

# The last eukaryotic common ancestor (LECA): Acquisition of cytoskeletal motility from aerotolerant spirochetes in the Proterozoic Eon

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We develop a symbiogenetic concept of the origin of eukaryotic intracellular motility systems from anaerobic but aerotolerant spirochetes in sulfide-rich environments. The last eukaryotic common ancestors (LECA) have extant archaeo-protist descendants: motile nucleated cells with Embden-Meyerhof glycolysis and substrate-level phosphorylation that lack the  $\alpha$ -proteobacterial symbiont that became the mitochondrion. Swimming and regulated O<sub>2</sub>-tolerance via sulfide oxidation already had been acquired by sulfidogenic wall-less archaeobacteria (thermoplasmas) after aerotolerant cytoplasmic-tubule-containing spirochetes (eubacteria) attached to them. Increasing stability of sulfide-oxidizing/sulfur-reducing consortia analogous to extant sulfur syntrophies (*Thiodendron*) led to fusion. The eubacteria–archaeobacteria symbiosis became permanent as the nucleus evolved by prokaryotic recombination with membrane hypertrophy, analogous to *Gemmata obscuriglobus* and other  $\delta$ -proteobacteria with membrane-bounded nucleoids. Histone-coated DNA, protein-synthetic RNAs, amino-acylating, and other enzymes were contributed by the sulfidogen whereas most intracellular motility derives from the spirochete. From this redox syntrophy in anoxic and microoxic Proterozoic habitats LECA evolved. The nucleus originated by recombination of eu- and archaeobacterial DNA that remained attached to eubacterial motility structures and became the microtubular cytoskeleton, including the mitotic apparatus. Direct LECA descendants include free-living archaeo-protists in anoxic environments: archamoebae, metamonads, parabasalids, and some mammalian symbionts with mitosomes. LECA later acquired the fully aerobic Krebs cycle-oxidative phosphorylation-mitochondrial metabolism by integration of the protomitochondrion, a third  $\alpha$ -proteobacterial symbiont from which the ancestors to most protoctists, all fungi, plants, and animals evolved. Secondarily anaerobic eukaryotes descended from LECA after integration of this oxygen-respiring eubacterium. Explanatory power and experimental predictions for molecular biology of the LECA concept are stated.

karyomastigont | kinetosome-centriole | mitotic apparatus | nucleus origin | *Thiodendron*

The cytoskeleton in all eukaryotes comprises the mitotic spindle (often its kinetosome-centrioles within the centrosome), protist karyomastigonts (1), and neurotubules; the kinetome of 10,000 species of ciliates; microtubules of foram granuloreticulopodia; submembranous microtubules of red blood cells, trypanosomes, and many algae; and the undulopodium (cilium-eukaryotic “flagellum” or intrinsically motile 0.25- $\mu$ m-diameter ninefold symmetrical intracellular “whip” generated from a kinetosome-centriole base). “This later structure is a highly complex organelle, composed of hundreds of proteins. It has no bacterial homolog, and undoubtedly evolved into its current form after the evolution of microtubules. However, it is a remarkably standardized organelle across diverse eukaryotic taxa, which indicates that it evolved once in the early evolution of eukaryotes” (2).

Movement inside cells, limited to eukaryotes, involves three classes of structures: actin filaments (6–8 nm in diameter)

associated with myosin-ATPases, intermediate filaments (12–14 nm in diameter), and microtubules (24 nm) with microtubule-associated proteins (MAPs) (dynein, kinesin, and other ATP- or GTPases). We detail only the microtubular cytoskeleton, but a complete account of cell evolution must consider other motile proteins. Three possibilities for the origin of the cytoskeleton include (i) ancient origin from our genetically-not-yet-annealed ancestors (3), (ii) direct filiation from a prokaryotic ancestor, or (iii) symbiotic addition to a different eukaryotic ancestor. Lack of precision of “genetically-not-yet-annealed” precludes investigation of the first. Continuous reevaluation of the assumed second reveals slim evidence and terminology misused (2). Even the accepted concept of FtsZ (filament temperature-sensitive Z protein) as prokaryotic ancestor of tubulin protein is debatable (2). Symbiogenetic origin of intracellular motility including mitosis, via addition of *Spirochaeta* to a *Thermoplasma*-like archaeobacterium (4–7), our concept, unlike other alternatives, explains superficially unrelated phenomena and generates details studiable by genomic, proteomic, cytologic, and geologic methods.

## Results and Discussion

Our evolutionary scenario, developed independently of molecular sequence data, unlike others (8–10) reconstructs evolutionary history from extant organisms (Fig. 1). Taken as representative clues, many of the steps have been videographed in live microbes. We show how both claims that no eukaryote “primarily lacked mitochondria” and “the evolutionary gap between prokaryotes and eukaryotes is now deeper, and the nature of the host that acquired the mitochondrion more obscure, than ever before” are based on protistological ignorance but rely on the kind of molecular data that will support or disprove our model.

New results mandate that aspects of our putative evolutionary (chronological) karyomastigont model be reconsidered and the only propositions disputed in scientific literature [(i) symbiotic origin of archaeobacterial cytoplasm from sulfidogens, not methanogens, and (ii) nucleus-associated microtubule cytoskeleton preceded acquisition of mitochondria] be defended.

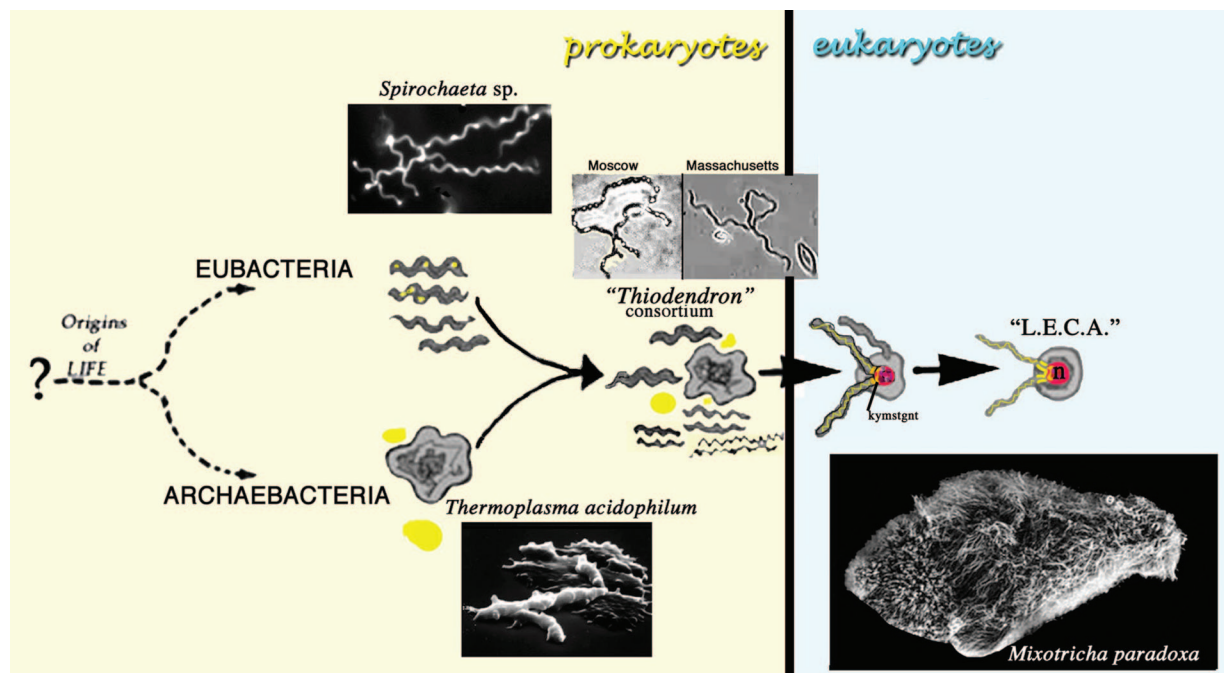
1. Hydrogen sulfide (H<sub>2</sub>S)-oxygen (O<sub>2</sub>) interface habitats abounded during the Proterozoic Eon (2,500–541 mya) throughout the world ocean as inferred from sedimentary, isotopic, and other geologic observation (11, 12). Worldwide localities show preservation of ancient eukaryotes mostly as microfossils in thin-section of cherty rock; nucleated cells evolved before 1,000 mya in sulfidic, muddy habitats periodically well lit, dominated by cyanobacteria and therefore rich

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Abbreviations: LECA, last eukaryotic common ancestor; MAP, microtubule-associated protein.

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**Fig. 1.** Karyomastigont model of origin of nucleated cells. The LECA evolved from eubacterial-archaeobacterial syntrophies in which sulfide-oxidizing spirochetes attached to sulfidogenic thermoplasmas to form a “Thiodendron”-like consortium. Archaeoprotist (trichomonad *Mixotricha paradoxa*, lower right), LECA analogue in termite *Mastotermes darwiniensis*, swims via motility symbiosis with 200,000 *Treponema* sp. surface spirochetes. Four distinctive surface spirochetes are detected by morphological and molecular techniques (36). Chromatin appears first in the karyomastigont (“kymstgnt”), the precursor cytoskeletal organellar system from which the tethered nucleus (“n”) was released.

in organics. Spirochetes, an ancient cohesive group of helical, motile chemoheterotrophic eubacteria, had evolved, in response to cyanobacterial oxygenesis, from their strictly anaerobic origin through aerotolerant-facultative aerobes, to microaerophils like *Leptospira* (13, 14). Spirochetes, active chemotactic swimmers, thrived in marine microbial mats, pond scums, and fresh and saline hot springs, where many, as now, lived syntrophically.

- The ancestors to which *Spirochaeta* were added were *Thermoplasma*-like sulfidogenic archaeobacteria. This postulate, critical to our model, was argued by Searcy (15–21) (Table 1). *Thermoplasma acidophilum*, heat- and acid-tolerant pleiomorphs that lack cell walls, are amenable to study as co-descendants of conserved archaeobacterial features in eukaryotes. *Thermoplasma* are predicted to enter metabolic associations more easily than walled relatives. Their DNA, unlike most prokaryotes, is protected from hot acid by “nucleosomes,” a coating of arginine- and lysine-rich histone-like proteins (20, 21), and thus, thermoplasmas in nature may constitute living refugia from the hydrolytic environmental habitat extremes in which they grow. Like eukaryotic cytoplasm *Thermoplasma* metabolizes glucose to pyruvate or lactate via Embden-Meyerhof enzymes anaerobically, but because its terminal electron acceptor is elemental sulfur (only oxygen at microoxic, <5% ambient concentrations), it tends to be sulfidogenic. Volcanoes, fumaroles, hot springs, and burning coal piles replete with elemental sulfur,  $H_2S$ , or sulfide ion ( $HS^-$ ) provided Proterozoic sulfatara and sulfureta environmental selection pressures (high sulfur concentration, heat, low pH, and cyclically high toxic oxygen gas and organic food from cyanobacteria). These led hungry oxygen-intolerant spirochetes to cohabit and attach to sulfidogenic thermoplasmas. The merger of incessantly motile spirochetes with sluggish acid-mediating thermoplasmas formed the earliest protists: amitochondriate, anaerobic mo-

tile nucleated cells with low levels of oxygen tolerance and utilization.

Spirochetes detoxified fluctuating ambient gaseous oxygen by its conversion with sulfide to elemental sulfur usable by *Thermoplasma* as terminal electron acceptor. Conferrence of motility on this redox syntrophy permitted both partners access to organic-rich habitats that augmented survival and growth. Modern descendants of syntrophic consortia became archaeoprotists, motile premitochondriates amenable to study. The Archaeoprotista phylum, members riddled with endo-, epi-, and even nuclear symbionts (22), includes the archamoebae (pelomyxids, mastigoamoebae), metamonads *Retortamonas* and *Giardia*, and parabasalids such as trichomonads, devescovinids, hypermastigotes, and calonymphids (23, 24). Distinctive features of eukaryotic cells (e.g., archaeobacterial-like transcription and translation in protein synthesis, histone-like proteins of nucleosomes and chromatin, glucose catabolism, sulfide generation, ATP- and GTP-based substrate-level energy transformation, details of ion regulation, membrane transport, and mechano-, chemo-, and photoreception), although recombined and refined, have been conserved such that their history can be reconstructed from ultrastructural, physiological, biochemical, and molecular data.

- The earliest symbiogenetic fusion that integrated thermoacidophilic archaeobacterial thermoplasmas with aerotolerant spirochetes produced the first protist, a swimming chimera that evolved into a stable nucleated protist cell: the last eukaryotic common ancestor, LECA. The nucleus in the karyomastigont was generated by recombination of eu- and archaeobacterial DNA that remained attached to membrane and to spirochete motility proteins. This three-part microtubular-mitotic apparatus-cytoskeleton-organellar system, the karyomastigont includes: (i) undulipodium connected to the (ii) nucleus by (iii) nuclear connector (rhizoplast). A “para-

**Table 1. Microtubular semes and their selective advantage**

Seme and its source	Putative selective advantage
Condensation reaction of acetate (carboxylic acid, from <i>Spirochaeta</i> fermentation) and thiol to form thioester [R <sub>1</sub> —C(=O)S—R <sub>2</sub> ] from <i>Thermoplasma</i>	Soluble energy transfer (14 kC/g molecule) and proton (H <sup>+</sup> ) generator as source of ATP via pyrophosphate phosphorylation (50)
Karyomastigont: kinetosome-centriole from spirochete attachment structure connected by protein (nuclear connector, rhizoplast) to recombined nucleic acid of syntrophic eubacterial-archaeobacterial partners	Swimming, other cell locomotion; chemo- and mechanosensitivity in feeding and defense; assurance of joint heritability of merged symbiont genomes
Nucleus (from syntrophic-motility integration) permanent metabolic and genetic integration of symbionts: sulfide-oxidizing <i>Spirochaeta</i> and sulfidogenic <i>Thermoplasma</i>	Sulfide oxidation by <i>Spirochaeta</i> for oxygen-hydrogen peroxide detoxification produces elemental sulfur for <i>Thermoplasma</i> 's terminal electron acceptor. Endomembrane system to distribute ion channels (ER, golgi). Energy generation by aerotolerant glycolysis to acetyl CoA, hydrogen, and oxidizable 3-C compounds (pre-adaptation for acquisition of mitochondrial ancestors)
Nuclear membrane (from motile syntrophic partnership integration) by fusion and proliferation of composite endomembrane system	Insure joint stable and permanent integration followed by segregation of symbiont DNA ( <i>Spirochaeta</i> + <i>Thermoplasma</i> ) each generation to produce offspring
Phagocytosis, cyclosis, pinocytosis, endocytosis, exocytosis (from spirochete innards) particulate feeding (bacteriovovery), intracellular motility and transport, facultative fertilization, and other forms of cell fusion	Feeding, cell "drinking," locomotion, prerequisite for mitotic reproduction and meiotic sexuality, complex sexual life histories, neuron-based sensory systems, ciliated retinal, olfactory, and auditory epithelial cells, and other eukaryotic features that require cytoskeleton
Synaptonemal complex (from bacterial conjugation protein structures)	Enzymatic and gene redundancy reduction, accurate recombination that guarantees euploidy: precursor to plant and animal tissue differentiation

basal body" (= golgi) is often also associated. Many forms of motility including cytoskeletal-based ingestion originated from the fusion. The eukaryotic cell née motility-sulfur syntrophy was selected for during the Archean or lower Proterozoic Eon by variables that fluctuated diurnally and seasonally: temperature, light, water abundance, pH, salinity, organics, and oxygen in chemically reduced, sulfide-rich anoxic habitats. The mitotic nucleus, evolved as part of the karyomastigont, preceded obligate aerobiosis because the acquisition of oxygen respiration occurred only after the endosymbiotic  $\alpha$ -proteobacterium became the mitochondrion. Eukaryotic-style evolution where entire genomes are ingestible preceded both the symbiotic acquisition of mitochondria-mediated aerobiosis and cyanobacteria that conferred photosynthesis on algal and plant ancestors.

- Origin of the cytoskeletal intracellular motility requires so many coordinated genes and biochemical activities that it can only be explained by symbiogenesis. Motile phenomena, visible in live cells by light microscopy, include undulipodial beating; "raft" transport along ciliary axonemes; chromosome movement by MAP motors on spindle microtubules; nuclear rotation; cyclosis, exocytosis, endocytosis, phagocytosis, and other particle translocation; evacuation of vacuoles, formation of vesicles, and cell process (pseudopod, axopod, nerve growth cone) extension; and fusion of cells in fertilization, myogenesis, and myriad other locomotion exclusive to eukaryotes.

The formation and function of the kinetosome-centriole-based undulipodium requires >360 different proteins (25). By contrast, large prokaryotic cells (e.g., *Lyngbya*, *Thiomargarita*,

and gliding myxobacteria) show no directed internal motility at highest magnifications ( $\times 1,000$ ) with differential interference or phase contrast microscopy. Sexual life history cycles require syngamic or karyogamic intracellular movement followed by meiosis. Microtubules and MAPs are so intrinsic to the eukaryotes that any hypothesis for the origin of the nucleus is fatally deficient unless it includes an account of the cytoskeleton (2).

- Histone orthologues have been isolated from methanogens and other archaeobacteria (26). However, the paucity of relict methanogenesis or its coenzyme biochemistry requires rejection of methanogens in eukaryotic ancestry. Methanogenesis, like oxygenic photosynthesis, is correlated with secondary acquisition of symbiotic bacteria in anoxic environments (27, 28). The absence of primary methanogenesis in anaerobic protists contrasts strikingly with the widespread, if not universal, detection of cytoplasmic sulfidogenesis (17) in all four eukaryotic lineages [protocists, plants, fungi, and animals including human erythrocytes (16)] consistent with the concept that sulfidogenesis was introduced by the *Thermoplasma* archaeobacterium symbiont (19).

**Mitochondria Evolved After Nuclei.** That hundreds of species of anaerobic eukaryotes lost mitochondria on return to anoxic habitats and/or that all eukaryotic lineages began with hydrogenosome-mitochondrial ancestry is more assumption than conclusion based on evidence (9).

"The genomes of *E. [Entamoeba] histolytica* and the amitochondrial protist pathogens *Giardia lamblia* and *Trichomonas vaginalis* share several metabolic adaptations. These include

reduced or eliminated mitochondrial metabolic pathways. Indeed the genome data are consistent with the lack of a mitochondrial genome. Tricarboxylic acid cycle and mitochondrial electron transport chain enzymes are lacking . . ." (29).

Yet many kinds of eubacterial genes are known in anaerobic eukaryotes (30, 31). The best available comparative genomic analysis suggests that mitochondria, hydrogenosomes, and related organelles evolved independently several times in various anaerobic ancestral eukaryotic lineages through evolutionary tinkering (32). The claim of Martin and Koonin (10) that the nucleus evolved in response to symbiotic acquisition of the  $\alpha$ -proteobacterial proto-mitochondrion ignores cytoskeletal data, protocist biology, and comparative organellar genetics. The abundance of introns in  $\alpha$ -proteobacterial relative to other eubacterial DNA required separation of the genome from the cytoplasm. But following the logic of those authors, initial acquisition of this intron-rich DNA would have been lethal to the cell (10).

So-called mitochondria "relict genes" in anaerobic protists (*Trichomonas*, *Giardia*) do not involve direct use of oxygen gas, the cytochrome *a/a3* (cytochrome-oxidase) terminal electron transport or other enzymes unique to mitochondria. The speciose amitochondriate protist taxa (i.e., trichomonads, calonymphids, and hypermastigotes) have no mitochondriate relatives; it is more likely they are descendants of Proterozoic evolution before the acquisition of protomitochondria than that all evidence of mitochondria has disappeared. Many, if not all eubacterial enzymes asserted to derive from relict mitochondria are common to eubacteria. Loss and/or dedifferentiation of mitochondria in isolated species is well known: the rumen protist *Neocallimastix*, a chytrid; plagiopylid ciliates with stripped bodies in the cytoplasm; and many sapropele ciliates (27, 28). Some fungi (glucose-repressed yeast) and marine worms have dedifferentiated mitochondria interpretable as legacies of local evolution: nearly all fungi and animals are mitochondriates in oxic habitats. Secondarily amitochondriate taxa are analogous to nongreen plants (e.g., *Monotropa*, *Cuscuta*, and *Corallorhiza*) whose cells contain proplastids or chromoplasts derived from plastidic ancestors. Nongreen plants, isolated rarities in the overwhelmingly green Kingdom Plantae, evolved where specialized habitat led to chloroplast loss. In stark contrast, hundreds of protists including archaeamoebae (e.g., *Pelomyxa* and *Mastigoamoeba*), metamonads (*Retortamonas* and *Hexamitus*) and parabasalids (Family Devescovinidae: *Devescovina*, *Mixotricha*, *Oxymonas*, *Pyronympha*, and *Trichomonas*; the Calonymphids, e.g., *Coronympha* and *Stephanonympha*), *Snyderella* and all hypermastigotes (including genera such as *Barbulanympha*, *Staurojoenina*, and *Trichonympha*), lack mitochondrial relict organelles and mitochondriate relatives (33).

Epi-, endo-, and intranuclear bacterial symbionts of protists tend to be eubacterial Gram-negative rods and coccoids. Whereas genus identification is rarely available, distinctive eubacteria regularly adorn the same anaerobic protist cell. In *Caduceia versatilis*, a devescovininid, more than four different bacterial types, including intranuclear, were detected (34); in the giant trichomonad, *Mixotricha paradoxa*, at least six different eubacteria were verified in Radek's electron microscopic analysis (35, 36), and more than seven were detected with molecular techniques (36). *Staurojoenina* associates with at least three distinguishable bacterial symbionts (37). Study of protists in low-oxygen insect tissue led Kirby to his masterpiece on bacteria living inside and on protists (38). Pervasive bacterial associations, including ones mistaken for mitochondria, is marked under anoxic relative to oxic conditions. Kirby's observations have been confirmed by transmission electron microscopy (39, 40).

Only if uniquely mitochondrial (not general eubacterial) genes are proved present in protists with taxon-specific cytoskeletons in which all species live anaerobically in anoxia and lack mitochondria

at all stages must we reevaluate the archaeoprotist concept. We explain the eubacterial enzymes [acetate kinase, phosphoglucosyltransferase, NADH oxidase, and NADH peroxidase (41)] as legacy of the aerotolerant spirochete not from the protomitochondrion.

Increasingly stable sulfide-oxidizing/sulfur-reducing consortia, analogous to "Thiodendron" syntrophies, were precursors to the LECA. As the *Thermoplasma/Spirochaeta* syntrophy became permanent, the nucleus evolved by prokaryotic recombination and intracellular membrane hypertrophy that led to a membrane-bounded nucleus analogous to the nucleoid of *Gemmata obscuriglobus* (42).

**Inadequacy of Other Nuclear Origin Hypotheses.** Three other symbiogenetic origin-of-eukaryotic cell hypotheses include the following: (i) the methanogen syntrophy (43), which postulates an original methanogen-proteobacteria symbiosis under anoxic conditions before mitochondria acquisition; (ii) the hydrogen hypothesis (9), where the nucleus and mitochondria originated concurrently—facultatively anaerobic mitochondrial metabolism provides the hydrogen ( $H_2$ ) and carbon dioxide ( $CO_2$ ) for methanogenic syntrophy; and (iii) overwhelming evidence, nearly exclusively drawn from amino acid residue sequence comparisons in many proteins, supports the chimera hypothesis—eukaryotes simultaneously have archaeobacterial and eubacterial ancestors (44, 45). Sequence analysis in anaerobes (*Giardia*, *Trichomonas*) suggests to Gupta that some nucleated lineages never acquired mitochondria, but he fails to specify which genera represent archae- and eubacteria that formed LECA. He depicts the endoplasmic reticulum origin by membrane motility yet postulates no origin for the intracellular capacity required for endocytotic in-folding. Even Rizzotti (46), who understands the importance of the cytoskeleton in his "cilium from peduncle" hypothesis, fails to account for the nuclear membrane, other endomembranes, or sulfidogenesis. Unlike the karyomastigont model (7), no previous hypothesis was documented by videography (35).

**Semes.** Although frequently inexplicit about methods, "traits in common" are used to reconstruct phyletic lineages by great evolutionists, e.g., Darwin, Haeckel, and Mayr (47). This is seme analysis (48) as used by Brogniart and many paleobotanists (49), Romer, Simpson, and other paleontologists. Semes, always determined by more than a single or even a few genes, are characteristics of clear selective advantage in given environments at specified times (tabulated in ref. 24, p. 106). A single mutation may cause a seme loss (of photosynthesis in plants or vision in cave animals) but never a gain. Semes, in order of evolution, include the following: heat-resistant spores, magnetosomes, dinitrogen gas fixation, oxygenic photosynthesis, cellulosic cell walls, desmosomes, actomyosin muscles, chitinous exoskeletons, amniote eggs, feathers, and speech. The karyomastigont, a seme that preadapted eukaryotes for mitosis, generated a descendant neoseme: the microtubular ninefold symmetrical shaft [9 (2) + 2] of the undulipodium that grows from a [9 (3) + 0] kinetosome-centriole. Selective advantages of semes from the symbiogenetic *Spirochaeta/Thermoplasma* syntrophy fusion are listed in evolutionary order (Table 1). The earliest probably is substrate-level phosphorylation that provided electron transfer and ATP for biosynthesis and motility since the beginning of the Archean Eon's anoxic world (50).

**Karyomastigont Evolved from Attached Symbiotic Aerotolerant Spirochetes.** The karyomastigont we place in its evolutionary context (4) was described in 1915 (51). Because a review of our model that emphasizes our protistological predecessors (H. J. Kirby, Jr., and L. R. Cleveland) appeared in *Paleobiology* [dedicated to S. J. Gould (7)], only newer work is presented here. Examples of explanatory power and experimental predictions of superficially unrelated phenomena include the fact that epitopes of gamma

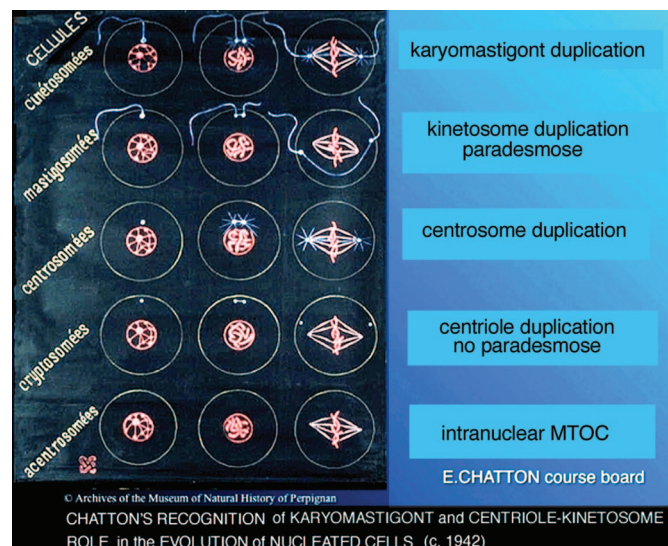
tubulin and a scleroderma antigen (anti-pericentrin serum) localized in the rotary motor zone in the archaeprotist (e.g., *Caduceia versatilis*) (5, 23).

Isolation of a filamentous bacterium from an abundant white sulfurous slime in marine coastal habitats associated with *Fucus* (rockweed, brown algae) led Perfiliev (52) to introduce the genus *Thiodendron latens* (“lazy sulfur tree”) to bacteriology. Thirty-five years of study by Dubinina (53, 54) proved that what Perfiliev had identified as a single bacterium with alternating motile-by-flagella with unicellular-stringy filamentous sulfur-ridden life-history stages was a syntrophic consortium. “*Thiodendron*” evolved convergently in at least six aquatic locations; it is a heterotrophic sulfate-reducing sulfidogen associated with a sulfur-oxidizing spirochete. The sulfate-reducing physiology suggested the spirochete’s partner was a *Desulfovibrio*. Further work identified at least two new genera (i.e., *Desulfobacter* and *Dethiosulfovibrio*) as the sulfidogens (41). In all “*Thiodendrons*” studied, the spirochete partners, based on swimming behavior, electron microscopic morphology, metabolism, and 16S rRNA, classify as *Spirochaeta* sp. However, the strains of *Spirochaeta* from distant sites (e.g., the White Sea, two Pacific Islands, and Moscow Staraya hot springs) differ in detail including 16S rRNA (5), which supports convergent origins of the partnership.

Dubinina’s work illuminates our karyomastigont model by provision of analogous spirochete syntrophies for field and laboratory investigation. The hypothetical syntrophic *Spirochaeta* sp. are physiologically identical to Dubinina’s aerotolerant ones: substrate-level phosphorylation generated by glycolysis pathways was enhanced by ambient oxygen in the spirochetes with their minimal oxygen metabolism—acetogenesis by pyruvate oxidation and exopolysaccharide production (41). The microoxic, more efficient glucose oxidation preadapted spirochetes for association with *Thermoplasma*. However, the marine sulfidogens (*Desulfobacter* and *Dethiosulfovibrio*) (55) differ from hypothetical archaeobacterial associates: *Thermoplasma acidophilum* (18) is found in fresher, hotter, and more acidic waters. Sulfide-sulfur redox metabolism was retained as intracellular physiological signal that enabled environmental expansion by LECA.

Homology of mitotic-microtubule variation was impossible to recognize until the development of glutaraldehyde fixatives after 1963, when by electron microscopy, microtubules became visible. Yet the prescient protistologist Edouard Chatton (56) anticipated this analysis. On “course boards” for students, he depicted cell evolution by centrosome-centriole-mitotic spindle morphology (“cellules cinetosomées” and “cellules mastigonemées”; Fig. 2). An expert marine protistologist and director of Laboratoire Arago, Banyuls sur Mer, France, he was first to tabulate all organisms as either “procariotique” or “eucariotique” (57, 58). His classification of “cells by centrosome behavior” genealogically organizes protist taxa (phyla, classes, and orders) in a valid manner consistent with our model.

**The Test.** A definitive proof of our origin-of-the-nucleus hypothesis requires complete genome sequence comparison of appropriate Dubinina “*Thiodendron*”-*Spirochaeta* with other hypothetical prokaryotic-ancestor-of-eukaryote co-descendants. An aerotolerant spirochete the size of undulipodia (0.25- $\mu\text{m}$  diameter by 10- to 14- $\mu\text{m}$  length) that oxidizes sulfide to intracellular elemental sulfur globules [that contains, as does *Hollandina* (14, 59), 24-nm-diameter cytoplasmic tubules] is predicted to contain DNA and protein sequences with greater homology to genes that code for cytoskeletal nucleic acid and proteins (e.g., MAPs) than do other prokaryotes. The identification of unique centrosome-specific RNA molecules in *Spisula* surf clams (60) helps identify potentially relevant homologous sequences. Indeed, all 30 million species of eukaryotes should have retained cytoskeletal nucleic acid and protein (MAPs) sequences. Appropriate “control” genome comparisons, in addition to any arbitrarily chosen bacterium, should



**Fig. 2.** Importance of the karyomastigont in the evolution of mitosis [Chatton’s 1938 course board (Left) Classification of cell types by the presence and localization of their centrosomes [Archives of the Museum of Natural History, Perpignan, France, bequest of André Lwoff (56)] corresponding to major taxa (Right)]. First row, mitosis including karyomastigont duplication, e.g., *Chlamydomonas* and *Trypanosoma*; second row, mitosis including “paradesmose” (pole-to-pole thin spindle) parabasalids—*Trichomonas*, devescovinids, and some hypermastigotes; third row, mitosis including centrosome duplication, animal cells; fourth row, mitosis includes duplication of intranuclear membrane-attached spindle-microtubule-organizing center (MTOC) of ciliates, red algae, conjugating green algae, and fungi (in ciliates and fungi with closed mitosis, the MTOC is attached to inner nuclear membrane); fifth row, acentrosomal mitosis typical of plants. [Reproduced with permission from Marie-Odile Soyer-Gobillard (56).]

include thermoacidophilic archaeobacteria (cytoplasm homologue),  $\alpha$ -proteobacteria (mitochondrial homologue), and cyanobacteria (plastid homologue). The full oxidation of elemental sulfur to sulfate should correlate with later acquisition of the  $\alpha$ -proteobacterium that became the mitochondrion. New techniques (e.g., genomics, proteomics, microbial physiology, geochronology, and geochemistry) are powerful enough to resolve this century-old evolutionary problem.

## Materials and Methods

The methods, mostly traditional, are published but ignored by molecular and microbiologists, namely seme analysis (48). The unit of evolutionary analysis is the “seme”: newly gained “neosomes” and changed “aposomes”; increase or decrease in the number or size of an existing seme (hyper-/hyposeme, respectively) (48). Other methods include microbial ecological and laboratory techniques (53, 58), standard electron microscopy (23), the fluorescent immunocytochemistry (5), and sulfidogen analysis by sulfidometer (16). To fill the largest evolutionary gap in the living world, we use knowledge of the organisms that bridged it: the unicellular eukaryotes in response to relevant environmental variables (fluctuating temperatures, salinities, pH, organic matter concentration, desiccation–rewetting cycles, and oxic-anoxic-sulfidic diurnal and seasonal variation). We attempt to recover an immense protistological literature: meticulous study of nucleated microbes in nature, their developmental life histories, and their morphological, cell biological, and ecological relationships.

**Notes Added in Proof.** Dubinina’s aerotolerant spirochetes differ significantly enough from *Spirochaeta* to warrant a new genus description. The two articles (i) that place at least six strains of these spirochetes in a new genus named for B. V. Perfiliev and (ii) that report DNA sequence

for one of them (White Sea strain) await the deposition of the pure cultures in two international culture collections (G. A. Dubinina, personal communication).

A first example of retention of microtubule-associated proteins (MAPs) has been published (61).

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