe
GCA_014384445.1	Aerobic	not tolerant	Aerobe
GCA_014384555.1	Aerobic	not tolerant	Aerobe
GCA_014523485.1	Aerobic	not tolerant	Aerobe
GCA_014523495.1	Aerobic	not tolerant	Aerobe
GCA_014523625.1	Aerobic	not tolerant	Aerobe
GCA_014523665.1	Anaerobic	not tolerant	Aerobe
GCA_014523685.1	Aerobic	not tolerant	Aerobe
GCA_015522695.1	Anaerobic	not tolerant	Anaerobe
GCA_016125975.1	Aerobic	not tolerant	Aerobe
GCA_016126025.1	Aerobic	not tolerant	Aerobe
GCA_016176745.1	Aerobic	not tolerant	Aerobe
GCA_016180375.1	Aerobic	not tolerant	Aerobe
GCA_016184315.1	Aerobic	not tolerant	Anaerobe
GCA_016190055.1	Aerobic	not tolerant	Anaerobe
GCA_016193005.1	Aerobic	not tolerant	Aerobe
GCA_016200025.1	Aerobic	not tolerant	Aerobe
GCA_016200035.1	Aerobic	not tolerant	Aerobe
GCA_016200045.1	Aerobic	not tolerant	Aerobe
GCA_016201435.1	Anaerobic	not tolerant	Aerobe
GCA_016206275.1	Anaerobic	not tolerant	Aerobe
GCA_016215465.1	Aerobic	not tolerant	Aerobe
GCA_016234975.1	Aerobic	not tolerant	Aerobe
GCA_016782225.1	Aerobic	not tolerant	Aerobe
GCA_016782245.1	Aerobic	not tolerant	Aerobe
GCA_016838785.1	Aerobic	not tolerant	Aerobe
GCA_016838795.1	Aerobic	not tolerant	Aerobe
GCA_016838825.1	Aerobic	not tolerant	Aerobe
GCA_016838845.1	Aerobic	not tolerant	Aerobe
GCA_016844685.1	Aerobic	not tolerant	Aerobe
GCA_016871975.1	Anaerobic	not tolerant	Aerobe
GCA_016871995.1	Aerobic	not tolerant	Aerobe
GCA_016872015.1	Aerobic	not tolerant	Aerobe
GCA_016872055.1	Aerobic	not tolerant	Aerobe
GCA_017855915.1	Aerobic	not tolerant	Aerobe
GCA_018128925.1	Aerobic	not tolerant	Aerobe
GCA_018263495.1	Anaerobic	not tolerant	Aerobe
GCA_018263505.1	Aerobic	not tolerant	Aerobe
GCA_900065925.1	Aerobic	not tolerant	Aerobe
GCA_900143675.1	Aerobic	not tolerant	Aerobe
GCA_900167955.1	Aerobic	not tolerant	Aerobe
GCA_900177045.1	Aerobic	not tolerant	Aerobe
GCA_900299125.1	Anaerobic	not tolerant	Aerobe
GCA_900620265.3	Aerobic	not tolerant	Aerobe
GCA_900696045.1	Aerobic	not tolerant	Aerobe
GCA_902826075.1	Aerobic	not tolerant	Aerobe

L188. The co-occurrence analysis relies solely on a binary presence/absence model

and fails to examine abundance dynamics. This is a critical limitation particularly for dominant taxa， which are often difficult to recover from metagenomic sequencing.

We see your point; however, the statement is just to indicate that today's Heimdallarchaeia co-occur with Alphaproteobacteria. The final statements of this paragraph have been revised as such "Collectively, metagenomes in this study and previous metagenomic data support that at least some Hodarchaeales and Kariarchaeaceae can tolerate aerobic environments, where they co-occur with various Alphaproteobacteria. It should be noted however that the observed co-occurrence does not confirm symbiotic interaction, and co-occurrence alone is not a reliable proxy for oxygen in the absence of biogeochemical measurements." We do not exaggerate the findings to the level of abundance-based analyses.

L194. Nearly all samples containing Hodarchaeales also harbor Alphaproteobacteria, yet sites lacking Hodarchaeales may likewise show high Alphaproteobacterial abundance. Provide comparative statistics to determine whether the association is significant.

We appreciate the reviewer's observation and agree that Alphaproteobacteria are not dependent on the presence of Hodarchaeales. Our intention is not to imply a strict dependency, which we acknowledge with "observed co-occurrence does not confirm symbiotic interaction". Rather, we are just highlighting that today's Hodarchaeales co-occur with Alphaproteobacterial lineages, and this may be interesting to investigate in the future with other approaches. Given our aim and this is just a minor point of the manuscript, we do not believe statistical testing of co-occurrence or co-abundance is necessary. Even if such an association was statically significant, it would not be sufficient to confirm symbiosis at the genomic level.

L198. If some Kariarchaeaceae and Hodarchaeales are truly adapted to aerobic environments, indicate their positions on the phylogeny. Are these taxa dispersed across the tree or concentrated in particular clades (e.g., guild 12) or in other guilds? Mapping oxygen-tolerant lineages onto the phylogeny would clarify this point.

We have mapped taxonomy, sampling location, guilds, hydrogenase types, cytochrome c oxidase (Cox123), nitrogenases (NarGHJ), catalases, catalase-peroxidases, peroxidases, superoxide dismutases, enzymes enriched in anaerobes (1.6.3.4, 1.3.3.13, 1.14.13.163, 1.14.11.6, 1.13.12.7, 1.13.12.5, 1.14.13.208) and total number of oxygen-utilizing enzymes on the Asgardarchaeota phylogeny from Fig. 1b (Supplementary Fig. 81) and then provided a zoomed in view of Heimdallarchaeia (Supplementary Fig. 82).

Table S10. I welcome the abundance information for Hodarchaeales, Alphaproteobacteria and other microbial lineages. However, the current data do not

clearly support an interaction between Hodarchaeales and Alphaproteobacteria, nor do Alphaproteobacteria appear to be a reliable proxy for oxygen.

We thank the reviewer for acknowledging our previous revisions, which added abundance information across NCBI Sequencing Read Archive (SRA) public metagenome sets (including binned and unbinned data) prior to 2021. We have further revised the text to explicitly state this is co-occurrence, and we are prudent not to use co-abundance.

Specifically, we removed "We also identify nearly all Asgard lineages co-occurring with aerobic bacteria, further supporting oxygen tolerance across the phylum," and modified "adapted to aerobic environments" to "can tolerate aerobic environments". Further, we end this discussion in the main text with "It should be noted however that the observed co-occurrence does not confirm symbiotic interaction, and co-occurrence alone is not a reliable proxy for oxygen in the absence of biogeochemical measurements."

The purpose of this statement is to provide evidence they can co-occur in today's environments, which is interesting based on our continued indication that these lineages are the descendants of the key players in eukaryogenesis. Based on the reviewer's suggestions, this is now more clearly reflected in our revised text.

For example, Kiloniellales are present in every Bohai sample but many of those samples lack Hodarchaeales or Kariarchaeaceae, whereas Hodarchaeales is present in most GE2 samples where Kiloniellales are absent. Additional evidence is needed, or the wording should be softened. More importantly, it is just a co-occurrence phenomenon, which does not provide evidence for symbiosis. I think it can be deleted but depend on authors' choice.

We agree and have revised the wording to soften the conclusions. We feel this now presents an interesting observation to be considered in future studies, but does not overstate our results.

Major comments 2

Metabolic reconstruction of Hodarchaeales, Kariarchaeaceae , and even LAECA rests largely on hydrogenases and on electron-transport chain complex IV. Hydrogenases – Please label the phylogenetic tree with catalytic direction (hydrogen-evolving, hydrogen-oxidizing, bidirectional) and assess critically how confidently type can be inferred from phylogeny and structural models, especially for the novel Asgard-specific enzymes.

We have confirmed the newly proposed Asgardarchaeota [NiFe]-hydrogenases are valid through examining signature cysteine-binding motifs via the sequence alignments and through comprehensive phylogenic analysis, as well as, with structural modeling. Our previous work has illustrated that hydrogenase directionality is strongly correlated with large subunit phylogeny and subunit

organization have conserved catalytic directions (primarily Greening et al., 2016). We have added this information for each group based on previously biochemically characterized hydrogenases in Supplementary Fig. 30 and legend, the legend of Fig. 3a, and provide additional details (some detailed below) in the Supplementary Discussion of the hydrogenases.

In the Asgardarchaeota genomes, we identified Group 1 (respiratory and hydrogen-oxidizing), 3 (cofactor-coupled and bidirectional), and 4 (energy-converting and typically hydrogen-evolving) (Søndergaard et al., 2016). Within our phylogenetic analysis (Supplementary Fig. 30), we see clear division between the four main hydrogenase groups (Group 2 not identified in Asgardarchaeota). We identified hydrogen-oxidizing Group 1 in Baldrarchaeia, Hermodarchaeia, Kariarchaeaceae, Njordarchaeales, Sifarchaeia, and Wukongarchaeia. Specifically, Kariarchaeaceae encodes subclade 1d, which has previously been characterized as oxygen-tolerant and used for electron input in aerobic respiration and oxygen-tolerant anaerobic respiration (Jespersen et al., 2025). Group 3 hydrogenases were identified in all Asgardarchaeota lineages, while Group 4 hydrogenases were identified in every Asgardarchaeota lineage except Lokiarchaeales.

However, it should be noted some hydrogenases vary in directionality depending on the physiology of their host. A few are even physiologically reversible and will switch directionality depending on factors such as cellular redox state. Group 3 and 4 [NiFe]-hydrogenases, especially, vary in their directionality. They often act in a hydrogen-oxidizing direction in autotrophic organisms, for example in methanogens, but instead primarily produce hydrogen in heterotrophic organisms including the cultured Asgard archaea. This is appropriately reflected in the main text and supplementary discussion in the current and previous version, e.g. "Two studies have alternatively proposed these hydrogenases mediate hydrogenotrophic acetogenesis, yet Asgard archaea culture-based studies reveal these enzymes enable H_2 production" and "They likely function by coupling the oxidation of reduced ferredoxin to the reduction of protons to H_2, resulting in the pumping of protons through its membrane arm, though a role in H_2 oxidation cannot be definitively ruled out."

Overall, it is unlikely that the Asgard archaeal lineages encoding both Group 3 and 4 [NiFe]-hydrogenases require a hydrogen-producing partner but are more likely are hydrogen producers. This is consistent with the phylogenetic and structural inferences made about these hydrogenases, especially when placed in conjunction of the broader metabolic inferences and the wider literature. We do not deny hydrogen could be provided by a partner, but that it is not a necessity for these lineages.

Maybe it is better to explain why these hydrogenases were missed in earlier surveys; and further re-analyzed public archaeal genomes with the new HMMs to see whether they are genuinely novel. Check whether homologous group 4 hydrogenases occur

outside Asgard and map their distribution among hydrogen-producing versus hydrogen-consuming lineages.

The expanded number of Asgardarchaeota MAGs presented in this study, along with the application of complex mixture models, enabled us to detect clades that do not cluster with any previously identified [NiFe]-hydrogenases groups. As reflected in our Cell 2024 paper (Greening et al., 2024), homology-based searches alone often miss the vast diversity of hydrogenases in nature. As explained above, our prediction of hydrogenase directionality is based on detailed consideration of phylogenies, structures, and wider physiology. Mapping the hydrogenases onto hydrogen-producing or hydrogen-consuming lineages will not be fruitful, since the vast majority of the hydrogenases in Asgard are unique, with a few abovementioned exceptions. Moreover, the variable environmental redox conditions and metabolic flexibility of archaea encoding multiple hydrogenase groups will not provide any further clarity.

Instead, we constructed an HMM profile based on our updated [NiFe]-hydrogenase phylogeny and used it to re-screen a phylogenetically diverse dataset, including 987 archaea and 1991 bacterial genomes (>50% complete and <10% contamination based on CheckM2). We filtered potential hydrogenases using the same steps described in the methods for the Asgard sequences and added 2126 sequences to our previous analysis. We checked these potential hydrogenases with a phylogeny, using the same complex mixture model chosen on previous analysis of the [NiFe]-hydrogenases. We used this phylogeny to identify potential non-Asgardarchaeota sequences grouping with these newly identified Asgard hydrogenases. Our results reveal that 0 bacterial lineages and 18 archaeal orders outside Asgardarchaeota also encode hydrogenases that group phylogenetically with some of these newly identified [NiFe]-hydrogenase subclades. We list the identified lineages below by hydrogenase subclade and provide the sequences in Supplementary Table 8. Though these lineages are found in archaea other than the Asgards, they remain novel and unclassified. Whereas subclades 1n, 3e, 3g, 3h, 3i, 4j, 4k, 4m, 4p, and 4r remain Asgard-specific based on our expanded analysis.

1m: Bathyarchaeia, Altiarchaeia, Syntropharchaeia, Halobaeteriota (UBA148), Archaeoglobi, Korarchaeia, Thermoproteota (JANJXX01), Thermoprotei, Syntropharchaeia, Methanocellia
3f: Korarchaeia, Bathyarchaeia
3j: Bathyarchaeia
3k: Korarchaeia, Hadarchaeota (B88-G9), Thermoproteota (EX4484-205), Thermoproteota (JANJXX01)

3l: Bathyarchaeia, Thermoproteota (JANQNL01), Thermoproteota (JANJXX01)
4l: Bathyarchaeia, Thermoproteota (QMWL01), Thermoprotei A, Methanomethylicia, Thermoplasmata, Nitrososphaeria A, Nitrososphaeria, Thermoprotei, Methanocellia
4n: Bathyarchaeia, Thermoproteota (EX4484-205)
4o: Bathyarchaeia, Thermoproteota (EX4484-205)
4q: Thermoproteota (EX4484-205), Thermoproteota (JANJXX01), Bathyarchaeia, Nitrososphaeria A, Nitrososphaeria, Thermoprotei, Thermoprotei A, Methanomethylicia

Additional biochemical analysis is necessary to better understand the catalysis and role of these numerous subgroups, but this study's focus is Asgardarchaeota and further analysis outside of this phylum is beyond the scope of our analysis. Given the suggestion by the reviewer, we now provide a more comprehensive characterization of [NiFe]-hydrogenase diversity in archaea.

Complex IV – This complex may also occur in Aigarchaeota and Halobacteriota. Among these, Aigarchaeota, not Poseidoniia, may be the nearest sister lineage of Hodarchaeales, though this needs testing. A previous study linked the Hodarchaeales complex IV to nitrate respiration, whereas in Halobacteriota it is thought to function in copper metabolism. Revisit screening criteria, survey its archaeal distribution again, and discuss whether the Hodarchaeales complex uses oxygen directly.

We appreciate the reviewer's insight. The phylogeny does include representatives from Aigarchaeota (d__Archaea; p__Nitrososphaerota) and Halobacteria. These two archaeal lineages are clustered together in the phylogeny. Please see Fig. 3b which highlights the positioning of Nitrososphaeria and Halobacteria. Neither is the closest lineage to the Heimdallarchaeia sequences.

A previous study did link complex IV to nitrate respiration, which we already discuss within the manuscript adding additional information on presence of nitrogenases. Further, we add structural analysis of these complexes, which is discussed below in response to the reviewer's other concerns.

Key functional genes should be mapped onto the species tree and examined for vertical versus horizontal inheritance. Only guild labels are shown at present, which is misleading: the basal lineage is Hodarchaeales, not Kariarchaeaceae (the main member of guild 12). The placement of GCA_022574115 is tricky; please show it clearly.

We have mapped taxonomy, sampling location, guilds, hydrogenase types, cytochrome c oxidase (Cox123), nitrogenases (NarGHJ), catalases, catalase-

peroxidases, peroxidases, superoxide dismutases, enzymes enriched in anaerobes (1.6.3.4, 1.3.3.13, 1.14.13.163, 1.14.11.6, 1.13.12.7, 1.13.12.5, 1.14.13.208) and total number of oxygen-utilizing enzymes on the Asgardarchaeota phylogeny from Fig. 1b (Supplementary Fig. 81) and then provided a zoomed in view of Heimdallarchaeia (Supplementary Fig. 82).

Finally, the manuscript focuses on the gain of aerobic traits but does not assess whether Hodarchaeales and Kariarchaeaceae have concurrently lost core anaerobic pathways. A genome-wide survey of canonical anaerobic markers would address this gap. To my knowledge, pyruvate formate-lyase—a hallmark gene of anaerobic metabolism—is widespread in Hodarchaeales.

As previously mentioned, enzymes enriched in anaerobes (1.6.3.4, 1.3.3.13, 1.14.13.163, 1.14.11.6, 1.13.12.7, 1.13.12.5, 1.14.13.208) and total number of oxygen-utilizing enzymes on the Asgardarchaeota phylogeny from Fig. 1b (Supplementary Fig. 81) and then provided a zoomed in view of Heimdallarchaeia (Supplementary Fig. 82). However, we note that aerobic and anaerobic markers are difficult to determine but provide additional details on our previous in-depth analyses.

Specific modifications needed
Line 24: "including 136 new Heimdallarchaeia (49 Hodarchaeales)" — please remove "(49 Hodarchaeales)"

Removed.

Line 77: "both the Hodarchaeales and the Heimdallarchaeia class" appears redundant since the latter encompasses the former

We removed Hodarchaeales here.

L110. As shown in Fig. 1, the purported "expanded genomic diversity" does NOT "increases support in the phylogeny for the deep-rooted clades". Please verify this point and delete or revise the statement and any related claims.

This statement was revised "adding additional representatives from deep-rooted clades (i.e., Frey/Jordarchaeia)".

L115. An increase in genome number does not automatically lead to an increase in phylogenetic diversity. Please quantify how much the additional genomes actually expand phylogenetic diversity, particularly for the Kariarchaeaceae and Hodarchaeales lineages.

We calculated phylogenetic diversity (PD) using the phylogeny shown in Fig. 1a, assessing the contribution of the genomes added in this study. We conducted this analysis across all Asgardarchaeota, and specifically within the Hodarchaeales

and Kariarchaeaceae lineages as requested. The results are now included in the "Phylogenetic diversity" section of the supplemental discussion, which is now cited in the main text after this statement. We provide the R script in the data files for Fig. 1 uploaded on FigShare. We also show the results in the table below.

Briefly, the genomes added in this study resulted in a 27.46% increase in overall Asgardarchaeota PD, a 44.43% increase within Hodarchaeales, and a 20.30% within Kariarchaeaceae. In addition to identifying a new Asgardarchaeota class, Ranarchaeia, and providing the first comprehensive metabolic description of Asgardarchaeia, these results further support the evolutionary distinctiveness of the genomes added by this study, particularly a substantial expansion in Hodarchaeales diversity.

Phylogeny	Clade	PD_public	PD_combined	PD_gain	PD_gain (%)
Fig. 1a	Asgardarchaeota	81.30	103.62	22.32	27.46
Fig. 1a	Hodarchaeales	8.16	11.79	3.63	44.43
Fig. 1a	Kariarchaeaceae	5.64	6.79	1.15	20.30

L123. The current guild classification separates Kariarchaeaceae and Hodarchaeales into distinct branches. Please clarify the rationale for this split. Was it driven by horizontal gene transfer affecting particular genes, and if so, which ones?

Our analysis split Kariarchaeaceae into guilds 9 and 12, and Hodarchaeales into guilds 11 and 12, with Guild 12 including genomes from both lineages.

As described in the methods section, we determined the presence-absence profiles of protein families across the Asgard catalog. We calculated Jaccard distance and applied Ward variance minimization to cluster MAGs, testing various maximum distance thresholds. A threshold of 0.35 resulting in biologically relevant guild division, capturing taxonomic relationships and environmental distributions.

This method is not designed to resolve the evolutionary origin of individual genes, and we did not assess whether horizontal gene transfer separated guilds 11 and 12. Instead these guilds reflect broad metabolic and ecological division, which is supported by comparative genomic analyses. These analyses identified unique functional annotations (e.g., PFAM, KEGG, InterPro), applying presence-absence thresholds (100%, 75%, 25%, 10%, and 5%) across taxonomy, environment, and guild. We used this analysis to describe differences in metabolism between guild 11 and 12 in the main text and further detailed in the supplemental.

Previously, we have chosen several genes/proteins in our analysis to run phylogenomic analyses. Yet to untangle the evolutionary origin of each gene across the guilds is beyond the scope of this study, as this is focused on the ecology

of these archaea, not their taxonomy. However, we do provide a separate classification for the entire Heimdallarchaeia class with the same presence-absence thresholds to determine features across taxa and environments.

L327. Please add a species phylogeny indicating the sampling location, predicted lifestyle (aerobic vs. anaerobic), and the presence or absence of hydrogenase, cox complex, narGHJ, and other key metabolic genes.

As mentioned previously, we now provide this in Supplementary Figs. 81 and 82.

L376. The authors state that the first Hodarchaeales co-cultures "are unlikely to be representative of all Heimdallarchaeia lineages." In reality, these two strains exhibit remarkable genome expansion, evocative of the genomic complexification associated with eukaryogenesis. The manuscript should offer a more balanced appraisal of the relevant studies.

We agree, these co-cultures provide incredible opportunities to understand Asgard physiology. However, we still believe these two lineages isolated from anaerobic environments and enriched under anaerobic conditions do not represent the entire Heimdallarchaeia class. Further, the genome expansion has previously been documented for Hodarchaeales genomes (Spang et al., 2017; Eme et al., 2023), but also does not represent the entire Heimdallarchaeia class. As such, we have revised the statement to reflect this advancement, while also preserving that environmental samples and metagenomics are vital in our understanding of these lineages.

We have revised the statements as follows "Of note, these two strains were isolated anaerobically from anoxic environments. Despite this advancement, these strains are unlikely to be representative of all Heimdallarchaeia lineages."

L424. The relationship between the coastal/deep-sea MAGs and guilds 11/12 should be clarified more explicitly.

This is thoroughly described in the Supplemental materials see "Guild 11 and 12" section for Hodarchaeales. However, we have now added additional details on Kariarchaeaceae.

L425. Please clarify how the structural models of Complex IV differ when configured for oxygen versus nitrate as the terminal electron acceptor.

There is no structural reconfiguration of Complex IV (cytochrome c oxidase) for nitrate reduction. Complex IV uses oxygen as the terminal electron acceptor. In contrast, nitrate reduction is carried out by nitrate reductase (NarGHI). When both complexes are encoded within a genome, they provide metabolic flexibility, allowing the organism to switch between aerobic and anaerobic respiration depending on environmental oxygen availability.

We discuss this metabolic flexibility in the main text "It is possible that like their relatives [Hodarchaeales] in variably oxygenated environments, such as coastal sediments, these cultures under other growth conditions are also facultative anaerobes, necessitating future culture-based studies" and further "Contrary to recent inferences that the LAECA was an obligate anaerobic H_2-dependent acetogen, our results suggest it was instead an aerotolerant or facultatively anaerobic H_2-producing microorganism with a hybrid fermentative and respiratory lifestyle."

Also, we discuss this transition in the main text see "Such a transition would necessitate the use of high-potential terminal electron acceptors like oxygen or nitrate. However, we found that nitrate reductase genes (*narGHJ*) were encoded exclusively in the deep sea and hot spring Hodarchaeales and Kariarchaeaceae MAGs, and were absent in the coastal Asgard MAGs, suggesting that these organisms do not use nitrate as a terminal electron acceptor (Supplementary Figs. 53-56)." Given the reviewer's suggestion we now have also mapped genes/proteins onto the phylogeny see Supplementary Figs. 81 and 82.

Line 455: "The presence of ETC components in the Asgard ancestor of eukaryotes would have" should be modified to "The presence of ETC components in the Heimdallarchaeial ancestor would have"

This paragraph has been revised to no longer include this statement.

Lines 472-474: "...especially in Hodarchaeales and Kariarchaeaceae, support that the Asgard archaeal ancestor of eukaryotes was likely…" should be modified to "...support that the Heimdallarchaeial ancestor was likely…"

This statement has been revised.

L484. I wonder if it might be best to remove Ran and Asgardaechaeia from Fig. 5. The order of GOE, eukaryogenesis, and FECA appears to be problematic. Figure 5 is still to complex for clearly show your idea. The left panel with blue background is not so important, I think it can be removed. For example, what is relationship with the deep-sea hydrothermal vents? Does the interaction only happen in coastal water column?

We have revised this figure to have multiple panels to avoid confusion in the timeline and simplified for clarity. This shows the metabolic transitions between deep-sea and coastal environments, which has now been simplified and updated.

Referee #3 (Remarks to the Author):

I would like to thank the authors; the revised version of the manuscript addresses my major concerns. My remaining suggestions would be to clarify some of the thinking, descriptions and figures regarding the evolutionary scenario presented, to investigate whether newly published information on Njordarchaeales MAGs might shed light on some of the phylogenetic analyses, and to ensure that methodological detail given is consistent between different parts of the manuscript (e.g., between the main text, figure legends, and supplementary materials).

We thank the reviewer for acknowledging our previous revisions and continued helpful suggestions. Our detailed responses for each point are described below.

Eukaryogenesis:

The abstract and the legend for Fig. 5 describe a 'Heimdallarchaeia-centric model of eukaryogenesis'; I find the use of '-centric' here confusing, it's unclear to me whether it's used because the model is developed based on inferences drawn from features in modern members of Heimdallarchaeia, or because it conflates eukaryogenesis with the mitochondrial symbiosis and focuses primarily on the evolution of the host. Either way, I feel that it would avoid confusion if either a more descriptive term were used, or if the evolutionary scenario were presented without the name.

We appreciate the reviewer's feedback. Yes, we were referring to this model developed from features in modern Heimdallarchaeia, not necessarily that focusing solely on the host. However, we have changed the caption to Eukaryogenesis in light of an expanded catalog of Asgard genomes. Now, we present the scenario without a name, which avoids the concern of using Heimdallarchaeia or the term "updated".

(Less importantly, use of the word 'updated' would also be more appropriate when referring explicitly to a previously described and named model: for instance, 'an updated version of the reverse-flow model of eukaryogenesis'.) Probably to maintain the linguistic link with this model name, the Fig. 4 legend title mentions 'Heimdallarchaeia", but since the diagram only presents the ETC of Hodarchaeales, it would be more accurate to change the title to either reflect this specificity, e.g. 'in Hodarchaeales (Heimdallarchaeia)'. The authors could also spell out the implications in the figure legend if they wished: that Hodarchaeales are the closest sister group to eukaryotes, and that the ETC of their shared ancestor with eukaryotes may have been similar.

As previously mentioned, we removed the name to avoid the concerns of using Heimdallarchaeia or the term "updated". We have also added the suggested "in Hodarchaeales" to Fig. 4 legend.

The timeline shown in Fig. 5 is not to scale, but even beyond that, the combination of

the timeline with some of the figure's other elements is still confusing. The relative positioning of Hodarchaeales and Kariarchaeaceae (both members of Heimdallarchaeia) and 'Heimdall ancestor' makes it look as though the former pre-date the latter. It took a bit of squinting at the figure on my part to see that the authors probably mean the right-hand portion of the figure to be an inset from the small red cell positioned under the word 'Aerobic'; but if so that's not really clear given the timeline, and in any case I think that the relative positioning of the cell representing Hodarchaeales and Kariarchaeaceae below this cell is still confusing. The inclusion of 'Heimdall' near the top right is also confusing: does this represent the emergence of the clade (if so, why is it positioned after the emergence of eukaryotes), or the simple existence of modern members of Heimdallarchaea? (It's also a confusing abbreviation given the existence of Heimdallarchaeaceae, see comment below.) Why show two different cells representing, respectively, 'Hodarchaeales and Kariarchaeaceae' and 'Contemporary Hodarchaeales and Kariarchaeaceae lineages'? Basically, I feel that it would be much easier to understand this figure if it were more clearly split into several distinct sections (especially if the number of sections could be minimised).

We have revised Fig. 5 accordingly.

The Fig. 5 legend suggests that 'LAsCA diversification allowed widespread niche expansion', and I think it's worth clarifying what the authors mean by this. Firstly, the meaning of 'diversification' isn't clear, and secondly, regardless of the meaning of this term, it's unlikely to apply to the LAsCA itself. I think that 'Genetic diversification in descendents of the LAsCA allowed their occupation of a variety of new niches' could be what the authors meant, but I may be wrong.

We have revised the legend, and the figure as suggested.

Line 89, changing "the archaeal host was an anaerobe" to "the host was an anaerobic archaeon" or "the host was an archaeon and an anaerobe" would be more accurate

We have revised to "the host was an anaerobic archaeon".

Line 99: The authors that FECA is the same thing as the first eukaryotes, but this is unlikely to be true: FECA is defined as the first descendant on the eukaryotic branch of the last common ancestor of eukaryotes and their closest archaeal relatives. It is therefore to be expected that many descendant lineages of the FECA went extinct in the interval between its existance, and the emergence of cells with key eukaryotic features. This is true whether one favours a 'mitochondria-late' scenario (in which the FECA might have had many descendents with increasing degrees of complexity in their endomembrane system and cytoskeletal organisation), or 'mitochondria-early' (in which the FECA would likely not have been a eukaryote at all, but rather a nucleus-lacking archaeon with an alphaproteobacterial endosymbiont). Likewise, the position of the FECA should be drawn much earlier along the timeline

shown in Fig. 5, immediately after the divergence of the Heimdall ancestor from the eukaryote ancestor

We have revised Fig. 5 accordingly.

Line 104-105, "it is plausible that the archaeal host and potentially some of its modern descendants" - This is confusing, as eukaryotes themselves are descended from this host and tolerate oxygen. Maybe something like, "it is plausible that the archaeal host and potentially some of its close modern archaeal relatives"?

We see the confusion and have revised as suggested.

Njordarchaeales MAGs:

The authors mention (in supplementary methods and review responses) potential artifacts introduced by sequences from the Njordachaeales clade. Some MAGs from this clade have recently been reported to be chimeric (Zhang et al. 2025 https://doi.org/10.1038/s41586-025-08955-7), so it might be helpful to investigate whether this affects the authors' dataset and might explain the apparent artifacts they report

Since the publication of Zhang et al., 2025, the placement of Njordarchaeales and potential artifacts has been re-analyzed in-depth by Huang et al., 2025 in Molecular Biology and Evolution (https://doi.org/10.1093/molbev/msaf201). This analysis supports the positioning of Njordarchaeales within Asgardarchaeota, supporting previous analysis of Eme et al., 2023. This study also found that the Njordarchaeales MAGs showed low/moderate levels of contamination, but not in the phylogenetic marker genes that were used to position eukaryotes (showing relationship with Hodarchaeales). Therefore, this "chimerism" would not impact the phylogenetic placement. Our study uses filters, multiple annotation pipelines, presence in numerous genomes, and phylogenetics to test the genes/proteins of interest.

Huang et al., 2025 concluded that poor-fitting phylogenetic models in Zhang et al., 2025, not chimerism resulted in the misplacement of Njordarchaeia within Korarchaeota (Korarchaeia). This supports the previous extensive analysis of Eme et al., 2023 and our phylogenetic analyses presented in this study. As this analysis has been completed, we now cite Huang et al., 2025, as well as Eme et al., 2023, in the supplemental methods and discussion.

Methodological detail:

The authors state in their response to one of my earlier comments (on the interpretation of ultrafast bootstrap values) that they are now showing only ultrafast bootstrap values equal to or higher than 95% for trees in Fig. 3c and supplementary figures, and that they are adding a note of caution regarding that ultrafast bootstrap

values should 'only be relied on when >=95%'. However, they have not done this for Fig. 1, which presents key findings on relationships between Asgard groups.

For Fig. 1a, we are only displaying bootstrap values >=95% for 1000 Ultrafast bootraps and >=80% SH-aLRT. In Fig. 1b, we are showing bootstrap values based on 100 nonparametric bootstrap pseudoreplicates distinguished with level of support into three categories of increasing support (70=<x<95, 70=<x<100, and =100). However, we see the potential confusion for the reader and have added into the legend "with 1000 ultrafast bootstraps and SH-ALRT tests" for Fig. 1a and "with 100 nonparametric bootstrap pseudoreplicates" for Fig. 1b. We also modified Fig. 1a adding UF for ultrafast bootstrap and ≥80% SH-aLRT and for Fig. 1b we leave Support values for nonparametric bootstrap values. As we are not showing ultrafast bootstrap values below 95, we have not added a note of caution for this figure.

The legend for Fig. 1 states that the species phylogeny was reconstructed with the WAG+C10 model, but the supplementary methods instead state that WAG+C10+G4 was used

We used WAG+C10+R4, which has now been correct in the legend and was accurately described in the supplementary methods.

Fig. 1 legend, do these include only genomes with >50% (as written) or also >45%?

Only genomes >50%.

Line 1460, were publicly available MAGs with >45% completeness included here, or only >50%?

This has been revised to >50% based on CheckM v1.2.1, which we present in Supplementary Table 4. We missed revising this text in our previous revision in which we updated the analysis of one MAG (D4993_C5_H3_Bin_385), which we reported in our first submission with the wrong marker set p__Euryarchaeota (UID4), but with the correct marker set k__Archaea (UID2) completeness was 51.87% and contamination 0.93%. Thank you for catching this oversight.

Minor points:

The authors mention which groups are included within Heimdallarchaeia, but for a general audience, it would really be helpful to show on Fig. 1b (not just on Suppl. Fig. 3) which groups are included within Heimdallarchaeia. Similarly, consistency should be used for the abbreviation 'Heimdall', which is used for Heimdallarchaeaceae in Fig. 1a but for Heimdallarchaeia in Fig. 4.

We agree this would help provide clarity within the field and have updated Fig. 1b to show the Heimdallarchaeia class similar to the supplementary figure.

Further, we leave Fig. 1a as the shorthand (to avoid reducing the font size) but have changed the other figures to only use Heimdall when referring to Heimdallarchaeaceae and to spell out Heimdallarchaeia. To make sure this is clear we have also added this in the legends of the main figures. We do not see Heimdall in Fig. 4 but have similarly altered Heimdallarchaeia in the updated Fig. 5.

Line 100, "between Asgard archaeon" should read "between an Asgard archaeon"

Revised.

Line 308, "expanding the known diversity" should read "expanding their known diversity"

Revised.

Line 314, "increase membrane potential" should read "increase in membrane potential"

Revised.

Line 543, "public metagenomes prior to 2021" should read "public metagenomes published prior to 2021"

Revised.

Line 552, "interest on" should read "interest in"

Revised.

*Line 554, the terms in brackets should be clarified, e.g. "that can break down carbohydrates (*using* CAZymes)"*

We have modified it to using CAZymes and now also write out the acronym.

Line 666, I would change "adopts a configuration like Complex I" to something like "adopts a configuration similar to that of Complex I"

Revised.

Line 1707, "The output was filtered to remove those sequences >200 amino acids..." - I think this should be 'extract' or 'retain' rather than 'remove'

Yes, thank you for catching this. The text has been updated to "retain those sequences >200 amino acids with two CxxC motifs 200 residues apart".

Line 1737, 'we chose a reference dataset' - I think this should read 'we constructed a reference dataset' (unless the dataset was an existing one, in which case a citation should be given)

Revised with "constructed".

Fig. 1. I think my point about roots in my earlier review was misunderstood: if the phylogenies were generated without specifying a root, then the trees can be displayed rooted on any clade (as the authors have appropriately done), but the little line representing the root itself should be removed (this can be done easily in a figure editing program such as Inkscape or Illustrator)

Thank you for your clarification and we apologize for our misunderstanding of your previous suggestion. We have gone back through and removed the line representing the root for Suppl Figs. 15-27; 30; 37-40; 53-59; 62-67.

Fig. 5 legend has a typo, "Heimall" instead of "Heimdall"

Revised.

Supplementary materials line 377, the wording of "for each marker a total of 1000 ultrafast bootstraps and SH-like approximate likelihood ratio tests" should be clarified as "for each marker a total of 1000 ultrafast bootstraps and 1000 SH-like approximate likelihood ratio test replicates"

This has been modified accordingly.

Supplementary materials line 640, a reference should be provided for CombFold.

Thank you, the reference has been added.

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have addressed all of my previous concerns in this revision. The manuscript represents an important step forward in our understanding of Asgard archaeal diversity and eukaryogenesis.

We appreciate the reviewer's suggestions and support throughout the revision of this manuscript.

Referee #2 (Remarks to the Author):

The authors have done an outstanding job addressing the previous points of critique. I have no further substantive comments.

We appreciate the reviewer's continued positive view of this manuscript.

Please remove any reference duplications that exist between the main article and the supplementary files.

We have updated the references as suggested by editorial comments.

Referee #3 (Remarks to the Author):

I would like to thank the authors for thoroughly addressing my concerns. I have no more major suggestions, and only a couple of minor ones:

We appreciate the reviewer's support and suggestions.

- that the same support criteria (>=80% SH-aLRT as well as 95% ultrafast bootstrap support) be applied for Figure 3 as for Figure 1, and similarly that the lines representing the roots be removed from these figures (in line with an earlier suggestion; ditto supplementary figure 82)

We have updated Figures 3ab to include only nodes with ≥80% SH-aLRT and ≥95% ultrafast bootstrap support. Additionally, for consistently we updated Supplemental Figures 15-27; 30; 37-40; 42-44; 57-60; 72-74 to highlight nodes ≥80% SH-aLRT and ≥95% ultrafast (UF) bootstrap support.

Figure 1a was accidently reported in the last round of revision to show SH-aLRT and ultrafast bootstrap support. However, Figure 1a shows the non-parametric pseudoreplicates as correctly described in the main methods and shown previously. We have updated the figure (removing the ≥80% SH-aLRT and changing UF Boot to Support values) and the legend to describe the support values as 100 non-parametric pseduoreplicates.

The roots were also removed for Figures 3ab and Supplementary Figure 82.

- the legend for Supplemenary Figure 83 states that "In Heimdallarchaeia (red), we identify..." if the red cell shown is Heimdallarchaeia, then it's not the FECA (as currently labelled)

Supplementary Fig. 83 legend has been updated to "The potential gain of ETC complexes (e.g., III and IV: yellow) prior to the first eukaryotic common ancestor (FECA; red) would have increased energy yield and reactive oxygen species (ROS) production. In Heimdallarchaeia, we identify the loss of nitrate reduction (NarGHJ) and…". Showing here we are referring to FECA.

- similarly, the same sentence appears in the figure legend for main Figure 5, where all of the descendants of the LAsCA, not just Heimdallarchaea, are shown in red

We have rewritten Fig. 5 legend to make this clearer that red cells highlight the archaeal ancestor from Last Asgard-Eukaryotic Common (LAECA) ancestor to modern Heimdallarchaeia and Eukaryotes.

- some of the features of Fig. 5b are described in the figure legend for Fig. 5a

We have rewritten Fig. 5 legend to improve clarity.

- both Fig. 5a and supplementary fig. 83a should show units (i.e. 'Ga' or similar) for the timeline

Units (Ga) have been added to the timelines in both Fig. 5a and Supplementary Fig. 83a.