

An ancestral bacterial division system is widespread in eukaryotic mitochondria

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Bacterial division initiates at the site of a contractile Z-ring composed of polymerized FtsZ. The location of the Z-ring in the cell is controlled by a system of three mutually antagonistic proteins, MinC, MinD, and MinE. Plastid division is also known to be dependent on homologs of these proteins, derived from the ancestral cyanobacterial endosymbiont that gave rise to plastids. In contrast, the mitochondria of model systems such as *Saccharomyces cerevisiae*, mammals, and *Arabidopsis thaliana* seem to have replaced the ancestral α -proteobacterial Min-based division machinery with host-derived dynamin-related proteins that form outer contractile rings. Here, we show that the mitochondrial division system of these model organisms is the exception, rather than the rule, for eukaryotes. We describe endosymbiont-derived, bacterial-like division systems comprising FtsZ and Min proteins in diverse less-studied eukaryote protistan lineages, including jakobid and heterolobosean excavates, a malawimonad, stramenopiles, amoebozoans, a breviate, and an apusomonad. For two of these taxa, the amoebozoan *Dictyostelium purpureum* and the jakobid *Andalucia incarcerationata*, we confirm a mitochondrial localization of these proteins by their heterologous expression in *Saccharomyces cerevisiae*. The discovery of a proteobacterial-like division system in mitochondria of diverse eukaryotic lineages suggests that it was the ancestral feature of all eukaryotic mitochondria and has been supplanted by a host-derived system multiple times in distinct eukaryote lineages.

mitochondria | mitochondrial division | Min proteins | MinCDE | mitochondrial fission

During bacterial division, septum formation is mediated by the Z-ring, a contractile ring structure made up of the polymerized tubulin homolog FtsZ (reviewed in refs. 1 and 2). The site at which FtsZ polymerizes is determined by the Min system (reviewed in refs. 1 and 3–5), comprising the three septum site-determining proteins MinC, MinD, and MinE. ATP-bound, dimerized MinD binds the inner cell membrane at the poles of the cell, forming aggregates. These MinD aggregates bind and activate dimerized MinC (6), which then inhibits local FtsZ polymerization (Fig. 14). Concomitantly, dimerized MinE forms a spiral ring whose constant polymerization and depolymerization causes it to oscillate across the cell (7, 8). Where MinE comes into contact with MinD, it causes the release of ATP and the subsequent liberation of MinD from the membrane (9, 10). In this way, MinD and MinC cannot inhibit FtsZ polymerization near the midpoint of the cell. The polymerizing Z-ring is stabilized and tethered to the membrane by FtsA, ZipA, and the nonessential ZapA and (in some organisms) ZapB (11–15). Maturation of the Z-ring into a complete septal ring continues with the subsequent recruitment by FtsA of further components of the divisome (i.e., FtsB, FtsE, Fts/PBP3, FtsK, FtsL, FtsN, FtsQ, FtsW, and FtsX), which proceed to stabilize FtsZ and

contribute to peptidoglycan synthesis (reviewed in refs. 3, 4, 16 and 17) (Fig. 1B) before Z-ring constriction and the completion of septum formation (Fig. 1C).

Plastids are known to possess FtsZ (18–20), MinD (21, 22), MinE (21, 23), and, in some cases, MinC (24) homologs of cyanobacterial endosymbiotic origin; in some cases, the latter are encoded on the plastid genome (21, 25). In contrast, only two examples of putative mitochondrial Min proteins have been reported, in the stramenopiles *Nannochloropsis oceanica* and *Ectocarpus siliculosus* (26). Indeed, although eukaryotic mitochondria are derived from an α -proteobacterial endosymbiont, the ancestral bacterial division machinery has been partly or wholly replaced by eukaryote-specific proteins in model system eukaryotes where mitochondrial division has been studied. Whereas Amoebozoa (27), stramenopiles (28, 29), and the red alga *Cyanidioschyzon merolae* (30, 31) have retained experimentally confirmed mitochondrial FtsZ, animals and fungi (opisthokonts) and plants examined to date lack this protein. In the latter taxa, an outer contractile ring is instead formed by Dnm1p/Drp1, a eukaryote-specific dynamin GTPase (32–34). This protein is implicated in mitochondrial division in organisms across the eukaryotic tree, including *Arabidopsis thaliana* (35–37), the parabasalid *Trichomonas vaginalis* (38), *Dictyostelium discoideum* (39), and *C. merolae* (40), suggesting that the outer contractile ring is a widespread eukaryotic feature. In *T. vaginalis* and *A. thaliana*, the nature of the inner contractile ring is not yet understood, although the presence of two Dnm1/Drp1 homologs in *A. thaliana* (37) raises the possibility that they form an outer and an inner contractile ring, respectively. Recent work (41) reconstructing the evolution of eukaryotic dynamins suggests that the ancestral mitochondrial dynamin was a bifunctional protein that also mediated vesicle scission. This protein underwent duplication events, followed by subfunctionalization, independently in at

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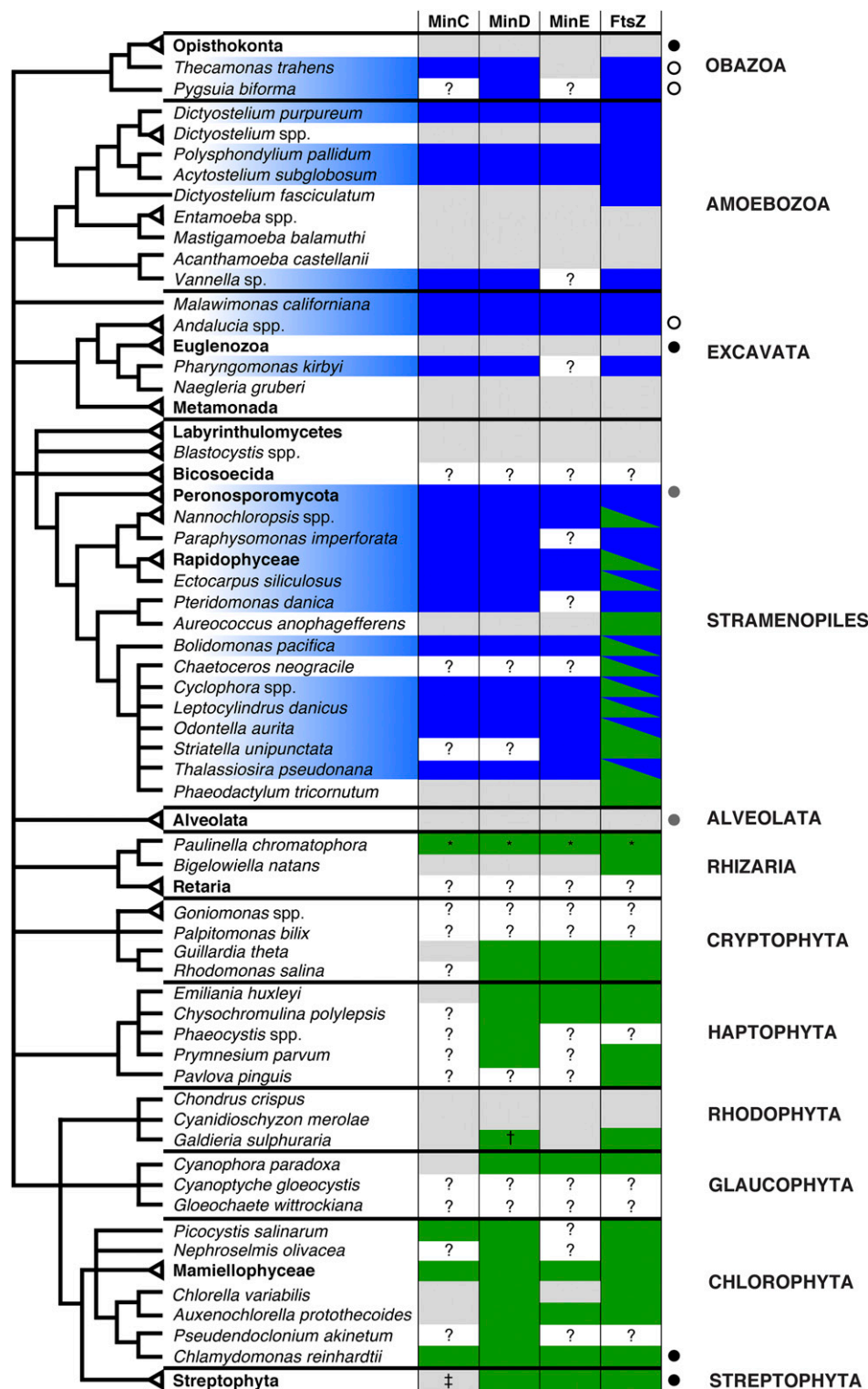


Fig. 2. Presence and absence of bacterial Min proteins and FtsZ in selected eukaryotic taxa. Blue, predicted mitochondrial proteins; gray, no protein found encoded in complete genome data; green, predicted plastid proteins; ?, no protein found encoded in transcriptome or incomplete genome data; *, chromophore protein; †, predicted pseudogene; ‡, with the exception of *Physcomitrella patens*. Boxes shaded half blue and half green represent multiple paralogs, predicted to be mitochondrial and plastid, respectively. In cases where only a transcriptome or incomplete genome is available, it should be noted that the presence of a plastid protein does not exclude the possibility of one or more mitochondrial paralogs also being present, and vice versa. Eukaryotic taxa possessing predicted mitochondrial Min proteins are shaded in blue. Mitochondrial or plastid predictions are based on phylogenetic affinity with previously localized proteins, predicted subcellular localization, and localization in yeast (*A. incarcerata*, *D. discoideum*). Black circles indicate taxa in which reticulate mitochondria have previously been described; gray circles indicate groups for which reticulate mitochondria have been described in at least one member; black-bordered white circles indicate taxa in which only single or unbranched mitochondria have been described. The schematic phylogeny reflects the current understanding of relationships based on multiple phylogenomic analyses. For a more complete table, see Table S1.

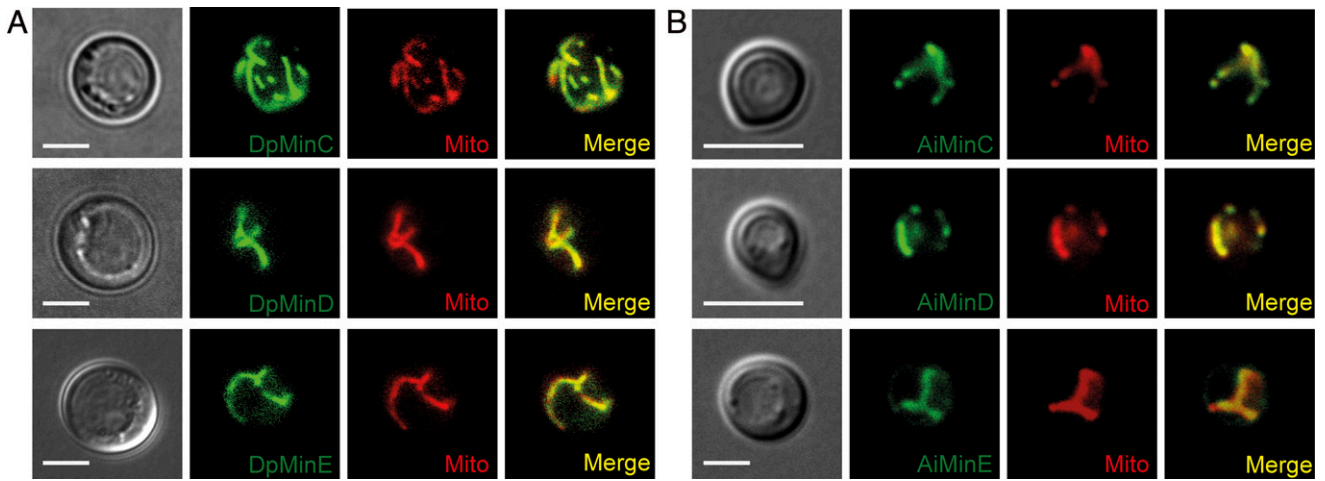


Fig. 3. Min proteins from *D. purpureum* (A) and *A. incarcerata* (B) expressed in *S. cerevisiae* to confirm predicted mitochondrial targeting. Differential interference contrast (DIC) images of *S. cerevisiae* cells expressing Min fusion proteins (Left); in green, MinC, MinD, or MinE expressed with the C-terminal GFP tag in *S. cerevisiae*; in red, mitochondria labeled with MitoTracker Red CMXRos (Mito); merged images (Merge) show mitochondrial localization of all Min proteins. (Scale bars: 5 μ m.)

least three lineages (opisthokonts, land plants, and alveolates), but the ancestral bifunctional form seems to have been retained in amoebozoa such as *D. discoideum*, the red alga *C. merolae*, and stramenopiles (and possibly additional eukaryotes that have currently less well-characterized dynamins). The distribution of ancestral-like bifunctional mitochondrial/vesicle fission dynamins thus seems to mirror that of mitochondrial FtsZ (41).

Here, we hypothesize that the complete loss of the α -proteobacterial division system is the exception, rather than the rule, for eukaryotes. We show that mitochondria-targeted homologs of bacterial Min proteins are patchily but widely distributed among diverse eukaryote lineages; and we further demonstrate that Min proteins from two of these lineages, the amoebozoan *Dictyostelium purpureum* and the jakobid excavate *Andalucia incarcerata*, localize to mitochondria when expressed in yeast.

Materials and Methods

Database Searches. Publicly available databases and sequencing projects were searched using the Basic Local Alignment Search Tools (BLAST) blastp and tblastn (42). A large number of databases containing eukaryotic sequences were screened with these tools using query sequences from *D. purpureum* (XP_003286111, XP_003292258, XP_003293637, XP_642499), *E. siliculosus* (CBJ32744, CBJ31561, CBJ28079, CBJ48312) *A. incarcerata*, *Pseudomonas fluorescens* (AEV64338, AEV64339, AEV64340, AEV64767), and *Anabaena* sp. 90 (YP_006998153, AFW94434, YP_006996248, YP_006996249). The databases searched included the Nucleotide collection (nr/nt), National Center for Biotechnology Information (NCBI) Genomes, Whole-Genome Shotgun contigs, Expressed Sequence Tags, High-throughput Genomic Sequences and Transcriptome Shotgun Assembly divisions of GenBank (43) (last accessed February 9, 2015); the Broad Institute project databases (44) (accessed April 23, 2014); the Joint Genome Institute (JGI) genome databases (45, 46) (last accessed February 9, 2015); dictyBase, 2013 release (47–49); the EnsemblProtists database (50) (last accessed February 9, 2015); the Eukaryotic Pathogen Database Resources (EuPathDB) (51) (last accessed February 9, 2015); and the Marine Microbial Eukaryote Transcriptome Sequencing Project (52) (accessed June 3, 2014), via the Community cyberinfrastructure for Advanced Microbial Ecology Research and Analysis (CAMERA) portal (53) (for a full list of sequences identified, see Table S1 and Dataset S1). In addition, we searched our own unpublished genome or transcriptome assemblies from several protist taxa of key evolutionary interest: two jakobids (*A. incarcerata* and *Andalucia godoyi*), the heterolobosean *Pharyngomonas kirbyi*, and *Malawimonas californiana*. Potential homologs identified were screened manually to exclude contaminants from bacterial or other eukaryotic sources, by searching for introns and excluding sequences with a notably high degree of similarity to bacterial or distantly related eukaryotic homologs. Subcellular localization and targeting peptides were predicted using TargetP, using “plant”

parameters for plastid-bearing taxa and “nonplant” parameters for taxa lacking plastids (54, 55).

Sequence Generation. *P. kirbyi* strain AS12B (56, 57) was cultivated at 37 °C in 10% (wt/vol) salt medium (NaCl 1.6 M, KCl 34.0 mM, MgCl₂ 44.2 mM, CaCl₂ 4.0 mM, MgSO₄ 4.5 mM) supplemented with *Citrobacter* sp. as a food source before RNA isolation. RNA was extracted using TRIzol (Life Technologies) following the manufacturer’s instructions and stored at –80 °C. The RNA sample was treated with Turbo DNase (Life Technologies) before conversion to cDNA using the GeneRacer kit with SuperScript III reverse transcriptase (Life Technologies) and stored at –20 °C. Primers were designed to amplify genes of interest using available sequences. Primer sequences were as follows: MinCF, 5'-ATGTCACGTGATGGTTAGT-3'; MinCR, 5'-TAATACAAAAAACA-3'; MinDF, 5'-ATGTATCGATCAACGAGTTC-3'; and MinDR, 5'-TTAGTTCCTGCTAAATAATC-3'. PCR reactions were done using the Phusion high-fidelity DNA polymerase (New England BioLabs) where the initial denaturation at 98 °C for 30 s was followed by 30 cycles of DNA denaturation at 98 °C for 10 s, primer annealing at 40 °C for 30 s, and strand elongation at 72 °C for 60 s, with a final extension at 72 °C for 10 s. PCR products were purified by gel extraction using the Nucleospin Extract II kit (Macherey-Nagel) and were directly sequenced using the PCR primers.

Phylogenetic Analyses. For each protein, alignments were generated from datasets including all known eukaryotic homologs and bacterial homologs harvested from NCBI using MUSCLE v.3.8.31 (58) or MAFFT-L-INSI v7.149b (59–61), and trimmed using BMGE 1.1 (62) (-m BLOSUM30; all other parameters default). Preliminary phylogenies were generated using FastTree, and datasets were manually refined. Twenty independent Maximum Likelihood (ML) tree estimates and 200 bootstrap replicates were generated using RAxML v.8.0.23 (63) under the PROTGAMMALG4X (64) model of amino acid substitution. Bayesian inference posterior probabilities were calculated using PhyloBayes v.3.3f (65) under the catf2 model of evolution. We tested whether specific phylogenetic hypotheses were rejected by the data using the approximately unbiased (AU) test implemented in CONSEL v.1.20 (66) (Table S2). Maximum-likelihood trees given specific constraints (i.e., corresponding to specific hypotheses) were generated using RAxML. In addition, the 200 trees from bootstrap replicates were included in the hypothesis-testing analyses performed with CONSEL.

Yeast Culture, Transformation, and Microscopy. *Saccharomyces cerevisiae* strain YPH499 was grown at 30 °C on YPD medium or selective medium without uracil after lithium-acetate transformation. For ectopic expression of AiMinC, -D, and -E, the complete AiMinC, -D, and -E ORFs were amplified by PCR from *A. incarcerata* cDNA. For ectopic expression of DpMinC, -D, and -E, the complete DpMinC, -D, and -E ORFs were amplified by PCR from synthesized DNA fragments, containing *Escherichia coli* codon-optimized sequences. The resulting PCR products were cloned separately into pUG35 using XbaI/ClaI restriction sites (AiMinD and DpMinC and -E) or BamHI/HindIII restriction sites

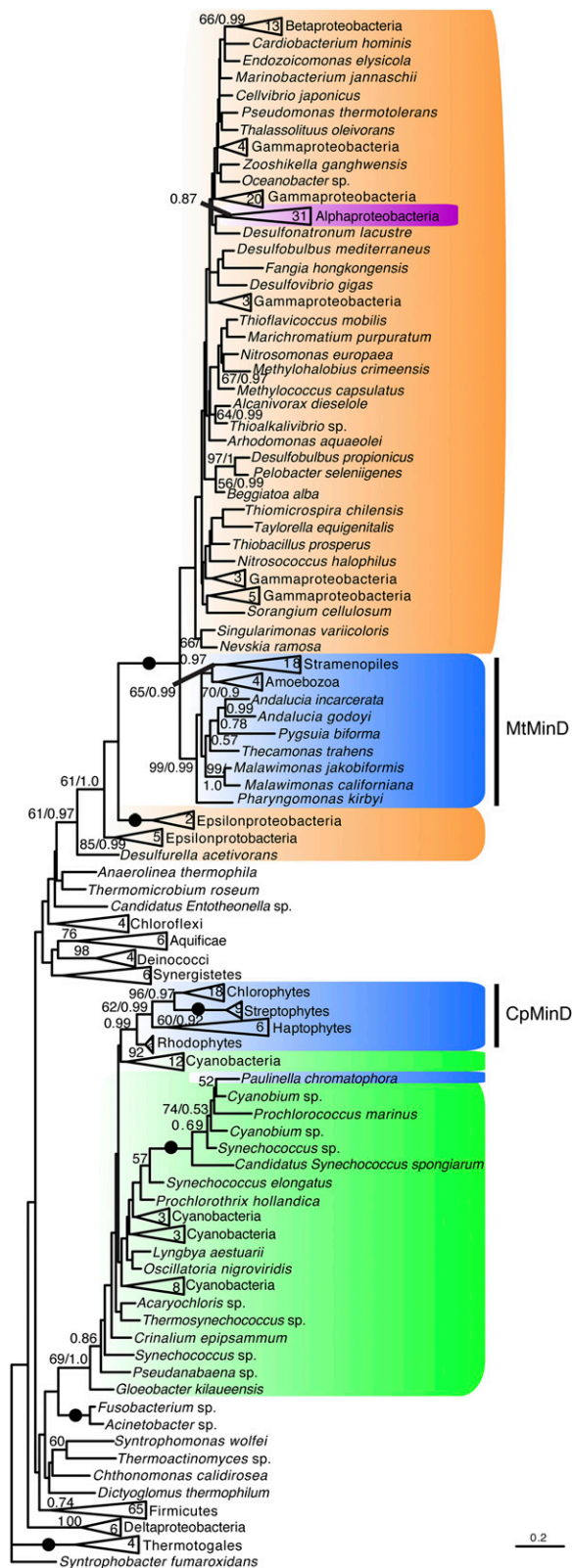


Fig. 4. Unrooted maximum likelihood (ML) tree of MinD sequences. Phylogenetic analyses were performed on 328 sequences and 226 sites, using RAXML and PhyloBayes. Bootstrap support values greater than 50% and posterior probabilities greater than 0.5 are shown. Branches with 100% bootstrap support and posterior probability of 1.0 are indicated by black circles. Eukaryotes are shaded blue, cyanobacteria green, proteobacteria orange, and α -proteobacteria magenta.

(AiMinC and -E and DpMinD), allowing the expression of GFP on the C-terminus of each protein. For fluorescence microscopy, cells were incubated with MitoTracker Red CMXRos (1:10,000) for 10 min, washed once in PBS, and mounted in 2% low-melting agarose. Cells were viewed using an Olympus IX81 microscope and a Hamamatsu Orca-AG digital camera using the cell'R imaging program at 100 \times magnification.

Results and Discussion

We identified sequences encoding at least one Min protein from a number of eukaryotic taxa (Fig. 2 and Table S1), including ancestrally plastid-lacking lineages such as the apusomonad *Thecamonas trahens*, the breviate *Pygusua biforma*, the jakobid excavates *A. godoyi* and *A. incarcerationata*, the malawimonad *M. californiana*, and several amoebozoan lineages, such as *D. purpureum*. Three previously reported FtsZ sequences identified in haptophytes (*Gephyrocapsa oceanica* and *Pleurochrysis carterae*) and a glaucophyte (*Cyanophora paradoxa*) (29) were excluded as probable α -proteobacterial contaminants, based on their position in preliminary phylogenies, their high degree of similarity to α -proteobacterial sequences, and, in the case of *C. paradoxa*, our inability to recover the reported mitochondrial FtsZ sequence from the genome sequence (67). All complete genomes encoding at least one Min protein also encoded at least one FtsZ homolog; however, the reverse was not true. Min proteins were retained not only in lineages with typical aerobic mitochondria, but also in lineages possessing mitochondrion-related organelles (MROs) such as *A. incarcerationata* (68) and *P. biforma* (69).

Most of these Min and FtsZ homologs possess predicted mitochondrial targeting peptides (Table S1). To confirm these predictions, we expressed GFP-tagged homologs of Min proteins in *S. cerevisiae*, in conjunction with the mitochondrial stain MitoTracker Red CMXRos (Fig. 3). We chose Min proteins from two representative taxa lacking plastids: the amoebozoan *D. purpureum* (Fig. 3A) and the jakobid excavate *A. incarcerationata* (Fig. 3B). In both cases, the GFP signal colocalized with the MitoTracker signal, supporting the predicted targeting of *A. incarcerationata* and *D. discoideum* Min proteins to the inside of the mitochondria.

Single-protein phylogenies of MinC, -D, -E, and FtsZ recover all predicted mitochondrial homologs as well-resolved clades, distinct from known and predicted plastid sequences (Figs. 4 and 5 and Figs. S1 and S2). For MinD (Fig. 4) and FtsZ (Fig. 5 and Fig. S3), the hypothesis that the plastid and mitochondrial homologs group in a monophyletic clade was rejected by AU tests (Table S2); however, this hypothesis could not be rejected for the more divergent MinC (Fig. S1) and MinE (Fig. S2). In all three Min phylogenies, mitochondrial homologs emerged within proteobacterial sequences although, because the resolution within that clade was too poor to identify the closest homologs (Fig. 4 and Figs. S1 and S2), we cannot exclude the possibility that these proteins originate from a group other than the α -proteobacteria.

An early study by Miyagishima et al. (70) reported the presence of two copies of plastid-targeted FtsZ in photosynthetic eukaryotes, as well as two copies of predicted mitochondrial FtsZ in *C. merolae* and *D. discoideum*. The authors hypothesized that duplication of FtsZ occurred early during primary plastid endosymbiosis and that a similar process might also have accompanied the establishment of the protomitochondrial endosymbiont. Our broader taxonomic sampling allowed us to confirm the presence of two types of mitochondrial FtsZ homolog in the majority of the eukaryotic taxa examined. They form two distinct phylogenetic clades, each of which contains one homolog from each eukaryote. In addition, although these clades lack strong statistical support, one encompasses copies retaining a variable C-terminal spacer domain that is also found in bacterial homologs (71) whereas sequences from the other clade lack this domain (Fig. 5 and Fig. S3). A robust grouping of α -proteobacterial and both putative mitochondrial FtsZ paralogs was recovered. We

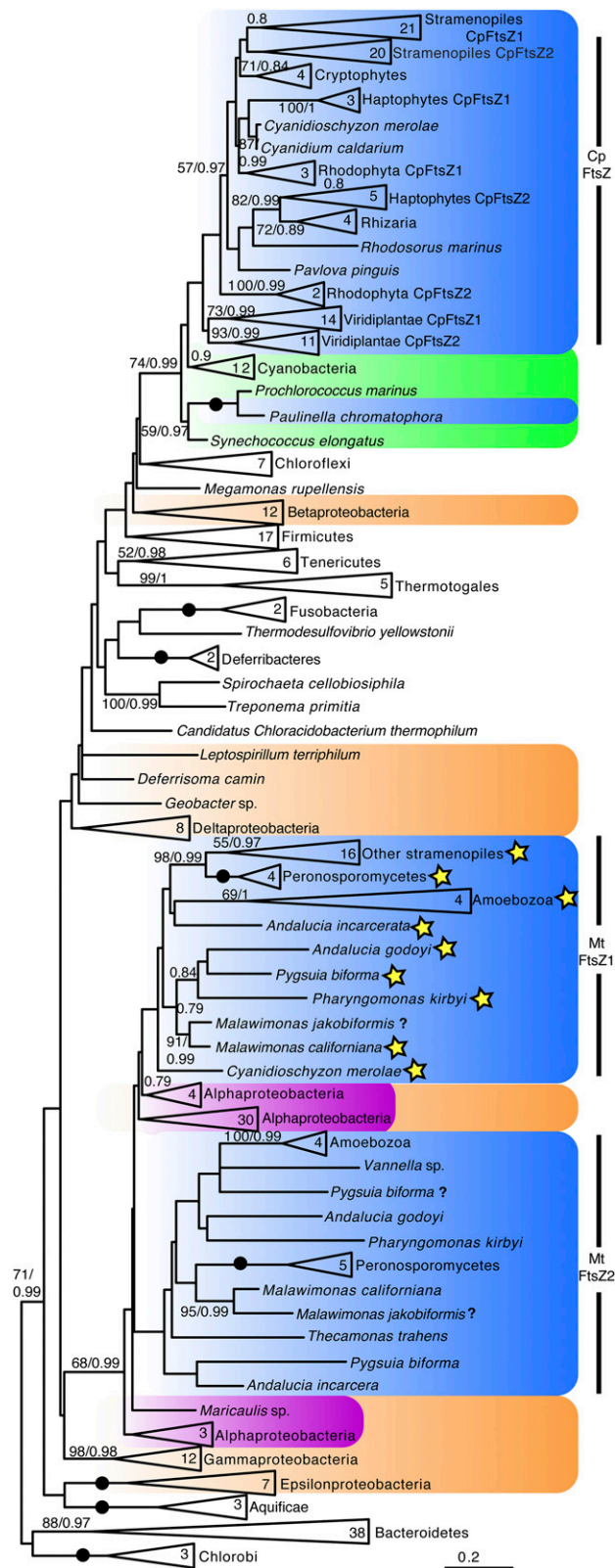


Fig. 5. Unrooted maximum likelihood (ML) tree of FtsZ sequences. Phylogenetic analyses were performed on 327 sequences and 257 sites, using RAxML and PhyloBayes. Bootstrap support values greater than 50% and posterior probabilities greater than 0.5 are shown. Branches with 100% bootstrap support and posterior probability of 1.0 are indicated by black circles. Eukaryotes are shaded blue, cyanobacteria green, proteobacteria orange, and α -proteobacteria magenta. Eukaryotic paralogs lacking the variable C-terminal spacer region are indicated by stars whereas those with

subsampled these sequences, using γ -proteobacterial sequences as an outgroup, and reanalyzed them in an attempt to better resolve this clade. We also excluded amoebozoan sequences because of their unusually high AT content and long branches in preliminary trees (Fig. S3). Unfortunately, we were unable to obtain better resolution of the branching order among α -proteobacterial and eukaryotic clades. Nevertheless, the C-terminally truncated FtsZ proteins were identified only in eukaryotes, and we did not identify more than one FtsZ homolog in α -proteobacteria. We therefore conclude that the duplication event that gave rise to the FtsZ paralogs found in extant eukaryotes likely occurred early in eukaryotic evolution, rather than earlier, in the α -proteobacterial lineage that gave rise to the mitochondrion.

Altogether, these lines of evidence are consistent with the hypothesis that the nuclear-encoded mitochondrial Min and FtsZ homologs of eukaryotes originated by endosymbiotic gene transfer from the ancestral mitochondrial endosymbiont.

Although found in diverse eukaryotes, the Min proteins are sparsely distributed, a pattern that can only partly be reconciled with taxonomic representation in the available data. A striking example of gene loss is seen in the Mycetozoa (*Dictyostelium* spp., *Acytostelium subglobosum*, and *Polysphondylium pallidum*). Here, *D. discoideum*, *Dictyostelium citrinum*, *Dictyostelium intermedium*, and *Dictyostelium firmibasis* have retained only FtsZ whereas their sister taxon *D. purpureum* and the more basal taxa *P. pallidum*, *Polysphondylium violaceum*, and *A. subglobosum* have additionally maintained all three Min proteins. Meanwhile, the yet more distantly related *Dictyostelium fasciculatum* (72, 73) seems to have independently lost the Min proteins and, like *D. discoideum*, only possesses FtsZ. This overall pattern raises the question of why Min proteins were retained in some taxa, yet lost in others. No correlation was found with mitochondrial cristae morphology, because Min proteins were found in organisms possessing discoid (e.g., *P. kirbyi*) (56) or tubular (e.g., *A. godoyi*) (74) cristae, as well as in lineages with MROs that apparently lack cristae entirely (e.g., *A. incarcerationata* and *P. biforma*) (68, 69). Nor is there any obvious difference in either overall mitochondrial morphology or lifestyle between lineages that possess Min proteins and lineages that do not. Kiefel et al. (29) have raised the possibility that FtsZ is lost in lineages with reticulate mitochondria, and thus the placement of the division site may not affect mitochondrial function. This hypothesis remains a plausible explanation that might apply to Min proteins; *A. godoyi* and *P. biforma* each possess a single mitochondrion or mitochondrion-related organelle (74, 75), and *T. trahens* is predicted to have discrete, nonbranching mitochondria based on 3D reconstructions (76) (Fig. 2). Meanwhile, a number of the lineages lacking Min proteins are known to possess reticulate mitochondria in at least one tissue and during at least one life stage, including opisthokonts (77), plants (78, 79), the euglenozoan *Euglena gracilis* (80), and apicomplexa (81, 82) (Fig. 2). One exception seems to be *Phytophthora cinnamomi*, an organism described in the literature as having 3–4 reticulate mitochondria per cell (83), and in which we found Min homologs. Unfortunately, there are relatively few taxa in our survey for which detailed microscopy data are available that would permit conclusions to be drawn about the 3D structure of their mitochondria. Furthermore, many organisms known to possess reticulate mitochondria may also possess unbranched mitochondria in some tissues, during some parts of their life cycle (79), or alongside reticulate mitochondria, as in *P. cinnamomi* (83). It is therefore also possible that the presence or absence of Min

incomplete sequence at the C-terminus are indicated by question marks. The exception to this pattern is a *Corethron hystrix* sequence that, despite branching with other stramenopiles in the MtFtsZ1 clade, possesses a C-terminal variable region (Fig. S3).

proteins reflects some unknown transient mitochondrial morphological feature specific to replication. Clearly, genetic and functional studies of mitochondrial Min systems are greatly needed to understand their precise roles.

Further questions are raised by the apparent absence of homologs of all other components of the bacterial divisome from the surveyed eukaryotes, including ZipA, ZapA, FtsA, FtsB, FtsE, FtsI, FtsK, FtsL, FtsN, FtsQ, FtsW, and FtsX. Searches of databases using α -proteobacterial and *E. coli* homologs of these proteins as queries yielded no candidate homologs. The bacterial divisome components recruited late in the division process (FtsB, FtsE, FtsI, FtsL, FtsN, FtsQ, FtsW, and FtsX) are primarily involved in facilitating peptidoglycan synthesis, and so their apparent absence is perhaps not surprising, given the lack of a peptidoglycan wall in any mitochondria. However, it is not clear how the Z-ring remains stabilized and anchored to the membrane in the absence of FtsA, ZipA, or ZapA. ZED, a coiled-coil domain protein with 25.8% sequence identity to ZapA, is reported to be involved in mitochondrial Z-ring formation in the red alga *C. merolae* (84). However, we were unable to identify any homologs of this protein in other eukaryotes. The two distinct FtsZ paralogs may form an alternating copolymer that forms the Z-ring; or the Z-ring might be composed of a single paralog whereas the second paralog might instead be involved in attachment of the Z-ring to the membrane. In either case, the anchoring mechanism of FtsZ remains a mystery.

Recent work (85) implicates the endoplasmic reticulum (ER) in the control of the mitochondrial division site location and subsequent Dnm1p recruitment in yeast. This type of external division site control contrasts with that of the Min protein system, which regulates division site location from the mitochondrial matrix. The contrast between these control mechanisms raise the questions of when the role of the ER in mitochondrial

division may have emerged; whether any taxa possess both Min proteins and Dnm1p/Drp1; and how these organisms (if they exist) recruit Dnm1p/Drp1 in the absence of ER-mediated division site control. Therefore, an important avenue of further study is the taxonomic distribution of mitochondrial Dnm1p/Drp1 and its functional interplay with FtsZ. Study of this distribution is hampered by the fact that multiple paralogs of dynamins have different functions within eukaryotic cells (41), including vesicular trafficking in yeast (86), and unknown functions in less-studied organisms such as *T. vaginalis* (38). These proteins lack N-terminal targeting peptides, and so, in the absence of localization data, a mitochondrial function cannot clearly be ascribed to any one of them based on sequence data alone. In any case, investigations into the molecular mechanisms governing the coordination of the various kinds of inner and outer contractile rings are critically needed in diverse eukaryote lineages to fully understand what are features of the division system of the last eukaryotic common ancestor and what are more recent lineage-specific innovations.

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