

The Neomuran Revolution and Phagotrophic Origin of Eukaryotes and Cilia in the Light of Intracellular Coevolution and a Revised Tree of Life

Thomas Cavalier-Smith

Department of Zoology, University of Oxford, Oxford OX1 3PS, United Kingdom

Correspondence: tom.cavalier-smith@zoo.ox.ac.uk

Three kinds of cells exist with increasingly complex membrane-protein targeting: Uni-bacteria (Archaeobacteria, Posibacteria) with one cytoplasmic membrane (CM); Negibacteria with a two-membrane envelope (inner CM; outer membrane [OM]); eukaryotes with a plasma membrane and topologically distinct endomembranes and peroxisomes. I combine evidence from multigene trees, palaeontology, and cell biology to show that eukaryotes and archaeobacteria are sisters, forming the clade neomura that evolved ~ 1.2 Gy ago from a posibacterium, whose DNA segregation and cell division were destabilized by murein wall loss and rescued by the evolving novel neomuran endoskeleton, histones, cytokinesis, and glycoproteins. Phagotrophy then induced coevolving serial major changes making eukaryote cells, culminating in two dissimilar cilia via a novel gliding–fishing–swimming scenario. I transfer Chloroflexi to Posibacteria, root the universal tree between them and Heliobacteria, and argue that Negibacteria are a clade whose OM, evolving in a green posibacterium, was never lost.

THE FIVE KINDS OF CELLS

The eukaryotic cell originated by the most complex set of evolutionary changes since life began: eukaryogenesis. Their complexity and mechanistic difficulty explain why eukaryotes evolved 2 billion years or more after prokaryotes (Cavalier-Smith 2006a). To understand these changes, we must consider the cell biology of all five major kinds of cells (Fig. 1); determine their correct phylogenetic relationships; and explain the causes, steps, and detailed

mechanisms of the radical transitions between them. Figure 1 highlights three fundamentally different kinds of prokaryote differing greatly in membrane topology and membrane and wall chemistry. In all cells, the major membrane lipids are glycerophospholipids having two hydrophobic hydrocarbon tails attached to a hydrophilic phosphorylated glycerol head, but glycerol-phosphate stereochemistry differs in archaeobacteria (*sn*-glycerol-1-phosphate) from that in all other cells (*sn*-glycerol-3-phosphate). Negibacteria and posibacteria (collectively called

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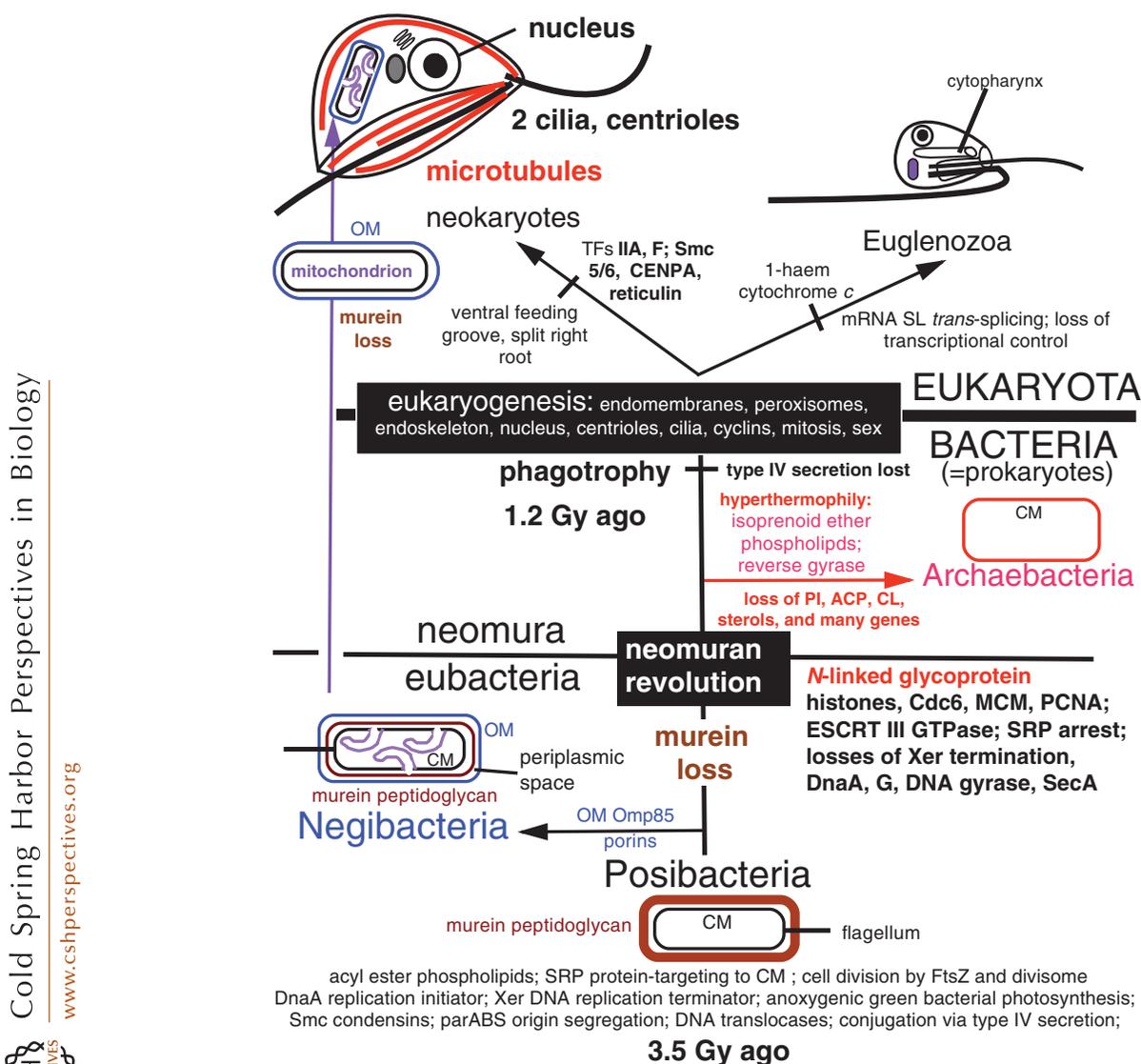


Figure 1. Relationships between the five major cell types, showing key evolutionary innovations in the transitions making them. Rigid murein cell walls originated before the ancestor of all life using both D- and L-amino acids in the first cell, a posibacterium with acyl ester glycerophospholipids that divided using FtsZ, possibly a photoheterotroph similar to *Heliobacterium*. Neobacteria evolved by acquiring an outer membrane (OM) with complex targeting of porins and other β -barrel proteins inserted by Omp85-dependent machinery never lost in the history of life, being retained when eukaryotes enslaved phagocytosed neobacteria to make mitochondria and subsequently chloroplasts (even kept in secondarily anaerobic DNA-free hydrogenosomes and mitosomes that evolved by drastically modifying aerobic mitochondria). The neomuran revolution was arguably a stabilizing response to traumatic loss of murein. Histones H3/4 ensured passive negative DNA supercoiling (making nucleosomes) to replace eubacterial ATP-driven supercoiling by DNA gyrase; this stabler DNA coiling forced drastic coevolutionary changes in RNA polymerase and especially DNA replication machinery: repair polymerase δ replaced DNA polymerase III, the β -clamp became PCNA, the replication fork helicase Mcm replaced DnaB, the unrelated Pol primase replaced DnaG primase, and Cdc6 replaced the replication initiator DnaA; Cdc6 possibly evolved from a gene duplicate of the eubacterial clamp loader DnaX, itself undergoing minor modification to neomuran RFC. (Legend continues on following page.)

“eubacteria”) are mutually closer in cell envelope chemistry and informational machinery than either is to archaeobacteria, whose basic informational machinery is eukaryote-like despite their cells being fully prokaryotic in structure and DNA segregation machinery. Eubacteria, unlike eukaryotes and archaeobacteria, generally have cell walls of the peptidoglycan murein that forms a covalently cross-linked bag (sacculus) completely surrounding the cytoplasmic membrane (CM). Murein hydrolase enzymes must repeatedly cleave, and other enzymes reseal, murein covalent bonds so that eubacteria can grow without bursting under high internal osmotic pressure (Egan and Vollmer 2013).

Some derived methanogenic archaeobacteria have covalently cross-linked walls of pseudomurein, a different peptidoglycan with similar cleavage-resealing growth. However, other archaeobacteria and all eukaryotes have cell surfaces of

non-cross-linked globular glycoproteins containing hydrophilic oligosaccharides covalently linked to asparagine (N) residues. N-linked glycoproteins are made cotranslationally, oligosaccharides being attached during *trans*-membrane protein secretion by membrane-associated ribosomes. I argued that this shared character evolved in the last common ancestor (cencestor) of eukaryotes and archaeobacteria, which jointly constitute the putative clade neomura. The neomuran theory of eukaryote origins (Cavalier-Smith 1987c; revised and updated: Cavalier-Smith 2002c, 2009, 2010c) has a phylogenetic part and a causal mechanistic part, as should any scientific explanation of megaevolutionary events. Many inadequate “theories” of eukaryote origin focus exclusively on phylogeny and have no explanatory part or only a cursory, unconvincing one. Phylogenetically, the neomuran theory asserts that (1) eubacteria are substantially older than and ancestors of neo-

Figure 1. (Continued) Novel TATA-box-binding transcription factors (TBP and others) (Ouhammouch et al. 2009) replaced the eubacterial transcription regulator CrtA. Murein loss freed MreB filaments that maintain eubacterial rod shape (or related ParM filaments that segregate some plasmids) to become the actin endoskeleton, conferring osmotic stability; new ESCRT-III filaments helped membrane division, allowing loss of FtsZ in eukaryotes and some archaeobacteria. Novel cotranslationally made N-linked glycoprotein enabled archaeobacteria to make rigid S-layer-like walls and eukaryotes a flexible cell surface coat, allowing phagotrophy and ingestion of prey cells to evolve, triggering a cascade of eukaryogenic changes associated with coated vesicle origins. These mediated endomembrane differentiation, internal digestion, targeted vesicle fusion, and nuclear envelope evolution to protect chromatin internalized by phagocytosis (see Fig. 2); α -tubulin, β -tubulin, and γ -tubulin evolved from posibacterial plasmid-segregating TubZ GTPase, enabling DNA segregation by mitosis, drastically changing chromosome organization; cohesins enabling mitosis and eukaryotic cell-cycle controls evolved from duplicated Smc condensins. Archaeobacteria replaced acyl ester lipids by heat-stable isoprenoid tetraethers to become the first extremophiles, but lost so many lipids and proteins that they could never have evolved directly into eukaryotes, as did the transient neomuran ancestor, retaining far more eubacterial characters. Archaeobacteria kept fatty acid (FA) synthesis (Lombard et al. 2012a) but lost acyl-carrier protein (ACP), which enables rapid bulk FA synthesis in eubacteria and eukaryotes, no longer needed for the trace FA amounts that sufficed after archaeobacteria lost acyl esters, including phosphatidylinositol (PI) and cardiolipin (CL). Neokaryotes retained bacterial transcription regulation but evolved new transcription factors (TFs). Soon after mitochondrially donated group II self-splicing introns became spliceosomal introns in the cenancestral eukaryote (Cavalier-Smith 1991c), Euglenozoa evolved *trans*-splicing of spliced-leader (SL) minioxons for all mRNAs (Cavalier-Smith 1993) and lost transcriptional control of gene expression. Neokaryotes alone replaced centromeric histone H3 by CENP-A and evolved Smc5/6 for DNA repair. Archaeobacterial flagella are not homologous to, and evolved independently of, eubacterial flagella, which must have evolved in Negibacteria, probably in early Gracilicutes (Cavalier-Smith 2006c); so if the tree is correctly rooted within Posibacteria, they were presumably acquired by Posibacteria by lateral gene transfer (LGT) subsequently, but before actinobacteria diverged from Teichobacteria (see Fig. 3). Ancestral green bacteria lacked flagella but could probably glide and thus make stromatolites, yielding the oldest fossil evidence for eubacteria. Absence of photosynthetic carbon fixation in archaeobacteria means that, unlike the much older eubacteria, they could never have fueled an extensive global ecosystem alone.

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mura; and (2) archaeobacteria are sisters of eukaryotes, not their ancestors. This explains why eukaryotes are a blend of eubacteria-like (e.g., membrane chemistry) and archaeobacteria-like characters (e.g., *N*-linked glycoproteins, information-processing machinery) plus entirely novel features (cell structure, mitosis). Eubacteria-like characters, ancestral for all life, were inherited vertically by eukaryotes, while archaeobacteria secondarily evolved unique lipids; shared archaeobacteria-like characters originated only in the neomuran cenancestor (last common ancestor); and uniquely eukaryotic characters evolved immediately after eukaryotes diverged from archaeobacteria. No other theory explains that as simply. Assuming that archaeobacteria were directly ancestral to eukaryotes (Van Valen and Maiorana 1980) requires non-parsimonious assumptions that eubacteria-like characters were regained during eukaryogenesis.

PHAGOTROPHY, CYTOSKELETON, MOTORS, AND THE COEVOLUTIONARY THEORY OF EUKARYOGENESIS

Three key innovations made eukaryotes: (1) an internal cytoskeleton of formin-associated actin filaments cross-linked by actin-related proteins (Arp 2/3) plus microtubules (α , β -tubulin) nucleated by γ -tubulin; (2) cytoskeleton-associated molecular motor ATPases: myosin for actin; kinesin and subsequently dynein for microtubules; (3) the eukaryote-specific endomembrane system (endoplasmic reticulum [ER], Golgi complex, and lysosomes—the bags of digestive enzymes discovered by De Duve that mediate intracellular digestion in eukaryotes but no bacteria). Only subsequently could the nucleus, centrioles, mitosis, and eukaryotic genetic system evolve, using and stimulated by this novel machinery. The decisive evolutionary mediator of eukaryogenesis was the origin of phagocytosis and intracellular prey digestion for the first time in history, which also enabled uptake and transformation of an α -proteobacterium into mitochondria, greatly improving aerobic utilization of intracellular digestion products simultaneously with the origin of the nucleus and cilia (Cavalier-Smith 2002c).

These ideas arose before archaeobacteria were recognized (Woese and Fox 1977). De Duve and Wattiaux (1966) suggested that rough ER evolved by budding from the CM of a wall-less bacterium when evolving an ability to ingest other cells by a primitive version of phagocytosis, with subsequent differentiation of internalized vesicles producing the Golgi apparatus and lysosomes for more efficient internal digestion. Stanier (1970) noted that such phagotrophy, absent from all prokaryotes, must have imposed novel selective forces favoring larger cell size and increased internal complexity, providing sufficient explanation of the greater internal complexity of eukaryotic cells, especially the origin of the internal cytoskeleton. I explained how phagotrophy, by internalizing CM with attached prokaryotic chromosomes, must have disrupted bacterial cell cycles and DNA segregation even more dramatically than wall-loss, so phagotrophy imposed novel selective forces causing evolution of mitosis, meiosis, and novel genetics and chromosome organization of eukaryotes (Cavalier-Smith 1975) and much larger genomes (Cavalier-Smith 1978b). John and Whatley (1975, 1977) argued that mitochondria originated from endosymbiotic purple–non-sulfur bacteria (now called α -proteobacteria).

I argued that these novel eukaryotic genetic features all evolved as coevolutionary responses to disruption of the prokaryotic cell-surface-located genetic system by the new endomembrane system, cytoskeleton, and associated motors. Moreover, the nucleus evolved by incomplete ER fusion around chromatin to protect it from shearing damage by cytoplasmic motors, and peroxisomes stemmed from subdivision and specialization of endomembranes. As murein-wall-loss necessitated osmotically and cell-cycle-stabilizing innovations, I proposed that microtubules evolved to stabilize DNA segregation by premitotic mechanisms, and actin was required for phagotrophy (not then known) and cytokinesis. I argued that actin was the primary and microtubules the next major molecular innovation enabling eukaryotes to evolve (Cavalier-Smith 1975). We now know that actin originated slightly before eukaryotes during the neomuran revolution, from MreB filaments

that help maintain shape for rod-like eubacteria (Löwe and Amos 2009; Wickstead and Gull 2011). However, changing MreB to actin was trivial, MreB sometimes being called “bacterial actin.” More radically, gene triplication made α, β, γ -tubulins subsequently, in the eukaryote ancestor, probably from posibacterial plasmid-segregating GTPase TubZ, rather than related FtsZ, to make spindle microtubules for mitotic DNA segregation (Cavalier-Smith 2010c).

I now suggest that the most fundamental eukaryogenic molecular innovation was the origin of formin rings (type A2) (Chalkia et al. 2008) to promote rapid actin polymerization (by encircling the filaments’ fast-growing barbed ends, drawing in monomers from profilin complexes) to extend pseudopodia around prey cells during phagotrophy, plus profilin to bind G-actin as a soluble store allowing sudden F-actin extension through formin-binding. Secondarily, actin duplicated, yielding Arp2/3 for branching by linking pointed ends to other actin filaments; with actin-capping proteins for barbed ends, branching made an osmotically stabilizing three-dimensional (3D)–gel meshwork. Figure 2 updates the intracellular coevolutionary consequences of phagotrophy. Like single-headed myosin I, actin proved essential for phagocytosis, but its involvement in cytokinesis in the contractile ring of podiate eukaryotes probably resulted from much later evolution of two-headed myosin II in early podiates (Fig. 3). Originally, eukaryote cytokinesis used the membrane-bending dynamin GTPase that evolved in the ancestral eubacterium (lost by crenarchaeotes) plus ESCRT-III GTPase filaments for membrane scission, which arguably evolved in the ancestral neomuran (Cavalier-Smith 2010c); crenarchaeotes lost ESCRT (and most lost FtsZ).

This coevolutionary phagotrophy theory of eukaryogenesis, enunciated before evidence for symbiogenetic origins of chloroplasts and mitochondria became compelling (Gray and Doolittle 1982; Gray 1992; Cavalier-Smith 2013b), initially wrongly assumed cyanobacterial ancestry of the whole eukaryotic cell (Cavalier-Smith 1975). I did not then appreciate the doubleness of the negibacterial envelope and likely mecha-

nistic impossibility of evolving unimembranous eukaryotes by wall-loss from any negibacterium. Although that defective phylogeny is consigned to history, the central logic of phagotrophy being the driving force behind the origin of endomembranes and cytoskeleton and of the radically transformative consequences of both novelties for eukaryotic cell cycles and genetics was almost certainly correct, providing the only logically coherent explanation for eukaryogenesis that is both mechanistically and selectively convincing.

Initially I argued that centrioles and cilia evolved from microtubules substantially after the first eukaryotes (Cavalier-Smith 1975, 1978a, 1981, 1982), but that was wrong. Almost certainly the first ciliated eukaryote had one cilium only (Cavalier-Smith 1975, 1978a), but the assumption that some primitively uniciliate eukaryotes still survive (Cavalier-Smith 1987c, 2002c) was wrong. Probably all uniciliates are secondarily simplified, and the eukaryote ancestor had two centrioles bearing dissimilar cilia, which take two cell cycles to develop to maturity, the anterior cilium being younger and forming in the first cell cycle, the older posterior one being modified in structure, position, and roots in the second cell cycle (ciliary transformation: Brugerolle 1992; Cavalier-Smith and Karpov 2012; Cavalier-Smith 2013a). Before each cell division, two new centrioles assemble beside old ones at the beginning of S phase when DNA is replicated, this being controlled by cyclin proteins unique to eukaryotes. Cyclins share a helical domain with the neomuran transcription factor TFIIB and perhaps evolved from it.

Proteolysis of proteins connecting the two older cilium-bearing centrioles allows their separation to opposite spindle poles during mitotic prophase. Possibly parent centrioles are held together by the same loop-like cohesin proteins as sister chromatids (Nasmyth 2011; Eichinger et al. 2013), both assembling at S-phase onset, and both cleaved by the enzyme separase, emphasizing the deep coevolution of chromosome and centriole cycles. However, in *Drosophila* at least, cohesin cleavage is inessential for centriole disengagement, which requires a drop in cyclin-

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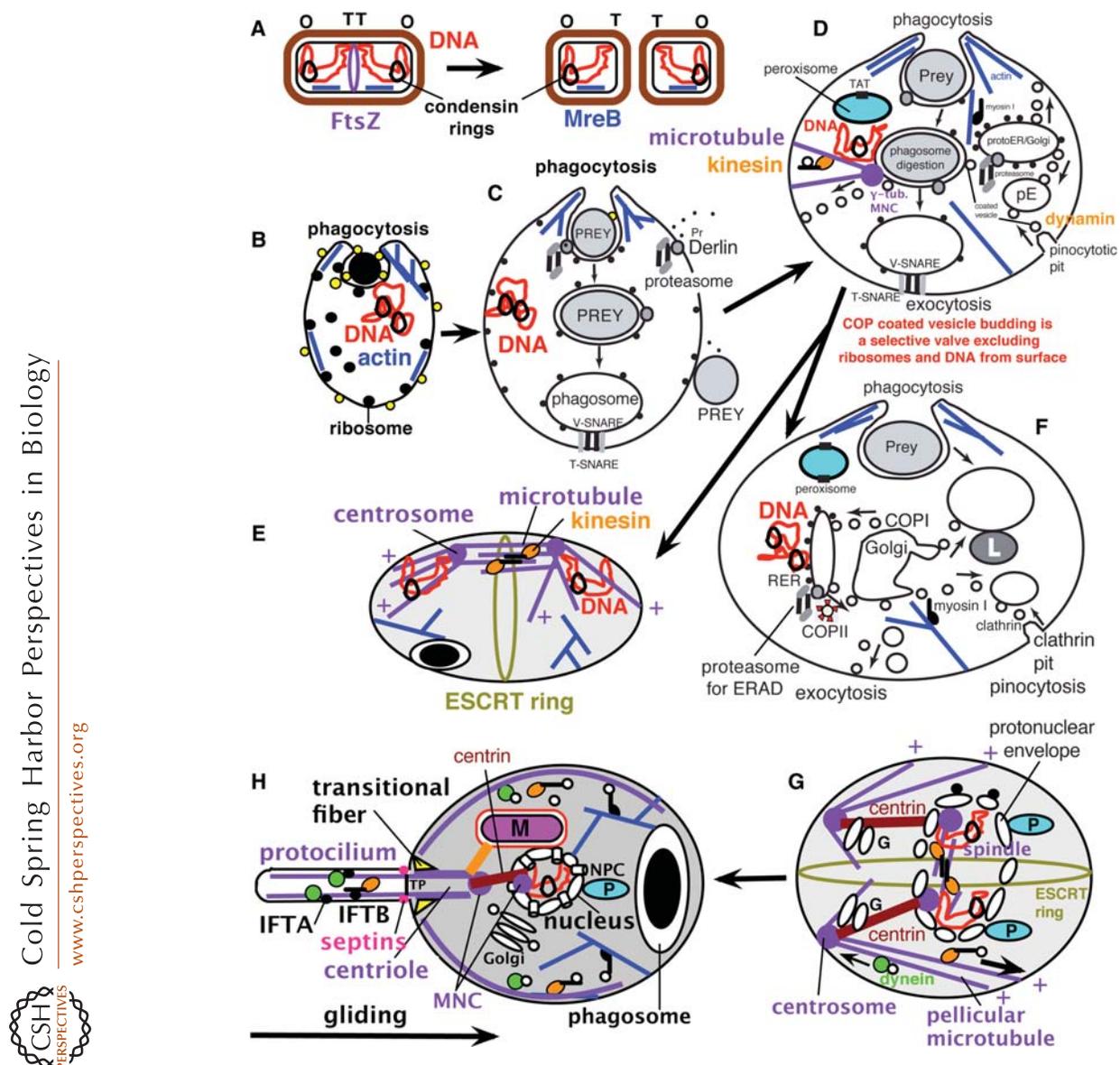


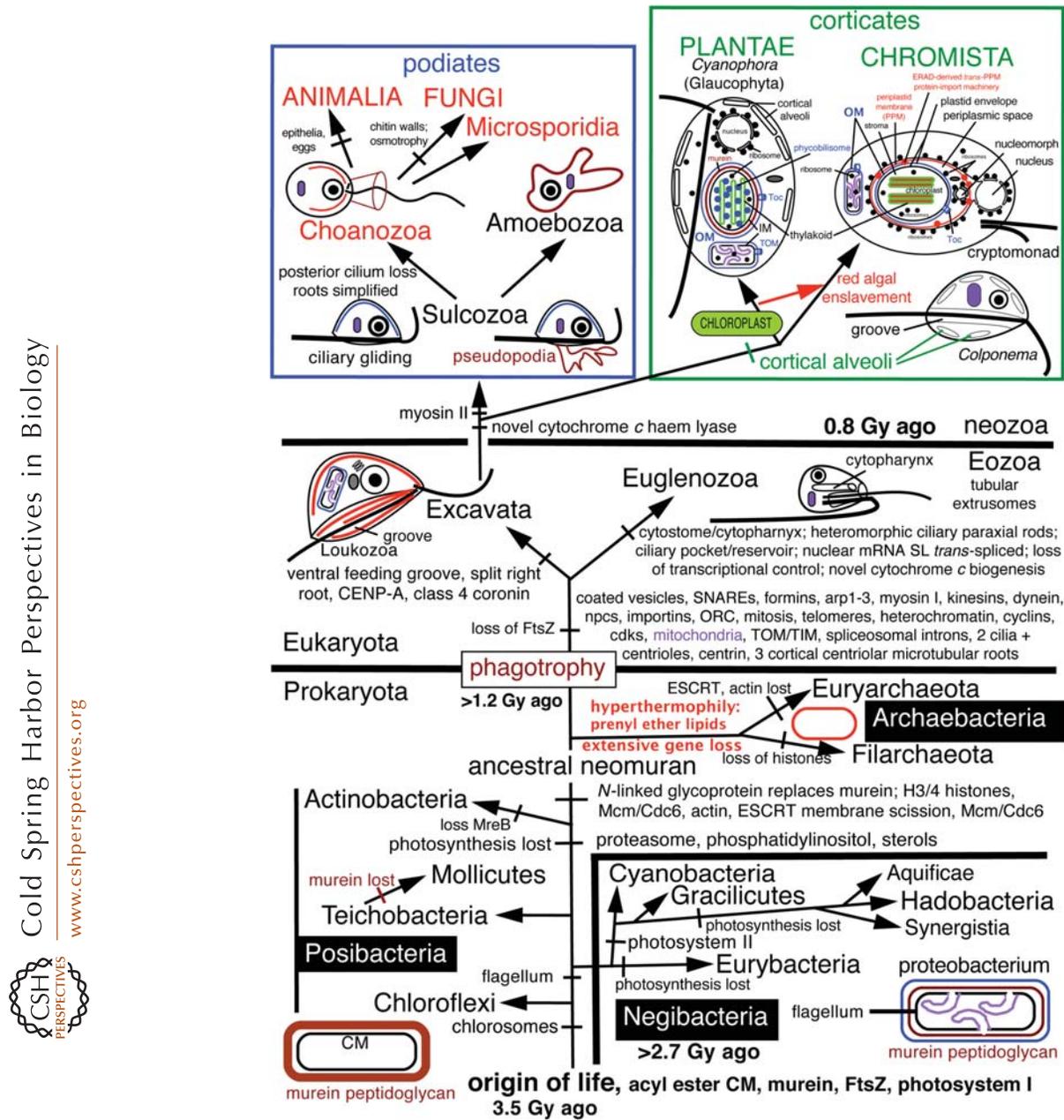
Figure 2. Intracellular coevolution during phagotrophy-driven eukaryogenesis. (A) Eubacteria segregate DNA by actively moving replicon origins (O) by ParABS machinery, DNA condensation by Smc condensin rings, and moving termini (T) by the DNA translocase FtsK anchored at the mid-cell nascent division site, marked by the GTPase FtsZ ring for membrane scission by the divisome after XerCD recombinase resolves daughter DNAs into covalently separate molecules. Neomuran loss of murein disrupted orderly linear arrangement of chromosome origins and termini on a rigid wall, causing FtsK loss and allowing replicon numbers per chromosome to increase, and ESCRT-III GTPase filaments replaced the eubacterial divisome; simultaneously, the posibacterial paracrystalline S layer became novel N-linked glycoproteins. (B) In eukaryotes only, these glycoproteins (yellow) became flexible and specialized for binding prey, initially digested by enzymes secreted externally by membrane-attached ribosomes. MreB (or its plasmid segregation ParM relative) (Yutin et al. 2009) evolved into actin, yielding linear filaments stimulated by formins for extending cytoplasm partly around prey, thereby increasing digestion product absorption, and duplications yielded Arp2/3, generating an osmotically stabilizing branching endoskeleton (blue). (*Legend continues on following page.*)

dependent kinase activity, and another centrosomal separase target may be the crucial linker of new and old centrioles (Oliveira and Namyth 2013); in *Caenorhabditis*, cohesin seems involved only in certain developmental stages (Cabral et al. 2013). In mammals, the giant coiled-coil kendrin is a separase target mediating centriole disengagement (Matsuo et al. 2012), but its sequence is highly conserved only in vertebrates, which have a clearly related family

of A-kinase anchor proteins important for many aspects of cell structure; but as neither is reliably traceable beyond vertebrates, it is possible that when early animals expanded centrosome size cleavage of more than one, possibly novel centrosome protein became necessary, even if cohesin may have been the ancestral target (still a conjecture). Cohesin rings comprise Y-shaped Smcs and kleisin cross-linkers that evolved (after pre-eukaryotes diverged from archaebacteria)

Figure 2. (Continued) (C) Evolution of a surface membrane protein channel (Derlin) enabled partially digested proteins to be pulled across the membrane and fully digested on its cytosolic face by cylindrical proteasomes. Digestion products of prey completely internalized into a phagosome (*center*) was most efficiently absorbed; phagosome-associated V-SNAREs and CM-associated T-SNAREs evolved to refuse phagosome membranes with the surface. (D) Accidental phagocytic internalization of membrane-attached DNA was made permanent by evolving COP-coated vesicles that returned membrane only to the cell surface; after exclusion of ribosomes and DNA from COP vesicles, continued phagocytosis removed all from the plasma membrane, and the internalized ribosome/DNA-associated membrane became protoER. Membrane fragmentations generated separate compartments specializing in β -oxidation of fatty acids (peroxisomes) and cytochrome P450 oxidation of aromatics and protein secretion (ER). Bacterial Sec61/SRP for extruding unfolded proteins was retained by ER and TAT machinery for unfolded proteins modified for peroxisome biogenesis (for more details, see Cavalier-Smith 2009). FtsZ-related posibacterial plasmid-segregated TubZ GTPase evolved by gene duplication into eukaryotic mitotic segregator (α - β -tubulin microtubules, nucleated at minus ends by γ -tubulin-containing centrosomes), microtubule rigidity mechanistically replacing peptidoglycan rigidity. Initially, microtubule polymerization forced sister centrosomes and associated DNA apart. COPs were also used for pinocytosis, and preexisting dynamin and ESCRT-III coopted for membrane scission generating protoendosomes (pE). Single-head myosin I and kinesin diverged from a common posibacterial ATPase ancestor to form motors for internalizing phagosomes along actin filaments or minus-to-plus movement of vesicles on microtubules for exocytosis, respectively. (E) Kinesin was coopted to push apart antiparallel microtubules from sister centrosomes, improving segregation, cytokinesis being by preexisting neomuran ESCRT-III GTPase ring orthogonal to the spindle. (F) Simultaneously, coordinate gene duplications of COP proteins and SNAREs multiplied the number of topologically and chemically distinct compartments developmentally interlinked by vesicle transport: copII for ER to Golgi, CopI for recycling membrane from Golgi, and clathrin for making protoendosomes (pE) and lysosomes (L) (Faini et al. 2013). (E and F) Aspects of the same stage. (G) A protonuclear envelope formed by partial ER cisternal fusion onto the surface of chromatin, centrosomes duplicating into centrin-connected distinct microtubule-nucleating centers (MNC) for cell-surface cortical microtubules and nuclear-envelope-associated spindle poles. COPII coats were retained by the protonuclear envelope, evolving into nuclear pore complexes (NPC: their origin and that of importin- and RanGTP-gradient-based nucleocytoplasmic protein import using nuclear localization signals [NLS] were fully explained in Cavalier-Smith 2010c). Novel rapid DNA segregation by mitosis in anaphase replaced two-stage rigid-wall-associated prokaryotic segregation via new spindle kinesins, causing chromosome linearization and telomeres. Minus-end-directed dynein ATPase motors evolved to move vesicles along microtubules to centrosomes, fusing to form centrosome-attached, stacked Golgi cisternae (G) specializing in subsequent glycosylation stages. (H) Transition fibers attached a ring of microtubules to the cell surface forming a protocilium, a novel heterotrimeric kinesin-2 evolving to move them relative to protociliary membrane glycoproteins adhering to the substratum, initiating protociliary gliding to carry cells to fresh prey; recruitment of septins to the protociliary base and evolution of a transition zone plate (TP) and collar, plus anterograde (IFTB) and retrograde (IFTA) transport particles from COPI coats, and modification of nuclear protein-targeting machinery for ciliary protein import established a discrete protociliary compartment. Figure 5 shows how this could have evolved into 9 + 2 cilia in the cenancestral eukaryote. Division of labor among coevolving peroxisomes (P: ancestrally attached to and segregated with the nuclear envelope in closed mitosis), endomembranes, and mitochondria (M: derived from phagocytosed, undigested α -proteobacteria) optimized aerobic metabolic utilization of phagotrophy digestion products.

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Figure 3. Expanded tree of life showing major subdivisions of ancestral eubacteria and derived neomura. Key innovations in cell evolution primarily involve membranes and cell skeleton. Unibacteria with single membranes evolved three different CM chemistries: Endobacteria (thick-walled Teichobacteria plus derived wall-free mycoplasmas and spiroplasmas [i.e., Mollicutes]) and Chloroflexi, the two most ancient posibacterial subphyla, retained ancestral hopanoids as membrane rigidifiers. Actinobacteria evolved sterols and phosphatidylinositol. Archaeobacteria, the youngest bacterial phylum, sister to eukaryotes, replaced acyl ester phospholipid bilayers by a stabler isoprenyl ether monolayer to become the first hyperthermophiles. Numerous proteins were lost during their origin and early diversification. (*Legend continues on following page.*)

by gene duplications from homologous condensin rings that universally mediate higher-order DNA folding (bacterial nucleoid-folding; eukaryote chromatin-folding). Anaphase chromosome separation is initiated later than centriolar disengagement by ubiquitin-controlled proteasome-mediated proteolysis of separase inhibitors and hundreds of other proteins, all marked by anaphase-promoting complex (APC)

ubiquitin ligase (Oliveira et al. 2010) and timed by changes in cyclin phosphorylation (a uniquely eukaryotic cell-cycle control principle) (Nasmyth 1995).

These universally shared cohesin-based features of higher-order DNA folding and segregation strongly argue against past wild speculation that neomura evolved DNA replication independently of eubacteria, quite apart from the

Figure 3. (Continued) The neomuran common ancestor probably arose from a stem actinobacterium by replacing covalently cross-linked cell-wall peptidoglycan by more flexible glycoproteins via an antibiotic-resistant but traumatically wall-less, DNA-segregationally defective intermediate that recovered through revolutionary change in ribosomes/SRPs and evolving histone-stabilized chromatin, causing radical changes to DNA-handling enzymes: the neomuran revolution. Eukaryotes arose by exploiting the new flexible glycoprotein surface to trap and phagocytose bacteria; phagotrophy internalized their digestive system (endomembranes) and genetic system, stabilized by additional histones, novel endomembrane attachments, and nuclear-pore complexes and a novel 3D internal cytoskeleton and novel motors, used for mitosis/cell division and vesicle and ciliary motility, and internalized an α -proteobacterium for enslavement as a mitochondrion (synergistically improving food-energy conversion). Eukaryotes diverged early into Euglenozoa (which retained ancestral ciliary gliding on surfaces and divergent DNA replication initiation and mitochondrial protein import machinery, but evolved specialized feeding apparatus for a surface-associated lifestyle) and Excavata, which lost gliding and evolved planktonic feeding by a posterior ciliary groove. Excavata comprise nonamoeboid Loukozoa, often with posterior cilium vanes, plus vane-free Percolozoa ancestrally with alternating amoeboid and flagellate stages (sometimes differentially lost). From a vanned *Malawimonas*-like loukozoan that simplified cytochrome *c* biogenesis by evolving unimolecular heme lyase stemmed two derived supergroups of contrasting morphology and lifestyle: (1) Corticates specializing on photic zone planktonic living by evolving cortical alveoli and enslaving cyanobacteria to form chloroplasts (first Plantae [almost all lost phagotrophy] then a secondary enslavement of a red alga to generate photophagotrophic Chromista) (Cavalier-Smith 2013b); many corticates evolved a fourth microtubular ciliary root (R4) absent from podiate and eozoan supergroups. (2) Exclusively heterotrophic podiates, by origin of ventral pseudopodia, and dorsal pellicle associated with reevolved posterior ciliary gliding, with subsequent loss of posterior cilium and its roots to create opisthokonts (names in red) with radically simplified cytoskeleton. Vanes were lost by all neozoa but *Colponema*, which retained the loukozoan feeding method. Ancestrally, chromists had four kinds of ribosome, four genomes, and novel membrane topology with nuclear-coded proteins imported across the periplastid membrane by novel mechanisms derived by duplications from the ERAD machinery that evolved to export unfolded proteins for proteasome digestion in the first eukaryote (Fig. 2C,D,F); many evolved tubular ciliary hairs that modified feeding in heterokonts (Cavalier-Smith and Scoble 2013). All except cryptomonads lost the nucleomorph and periplastid ribosomes. Long-tailed myosin II that forms antiparallel aggregates mediating contraction of podiate pseudopodia and cytokinetic contractile actomyosin rings probably evolved near the ancestral podiate, assuming that the percolozoan *Naegleria* got myosin II by LGT from podiates. Very different reticulose/filose pseudopodia evolved in the chromist infrakingdom Rhizaria. Amoebozoa and opisthokonts, formerly grouped as “unikonts,” evolved from biciliate Sulcozoa by independently losing gliding (eukaryote cytoskeletal diversification and its coevolution with changing feeding modes are detailed elsewhere: Cavalier-Smith and Chao 2012; Cavalier-Smith and Karpov 2012; Cavalier-Smith 2013a). Ancestrally, photosynthetic Negibacteria retained hopanoids and diversified into eight phyla differing in IM photosynthetic machinery, OM chemistry, and flagellar organization. Eurybacteria comprise Negativicutes (Marchandin et al. 2010) (formerly Selenobacteria: Cavalier-Smith 1992, 2002b, 2006c), Fusobacteria, and Thermotogales. Filarchaeota comprise crenarchaeotes, thaumarchaeotes, and korarchaeotes. Although the origin of the first (stem posibacterial) cell was probably as early as 3.5 Gy ago, the major eubacterial radiation producing their modern (crown) phyla likely occurred subsequently, possibly ~2.7–2.5 Gy ago; its essential simultaneity accounts for almost nonexistent resolution at the base of the eubacterial tree (Pace 2009), which coupled with a quantum-evolution-stretched neomuran stem in many sequence trees makes it very hard to place neomura anywhere robustly within the eubacterial tree.

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fact that the genetic complexity of any eubacterial/neomuran intermediate with probably at least a thousand genes could not have been supported by a purely RNA-based genome without the conservatism and evolutionary stasis allowed by efficient DNA repair. The key importance of cell-cycle continuity during eukaryogenesis has too often been ignored; many imaginary intermediates could not have reproduced.

The complex origin of phagotrophy was possibly simplified before full phagocytosis by an intermediate stage importing extracellular prey proteins through Derlin surface-membrane pores, coupled with digestion internally by surface-attached proteasomes before lysosomes evolved (Fig. 2C) (Cavalier-Smith 2009). Proteasomes, originating before actinobacteria and neomura diverged (Cavalier-Smith 2006c), were radically complicated by numerous gene duplications in the eukaryote ancestor when first participating in novel eukaryotic cell-cycle controls.

L-FORMS AND THE NEOMURAN REVOLUTION AND EUKARYOGENESIS: CELL CYCLE/STRUCTURE COEVOLUTION

The neomuran revolution was initiated by accidental loss of the posibacterial murein wall. Posibacteria can spontaneously lose murein to become naked L-forms; similar protoplasts can be generated in the laboratory by penicillin, preventing enzymes from resealing murein during growth (Dominguez-Cuevas et al. 2012). Such naked cells survive without bursting in osmotically protected environments and undergo multifarious drastic changes, far more extensive than the effects of most DNA mutations: experimentally tractable analogs for the initiating phase of the neomuran revolution.

During eubacterial cell cycles, coordinated DNA segregation, cell growth, and division depend on CM attachment of sister chromosomes, cell wall rigidity, and its geometrically controlled growth and division (Fig. 2A) (Egan and Vollmer 2013). The fission site for divisomes is marked by a GTPase FtsZ filament ring (Adams and Errington 2009; Buske and Levin 2013);

ParA ATPase filaments move replicon origins via ParB-binding condensin-rich centromere-like parS DNA segments (Gruber and Errington 2009; Sullivan et al. 2009); after replication terminates with eubacteria-specific dimer resolution by Xer-recombinase, surface-associated DNA translocases separate termini (Kaiser et al. 2009; Sherratt et al. 2010; Grainge et al. 2011). Murein loss grossly disrupts that elaborate cell-cycle mechanism and osmotically destabilizes cells. L-forms therefore bleb membranes, yielding DNA-less gobbets and polyploid cells with several chromosomes. These traumatized physically and genetically unstable cells over weeks or months can spontaneously become stable L-forms without murein, which can multiply for years and have even been isolated from nature; that indicates extremely strong selection for cell-cycle stabilization in accidental L-forms. Eubacterial chromosome organization (a single replicon with one origin and terminus only) is fundamentally coadapted to progressive DNA segregation throughout the cell cycle and active movement of origins separately from termini (Cavalier-Smith 1987a); that originally unfashionable idea of active movement and orderly cell-surface association of eubacterial DNA is vindicated (Stouf et al. 2013).

Simultaneously, I argued that unique features of eukaryote genetics and cell cycles were caused by rescuing segregationally defective L-forms by evolving mitosis with one-step sudden anaphase segregation of all DNA, necessitated by phagotrophy-induced internalization of membrane DNA-attachment sites (Cavalier-Smith 1987c). Indirectly, novel cell structure changed genetics by shifting selective forces. As adumbrated for eukaryotes alone (Cavalier-Smith 1975), nucleosomes with histones H3/4 evolved immediately following murein wall loss to stabilize neomuran chromosomes. Histones H2a,b and H1 evolved in eukaryotes only for mitotic chromosome condensation cycles: prophase compaction to avoid shearing in sudden anaphase, plus telophase loosening for interphase transcription (Cavalier-Smith 2010c). In its neomuran form, the unified phagotrophy and coevolutionary theory of eukaryogenesis coherently explained 48 eukaryotic innovations (Cavalier-

Smith 1987c), and subsequently still more (Cavalier-Smith 2002c, 2010c).

THE NEOMURAN REVOLUTION, EUKARYOGENESIS, AND ARCHAEBACTERIAL DIVERGENCE

Mechanistically, the neomuran theory comprises three distinct, interlinked theories, explaining (1) the origin of eukaryotes; (2) the origin of archaeobacteria; and (3) that both origins were caused by a single dramatic initiating event, the “neomuran revolution”: a radical change in eubacterial cell structure generating a transient, unstable L-form-like, evolutionary intermediate—the neomuran cenancestor (Cavalier-Smith 1987c). The core ideas of the neomuran theory are as follows: (1) Murein loss and cell-cycle destabilization happened immediately before Eukaryote/Archaeobacterial divergence. (2) Stabilization was rapid (over a few years, not thousands or millions) but extended long enough for some changes to occur in common before the phylogenetic split (Table 1) and some (in radically divergent directions) subsequently, separately in eukaryote and archaeobacterial sister lineages (Fig. 1). The neomuran revolution was a rescue from trauma that changed cells more radically than standard evolutionary divergence by single-gene mutations or getting preexisting genes from foreign bacteria by LGT. (3) Evolution of phagotrophy simultaneously explains the novel cell structure and genetic system of eukaryotes, both mechanistically and selectively. Transient instability and polyploidy before mitosis was perfected made innumerable simultaneous gene duplications (allowing major genetic innovations) and gene losses. (4) The evolutionary success and cellular divergence of eukaryotes and archaeobacteria

were because the stabilized neomuran’s immediate descendants adopted two entirely novel, contrasting life styles, freeing them from competition with their long-established eubacterial ancestors: phagotrophy and hyperthermophily.

The naked protoeukaryotic lineage invented phagotrophy by phagocytosis and internal digestion of other cells, thereby becoming more efficient predators than any eubacteria, and sexual cell fusion, thereby creating more sharply defined “biological species” with radically different population structure from typically clonal prokaryotes (Cavalier-Smith 1991a). Archaeobacteria invented hyperthermophily via novel heat-stable lipids: isoprenoid ethers with two glycerophospholipids covalently linked by their hydrophobic tails, making double-headed tetraether lipids forming a stable membrane monolayer. Life originated as eubacteria under easy mesophilic conditions, although Thermotogales and Aquificales became secondary hyperthermophiles by thermostable-protein LGT from archaeobacteria. Fatty acid and isoprenoid synthesis evolved in the cenancestral cell (Lombard and Moreira 2011; Lombard et al. 2012a,b; like many others, they incorrectly root the tree between neomura and eubacteria). Younger archaeobacteria were ancestrally hyperthermophiles, although some subsequently became mesophilic by LGT from eubacteria, reverting to lipid bilayers, unavoidably retaining isoprenoid diethers because their hyperthermophilic cenancestor irretrievably lost ancestral acyl ester biosynthetic machinery. Because evolving archaeobacterial heat-stable lipids was enzymatically simple, they became the world’s first hyperthermophiles by gradually modifying numerous proteins enabling spread into ever-hotter habitats free of competitors. The neomuran mevalonate pathway for isoprenoid synthesis was

Table 1. Key stabilizing innovations during the neomuran revolution

1. MreB becomes actin; stabilizes against osmotic stress
2. ESCRT-III filaments for membrane division during cytokinesis
3. DNA gyrase (ATP-using active supercoiler) lost; replaced by histones H3/4 (later lost by crenarchaeotes); passive wrapping of DNA around nucleosomes protects; Mcm proteins
4. SRP adds cotranslation arrest domain
5. N-linked glycoproteins

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already present in their posibacterial ancestor (archaebacteria replaced some enzymes) as was a relative of the enzyme making *sn*-glycerol-1-phosphate to which archaebacteria alone attach isoprenoid tails (Peretó et al. 2004). Probably mevalonate and methylerythritol phosphate (MEP) isoprenoid synthesis pathways both originated in early posibacteria and were differentially lost (notably MEP during the neomuran revolution and mevalonate by many eubacteria); LGT was rarer than earlier claimed (Lombard and Moreira 2011).

Membrane stability also involved retaining contiguous laterally interacting semicrystalline surface glycoproteins as an S layer, like the nonglycosylated S layer outside the thick murein walls of their posibacterial ancestors. Wall rigidity enabled some archaebacteria to retain the ancestral eubacterial FtsZ cell division machinery but prevented phagotrophy or sexual cell fusion evolving. Therefore archaebacterial cell structure, growth, division, and genetics remained fundamentally bacterial or prokaryotic. Early claims that archaebacteria are a “third form of life” in addition to eukaryotes and prokaryotes/bacteria are thus falsified, despite misleading, confusing, purely propagandistic name changes that some of us never accepted (deleting “bacteria” from archaebacteria and “eu” from eubacteria); as *Archaea* Koch and Baerendt, 1854 is a genus of Madagascan spiders first discovered in Baltic amber (Dippenaar-Schoeman and Jocqué 1997), it was doubly confusing to use the same name for a group of bacteria and to make it ambiguous whether “bacteria” refers to all prokaryotes, as it properly does (Cavalier-Smith 2007a), or just eubacteria (Woese et al. 1990). In fact, *archaebacteria are a third form of bacteria* (Figs. 1 and 3). Contrary to early misconceptions, still sadly widespread, their discovery was irrelevant to the origin of life, yet crucial for understanding eukaryote origins because their striking molecular differences from eubacteria and marked partial similarities to eukaryotes enabled four strong deductions, universally accepted:

1. In conjunction with strong similarities of mitochondria to α -proteobacteria (John and Whatley 1975; Gray 1992), it showed that mi-

tochondria could not have evolved from the same ancestor as the rest of the eukaryotic cell. One must accept that eukaryotes are evolutionary chimeras of a moderately changed α -proteobacterium (negibacterium) and far more complex, radically different host cell (Cavalier-Smith 2002c).

2. That host was more closely related to archaebacteria than to eubacteria.
3. Differences between it and eubacteria evolved in two stages: those shared with archaebacteria first; organizationally more radical, uniquely eukaryotic inventions subsequently.
4. In conjunction with marked similarities between chloroplasts and cyanobacteria, one must accept that the host component was a heterotroph, chloroplasts being implanted subsequently by phagocytosis and radical transformation of cyanobacteria (Cavalier-Smith 2000, 2013b).

ACTINOBACTERIA, LIKELY NEOMURAN SISTERS

The neomuran theory identified exospore-forming actinobacteria (e.g., *Mycobacterium*, *Streptomyces*), all possessing phosphatidylinositol (unlike other prokaryotes), crucial for eukaryote cell signaling, and cholesterol, and many with 40S proteasomes (unlike other eubacteria), as more likely posibacterial ancestors of neomura than Teichobacteria, the posibacterial class with thick teichoic-acid-containing walls and endospores (e.g., *Bacillus*), although Valas and Bourne (2011) gave reasons favoring Teichobacteria instead. Their and my arguments are simultaneously satisfied if the ancestor was a phylogenetic intermediate, sister to actinobacteria but derived from a Teichobacteria-like ancestor (Fig. 3); this also reconciles neomuran theory with sequence trees that typically place neomura in an unresolved position close to the base of the eubacterial radiation in which branching orders among the three main posibacterial groups Actinobacteria, Endobacteria, and Chloroflexi and negibacterial phyla are ill-resolved (Pace 2009; Woese 2013), as well as with actinobacteria-specific signatures

(Gao and Gupta 2005, 2012; Gao et al. 2006, 2009; Servin et al. 2008). Actinobacteria are immediately adjacent to archaeobacteria on two of the five protein-family splits networks of Dagan et al. (2010) based on 191 bacterial genomes and separated from them by Endobacteria on a third, so the majority support a neomuran relationship with Posibacteria, with actinobacteria favored as the closest. Cyanobacteria are also adjacent on three, although their negibacterial cell structure makes a direct relationship less likely, whereas Gracilicutes are overall more distant; thus, of the limited bacterial megagroups sampled, posibacteria and cyanobacteria were the best candidates for the closest relatives to neomura. It is a pity that the analyses excluded Eurybacteria and Hadobacteria (data for Chloroflexi were then unavailable). Actinobacteria alone in prokaryotes have a CTP-dependent cardiolipin synthetase needed for mitochondria (Sandoval-Calderón et al. 2009).

COTRANSLATIONAL SECRETION DURING THE NEOMURAN REVOLUTION AND EUKARYOGENESIS

The neomuran ancestor evolved shared features of ribosomes (anisomycin inhibition of peptidyl transferase; chloramphenicol resistance; new proteins), ribosomal rRNA processing (fibrillarin; U3 and C/D- and H/AC-box snoRNA guides for pre-rRNA cleavage and base modification), tRNA protein-spliced introns, and signal recognition particle (helix 6; SRP19 protein) that are absent in eubacteria and evolved from the simpler system of Posibacteria (Cavalier-Smith 1987c, 2002b). Neomuran glycoproteins are made by ribosomes docked onto a membrane by the ribonucleoprotein signal recognition particle (SRP) that recognizes their amino-terminal hydrophobic signal sequence and membrane-embedded SRP receptors and transfers the signal hairpin into the membrane. The rest of the unfolded protein then crosses the membrane via an openable protein channel, folding correctly on the other side. Before signal peptidase removes its signal peptide, oligosaccharyltransferase attaches a presynthesized hexose-rich oligosaccharide by its basal *N*-acetyl-

glucosamine to one or more asparagines two amino acids upstream of a serine or threonine. The oligosaccharide is synthesized sequentially on the ribosomal face of the membrane while covalently attached (in eukaryotes always via *N*-acetylglucosamine; in archaeobacteria ancestrally thus) to a phosphorylated polyisoprenol carrier (dolichol phosphate or pyrophosphate). This hydrophilic core oligosaccharide is flipped across the membrane by flippase protein. This complex machinery is strongly conserved and homologous between archaeobacteria and eukaryotes, proving the central thesis of the neomuran theory and refuting the progenote idea (Woese and Fox 1977). The common ancestor of neomura, undeniably with all of these mechanisms, was an advanced cell with extremely sophisticated translation, transcription, DNA replication and segregation, and a lipid membrane with numerous embedded proteins. There certainly was an historical changeover between acylester membrane lipids (eukaryotes and eubacteria) and isoprenoid ethers of archaeobacteria: one was not converted into the other (chemically impossible). Instead, one replaced the other, hyperthermophily being the strong selective advantage for replacing eubacterial by archaeobacterial lipids (Cavalier-Smith 1987b,c), there being none for the reverse; the transient intermediate had both types (Cavalier-Smith 2002b).

Eubacterial murein peptidoglycan is analogous to neomuran *N*-linked glycoproteins: both comprise amino acids, sugars, and amino sugars; synthesis involves transfer of hydrophilic core units containing *N*-acetylglucosamine across the hydrophobic CM, after being synthesized on its cytosolic face by sequential covalent attachment to a phosphorylated polyisoprenol. Murein consists of an alternating copolymer of amino sugars *N*-acetylglucosamine and *N*-acetyl muramic acid covalently cross-linked by oligopeptides: its precursors flipped across CM on undecaprenol are muramopeptides with two amino sugars and four amino acids. Common biogenetic involvement of *N*-acetylglucosamine and isoprenoid carriers suggested an evolutionary relationship (Cavalier-Smith 1987c); I proposed that the ancestral neomuran posibacte-

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ria-derived L-form lost muramic acid, omitted the amino acids, and added extra hexoses, and the same oligosaccharyltransferase added it to preexisting S-layer proteins, yielding the first N-linked glycoproteins (Cavalier-Smith 1987c). BLAST analysis now strongly supports that: as predicted, eukaryotic and archaeobacterial dolichol-dependent oligosaccharyltransferases making N-linked glycoproteins are homologous, strongly conserved, and homologous with, but somewhat less similar to, the undecaprenol-associated oligosaccharyltransferases of teichobacterial and actinobacterial posibacteria that make murein. Therefore, almost certainly, neomuran N-linked glycoproteins did evolve from preexisting posibacterial oligosaccharyltransferases that previously made murein. The similarity of neomuran transferases is much greater to those of posibacteria than to any Negibacteria except a few δ -proteobacteria, for example, the myxobacterium *Stigmatella*; these few δ -proteobacterial oligosaccharyltransferases perhaps underwent LGT from neomura to myxobacteria.

The eukaryote rough ER cotranslational protein channel comprises Sec61 and ancillary α, γ -subunits; these evolved from the SecYEG prokaryote translocon when phagotrophy internalized the CM during eukaryogenesis. The plasma membrane also kept prokaryotic ABC transporters for rare posttranslational protein secretion, for example yeast mating hormones, but other protein secretion mechanisms were lost or transformed beyond confident recognition during eukaryogenesis. Loss of type IV secretion (responsible for bacterial conjugation in archaeobacteria and eubacteria) largely explains why LGT is much rarer in eukaryotes than bacteria. The TatAC bacterial pathway for exporting folded proteins (using the same signal peptidase as Sec for unfolded ones) (Palmer and Berks 2012) is absent in eukaryotes, but the more complex homologous negibacterial TatABC was retained by chloroplast thylakoids.

Universal Sec arose in the first cell, as did TAT and ABC transporters, and two types of signal peptidases: type I for most proteins and type II specific for lipoproteins. Type III, used for secreting prokaryotic flagellar proteins and

pilins and in archaeobacteria also for many other proteins, probably evolved subsequently with flagella. Types II and III signal peptidases were lost during eukaryogenesis. Eubacterial SecA that unfolds folded proteins for threading through SecY was lost when the neomuran ancestor made Sec more cotranslational, by the novel neomuran arrest domain of SRP (Cavalier-Smith 2002b). Bacterial TAT secretes folded globular proteins across CM. Cavalier-Smith (2006a, 2009) suggested that eukaryotes modified TAT for protein import into peroxisomes. Import of soluble peroxisomal enzymes uses two alternative topogenic sequences: a nonapeptide recognized by type II receptors and the carboxy-terminal (type I) SKL motif by receptors; although TAT recognizes a carboxy-terminal RR motif, Pex5, the SKL receptors like TAT use tricopeptide repeats (Rucktäschel et al. 2010) and thus might be related.

COEVOLUTION OF MITOCHONDRIAL, ER, AND PEROXISOMAL RESPIRATION AND SEGREGATION

These three oxygen-consuming respiratory organelles—the mitochondrion, the ER, and the peroxisome—coevolved in early eukaryotes, partitioning aerobic metabolism among them, making aerobic phagotrophy more efficient. Early mitosis was open or semiopen, with peroxisomes segregated to daughter cells by attachment to the nuclear envelope through the cell cycle. Space constraints prevent discussing the origin of mitochondria, probably from a photosynthetic α -proteobacterium contemporaneously with the origin of cilia and nucleus (Cavalier-Smith 2006b, 2007b), and how its negibacterial reproduction (originally using FtsZ-associated division) was modified and integrated into the eukaryotic cell cycle, partly divergently in excavates and Euglenozoa (Kinetoplastea segregate mitochondria by centriole attachment) (Gluenz et al. 2011); they and diplomonids evolved glycosomes from peroxisomes (Cavalier-Smith 1997; Gualdrón-López et al. 2012). Peroxisomes originated marginally before mitochondria, not long before, as De Duve once thought. Although they probably evolved by pri-

mordial divergence from the ER (Cavalier-Smith 1975; Gabaldon 2010), not by symbiogenesis of bacteria as De Duve proposed, they evolved novel division machinery, multiplying mainly by growth and division (Pieuchot and Jedd 2012), thus becoming effectively distinct genetic membranes (Cavalier-Smith 2004). Mitochondria, and subsequently plastids, apparently coopted this division machinery (Pan and Hu 2011), just as mitochondria arguably duplicated the peroxisomal ADP/ATP exchanger when evolving IM carriers, the key to their enslavement (Cavalier-Smith 2006b), subsequently donating duplicates to chloroplasts (Cavalier-Smith 2000). Ancestrally, peroxisomes were probably segregated through specific nuclear-envelope attachments, retained by diverse protists (Cavalier-Smith and Oates 2012).

Separating ATP-generating and degradative respiratory cytochromes in mitochondria and ER following the differentiation of peroxisomes and endomembranes (Fig. 2) made aerobic exploitation of prey more efficient, but, contrary to Lane and Martin (2010), mitochondria did not cause large eukaryote genomes. Increased genome size resulted from the origin of the novel phagotrophy-driven cytoskeleton, eukaryotic cell cycle, and nuclear architecture with nuclear pores, which collectively transformed the machinery and selective forces acting on chromosomes, and by allowing marked (initially phagotrophy-driven) increases in cell size imposed upward coevolutionary pressures on genome size, which controls nuclear volume, which invariably coevolves with cell size (Cavalier-Smith 2005, 2010c). Symbiogenesis did not drive these innovations; for its importance to eukaryote cell evolution, see Cavalier-Smith (2013b).

DIVERSE CELL BIOLOGY OF FILARCHAEOTA CLARIFIES NEOMURAN EARLY EVOLUTION

Archaeobacterial multigene trees show Korarchaeota and Thaumarchaeota forming a clade with Crenarchaeota (Podar et al. 2013), all here grouped as subphylum Filarchaeota (Table 2) to emphasize that they ancestrally had two eukaryote-like filamentous cytoskeletal proteins: actin (Yutin et al. 2009) and ESCRT-III filaments for

cytokinesis (Hobel et al. 2008; Samson et al. 2008; Ettema and Bernander 2009; Makarova et al. 2010). A direct eukaryotic origin from filarchaeotes is implausible because they lack the right lipids and scores of proteins (e.g., dynamin, Hsp90) vital for making eukaryotes. The archaeobacterial ancestor was not a crude gene-poor progenote but had >1000 genes, undergoing extensive genomic reduction after diverging from eukaryotes (Cavalier-Smith 2002b, 2007b): differential losses during archaeobacterial radiation reduced genes from ancestrally ~2000 (Csürös and Miklós 2009; Wolf et al. 2012), yielding lineages lacking different subsets of proteins necessary for making eukaryotes, all originally present in the more complex ancestral neomuran. The mechanistically implausible idea of host archaeobacterial lipid replacement by mitochondria (Martin and Müller 1998) can “explain” hypothetical loss of isoprenoid ether lipids but not acquisition of phosphatidylinositol, cardiolipin (actinobacteria alone in prokaryotes have CTP-dependent cardiolipin synthetase), and sterols, all readily supplied by the neomuran ancestor. It is far simpler that this ancestor stabilized its genome by evolving chromatin, its cytoplasm by actin, and division by ESCRT before eukaryotes and archaeobacteria diverged, filarchaeotes losing dynamin, and euryarchaeotes losing actin and ESCRT. Many trees suggest archaeobacterial holophyly (Yutin et al. 2008), but despite incontrovertible evidence for multiple losses within Filarchaeota, some investigators ignore the possibility of eurybacterial actin/ESCRT loss and suppose that eukaryotes evolved from filarchaeotes. Contradictorily, others assumed that eukaryotes evolved from euryarchaeotes (where eukaryotic-like glycosyltransferase is more widespread than in crenarchaeotes) because of histone similarities (Reeve et al. 2004; Cubonova et al. 2005) or the phylogenetically refuted and mechanistically implausible hypothesis that eukaryotes evolved from a methanogenic euryarchaeote (Martin and Müller 1998).

Given huge problems in cell biology that massive replacement of host lipids and proteins entails in a hypothetical archaeobacterial origin, one must not uncritically accept certain mul-

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Table 2. Classification of new archaeobacterial subphylum Filarchaeota^a**Class 1 ‘Korarchaeota’**

Name suggested by Barns et al. (1996) at the grossly inflated ‘kingdom’ rank; should be a class, which cannot be made formally until ‘*Candidatus* Korarcheum’ or another contained genus is legitimately published.

Class 2. ‘Thaumarchaeota’

Name suggested by Brochier-Armanet et al. (2008) at the unwisely inflated rank of ‘phylum’; should be a class, but not formally possible until a genus within it is legitimately published.

Order 1. ‘Cenarchaeales’ Cavalier-Smith, 2002 was illegitimate as the widely used ‘type genus’ ‘Cenarchaeum’ is still not formally published and thus illegitimate.

Order 2. ‘Caldiarchaeales’ may be a suitable name for the clade containing ‘*Candidatus* Caldiarchaeum’, which does not deserve the phylum rank suggested by Nunoura et al. (2011) as ‘Aigarchaeota’.

Class 3. Crenarchaeota Cavalier-Smith, 2002 (later synonym: Thermoprotei [Reysenbach 2002])

Their stronger hyperthermophily than other archaeobacteria probably caused more extensive loss of ancestral proteins (e.g., histones).

^aDiagnosis: Archaeobacteria containing actin and/or ESCRT-III filaments, unlike Euryarchaeota. Type order Thermoproteales Zillig and Stetter, 1982. Etymology: *Filum* L. thread, to show they have one or both of these cytoskeletal filaments: *archae* Gk ancient. Comment: Equivalent to the too highly ranked informal ‘TACK superphylum’ (Guy and Ettema 2011), who demonstrate numerous independent losses of neomuran proteins within Filarchaeota and Euryarchaeota, postulating loss of actin and ESCRT-III by Euryarchaeota, which retained FtsZ and histones, unlike most Filarchaeota, is much more parsimonious than accepting that eukaryotes are sisters to Filarchaeota, as some trees suggest (Williams et al. 2012). I give a formal definition despite no taxa above classes currently having standing in prokaryotic nomenclature; the code needs changing to encompass phylum and kingdom names (as for classes, do not apply priority to them). Clades DSAG/AAG/MHVG of Guy and Ettema (2011) should not be a separate phylum, but may belong in Thaumarchaeota when better characterized. Filarchaeota contains all archaeobacteria except subphylum Euryarchaeota (with five classes: Cavalier-Smith 2002b), in which I now place ‘Nanoarchaeum’, which will not deserve separation from other archaeobacteria at higher rank than an order after being formally named, contrary to those who treat it as a ‘phylum’; its deep divergence from other euryarchaeotes looks like a typical artifact of rapid evolution after parasitic reduction, as in mycoplasmas, microsporidia, and *Mikrocytos*.

tigene trees emphasizing ribosomal proteins showing eukaryotes as sisters to Filarchaeota only, not to all archaeobacteria (Williams et al. 2012), as refuting the neomuran theory. As Valas and Bourne (2011) explain, most of those 31 proteins (Williams et al. 2012) underwent rapid (possibly tree-biasing) quantum-evolutionary changes in ribosomes during the origins of eukaryotes and archaeobacteria, as earlier argued for rRNA (Cavalier-Smith 2002b); the very long stem at the base of eukaryotes in these trees is precisely the condition most likely to ensure that a genuine outgroup (sister) is misplaced within the ingroup (here archaeobacteria), as decisively shown by Shavit et al. (2007). I therefore consider this probable artifact the simplest way to reconcile contradictory trees showing archaeobacterial holophyly (probably right for reasons previously detailed) (Cavalier-Smith 2010c) and paraphyly (probably this long-stem artifact)—see also Gribaldo et al. (2010), who do not yet take seriously the possibility

that neomura arose from eubacteria, despite it simply resolving the impasse that they discuss (although unlike most overinfluenced by the progenote myth that ignored cell biology and palaeontology, they are open-minded enough to mention it as a potential solution to which they may eventually be driven!). Even the Williams et al. tree suggests that eukaryotes and archaeobacteria arose *nearly* contemporaneously, reconcilable with fossil dates and eukaryote multigene trees only by accepting that archaeobacteria are at least twice as young as eubacteria and evolved from them (Cavalier-Smith 2006a, 2013a). Neomuran theory holds that eukaryotes and archaeobacteria diverged almost immediately after neomura originated, and euryarchaeotes and filarchaeotes diverged immediately thereafter. It therefore predicts that it should be extremely hard for multigene trees to decide whether eukaryotes are sisters to or nested deeply within archaeobacteria, expecting some trees to show one and some the other, exactly

as is observed. Above all, these trees provide no evidence that archaeobacteria are as old as eubacteria. If they were, they would be two to three times older than eukaryotes, which should make multigene trees nest eukaryotes *shallowly* within archaeobacteria, which they do not.

SUCCESSIVE GLIDING, FISHING, AND SWIMMING STEPS IN THE PHAGOTROPHY-RELATED ORIGIN OF CILIA

Ciliary biogenesis and locomotory undulation are extremely complex, requiring more than 660 genes (Carvalho-Santos et al. 2010, 2011), and evolved autogenously by modifying the microtubular cytoskeleton and associated motors. Early theories inadequately explained how that complexity evolved (Cavalier-Smith 1978a, 1982). Centrioles are so intricately constructed (Li et al. 2012) that their ontogeny probably closely recapitulates phylogeny: amorphous germinative discs containing γ -tubulin initiate assembly of ninefold cartwheels, then singlets, then doublets/triplets (Cavalier-Smith 1974; Jerka-Dziadosz et al. 2010; Gogendeau et al. 2011). Intraflagellar transport (IFT) machinery for intraciliary protein movement evolved from COPI-coated vesicles (Jékely and Arendt 2006; van Dam et al. 2013), probably contemporaneously with nuclear pore complexes from COPII vesicle coats (Cavalier-Smith 2010c); ciliary gliding, using the same machinery, probably arose before swimming (Cavalier-Smith 2009). In remarkable harmony with the simultaneous coevolutionary origin of cilia and nuclei thesis (Cavalier-Smith 1987c), cilia are a distinct cell compartment into which ciliary proteins are targeted across a basal “ciliary pore complex” by machinery containing importin and nucleoporins used for nuclear import, similarly governed by a RanGTP/GDP gradient, and recognizing ciliary localization signals (CLS) related to nuclear localization signals (NLS) (Fan et al. 2011; Kee et al. 2012). Nuclear import machinery was either partially coopted and modified for cilia or nuclear and ciliary import diverged simultaneously from vesicle coats.

Like bird feathers, which originated to insulate small running dinosaurs (easier), subse-

quently modified for flight (harder), Figure 4 outlines an autogenous origin of centrioles and cilia with two functional shifts and three successive selective forces: (1) kinesin-driven ciliary gliding and compartmentation for surface motility; (2) protociliary duplication and transformation making a differentiated prey trapping anterior ciliary fishing rod (as *Phalansterium* cilium does today: Smirnov et al. 2011) and a posterior gliding cilium for enhancing phagotrophy; and (3) ciliary bending, initially to improve fishing and subsequently allow swimming. Thus, improved phagotrophy probably preceded swimming as a selective advantage, and final stages in centriole and ciliary evolution followed ciliary duplication, unlike previous theories. The resulting cenacestral eukaryote immediately diverged into (a) swimming excavates by losing gliding, evolving a ventral groove with a split right centriolar root and a posterior ciliary undulation drawing in food; and (b) posteriorly gliding Euglenozoa by evolving a ventral root-supported cytostome for surface feeding, consistently with rooting the tree between excavates and Euglenozoa (Figs. 1 and 3), with contrasting centriole-anchoring cytoskeletons (Cavalier-Smith 2013a). Because gliding and ciliary surface feeding are mechanistically simpler, and gliding could be initiated by preexisting cytoskeletal properties (Fig. 4A), their historically preceding ciliary beating allowed the seemingly irreducible complexity of ciliary beating to evolve in simple stages; each had clear selective advantages, improvable gradually by blind mutations, needing no symbiogenesis (Margulis 1970), no viral input (Satir et al. 2008), and no intelligent design.

Centriolar duplication and mitosis co-evolved with new cell-cycle controls (Fig. 5). Although archaeobacteria share eukaryote-like DNA replication machinery, cycle controls remained prokaryotic because they retain cell-surface-attached DNA, ParA P-loop ATPase filaments, and typically ParB centromere-like proteins for moving chromosomes (Bernander et al. 2010), although the ancestral parS DNA sites that they bind are rarely recognizable (Livny et al. 2007), probably through histone-stimulated divergence. Eukaryotic cycle control

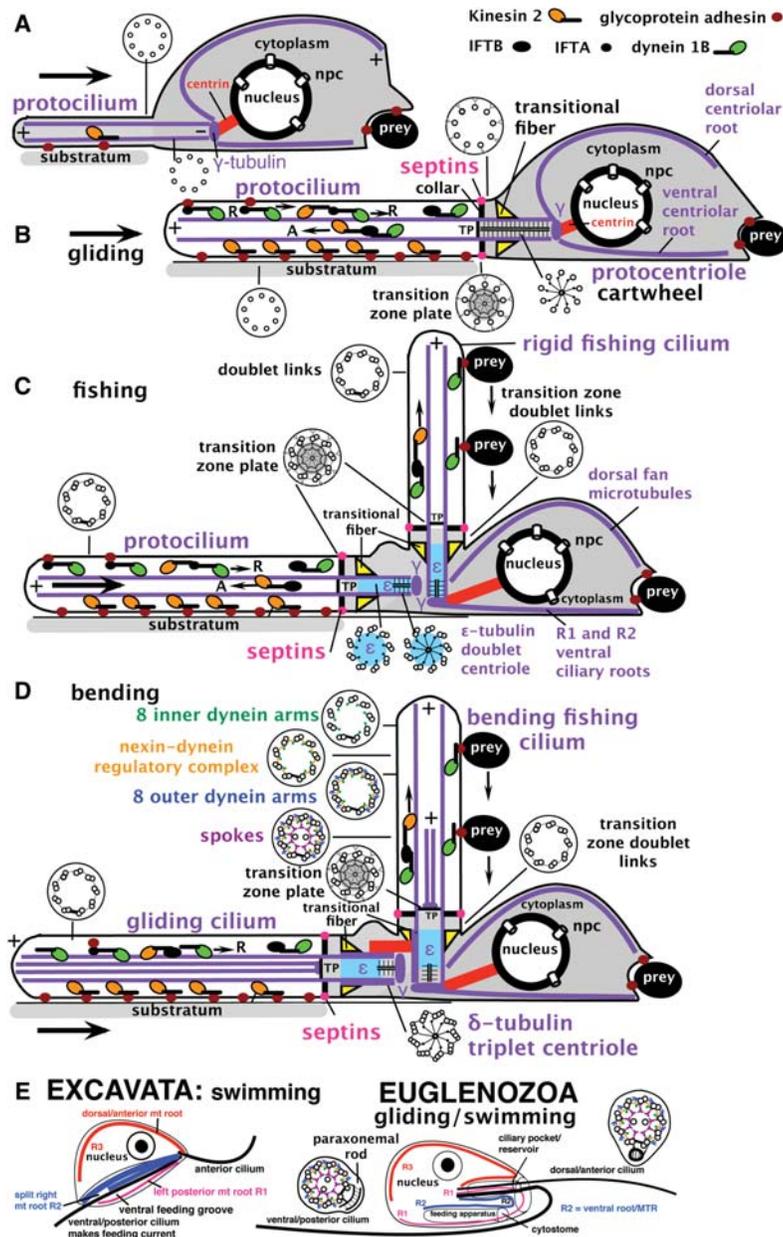


Figure 4. Autogenous origin of cilia with successive origins of different ciliary components under three contrasting selective forces. (A) During or just after the origin of nuclear pore complexes (npc Fig. 2), singlet microtubules from the γ -tubulin proto-centrosome push out the plasma membrane as a protocilium by which plus-end-directed kinesin-2 motors (Verhey et al. 2011) attached by their tails to glycoprotein surface adhesins sticking to the substratum propel its microtubules forward (arrow) in primitive gliding motility, enabling cells to find fresh prey on the substratum as phagotrophy locally depletes it. (B) Posterior ciliary gliding was improved by attaching microtubules firmly to the cell surface by proto-centriolar transitional fibers (proximally) and Y-shaped membrane connectors (slightly distally) plus ciliary compartmentation dependent on novel diffusion barriers (septin filaments in ciliary membrane base: Carvalho-Santos et al. 2011), central distal transition plate (TP), and a peripheral dense collar at the distal end of the Y-connector region, associated with npc proteins with novel ciliary import machinery using npcs and importin- β 2 and NLS-related CLS targeting sequences (located in TP and/or collar). (Legend continues on following page.)

(including cyclins, origin of ubiquitin from ancestral neomuran ubiquitin-like proteins [Pearce et al. 2008; Maupin-Furlow 2013], and proteasome complexification), were therefore consequences not of novel neomuran chromatin and DNA-replication machinery but

of radical eukaryote-specific cell structural innovations, especially phagotrophy-driven DNA internalization to endomembranes and origins of TubZ-GTPase-related microtubules, γ -tubulin centrosomes, centrioles/cilia, and mitosis.

Figure 4. (Continued) Dynein 1b, the first dynein, evolved (by gene-duplicating its common ancestor with the related AAA⁺ ATPase midasin/REA1 that mechanically strips biogenesis factors from 60S preribosomal subunits just before they exit the nucleus: Garbarino and Gibbons 2002; Kressler et al. 2012) to recycle distal adhesins left at the protociliary tip by axoneme gliding. Anterograde movement of dynein improved by carriage on kinesin-2-driven IFTB particles that evolved from CopI coat proteins, and special SNAREs evolved for basal delivery of ciliary membrane precursor vesicles with distinctive proteins and lipids. Protocentrioles were rigidified by a hub-spoke core, microtubules fixed at nine by ninefold SAS-6 hub assembly (Guichard et al. 2012). Cross-links rigidified the axoneme, the two on its substratum face having numerous extra-rigid linkers linking them like sled runners (subsequently becoming doublets 1 and 2 of *Chlamydomonas*: Lin et al. 2012). Novel proteins (e.g., Rib43a: Norrander et al. 2000) stabilized A-tubules and the bases of centriolar root microtubules (dorsal and ventral fans) compared with transient spindle microtubules. (C) Ciliary duplication produced a younger “fishing” cilium projecting into the medium for trapping swimming bacteria, pulling them baseward by minus-directed dynein 1b, for phagocytosis. Centrin plus novel proteins orthogonally rigidly connected the two protocentrioles now with doublets (architecture [Nicastro et al. 2011] perhaps determined by novel microtubule inner proteins, for example PACRG [Ikeda et al. 2007; Ikeda 2008], stabilized by a scaffold containing ϵ -tubulin [blue]). PACRG-interacting Rib72 [Ikeda et al. 2003] differentiated ciliary from centriolar doublets. Centriolar transformation temporally and physiologically differentiated the two cilia and made separate left and right ventral microtubular roots. (D) Successive dynein duplications generated inner arms (*top*: sufficient for bending [Heiss et al. 2013] as doublet 1–2 linker excluded arms from doublet 1, destroying ninefold symmetry; then the nexin–dynein regulatory complex [Heuser et al. 2009, 2012; Lin et al. 2011; Bower et al. 2013] for calcium regulation of beat; then outer dynein arms for greater power, and the center pair [nucleated by γ -tubulin on TP, and fixed so did not rotate] with new arms [Carbajal-Gonzalez et al. 2013] and kinesin-9 [Wickstead et al. 2010] and spokes [Barber et al. 2012] to modulate beat mechanics [not inherently needed for planar beat: Idei et al. 2013]) to draw in more prey by water currents. For pictorial simplicity, the cell body is proportionally too small and ahead of the cilia, but probably at stage b/c the cytoskeleton geometrically rearranged to lift the cell body from the substratum and put the ciliated cell apex at the front, which is mechanically stabler (found in all extant posterior ciliary gliding eukaryotes), entailing a basal stable bend ([E] mechanism unknown) for the posterior gliding cilium. Many complexities of present cilia (Mizuno et al. 2012) probably evolved subsequently to improve efficiency but would not have been essential for their origin, for example, association of IFTA/B into one complex and of these into distinct anterograde and retrograde trains (Pigino et al. 2009; Buisson et al. 2013), and addition of BBsomes, likely adaptors for improving retrograde transport of some proteins (Lechtreck et al. 2013) and sensory functions (sensation [Jékely and Arendt 2006] is less plausible than gliding for the original function), and beat pattern modulators. (E) The cenacestral eukaryote diverged to form swimming excavates that abandoned gliding and undulate the posterior cilium to draw prey into the ventral groove supported by a split right ventral centriolar root R2 (blue), and Euglenozoa that ancestrally retained gliding, added a cytopharynx supported by ancestrally unsplit R2 and dissimilar paraxonemal rods (probably attached to the specially linked doublet 1–2 homologs) to broaden and further rigidify the posterior cilium for stabler gliding, and parallelized their centrioles within a ciliary pocket. After losing phagotrophy, saprophytic, parasitic, and photosynthetic Euglenozoa lost gliding and developed swimming by the anterior (Euglenophyceae) or posterior (trypanosomatids) cilium; some bacterivores (petalomonads) lost the posterior cilium, presumably recruiting dynein 1b for anterior ciliary gliding. All other eukaryotes evolved from excavates (Fig. 3): Apusozoa, Cercozoa, and the heterokont *Caecitellus* reevolved posterior ciliary gliding, presumably using kinesin-2. The V-fiber, with associated acorn-base attached distally to centriole 1–2 triplets (at least in neokaryotes: Geimer and Melkonian 2005), demarcates the centriole from the transition zone and perhaps evolved in the cenacestral eukaryote; its rotational asymmetry and that of centriolar root attachments to specific triplets probably reflect an asymmetric doublet “numbering machinery” that probably evolved in the earliest gliding protocilium (B).

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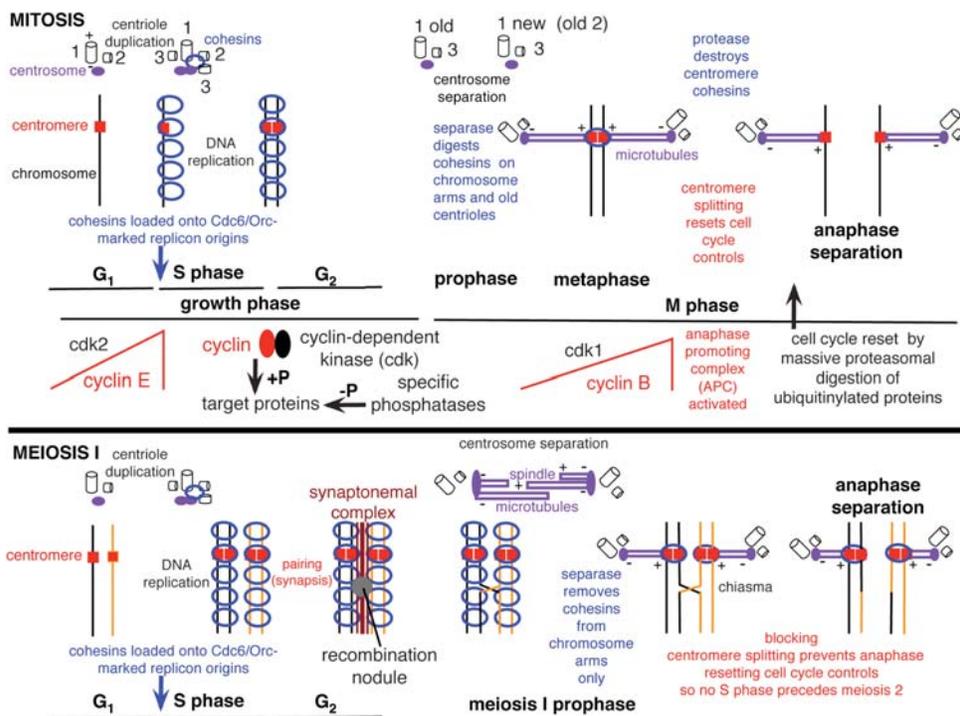


Figure 5. Eukaryote cell-cycle logic and evolution. Complexes of cyclin-dependent kinase (CDK) and cyclins control the eukaryotic cell cycle by phosphorylating numerous proteins, timed by growth-dependent increases in cyclins and their sudden proteolysis (red curves) (Nasmyth 1995). Cyclins share a domain with neomuran-specific transcription factor TFII, and CDKs evolved from posibacterial serine-threonine (S/T) kinases. Originally one cyclin could have controlled S-phase initiation and anaphase onset using a lower threshold for the former switch (Novak et al. 1998; Tyson and Novak 2008; Harashima et al. 2013); gene duplication enabled better control by cyclinE/cdk2 (for DNA replication and centriole duplication) and cyclin B/cdk1 to activate the anaphase promoting complex (APC), the ubiquitin ligase that initiates anaphase resetting of cell-cycle controls via proteasome degradation of cyclins and numerous key cycle proteins. Phosphorylation-cum-proteolytic cell-cycle controls originated in posibacteria, but a novel Cdc-6-mediated control over replication initiation evolved in ancestral neomura after histones H3/4 and MCM DNA helicase, replacing eubacterial DnaA/CrtA control; proteolysis by proteasomes that originated in the neomuran/actinobacterial cenancestor replaced eubacterial ClpXP proteolysis. Ancestral eukaryotes evolved origin recognition complexes (ORCs) more complex than the single protein Cdc-6 of archaeobacteria, probably because the suddenness of mitotic anaphase (rapidly segregating all parts of chromosomes at once, unlike the temporally separate segregation of origins and the generally single terminus in bacteria) required concerted replication initiation at hundreds of origins, ensuring replication completion and tighter chromosome folding (using extra histones and novel heterochromatin machinery: Cavalier-Smith 2010c) well before mitosis; uniquely in eukaryotes, mitosis demands a temporally discrete S phase. Formerly, only neokaryotes were thought to have ORCs (Cavalier-Smith 2010b), but extremely divergent versions of most constituents are now known in trypanosomes, whose cell-cycle controls are the most divergent within eukaryotes (Li 2012), consistent with eukaryotic rooting between Euglenozoa and neokaryotes (Figs. 1 and 3). Successively more complex controls and checkpoints evolved with novel polo-like and aurora S/T kinases playing multifarious roles in mitosis and cytokinesis and a multiplicity of kinesins evolving to improve spindle assembly and function. Probably all proteins shared by trypanosomes (Li 2012) and opisthokonts evolved before the eukaryote cenancestor. Mitosis (*upper panel*) was converted to meiosis by two innovations (*lower panel*): homologous chromosome pairing by the synaptonemal complex and blocking centromeric cohesin digestion at meiosis I anaphase, which automatically bypassed cell-cycle resetting caused by anaphase centromere splitting so that the next interphase had no S phase as previously explained (Cavalier-Smith 1981), thereby halving ploidy—the original meiotic function (Cavalier-Smith 2002a, 2010c). (*Legend continues on following page.*)

MISUNDERSTANDINGS OF THE NEOMURAN THEORY OF CELL EVOLUTION

Acceptance that archaeobacteria are not a primary domain but evolved from posibacteria is growing. Indel analysis (Skophammer et al. 2006, 2007; Lake et al. 2009; Valas and Bourne 2009, 2011) supports that and that negibacteria are a derived clade. Palaeontology and multi-gene trees when critically combined prove that eubacteria historically preceded neomura (Cavalier-Smith 2002b), and eubacteria are about three times as old as neomura (Cavalier-Smith 2006a, 2013a). Thus, there is only one primary “domain” of life: eubacteria (Cavalier-Smith 1987b,c, 2006a,c). Archaeobacteria are certainly more recent. The neomura theory is probably cladistically historically correct and coherently logically explains the origins of both eukaryotes and archaeobacteria. The transformations it entails are cell-biologically reasonable, selectively comprehensible, and unexplained by alternative ideas. A stimulus through antibiotic warfare among bacteria is likely because penicillin can cause wall loss and many antibiotics target ribosomes and lead to resistance through changes in their structure (Cavalier-Smith 1992). Despite its unequalled explanatory power, the neomuran theory was repeatedly ignored, misunderstood, or misrepresented, but remains unrefuted. Most discoveries since 1987 greatly strengthen its core ideas.

Yet, the lingering dogma/prejudice that archaeobacteria are a “primary domain,” never with any sound evidence from paleontology or phylogeny, still makes some reluctant to accept that archaeobacteria are younger than and evolved from eubacteria. Some still think archaeobacteria are ancestors, not sisters of eukaryotes, an idea subconsciously favored by wrongly calling shared features “archaeobacterial” not

“archaeobacteria-like.” Some skeptics confuse fundamental tenets of the neomuran /phagotrophy/coevolutionary theory of eukaryote origins with the logically independent, now-disproved idea that some eukaryotes were primitively without mitochondria, proposed earlier when classifying amitochondrial eukaryotes as Archezoa and arguing for the first time (correctly it turned out) that the mitochondrial OM evolved from that of α -proteobacteria (Cavalier-Smith 1983a,b). Discoveries of mitochondrial relics in all “Archezoa” showed that no primitively amitochondrial eukaryotes persist, so I abandoned that idea long ago (Cavalier-Smith 1998, 2002c). That disproved “archezoan hypothesis” concerned the root of the eukaryote tree, not the *causes* of eukaryogenesis; causal explanation of eukaryogenesis by the probably correct phagotrophy/coevolutionary theory is logically independent, as is the now-associated neomuran interpretation of the phylogenetic relationship between eukaryotes, archaeobacteria, and posibacteria, which also I argue is correct. What differs in my present interpretation (Fig. 1) is the root position for (a) eukaryotes, and (b) the whole tree of life. The position of the eukaryotic root between Euglenozoa and neokaryotes (excavates and their descendants) is arguably more secure than previously, being based on a dozen independent reasons, detailed elsewhere (Cavalier-Smith 2010a,c, 2013a), not just one as in previous guesses, and also strongly supported by all prokaryote-rooted ribosomal multiprotein trees using 13,432 amino-acid positions (Lasek-Nesselquist and Gogarten 2013). An alternative, slightly different position of the eukaryote root within loukozoan excavates, between jakobids and *Malawimonas*, based solely on mitochondrial protein 42-gene trees (Zhao et al. 2013), risks being somewhat inaccurate because of long-branch artifacts (Shavit et al. 2007).

Figure 5. (Continued) It is unclear whether centromeres evolved from posibacterial plasmid partition proteins associated with GTPase TubZ (Aylett et al. 2010, 2013) or de novo; CENP-A was probably added only in neokaryotes after they diverged from Euglenozoa (Fig. 3) (Cavalier-Smith 2010c). As the text notes, recent evidence suggests that cohesin digestion is not invariably necessary for centriole separation in animals, raising doubt as to the ancestral eukaryotic process.

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Differences in ribosomal RNA sequences that separated the so-called ‘three domains’ are superficial compared with deep similarities in cell secretory machinery. Shared glycoprotein synthesis involving dolichol phosphate carriers was only one of 16 supporters of the neomuran clade (Cavalier-Smith 1987c); since then at least as many more have been discovered (Cavalier-Smith 2010c); even those few still imagining that archaeobacteria and eubacteria evolved separately from a progenote (Martin and Russell 2003) accept neomura as a clade and that archaeobacteria and eukaryotes cannot both be “primary domains.” Cavalier-Smith (1987c) listed 24 cellular and molecular characters shared by eubacteria and archaeobacteria that decisively refuted the progenote hypothesis, proving a direct transition between eubacteria and archaeobacteria via a highly developed cellular intermediate with lipid membrane and cytochrome-based membrane-dependent bioenergetics. Such complexity required reliable translation and DNA genomes with efficient replication, repair, and transcription. Subsequently, many others also proved the last common ancestor of life must have been a complex bacterium with well-developed membrane bioenergetics, growth, and division machinery (e.g., Jékely 2006; Lombard et al. 2012b), refuting the entirely untenable idea of separate precellular origins of neomura and eubacteria (Martin and Russell 2003).

Thiergart et al. (2012) tendentiously falsely attributed the branching of only 15 eukaryotic proteins with actinobacteria as the “level of support” for the neomuran theory, seriously misrepresenting its predictions. In fact, their 145 genes branching closest to archaeobacteria strongly support it; given equal evolutionary rates and gene losses within euryarchaeotes and filarchaeotes, it predicts that an equal number should appear as sister to each subphylum, assuming that they and eukaryotes diverged at virtually the same time; purely by chance, some will group with one and some the other (Cavalier-Smith 2007b). Because crenarchaeote proteins mostly evolve faster and are more often lost, one expects some bias toward euryarchaeotes, exactly as found: 68 trees put eukaryotes

closer to filarchaeotes and 77 closer to euryarchaeotes. If eukaryotes evolved substantially more recently than archaeobacteria from an ancestor in either archaeobacterial subphylum, all 145 trees should put them *within* that one group. Unwittingly, Thiergart et al. (2012) supplied the strongest evidence yet that archaeobacteria cannot be substantially older than eukaryotes, contrary to Martin’s assumptions (Martin and Müller 1998; Martin and Russell 2003). Thiergart et al. (2012) misunderstood and misrepresented the neomuran theory by asserting that it originally postulated that eukaryotes evolved from cyanobacteria, citing Cavalier-Smith (1975). That paper, predating archaeobacterial recognition (Woese and Fox 1977) did *not* propose the neomuran idea.

Very detailed explanations of stepwise origins of endomembranes, nucleus, mitosis, cell cycle, and sex (both mechanisms and selective advantages) in earlier publications (Cavalier-Smith 2009, 2010c), including detailed criticisms of less plausible alternatives, cannot be adequately summarized here. The rest of this article outlines new insights into bacterial cell evolution that sharpen distinctions between Negibacteria and Posibacteria, making me conclude that Negibacteria and neomura both evolved from Posibacteria, the first cells. This is necessary to explain how bacterial phylogeny maps onto the fossil record, the only direct evidence for evolutionary timing, and thereby perhaps more convincingly than before defend the well-substantiated recency of archaeobacteria compared with eubacteria, and simultaneous origin of archaeobacteria and eukaryotes from a radically transformed actinobacteria-related posibacterium.

EVOLUTIONARY SIGNIFICANCE OF THE NEGIBACTERIAL CELL ENVELOPE

The names “Negibacteria” and “Posibacteria,” coined to distinguish eubacteria with double or single bounding membranes (Cavalier-Smith 1986, 1987b,c), are phylogenetically more precise than the older “Gram-negative” and “Gram-positive” that were evolutionarily confusing because some Posibacteria stain Gram-negatively



(e.g., mycoplasmas) and some negibacteria (e.g., *Deinococcus*) stain Gram-positively. I argued that membrane number and biogenesis were fundamentally more important than staining, that OM evolved only once, and was lost at most once since life began. I argue here it was never lost. My key distinction was ignored for decades, partly because Woese's repeated historically inaccurate, naïve tirades against morphology (Woese 1994) led many biochemists to ignore bacterial envelope biology. Confusions caused by such myopia are clearing; renaissance of molecular cell ultrastructural anatomy and membrane biogenesis confirms the fundamental negibacterial/posibacterial dichotomy (excellent reviews: Desvaux et al. 2009; Sutcliffe 2010). Gupta (1998a,b,c) made the same distinction much later, inventing informal terms "diderm" and "monoderm," but astoundingly wrongly asserted that their fundamental phylogenetic distinction was not recognized before 1998 (Gupta 2011). OMs are simple phospholipid bilayers in Hadobacteria (*Deinococcus*, *Thermus*) and Thermotogales, which I therefore now place in that phylum, but in all other Negibacteria (as here revised by excluding Chloroflexi), only its inner leaflet is phospholipid (its outer is lipopolysaccharide).

The inner membrane (CM) of negibacteria is homologous with CM of unibacteria in bearing SecYEG channels, SRP receptors, TAT, ABC transporters, type IV secretion, and membrane-bound respiratory and/or photosynthetic electron transport chains, supporting proton gradients and proton-driven phosphorylation by partially membrane-embedded ATP synthetases. Contrastingly, negibacterial OMs lack all those and are penetrated by large cylindrical proteinaceous pores (porins), allowing free traverse of small molecules, and have β -barrel proteins, not α -helical globular proteins of the CM and eukaryotic plasma membrane, endomembranes, and peroxisomes. OM β -barrel proteins move across the CM via Sec or ABC carriers, then insert into OM by using the β -barrel OM protein Omp85 (attached to the ABC transporter by a "membrane fusion" protein connecting CM and OM). The complexity and conservation of this β -barrel insertion machin-

ery mean that negibacterial OMs had a single origin and were inherited by membrane heredity (Cavalier-Smith 2004) for >2.5 billion years, being kept throughout mitochondrial and chloroplast evolution (Cavalier-Smith 2013b). Negibacteria evolved four other secretion mechanisms absent in Unibacteria: types II, V, VI, and VII using different OM porins.

The negibacterial cell plan is outstandingly stable. If the universal tree is rooted in Unibacteria (Fig. 1), the first cell had one membrane, and OMs were never lost in the history of life. If the first cell were negibacterial (Blobel 1980), the OM arose with the first cell (for possible mechanisms, see Cavalier-Smith 2001) and must have been lost by Posibacteria, for which the only plausible mechanism proposed is hypertrophy of murein, thickening the wall enough to break mechanically all contacts between the CM and OM, preventing OM biogenesis (Cavalier-Smith 1980). No theory of negibacterial eukaryote origins can be taken seriously without a physically plausible mechanism of OM loss, sufficient reason for firmly rejecting evolution of a phagotrophic wall-free eukaryote from a cyanobacterium (Cavalier-Smith 1975), δ -proteobacterium (López-García and Moreira 1999), or planctobacterium (similarities between eukaryotes and planctobacteria are superficial misinterpretations: Cavalier-Smith 2010c; Santarella-Mellwig et al. 2013); Planctomycetes independently lost murein and FtsZ and some acquired tubulins and a kinesin by LGT. Elsewhere, I explained other fatal flaws of the δ -proteobacterium fusion theory (Cavalier-Smith 2010c).

The unrelated extra membrane in the crenarchaeote *Ignicoccus* without Omp85 machinery (Nather and Rachel 2004) is a trivial convergence irrelevant to the monophyly and uniqueness of negibacteria, contrary to superficial assertions that it invalidates the fundamental importance, evolutionary stability, and uniqueness of negibacterial architecture, and complex biogenesis. More important exceptions to the usual posibacterial body plan are Mycobacteria, actinobacteria that probably convergently with Negibacteria evolved an OM. Mycobacteria have a very thick murein wall, fur-

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nished with posibacterial sortases for lipoprotein secretion like related teichobacteria (Cavalier-Smith 2006a); their OM is a lipid bilayer, not phospholipid, but outer leaflet glycolipid and inner mycolic acid linked by arabinogalactan to underlying peptidoglycan (Silhavy et al. 2010). Presumably, it originated by secretion across the wall like the waterproofing lipid cuticles of land plants and insects. The key biophysical problem in its origin was permeabilizing it to hydrophilic molecules: by β -barrel porins (Song et al. 2008)—but did these originate independently of negibacterial porins or via LGT from them? I predict that Omp85 insertion machinery is absent and consider them Posibacteria.

Theories of negibacterial origin generate their OM contrastingly: from endospore-forming posibacteria (Endobacteria) by prespore engulfment by its sister cell (Dawes 1981); by folding up an inside-outcell with external genes and ribosomes to form the first cell (Blobel 1980). They give radically different answers to the fundamental question: Did posibacteria precede negibacteria (Fig. 1), or vice versa (Blobel 1980)? Neomura remain a clade in either view. Because Blobel's idea offered a way of evolving cells unhampered by phospholipid bilayer impermeability to nucleotides and amino acids, I initially preferred it (Cavalier-Smith 1987b, 2001). However, that problem is now circumvented because FAs probably originally were shorter than today (Budin et al. 2009, 2012), making a single membrane more plausible for the first cell because it avoids Blobel's sealing-off gastruloid phase, which the OM biogenesis complexity now revealed makes unlikely. Recent endobacterial discoveries also support Dawes's idea, although some (Errington 2013) overlook his priority. Overall, a unibacterial root for prokaryotes now seems mechanistically simpler for envelope evolution.

GREEN BACTERIA AND THE ORIGIN OF LIFE

Chloroflexi (chlorobacteria: non-sulfur green bacteria plus non-photosynthetic relatives) are not Negibacteria, despite their Gram-negative

staining and some electron micrographs suggesting an OM, but divergent Posibacteria (Sutcliffe 2011). A long-running mistake considered them negibacteria (Cavalier-Smith 1987b,c, 1991a,b, 1992, 2002b, 2006a,c, 2010a). Their early 16S rDNA grouping with Hadobacteria (oddly still believed by Woese 2013) seems to have been a misleading long-branch problem. A 31-protein tree (Ward et al. 2009) groups them with Teichobacteria and Actinobacteria (Fig. 3). Transfer of Chloroflexi to Posibacteria makes Negibacteria coterminous with Omp85 OM biogenesis machinery, more sharply defining the subkingdom. Accepting Chloroflexi as Posibacteria removes the polarization wrongly placing them at the base of Negibacteria (Cavalier-Smith 2006c) but does not alter earlier arguments for their being the most primitive of all bacteria.

Indeed, a novel aerobic green photoheterotrophic bacterium *Candidatus Chloracidobacterium* (Bryant et al. 2007), a Negibacterium in deep-branching class Acidobacteria of Proteobacteria sensu (Cavalier-Smith 2002b), reinforces the view that green bacteria were at the root of the universal tree (Cavalier-Smith 1985, 1991a,b, 1992). Because Chloracidobacterium has chlorosomes and a type I photosynthetic reaction center, like Chloroflexi and Chlorobi (green sulfur bacteria: negibacteria) (Garcia Costas et al. 2011; Tsukatani et al. 2012), and Acidobacteria are sisters to purple proteobacteria and their colorless relatives (Ward et al. 2009), the lineage connecting negibacteria and posibacteria clearly had chlorosomes and type I reaction centers, as also did that joining Chlorobi to the base of Proteobacteria, assuming vertical inheritance of photosynthesis. Congruence of type I reaction-center (Bryant et al. 2007) and 31-protein (Ward et al. 2009) trees contradicts invoking photosynthetic machinery LGT to negate that.

A universal root in Negibacteria, which a flagellar polarization argument favored (Cavalier-Smith 2006c), would most plausibly be within Gracilicutes between Chlorobi and Chloracidobacterium. Were it in Posibacteria as I now prefer (Fig. 3) and some indels suggest (Skophammer et al. 2007), it is probably be-



tween Chloroflexi and Teichobacteria (which include the green chlorosome-lacking photoheterotrophic Heliobacteria as well as heterotrophic Bacillales and Clostridiales), and LGT from gracilicute Negibacteria presumably gave Posibacteria flagella. Thus, ancestral cells were probably photosynthetic green bacteria, whether the universal root is in Posibacteria or Negibacteria. Unless this root is within Gracilicutes, the unique purple bacterial photosynthetic machinery must have green bacterial ancestry, as probably do cyanobacterial photosystems. The 31-protein tree and posibacterial root together fit conclusions from photosystem evolution that heliobacterial photosynthesis is the most primitive, followed by Chloroflexi, then Cyanobacteria, gracilicute photosynthesis being more derived (Gupta 2012). If cell lineages diverged as in Figure 3, gene duplications probably created photosystem II (Sousa et al. 2013) in the common ancestor of Cyanobacteria and Gracilicutes, system II being lost by Chlorobi and Chloracidobacterium (and type I reaction centers by Proteobacteria); galactolipid synthesis for photosynthetic membranes probably originated in green posibacteria (monogalactosyl-diacylglycerol synthase homologs are conserved throughout posibacteria) (Yuzawa et al. 2012). Multiple losses during photosynthetic diversification were probably more and LGT less frequent than many assume. Whether the first cell was a *Heliobacterium*-like posibacterium (most likely) or *Chlorobium*-like Negibacterium, it lacked flagella and RuBisCo.

INTEGRATING MULTIGENE SEQUENCE TREES AND FOSSIL DATA FOR REAL TIMING OF CELL MEGAEVOLUTION

The eubacterial radiation is extremely bush-like with very little basal resolution even on multi-gene trees (Battistuzzi et al. 2004; Ciccarelli et al. 2006). The 25-protein tree of Battistuzzi et al. (2004) showed clear bipartition between Gracilicutes (phyla Spirochaetae, Proteobacteria, Sphingobacteria, Planctobacteria, including the monophyly of each except Planctobacteria) and all other bacteria, but incorrectly placed cyanobacteria and *Deinococci* (with *Thermus*

constituting the phylum Hadobacteria) within Posibacteria, suggesting that they might be more closely related to Posibacteria than are Gracilicutes. The 31-protein tree of Ward et al. (2009) groups Hadobacteria (bootstrap support 100% for their monophyly) and *Thermotoga/Aquifex* as sisters with 69% support, but wrongly places Cyanobacteria and Fusobacteria deeply within Posibacteria (insignificant 12% support); that tree showed monophyly of Gracilicutes (81%, support), 97% support for monophyly of Planctobacteria, 87% for Proteobacteria sensu Cavalier-Smith (2002b) (including Acidobacteria), and 100% for Spirochaetae and for Sphingobacteria (Chlorobi + Bacterioidetes and relatives), and for the latter two being sisters. From cell evolution perspectives, that multigene tree is the best I know for Eubacteria.

To eliminate potential LGT problems, Baptiste et al. (2008) selected 22 compatible markers from 31, unfortunately with narrower taxonomic sampling and all branching with <50% support unwisely collapsed; they found just four eubacterial clades: Gracilicutes (72%), Endobacteria (lowGC Posibacteria: 58%), both with major anaerobic and aerobic lineages, and two aerobic clades, each with 100% support (Cyanobacteria, Actinobacteria). Higher support for aerobic clades is because they branch less deeply than mixed aerobic/anaerobic clades, with a longish bare stem. That contrast has a simple evolutionary explanation, their respective radiation timings: crown cyanobacteria arguably radiated near the time of the great oxygenation event they caused (2.4 Gy ago), whereas actinobacteria (with proportionately longer bare stem) radiated somewhat later, after oxygenation enabled their aerobic heterotrophy. In contrast, Gracilicutes and Endobacteria, with anoxygenic photosynthesizers and numerous anaerobic heterotrophs or chemotrophs, radiated earlier, probably >2.7 Gy ago, but <3.5 Gy ago. Stem lineages of both aerobic groups were probably anaerobes; I suggest anaerobic green bacteria for cyanobacteria and anaerobic heterotrophic endobacteria for actinobacteria. Mycoplasmas are sisters to Bacillales within Teichobacteria, confirming their murein loss; they have longer branches than any other Posibacte-

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ria, showing that wall loss and/or genome miniaturization somewhat released their proteins from stabilizing selection, analogously to the elevated evolutionary rates of neomura. Evolutionary rates in Bapteste et al. (2008) are similar within other major clades, slightly greater in Gracilicutes than Cyanobacteria or Posibacteria (Posibacteria apparently a weakly supported clade collapsed into two during figure preparation). Applying a crude evolutionary clock to eubacteria indicates that cyanobacteria initially radiated almost contemporaneously with purple bacteria. Although backward-extrapolating clock estimates are problematic because they do not allow for quantum evolution that can inflate ages (Cavalier-Smith 2002b) or for systematic differences between rates across domains and phyla, Feng et al. (1997) estimated 2.1–2.5 Gy ago for the date of the Posibacteria/Cyanobacteria/other Negibacteria radiation, in surprisingly close harmony with the robust palaeontological date of ~ 2.4 Gy ago for the great atmospheric oxidation event (Schirmermeister et al. 2013).

Eukaryotes cannot have originated significantly before α -proteobacteria, which must be younger than purple bacteria generally. The α -proteobacterial radiation on the 22-gene tree is about half as old as for purple bacteria collectively. That implied age of ~ 1.25 Gy ago must be somewhat too low because of limited taxon sampling. From the more taxon-rich 191-genome tree of Ciccarelli et al. (2006), the same argument gives a maximal eukaryote age of ~ 1.8 Gy ago. Given the resolution within joint mitochondrial/ α -proteobacterial gene trees (Derelle and Lang 2012; Zhao et al. 2013), mitochondria probably did not arise from the first α -proteobacteria, but are probably $< 80\%$ as old as α -proteobacteria, perhaps younger still. That gives ~ 1.44 Gy ago as a reasonable upper limit for eukaryote age, close to the oldest fossils (1.45 Gy ago) that several palaeontologists regard as eukaryotic (Javaux et al. 2001), although I consider them more likely all misinterpreted bacteria (Cavalier-Smith 2006a). Independent estimates of minimum eukaryote age from the morphological fossil record and eukaryote multigene trees (Cavalier-Smith 2013a) of

$\sim 1.2 \pm 0.25$ Gy are congruent and surprisingly close. These estimates bracket the origin of eukaryotes between 0.95 and 1.45 Gy ago, making them $2.4\text{--}3.7\times$ younger than stem eubacteria (~ 3.5 Gy), although possibly no more than twice as young as the probably explosive crown eubacterial radiation (here estimated as ~ 2.7 Gy ago). The archaeobacteria branch is far longer than all eubacterial clades: more than three times as long as that of most eubacteria and about five times longer than cyanobacteria. Because cyanobacteria are > 2.4 Gy old (Schirmermeister et al. 2013), more likely ~ 2.7 Gy (Bosak et al. 2009, 2012; Petroff et al. 2010; Bosak et al. 2012), that date objectively calibrates the mean length of crown cyanobacterial branches; because the stem connecting the base of crown eubacteria to the base of crown archaeobacteria is just over five times longer, it would represent 12 Gy of evolution (conservatively assuming 2.4 Gy: $2.6\times$ the 4.6 Gy age of the Earth and $3.4\times$ the age of all life: 3.5 Gy) if these 22 proteins were then evolving at the same rate as in cyanobacteria for the past 2.4 Gy. Likewise, crown Archaeobacterial branches are substantially longer than crown cyanobacterial ones and would represent ~ 6.4 Gy of evolution; stem plus crown would represent 18 Gy, greater than the 13 Gy age of the universe. From these necessarily underestimates, the logically inescapable conclusion is that these proteins evolved substantially faster in crown eukaryotes and crown archaeobacteria than in eubacteria, and that neomuran stem lengths on most sequence trees are hugely inflated by quantum evolution, causing false conclusions about timing if that is overlooked (e.g., Doolittle 1995).

That gross rate inequality invalidates midpoint rooting of prokaryotic distance networks (using subsets of gene families from 191 genomes) by Dagan et al. (2010). Their favorite network (Fig. 5) shows the archaeobacteria stem much longer than the whole cyanobacterial branch and stem plus crown $\sim 3\times$ longer, so their statistical argument for uniform evolutionary rates is fallacious and their rooting no better than “reading chicken entrails,” to use the apposite phrase introduced by the senior author (Graur and Martin 2004). Dagan et al. falsely



claim that I “dismiss molecular data” “where convenient” by invoking quantum evolution. On the contrary, I *extensively used molecular data* combined with *objective fossil evidence on timing*, which proved the reality of quantum evolution in many molecules and disproved their gradualist assumptions. They selectively interpret fossil evidence that provides no hard evidence that archaeobacteria are older than 1.5 Gy, notwithstanding earlier carbon isotope ratios overconfidently interpreted as evidence for archaeobacterial methanogenesis (Cavalier-Smith 2006a). Their Figure 4C net for the gene family tranche conserved 35%–40% clearly bipartitions Negibacteria from Unibacteria.

Compared with the sophisticated multigene trees discussed above, the three-domain single-gene rDNA trees shown by Pace (2009), Woese (2013), and Fournier and Gogarten (2010) are archaic, of only historical interest representing a pioneering but primitive three-decades-old phase of phylogenetics, suitable only for Pace’s T-shirts. In all, and even the technically better but taxonomically undersampled concatenated rDNA trees of Williams et al. (2012), the branching within eukaryotes is certainly substantially wrong (judged by numerous multigene trees based on many scores of proteins), that within eubacteria completely unresolved bush, and assumptions of rooting probably erroneous, naïvely relying on probably artifactual paralog duplicate trees. Referring to them as the universal tree of life (Pace 2009) is more confusing than enlightening.

The trees based on 34 universal ribosomal proteins in Lasek and Gogarten (2013) are much better in emphasizing a two-domain structure (eubacteria versus neomura; neomura share 30 new proteins entirely absent in the ribosomally simpler and older eubacteria) and having an almost correct branching order within eukaryotes (their root constantly between Euglenozoa and neokaryotes). They have no basal resolution within eubacteria, as expected if the universal root is among them followed by extremely rapid radiation and or saturation effects. Williams et al. (2012, 2013, 2014), though accepting that the three primary domains idea is wrong (they correctly treat eukaryotes as derived, not primary)

and that neomura are a clade (oddly without using that name), still argue for two primary domains, mistakenly viewing eubacteria as a clade and regarding eubacteria and archaeobacteria both as primary domains. I suspect that widespread failure to accept eubacteria alone as the single primary domain of life, as I have consistently argued since 1987, owes more to the mistaken dogmas briefly discussed below than to the evidence, which is admittedly hard for many specialists to evaluate as it depends on critical integration of palaeontology and neontology, both phylogenetics and molecular and especially cell biology of both bacteria and eukaryotes, as well as critical judgments about the mechanistic and selective plausibility of the various transitions theoretically possible among the five major cell types shown in Figure 1.

METHODOLOGICAL BIASES AND MOLECULAR CHRONOMETRIC MYTHS

It is important to note that even if there is no resolution whatever in input data for sequence trees, as would be the case if (as I argue) eukaryotes, filarchaeotes, and euryarchaeotes diverged essentially simultaneously, phylogenetic algorithms can be biased to introduce apparently strong support for meaningless conclusions (Yang 2007). Too often sequence trees are treated as factual data, rather than indirect inferences based on oversimplified evolutionary assumptions, because they always inevitably are, and given excessive emphasis compared with other evolutionary evidence, morphological, cell biological and palaeontological; all kinds of evidence must be treated critically and integrated if we are to solve such difficult historical problems.

The very long eukaryotic stems in the 29-protein trees of Williams et al. (2012), strongly dependent on ribosome-associated proteins, are attributable substantially to exceptionally rapid quantum evolution consequential on the origin of the nucleus, as explained previously (Cavalier-Smith 2002b); as also explained there, similar quantum evolution during the neomuran revolution in SRP cotranslational secretion may partially explain the comparably

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long stem separating eubacteria and Neomura, whose length (whatever its causes) arguably virtually ensures a Shavit et al. (2007) type of artifact, failing to group neomura with the single (!) included posibacterium. Neither of these long stems accurately reflects long evolutionary times, but rapid quantum evolutionary stretching; proteins chosen for many three-domain trees are the least clock-like of all and are biased toward the minority that clearly differentiate archaeobacteria and eubacteria (Cavalier-Smith 2002b). The two posibacterial lineages (Actinobacteria, Chloroflexi) that are closest to Neomura on classical rRNA trees (Woese 2013) and closer than any sampled were not even included by Williams et al. (2012), and the latter (the closest) was absent from Fournier and Gogarten (2010), making both poor tests of neomuran relationships.

One need only look at the wildly high evolutionary rates of 16S rDNA in microsporidia that led to their being misconstrued as the earliest eukaryotes (Vossbrinck et al. 1987), when they really are advanced derivatives of Choanozoa of comparable recency to fungi (Cavalier-Smith 2013a; James et al. 2013), or of chloroplast 16S rDNA in dinoflagellates (Zhang et al. 2000), to appreciate the absurdity of the claim for small-subunit ribosomal rRNA as a molecular chronometer, or the falseness of the assumption that big changes in its structure must necessarily be ancient (Woese and Fox 1977). The whole Woesian “three-domain” perspective was biased and fundamentally misled by his profoundly mistaken assertion that rRNA is a “molecular chronometer”; the precise opposite is true, rRNA being possibly the least clock-like of all molecules in evolutionary rates. The extraordinarily elongated branch lengths for Microsporidia (James et al. 2013) and for an independently ultrarapidly evolving haplosporidian, the rhizarian parasite *Mikrocytos mackini* (Burki et al. 2013), on multigene trees emphasize that no proteins universally evolve in a uniform clock-like manner. One must use fossils and genuine atomic decay clocks in igneous rocks for objective timing, as Woese never did.

The foundation of the three-domain dogma is excessively narrow, depending mainly (not

quite exclusively) on ribosome-related characters subject to temporally misleading quantum-evolutionary biases during the neomuran revolution and eukaryogenesis (Cavalier-Smith 1981, 1987c, 2002b). In a survey of 80 universally conserved proteins, Harris et al. (2003) found that of the 50 whose trees agreed with that of rDNA in showing archaeobacteria and eukaryotes as sisters, all except two transcriptional and four DNA replication/repair genes were ribosome-associated; none of the 30 proteins that disagreed with their rRNA tree were ribosome-associated. They unwisely dismissed all 28 of those showing phylogenetically intermixed eubacteria and neomura from serious consideration as likely to have undergone LGT at some stage, downplaying and almost ignoring the alternative reasonable explanation that metabolic genes generally did not undergo quantum evolution during the neomuran revolution, and therefore lack the stretched stem that through grossly violating molecular-clock assumptions greatly enhances ribosome-related sequence tree evidence for the monophyly of neomura (see Cavalier-Smith 2002b). In fact, both their and my explanations of this important, sub-carpet-swept, incongruence between metabolic and ribosomal genes are probably correct for different branches; I suspect that inherently poor resolution caused by lack of resolution-enhancing quantum evolution (combined with archaeobacteria actually being younger than their eubacterial ancestors) is the major reason and LGT the minor one.

Two more open-minded devotees of the Woesian perspective (Doolittle and Zhaxybayeva 2013) emphasize that “a much larger number [than the few informational genes that differentiate archaeobacteria and eubacteria], many hundreds, of ‘operational’ genes make up a shared resource that is common to Bacteria and Archaea” and that all prokaryotes have “a vast shared pool of genes encoding diverse metabolic functions seldom if ever used by eukaryotes.” They candidly admit that for “metabolism, regulation, population genetics, and ecology. . . without knowing in advance it would, in many instances, be hard to say whether a particular published paper on these topics



had a bacterium or an archaeon as its study object.” Differences between archaeobacteria and eubacteria have been grossly exaggerated. Doolittle and Zhaxybayeva (2013) cogently argue that the notion of a fundamental dichotomy between ancestral prokaryotes and derived eukaryotes in cell organization remains perfectly valid and evolutionarily sound, despite overconfident and superficial propaganda against it from archaeologists, mainly Pace. They also highlight the tacit refusal by Woese and Pace to recognize neomura as a clade, a failure that Doolittle and Zhaxybayeva (2013) emphasize can be understood only in sociological, rhetorical, or propagandistic, not scientific terms, because it would entail explicitly admitting the undoubted fact that the idea of *three* primary domains was simply wrong. (So is the idea of *two* primary domains, which Doolittle and Zhaxybayeva still espouse.) They equally overlooked the key concept of Negibacteria; neither Woese (2013) nor Pace (2009) (nor even Doolittle and Zhaxybayeva) mentions the two-membrane character of their envelope, vital for understanding bacterial evolution and eukaryogenesis.

As far as I know, Woese and Pace never cited any of my papers or attempted to refute their arguments; under a tree in Cold Spring Harbor in 1987, George Fox (coinventor of archaeobacteria) told me not to send my first neomuran paper to Woese, despite complimenting its “many good arguments,” because “he would not bother to read it.” Even his swansong (Woese 2013) restricted discussion of the archaeobacteria–eukaryote relationship to one extremely superficial paragraph that merely (rightly) rejected the ideas of Hyman Hartman and Martin and Russell (2003) (sadly, Woese’s own attempts at “explaining” cell evolution—latterly by vague invocation of rampant LGT—were devoid of significant cell biological content or logic). His assertion that “Evolution does not proceed by suddenly and drastically altering a given cell design (at least a fairly advanced one)” could hardly be more wrong for eukaryogenesis; but that prejudice simply explains why he was unprepared even to contemplate the possibility of a derived nature for archaeobacteria, as some other influential and otherwise good scientists

sadly still are. Nonetheless, Woese (2013) greatly pleased me by advocating “bacteriology” not “microbiology” for the whole science of prokaryotes (a name he also used without objection, unlike Pace), and using “bacteria” in his text exactly as I long have to refer to all prokaryotes, including “archaea,” and reinstating both “eubacteria” and “eukaryotes” (but his figure contradictorily used “bacteria” and pointless “eukarya”)—welcome partial recantation of earlier nomenclatural unwisdom (Woese et al. 1990). Likewise, his fundamental misinterpretations of the tree of life and the evolutionary significance of archaeobacteria that so severely misled a generation of researchers will not maintain their distorting stranglehold over phylogenetic thinking, if the rising generation of vigorous, less-prejudiced younger researchers demands proper emphasis on cell-biological, palaeontological, and 3D ultrastructural and crystallographical evidence of molecular morphology (Jékely 2006, 2008; Valas and Bourne 2009; Keeling 2013), in addition to the limited presently dominant one-dimensionality of sequences, when reconstructing the history of life. Morphology at all levels underlies evolution: electrical attractions and repulsions between different shapes vivify cells, not mathematical abstractions like sequences.

Doolittle (2000), especially, has much exaggerated the degree to which LGT confuses the tree of life, best seen as an *organismal* tree based on predominantly vertically inherited cell lineages (Fig. 1), not a *multigene* tree. Even in prokaryotes, cell lineages devoid of anastomoses by sex or symbiogenesis were simply tree-like for 2 billion years before eukaryotes.

Vertical inheritance of distinctive prokaryotic cell structures and integrated cell cycles was the phylogenetic framework within which LGT fiddled around with relatively restricted metabolic details, creating a genic but not organismal web: both by near-neutral replacements of roughly equivalent enzymes catalyzing a standard reaction and by truly novel acquisition of adaptively valuable, phylogenetically distant catalysts. But these gene-by-gene transfers do not alter the fact that the basic differences in cell structure between eubacterial phyla like

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Cyanobacteria, Spirochaetae, Posibacteria, and Proteobacteria have been stable for billions of years despite LGT, as has their distinctiveness from Archaeobacteria for more than a billion, and can be used in interpreting bacterial organismal megaevolution (Fig. 3) in much the same way as we can reliably and meaningfully for eukaryotes despite the temporarily confusing effects of sex and symbiogenesis (although LGT, greater substitutional saturation over the longer timescale, and paucity of ultrastructural complexity do make it harder for the truly ancient eubacteria than for the younger neomura). On the neomuran interpretation, the primary phase of “progressive Darwinian evolution” that improved the basic machinery of life after cells first evolved, as speculatively discussed by Doolittle and Zhaxybayeva (2013) and hordes of others (including me: Cavalier-Smith 1987b, 2001) in the past three decades, took place not in the stem connecting neomura and eubacteria, as they and so many others wrongly assume, but much earlier in the stem eubacteria necessarily missing from sequence trees, during the early Archaean period 3.5–2.7 Gy ago (which does *not* mean the period when “Archaea” lived—they are purely Protoerozoic [mid and late, not even early] and Phanerozoic bacteria). Some of these speculations are sensible if applied to stem bacteria but totally misleading if applied to stem neomura, whose evolution we can reliably infer by critical and multievidential comparative biology. Contrary to pervasive, often dogmatic, misconceptions stemming primarily from Woese’s chronometric myth and aversion to accepting megaevolutionary change in advanced cells, the last universal common ancestor of life (LUCA) did not lie on the stem connecting neomura and eubacteria, but within the eubacterial radiation.

The derived nature and recency of archaeobacteria have been long concealed by pervasive biases in citing or interpreting contradictory paralog duplicate trees, as explained previously (Cavalier-Smith 2002b, 2006c); numerous paralog trees, which place the root within eubacteria, as I consider correct, and thus contradict the Woesian paradigm, are either ignored or dismissed by vaguely invoking LGT (Zhaxy-

bayeva et al. 2005), and those that agree, which are probably dominated by misleading long-branch artifacts (Cavalier-Smith 2006c), are cited as support! Trees that place Archaeobacteria closer to Posibacteria than to Negibacteria, in concordance with neomuran theory, are too lightly dismissed as implying LGT (e.g., Feng et al. 1997). The seemingly objective but probably artifactual rooting by the first paralog duplicate trees (Gogarten et al. 1989; Iwabe et al. 1989) eclipsed my still-cogent earlier arguments for a eubacterial root (Cavalier-Smith 1987c), and subsequent dogma and uncritical inertia allowed Doolittle and Zhaxybayeva (2013) to write that “most biologists today” would believe one of their Figures 2.3f–h, which all wrongly (as argued here and since 1987) show archaeobacteria as old as eubacteria. Contrary to that widely held assumption there is *no* historically convincing evidence for archaeobacteria being as old as Eubacteria; instead, their Figures 2.3b,d (elaborated in my Figs. 1 and 3) are probably correct for reasons summarized above. Another attempt at rooting the universal tree using amino acid composition biases depends on several highly questionable assumptions (Fournier and Gogarten 2010); from my perspective, their data simply indicate that there was a bigger change in ribosomal protein composition during the neomuran revolution than during either eukaryogenesis or the origin of archaeobacteria, and fall short of proving where the root lies.

Two other biases prejudice bioinformatics interpretations: the prevalence of gene loss (underestimating it inflates LGT estimates and grossly deflates estimates in the number of genes present in the cenancestor of each domain); and the prevalence in megaevolution like eukaryogenesis of radical gene evolution beyond bioinformatics recognition, which led to hugely wrong conclusions about the relative contributions of neomura and α -proteobacteria to the eukaryotic chimera (see Cavalier-Smith 2007b).

I must stress that if more convincing evidence than now exists were to appear and establish that eukaryotes are really derived from archaeobacteria (Williams et al. 2012, 2013, 2014), contrary to my arguments that they are sisters, that would prove even more strongly that neo-



mura are a clade. But it would not provide any evidence that archaeobacteria are a primary domain as old as eubacteria. Nor would it alter the essential cell biological or selective bases for the major innovations in cell biology during eukaryogenesis discussed here. It would just make a mechanistically rather trivial change in the precise phylogenetic origins of some of the precursor proteins for these dramatic megaevolutionary changes, and introduce a slightly greater time interval between the origin of eukaryotes and archaeobacteria than I have assumed. The most innovative core features of my cell evolutionary arguments for the secondary origins of both groups would remain valid.

CONCLUDING REMARKS

Understanding eukaryote origins requires balanced synthesis of cell biology, palaeontology, and phylogeny into a complete logically consistent picture, here outlined. Partial approaches from single perspectives spawned many oversimplified or unnecessarily complex ideas, often biasing interpretations or obscuring the basic simplicity of cell history. Especially difficult has been determining the root of the tree for all life and eukaryotes (Figs. 1 and 3), essential for deducing phenotypes for early eukaryotes and their ancestors and sound reasoning about eukaryogenesis. Errors ensure false interpretations, hard to correct because tree-root positions are often taken as “obviously true” when interpreting other data. We must be ready to change conclusions radically if new evidence or insights demand it.

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