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CORRESPONDENCE

# The LECA had a conventional kinetochore and the kinetoplastid kinetochore is a derived feature – a critical evaluation of Akiyoshi, 2025

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## Introduction

Ever since the first genomes of divergent protist lineages were sequenced, inferences of the gene repertoire of the last eukaryotic common ancestor (LECA) have led to what has been dubbed our "incredible expanding ancestor"; a highly gene-rich and molecularly complex LECA (Koonin, 2010). To make sense of this perhaps counterintuitive ancestral complexity – and to better understand the prokaryotic origins and cell biological nature of the LECA, concepts such as the LECA pangenome have been posited (O'Malley et al., 2019) and much effort has gone into pinpointing the root of the eukaryotic tree of life (eToL). Yet the inferred large proteome of the LECA suggests that many hallmark features of eukaryotic cell biology are ancestral despite being absent from prokaryotes, highlighting the vast evolutionary and cell biological gap between prokaryotic and eukaryotic cells, one of the major enigmas of present-day biology (Richards et al., 2024). In this context, the recent Hypothesis article by Akiyoshi (2025) published in Journal of Cell Science is a welcome attempt to contribute to the LECA debate. Drawing on the presence of a unique core cellular system in the flagellated protist lineage of kinetoplastids, which includes medically relevant human parasites, Akiyoshi presents a hypothesis with far-reaching implications on the placement of the root of the eToL and our subsequent interpretation of the nature of the LECA. Most notably, this hypothesis entails that the LECA lacked a conventional kinetochore. Here, we critically evaluate Akiyoshi's hypothesis and conclude that it leads to a highly implausible evolutionary scenario.

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## The hypothesis of a kinetoplastid-first scenario in the eToL

The first node on the eukaryotic tree of life is the root of the eToL and thus represents the LECA. Consequently, identifying the root of the eToL is crucial in our quest to understand the nature of the LECA, including the origin of the hallmark molecular systems of eukaryotic cells, such as those required for meiosis and mitosis. Inferences of gene repertoire for the LECA are based on the presence of orthologs of a gene in extant representatives of lineages on both sides of the eukaryotic root (Richards et al., 2024). Under multiple plausible positions for the root of the eToL (see Box 1 in Akiyoshi, 2025), this logic has led to the inference that many key eukaryotic systems were already present in the LECA, including the conventional kinetochore, which is found across virtually all lineages of the eToL (van Hooff et al., 2017). Despite its pivotal role in understanding early eukaryotic evolution, the placement of the root is subject of an ongoing debate, as pointed out by Akiyoshi (2025).

Akiyoshi's hypothesis places the root of the eToL between kinetoplastids and all other eukaryotic species. This is based on the absence of a conventional kinetochore in kinetoplastids, which instead have a unique and near-analogous kinetochore system with only few remote homologs to canonical kinetochore components (D'Archivio and Wickstead, 2017; Ballmer et al., 2024). Akiyoshi deems it improbable that an essential and complex cellular machine such as the conventional kinetochore was lost and subsequently replaced in kinetoplastids. From this basis, Akiyoshi argues that kinetoplastids diverged from all other eukaryotes before the emergence of the conventional kinetochore, with the implication that the LECA did not have a kinetochore and that the conventional kinetochore and the kinetoplastid-specific kinetochore evolved independently. This scenario also raises the possibility that during eukaryogenesis, meiosis and mitosis evolved in the absence of a kinetochore system.

This line of reasoning is reminiscent of an earlier debate on the phylogenetic position of eukaryotes that purportedly lack mitochondria. These 'amitochondriate' eukaryotes were once believed to have diverged from other eukaryotes before the acquisition of mitochondria during eukaryogenesis (Cavalier-Smith, 1983). However, these organisms have now been firmly established as relatives of species possessing mitochondria, and most have been shown to possess highly reduced mitochondrion-related organelles, proving that the absence of classical mitochondrial pathways in these organisms is the result of secondary loss (Embley et al., 2003). Thus, it has historically been shown that interpreting apparent absences of core cell biological features as ancestral, rather than as the result of secondary loss, can lead to spurious inferences on the phylogenetic position of taxa at the root of the eToL.

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Indeed, patterns of dramatic loss and even subsequent replacement of core eukaryotic cellular systems seems to be not so unique after all. Notably, in dinoflagellates the conventional histone-based system of chromatin packaging is largely replaced by viral-derived proteins (Gornik et al., 2012), and in the metamonad *Giardia lamblia*, the entire complement of conventional actin-binding genes is absent (Morrison et al., 2007). A recent characterisation of actin interactors in *G. lamblia* has revealed numerous actin-interacting proteins, many without apparent homologs in other eukaryotic lineages (Steele-Ogus et al., 2021). This mode of evolution has been dubbed 'backfilling', and further examples are outlined in a recent review (Prokopchuk et al., 2023). Furthermore, it is likely that there exist numerous other, yet-elusive examples of backfilling across the eToL, given that a large number of eukaryotic lineages lack representation in current cell biological studies, whereas comparative genomics analyses routinely identify absences of otherwise-conserved genes in select lineages (Prokopchuk et al., 2023). Importantly, kinetoplastids are not the only eukaryotic lineage that lacks a conventional kinetochore – the genome of the metamonad *Carpediemonas membranifera* has also been found to be devoid of orthologs of kinetochore subunits (Salas-Leiva et al., 2021). One should thus be wary of inferring phylogeny based on (the absence of) a limited set of characteristics, especially in the face of pervasive loss of complexity and potential for replacement among even highly conserved features found across the breadth of eukaryotic diversity. Based on these observations, we argue that Akiyoshi's hypothesis, which infers the location of the root of the eToL based on the absence of the conventional kinetochore in kinetoplastids, is unlikely to hold up to critical evaluation.

## The undisputed monophyly of Euglenozoa precludes a kinetoplastid-first scenario

The suggestion that kinetoplastids were the earliest-diverging eukaryotic lineage goes beyond the debate regarding the eToL root, as it is inconsistent with the well-established monophyly (i.e. forming a phylogenetic clade with a common ancestor to the exclusion of all other taxa) of the Euglenozoa, a phylum comprising kinetoplastids, diplonemids and euglenids (Kostygov et al., 2021). Indeed, if kinetoplastids – but not other Euglenozoa – split off from all other eukaryotes at the primary node of the eToL, as suggested by Akiyoshi, the implication is that most Euglenozoa, such as *Euglena*, are more closely related (i.e. having a more recent common ancestor) to all other eukaryotes, than to kinetoplastids (Fig. 1) – indeed violating the well-established monophyly of Euglenozoa. This is an unsustainable proposition for which no phylogenetic or morphological evidence exists. Instead, there is very strong evidence for a close relationship between kinetoplastids and other euglenozoans (Kostygov et al., 2021). Akiyoshi acknowledges this by noting the large number of unique cellular features shared across euglenozoans (Akiyoshi, 2025). Crucially, Akiyoshi's hypothesis does not question the monophyly of the Euglenozoa and does not point out the inconsistency in referring to kinetoplastids as Euglenozoa, while simultaneously arguing for a hypothesis that requires a kinetoplastid-first scenario – thus breaking up Euglenozoan monophyly (Fig. 1).

The kinetoplastid-first placement of the eToL root entails that the LECA itself must have been a euglenozoan-like cell. This is highly improbable given the numerous traits that are shared across euglenozoans but are not found in any other extant eukaryotes (Kostygov et al., 2021). Thus, this scenario would necessitate a large number of secondary losses of these traits in all other eukaryotes. Another possibility that is briefly raised by Akiyoshi is a glycomonad-first scenario, where kinetoplastids branch together with diplonemids (together known as glycomonads) away from all other eukaryotes. This alternative hypothesis is again based on the observation that, in addition to kinetoplastids, diplonemids also appear to lack conventional kinetochore proteins (Akiyoshi et al., 2025; Benz et al., 2024). We note that the arguments raised above apply equally to this variant scenario.

In a 2010 paper, Cavalier-Smith suggested that Euglenozoa represent the first split in the eToL (Cavalier-Smith, 2010), an idea which Akiyoshi uses as further support for a kinetoplastid-first scenario. However, Cavalier-Smith did not propose a kinetoplastid-first scenario but rather a Euglenozoa-first scenario, which, as explained above, is not equivalent – and is even contradictory to – Akiyoshi's hypothesis (Fig. 1). Furthermore, the evidence on which Cavalier-Smith based the Euglenozoa-first scenario – namely, the absence of key eukaryotic genes in Euglenozoa, which was suggested to represent an ancestral intermediate eukaryotic state – is no longer supported. Indeed, numerous genes that Cavalier-Smith believed to have been absent in Euglenozoa have since been identified in extant Euglenozoa (Ocaña-Pallarès et al., 2020; Zarsky et al., 2012; Ebenezer et al., 2019). It is worth pointing out that Cavalier-Smith raised a multitude of mutually exclusive hypotheses on the root of the eToL, and that the Euglenozoa-first scenario was later discounted by Cavalier-Smith himself (Cavalier-Smith, 2021).

Taken together, although it is at the core of Akiyoshi's hypothesis, a kinetoplastid-first scenario for the root of the eToL is not supported. It carries the highly unlikely implication that the groups that comprise Euglenozoa are paraphyletic (i.e. are not each other's closest relatives) in the eToL, and also implies that the LECA was a euglenozoan-like organism, a scenario which cannot be reconciled with the abundance of evidence for euglenozoan monophyly.

## Euglenozoan monophyly results in a robust inference of a conventional kinetochore in the LECA

The established monophyly of Euglenozoa – and thereby the rejection of the kinetoplastid-first scenario – has major implications for Akiyoshi's inferences regarding kinetochore evolution. Recently, various lineages of euglenozoans were shown to possess core components of a conventional kinetochore (Ebenezer et al., 2019; Benz et al., 2024). Consequently, the ancestor of Euglenozoa is likely to also have had a conventional kinetochore. The absence of a

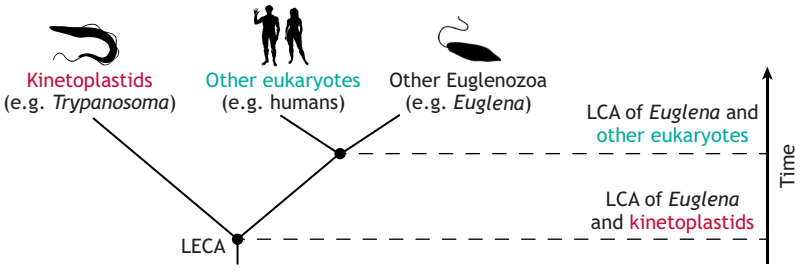


**Fig. 1. A kinetoplastid-first scenario requires the rejection of Euglenozoa monophyly.** Schematic representation showing that a Kinetoplastid-first scenario entails that other Euglenozoa, such as Euglena, are more closely related to all other eukaryotes than to kinetoplastids. This scenario thus rejects Euglenozoa as a monophyletic group. LCA, last common ancestor; LECA, last eukaryotic common ancestor. Silhouettes of *Trypanosoma brucei* and humans were taken from PhyloPic (https://www.phylopic.org/) and are public domain.

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conventional kinetochore in kinetoplastids is thus likely the result of secondary loss and replacement by the kinetoplastid-specific kinetochore. This inference is further supported by the presence of functional components of the spindle assembly checkpoint (SAC) proteins in kinetoplastids (Ballmer et al., 2024). As the SAC is a core kinetochore-based cell cycle checkpoint, the presence of some of its components in kinetoplastids likely represent remnants of a former conventional kinetochore system that was present in their ancestors. It has even been suggested that one kinetoplastid kinetochore component (KKIP1) might be homologous to components of the conventional outer kinetochore (Ndc80 and its homolog Nuf2), implying that the kinetoplastid kinetochore could be partially derived from an ancestral conventional kinetochore (D'Archivio and Wickstead, 2017).

Given this evidence, projecting the trajectory of kinetochore evolution on a Euglenozoa-first version of the eToL, as proposed by Cavalier-Smith (Cavalier-Smith, 2010), makes it clear that the LECA had a conventional kinetochore (Fig. 2A). The inference that ancestral Euglenozoa possessed a conventional kinetochore is further supported by the fact that a group of species known as jakobids have been confidently placed as sister clade to Euglenozoa (Rodríguez-Ezpeleta et al., 2007). Jakobids have a complete conventional kinetochore, bolstering the notion that the ancestor of this lineage possessed a conventional kinetochore (Benz et al., 2024). Finally, due to the broad distribution of the conventional kinetochore across all other major eukaryotic lineages, the inference that the LECA possessed a conventional kinetochore remains robust in all other proposed eToL rooting scenarios. This includes the well-supported root position between lineages that are classically referred to as 'excavates' (Fig. 2B) (Williamson et al., 2025). Hence, it is highly likely that the kinetochore of kinetoplastids became derived secondarily. Indeed, kinetoplastids have an extraordinary capacity to evolve extremely diversified biological features (Lukeš et al., 2023).

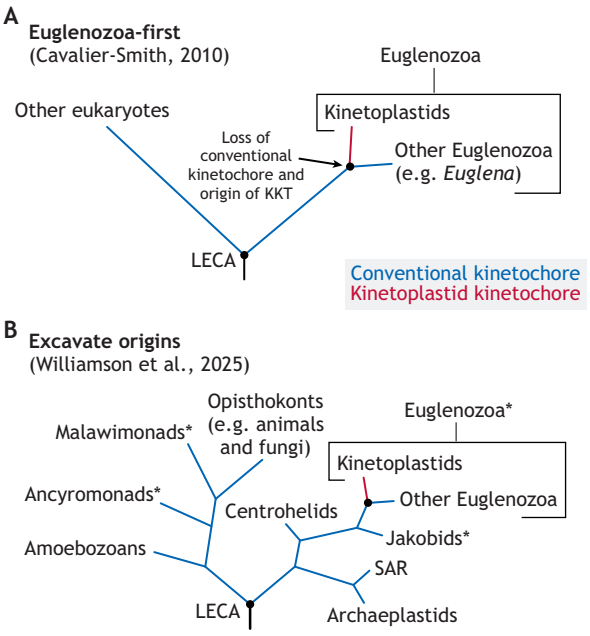


**Fig. 2. Alternative eToL rooting hypotheses unequivocally lead to a conventional kinetochore in the LECA.** (A) Schematic representation of kinetochore evolution following the scenario in which Euglenozoa represent the first diverging lineage in eToL. This topology, as suggested by Cavalier-Smith (Cavalier-Smith, 2010), supports the idea that the LECA did have a conventional kinetochore and that the kinetoplastid kinetochore was derived secondarily. KKT, kinetoplastid-specific kinetochore. (B) Similar to A, but here following the topology and root placement in accordance with Williamson et al., which show lineages with an excavate-type cell 'body' plan on both sides of the root, nested within much larger groups as opposed to a single lineage branching separately (Williamson et al., 2025). A select number of taxa are shown for simplicity. This topology also supports the LECA possessing a conventional kinetochore. Note: kinetoplastids were not included in this analysis but can here be placed in Euglenozoa due to their undisputed monophyly, as outlined above. *Lineages classically considered 'excavates'. SAR refers to Stramenopila-Alveolata-Rhizaria.

## Meiosis and mitosis likely co-evolved with the conventional kinetochore

The inference that the LECA possessed a conventional kinetochore strongly questions the validity of the hypothesis that either meiosis or mitosis evolved in the absence of a kinetochore. The kinetochore is essential to all known forms of meiosis and mitosis across eukaryotic diversity, implying its ancestral involvement in both these processes. Rather than the scenario that meiosis might have preceded mitosis, we pose it is much more likely that these processes originated together along with the emergence of the kinetochore and the spindle apparatus during eukaryogenesis, and therefore prior to the LECA. Consequently, we argue that hypotheses on the timing of the emergence of meiosis and mitosis should be put in a pre-LECA framework – i.e. occurring during the transition from a prokaryotic ancestor to the LECA – rather than speculating on the nature of the later LECA. Indeed, the LECA was most likely a bona fide eukaryote that already had a large repertoire of quintessential eukaryotic systems (Richards et al., 2024), including a conventional kinetochore that facilitated both mitosis and meiosis. Thus, although we do not discount the possibility that aspects of meiosis emerged prior to the emergence of mitotic mechanisms during eukaryogenesis, we argue that it is likely that these processes co-evolved together and in the presence of the conventional kinetochore, rather than meiosis and mitosis emerging in a step-wise, sequential evolutionary process in which the kinetochore was not involved. Finally, insights on the order of events that led to the evolution of meiosis and mitosis can only come from investigations into eukaryogenesis itself – during which these transitions occurred – and cannot come from arguments based on gene content in the LECA, given that it already possessed many genes involved in both processes (Richards et al., 2024).

## Conclusions

Considering the abundance of evidence for the monophyly of Euglenozoa and the implications this has on inferences regarding the LECA kinetochore, we argue that fundamental aspects of the hypothesis put forth by Akiyoshi are implausible. Numerous lines of evidence argue against a kinetoplastid-first eukaryotic root. Instead, recent analyses place the root of the eToL in a position that implies that the LECA must have possessed a conventional kinetochore, negating the notion that either meiosis or mitosis evolved in the absence of a kinetochore system. Finally, we stress that a comprehensive view of the topology of the eToL is crucial for making inferences on the early evolution of eukaryotic cell biology.

### Competing interests

The authors declare no competing or financial interests.

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**See next page for a response by Akiyoshi.**

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# Euglenozoa are paraphyletic if the eukaryotic root lies between glycomonads and the rest of eukaryotes. Response to 'The LECA had a conventional kinetochore and the kinetoplastid kinetochore is a derived feature'

**Bungo Akiyoshi***

## Introduction

In a recent Hypothesis article (Akiyoshi, 2025), I speculated on the origin of kinetochores, macromolecular protein machines that are essential for chromosome segregation in eukaryotes. In this article, I proposed that kinetoplastids or glycomonads (kinetoplastids and diplonemids) are the earliest-branching eukaryotes, which would mean that Euglenozoa are paraphyletic. In their Correspondence article, Raas et al. (Raas et al., 2026) argue that my hypothesis is highly implausible because Euglenozoa are believed to be monophyletic in the phylogeny field. Here, I maintain my hypothesis and clarify a few concepts that the authors might have failed to appreciate.

There are two types of kinetochores known to date – canonical kinetochores, whose components are widely conserved among eukaryotes (Earnshaw and Rothfield, 1985), and unconventional kinetochores found exclusively in kinetoplastids (Akiyoshi and Gull, 2014). The fact that prokaryotes do not have kinetochores means that both kinetochore systems evolved at some point during the transition from prokaryotes to eukaryotes. In my Hypothesis article, I proposed that the last eukaryotic common ancestor (LECA) did not have a kinetochore and that the canonical and unconventional kinetoplastid kinetochore systems evolved independently after kinetoplastids branched off from the rest of eukaryotes. This is based on two lines of reasoning. First, because prokaryotes can segregate their chromosomes without kinetochores, early eukaryotes might well have proliferated without kinetochores. Second, as a cell biologist focused on understanding the mechanism of chromosome segregation in various organisms, I find a scenario in which kinetoplastids ancestrally possessed canonical kinetochores that were lost and replaced by a novel kinetochore system to be difficult to imagine. In this scenario, an organism with an established kinetochore system would have abandoned a molecular machine absolutely essential for its survival. Rather, I argue that it would make more sense that the ancestor of kinetoplastids never had a canonical kinetochore. One implication of this hypothesis is that kinetoplastids would be the earliest-branching eukaryotes, an idea I initially proposed in 2014 (Akiyoshi and Gull, 2014).

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## Kinetoplastids have unconventional kinetochores

In response to the hypothesis that kinetoplastids represent the earliest-branching eukaryotes, Raas et al. criticize that "this line of reasoning is reminiscent of an earlier debate on the phylogenetic position of eukaryotes that lacked mitochondria", in which 'amitochondriate' eukaryotes were suggested to be the earliest-branching (Cavalier-Smith, 1983), a hypothesis that was later refuted. They also argue that kinetoplastids are not the only eukaryotic lineage that lacks a conventional kinetochore, referring to *Carpediemonas membranifera*, for which no obvious homolog of known kinetochore proteins has been identified using bioinformatics approaches (Salas-Leiva et al., 2021). I fully agree that it is improper to make a strong conclusion based on absence of evidence. In fact, there remains a possibility that *C. membranifera* has highly divergent canonical kinetochore proteins that have escaped detection even by state-of-the-art bioinformatics methods. Regardless, there is a fundamental difference between the amitochondriate debate and the lack of canonical kinetochores in kinetoplastids – there is strong evidence that amitochondriates ancestrally did possess mitochondria (Embley and Martin, 2006); in contrast, there is no evidence that kinetoplastids ancestrally had canonical kinetochores. Kinetochore components in kinetoplastids have been experimentally identified and characterized, revealing that they are fundamentally different from canonical kinetochores in terms of their composition (Akiyoshi and Gull, 2014; Nerusheva and Akiyoshi, 2016; Llauró et al., 2018; Nerusheva et al., 2019; Ishii and Akiyoshi, 2020; Marcianò et al., 2021; Tromer et al., 2021; Ishii et al., 2022; Ludzia et al., 2025) and overall design (Akiyoshi and Gull, 2013; Tromer et al., 2021).

It is also important to stress that there is no strong evidence that kinetoplastids have conventional kinetochore proteins or a canonical spindle checkpoint (see below). Raas et al. cite a study that argued that a structural component of kinetoplastid kinetochores, KKIP1, has distant similarity to the canonical kinetochore proteins Ndc80 and Nuf2 based on weak sequence similarity in their coiled-coil regions (D'Archivio and Wickstead, 2017). However, additional studies suggest that the evidence that KKIP1 is a Ndc80 or Nuf2 homolog remains inconclusive (van Hooff et al., 2017; Butenko et al., 2020). In fact, KKIP1 does not have a calponin homology (CH) domain, a region essential for the microtubule-binding activity of the Ndc80 complex, and there is no experimental evidence that KKIP1 has microtubule-binding activity. Furthermore, KKIP1 is not well conserved among kinetoplastids (Nerusheva et al., 2019; Butenko et al., 2020), whereas Ndc80 is widely conserved in other eukaryotes (van Hooff et al., 2017). Taken together, these data suggest it is highly unlikely that KKIP1 is a bona fide homolog of Ndc80 or Nuf2. Therefore, there is no evidence that any canonical kinetochore protein is present in kinetoplastids.

### Kinetoplastids lack a canonical spindle checkpoint system

The spindle checkpoint (or spindle assembly checkpoint, SAC) is a feedback control mechanism that regulates cell cycle progression by monitoring the status of kinetochore–microtubule attachment (Kops et al., 2020). Interestingly, two kinetoplastid kinetochore proteins, KKT14 and KKT15, have been identified as divergent homologs of the spindle checkpoint proteins Bub1 and Bub3, respectively (Ballmer et al., 2024a). Raas et al. say that this finding indicates "the presence of functional components of the SAC in kinetoplastids" and supports the idea that "…SAC components in kinetoplastids likely represent remnants of a former conventional kinetochore system which was present in their ancestors", but I argue that this is a mis-interpretation. It is unlikely that KKT14 and KKT15 function in a canonical SAC because there is no evidence that kinetoplastids have a functional SAC. The parasite *Trypanosoma brucei*, a model kinetoplastid, fails to delay the metaphase-to-anaphase transition in response to spindle defects (Ploubidou et al., 1999; Hayashi and Akiyoshi, 2018). Furthermore, in *T. brucei*, Mad2, a key SAC component, localizes near basal bodies, not kinetochores (Akiyoshi, 2020 preprint). Available data suggest that KKT14 and KKT15 might instead directly regulate the activation of the anaphase-promoting complex (APC/C) (Ballmer et al., 2024b), which could represent a primitive cell cycle control mechanism that existed prior to the evolution of a feedback control system that requires localization of Mad2 at unattached kinetochores. Therefore, the finding that KKT14 and KKT15 are divergent homologs of Bub1 and Bub3 does not necessarily support the idea that kinetoplastids ancestrally had canonical kinetochores.

### Aurora B and INCENP are conserved across eukaryotes

The Aurora B protein kinase and its activator inner centromere protein (INCENP) are subunits of the chromosomal passenger complex, which plays key regulatory roles in chromosome organization and segregation during mitosis and meiosis (Carmena et al., 2012). Unlike structural kinetochore proteins, these proteins are found in essentially all sequenced eukaryotes, including kinetoplastids (Li et al., 2008; Hochegger et al., 2013), meaning that they were most likely present in the LECA. Based on the fact that Aurora B and INCENP localize at kinetochores in diverse eukaryotes including kinetoplastids, one might argue that the LECA had kinetochores that were regulated by these proteins. However, Aurora B and INCENP have many additional functions (e.g. regulation of cytokinesis and synaptonemal complexes; Jordan et al., 2009; Carmena et al., 2012; Wellard et al., 2020). Thus, it remains unknown for which purpose these proteins initially evolved. For example, under my primitive-meiosis-first hypothesis (Akiyoshi, 2025), their original function might well have been regulation of synaptonemal complexes (or chromosome axes) rather than of kinetochores. Therefore, the wide conservation of Aurora B and INCENP among eukaryotes does not necessarily mean that the LECA had kinetochores.

### Glycomonads share similarity in chromosome segregation mechanisms

Phylogenetic trees place kinetoplastids and diplonemids next to each other (together, they are called glycomonads) (Cavalier-Smith, 2016). Interestingly, no kinetochore components have been identified in diplonemids, whereas Aurora B and INCENP are conserved (Benz et al., 2024; Akiyoshi et al., 2025). Importantly, the localization module proteins that recruit Aurora B and INCENP to different locations are unique in glycomonads. In *T. brucei*, a kinesin-like motor protein KIN-A recruits Aurora B and INCENP onto kinetochores in metaphase and to the central spindle in anaphase (Ballmer and Akiyoshi, 2024; Ballmer et al., 2024b). A recent finding that a KIN-A homolog is a component of the chromosomal passenger complex in a model diplonemid *Paradiplonema papillatum* (also known as *Diplonema papillatum*) implies similarity between kinetoplastids and diplonemids (Akiyoshi et al., 2025). Similar to in *T. brucei*, *P. papillatum* Mad2 also localizes near basal bodies (Akiyoshi et al., 2025). Together with other molecular features that are unique to glycomonads (see below), these organisms appear to be fundamentally different from the rest of eukaryotes. I therefore proposed that kinetoplastids or glycomonads are the earliest-branching eukaryotes (Akiyoshi and Gull, 2014; Akiyoshi, 2025), although it important to acknowledge that these possibilities are not currently supported by any results obtained by standard molecular phylogenetic approaches.

### Inconsistencies in the root position inferred by molecular phylogeny approaches

Determining the position of the root of the eukaryotic tree of life remains a major challenge in the phylogeny field (Embley and Martin, 2006; Walker et al., 2011; Burki et al., 2020). Many competing hypotheses have been proposed (Stechmann and Cavalier-Smith, 2003; Richards and Cavalier-Smith, 2005; Rogozin et al., 2009; Cavalier-Smith, 2010; Derelle and Lang, 2012; Katz et al., 2012; He et al., 2014; Derelle et al., 2015; Cerón-Romero et al., 2022; Al Jewari and Baldauf, 2023; Williamson et al., 2025). As I explained in my Hypothesis article (see Box 2 in Akiyoshi, 2025), to determine root organisms within a given group, sequence-based molecular phylogenetic approaches require comparison to an outgroup that does not belong to that group but is reasonably closely related to it. It is thought that eukaryotes evolved from an archaeal host that incorporated a bacterial symbiont (Alphaproteobacteria) (Vosseberg et al., 2024). However, any known archaeon is extremely divergent from any known eukaryote. Therefore, the use of archaeal-origin proteins for sequence-based molecular phylogenetic analysis is currently limited (Al Jewari and Baldauf, 2023), although structure-based phylogenetic approaches might help with this challenge (Moi et al., 2025; Puente-Lelievre et al., 2025).

Several studies, including Williamson et al. (2025), instead used bacterial-origin proteins that are conserved among eukaryotes to infer the root position using Alphaproteobacteria as an outgroup. I argue that there are several issues with this approach. First, if archaeal-origin proteins cannot be used for such analyses, the validity of using bacterial-origin proteins is unclear. Second, the choice of proteins and organisms used in molecular phylogeny studies remains, in my view, subjective. For example, neither kinetoplastids nor diplonemids were included in the above analysis (Williamson et al., 2025). Third, the evolutionary pressure on mitochondria might differ significantly in different eukaryotic lineages, which could skew the evolutionary trajectory of these bacterial-origin proteins. In fact, this approach did not work well for organisms that have highly reduced mitochondrion-related organelles (Williamson et al., 2025). Given these issues, my position remains the same – current phylogenetic methods using protein sequences might not be able to resolve the position of the root of the eukaryotic tree simply due to the massive evolutionary distance between any existing eukaryote and prokaryote (Akiyoshi, 2025).

### Re-evaluating the Cavalier-Smith Euglenozoa-first hypothesis

Cavalier-Smith appreciated the above difficulties and took a drastically different approach to tackle the question of the eukaryotic root. He reasoned that the earliest-branching eukaryotes should have unique molecular and cellular features that are not present

in any other eukaryotic lineages, and proposed several competing ideas on the root position during his career (Cavalier-Smith, 1983, 2010, 2022; Stechmann and Cavalier-Smith, 2002; Richards and Cavalier-Smith, 2005), one of which is the Euglenozoa-first hypothesis (Cavalier-Smith, 2010). Based on the observation that Euglenozoa differ immensely from other eukaryotes – for example, in the organization of their nuclear genomes (Clayton, 2002) and in cytochrome *c*-type biogenesis (Allen et al., 2008) – Cavalier-Smith stated "I now argue that the root is between Euglenozoa and all other eukaryotes (or possibly deeply within Euglenozoa)" (Cavalier-Smith, 2010). This idea was not taken up by others in the field (Roger, 2021), partly due to the subjective nature of his approach. It is noteworthy that Cavalier-Smith abandoned the Euglenozoa-first hypothesis in his very last paper (Cavalier-Smith, 2022), which was published after his death. Regardless, it is important to emphasize that in his 2010 paper Cavalier-Smith did not exclude the possibility that the root might lie deeply within Euglenozoa (because additional unique features, if discovered, could split Euglenozoa into two or more groups), a crucial point that Raas et al. do not appear to appreciate in their discussion.

## Challenging the 'undisputed monophyly' of Euglenozoa

Euglenozoa are a highly diverse group of flagellates which consists of kinetoplastids, diplonemids, euglenids and symbiontids (Cavalier-Smith, 2016). As Raas et al. point out, it is currently thought that Euglenozoa are monophyletic, meaning that these four groups descended from a common ancestor and that Euglenozoa includes every single descendant of that ancestor. It is clear that Euglenozoa share several morphological features, such as unique paraflagellar rods (Simpson, 1997; Cavalier-Smith, 2016; Kostygov et al., 2021). Molecular phylogenies also support the close relationship among euglenozoans (Cavalier-Smith et al., 2016; Lax et al., 2021). However, it has become increasingly evident that fundamental differences do exist among these diverse flagellates. In fact, based on the unique kinetochores found in kinetoplastids, I previously questioned the monophyly of Euglenozoa (Akiyoshi, 2016). Another striking feature that is uniquely found in kinetoplastids and diplonemids is compartmentalization of most glycolytic enzymes within an organelle called the glycosome (Opperdoes and Borst, 1977; Makiuchi et al., 2011; Gabaldón et al., 2016). The term 'glycomonads' was therefore established to encompass these two groups (Cavalier-Smith, 2016). Additional features that distinguish glycomonads from euglenids include the organization of their mitochondrial DNA (Dobáková et al., 2015; Faktorová et al., 2016), complex RNA editing (Butenko et al., 2021), a lack of pellicles (Cavalier-Smith, 2017), the presence of a unique nuclear lamina protein NUP-1 (Prokopchuk et al., 2023) and absence of the key mRNA export factor DBP5 (Butenko et al., 2021). These unique features highlight that glycomonads are fundamentally different from the rest of eukaryotes, supporting the possibility that the root of the eukaryotic tree of life lies between glycomonads and the rest of eukaryotes (Fig. 1) (Akiyoshi, 2025). Importantly, this proposed tree does not contradict the close relationship between glycomonads and euglenids (Fig. 1). However, the topology of this tree would inevitably indicate that Euglenozoa are paraphyletic.

## Conclusions

Raas et al. conclude their correspondence by saying "we stress that a comprehensive view of the topology of the eToL [eukaryotic tree of life] is crucial for making inferences on the early evolution of eukaryotic cell biology". However, they dismiss the possibility that the root lies deeply within Euglenozoa because of its undisputed monophyly. Furthermore, they seem to overlook the importance of understanding cell biological differences to gain insights into deep phylogeny and instead focus only on molecular phylogenetic approaches (bioinformatics). However, such bioinformatic approaches have resulted in many competing ideas on the root position (Stechmann and Cavalier-Smith, 2003; Richards and Cavalier-Smith, 2005; Rogozin et al., 2009; Derelle and Lang, 2012; Katz et al., 2012; He et al., 2014; Derelle et al., 2015; Cerón-Romero et al., 2022; Al Jewari and Baldauf, 2023; Williamson et al., 2025). This lack of consensus among phylogenetics experts makes it difficult for non-experts to evaluate which root position is most plausible. Based on the unique features of glycomonads discussed above, I would like to challenge the widely accepted monophyly of Euglenozoa and propose that the root lies between glycomonads and all other eukaryotes. Compared to kinetoplastids, much less is known about the biology of diplonemids despite the fact that they are highly abundant and diverse in the world's oceans (de Vargas et al., 2015; Flegontova et al., 2016). A deeper understanding of diplonemids, which historically received very little attention from the scientific community, could shed further insights into the deep phylogeny of eukaryotes as well as the mysterious process of eukaryogenesis.

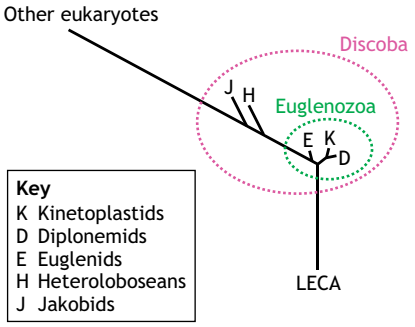


**Fig. 1. Hypothesis that glycomonads are the earliest-branching eukaryotes.** This proposed position for the root of the eukaryotic tree of life, inferred from the unique biological features of glycomonads (kinetoplastids and diplonemids), challenges the idea that Euglenozoa are monophyletic. In this scenario, the last eukaryotic common ancestor (LECA) did not have kinetochores (see Akiyoshi, 2025), and the canonical and unconventional kinetoplastid kinetochore systems evolved independently after the split of glycomonads from the rest of the eukaryotic lineages: unconventional kinetochores evolved in the ancestor of kinetoplastids, and canonical kinetochores evolved in the ancestor of non-glycomonads. Note that it remains unknown what type of kinetochores are present in diplonemids. Symbiontids, another group that falls within Euglenozoa, are not shown due to lack of genome information. Branch lengths are arbitrary.

### Competing interests

I declare no competing or financial interests.

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