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The *Naegleria* genome: a free-living microbial eukaryote lends unique insights into core eukaryotic cell biology

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Abstract

Naegleria gruberi, a free-living protist, has long been treasured as a model for basal body and flagellar assembly due to its ability to differentiate from crawling amoebae into swimming flagellates. The full genome sequence of *Naegleria gruberi* has recently been used to estimate gene families ancestral to all eukaryotes and to identify novel aspects of *Naegleria* biology, including likely facultative anaerobic metabolism, extensive signaling cascades, and evidence for sexuality. Distinctive features of the *Naegleria* genome and nuclear biology provide unique perspectives for comparative cell biology, including cell division, RNA processing and nucleolar assembly. We highlight here exciting new and novel aspects of *Naegleria* biology identified through genomic analysis.

Keywords

Naegleria; Genome; Evolution; Ploidy; Mitosis; Nucleolus

1. Introduction

Members of the protistan genus *Naegleria* are easily isolated from wet soil and freshwater from around the world (De Jonckheere, 2002; Fulton, 1970, 1993), and have been found closely associated with freshwater organisms, including fish (Dykova et al., 2001). *Naegleria* are best known for their ability to form three types of cells: amoebae that divide by mitosis, transient non-dividing flagellates and resting cysts (Fig. 1) (Fulton, 1970). The amoeba-to-flagellate transition requires the formation of an entire microtubule cytoskeleton, including

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de novo assembly of two basal bodies (equivalent to centrioles) and flagella (equivalent to cilia) (Fulton, 1993). Despite a phylogenetic diversity perhaps as broad as that of vertebrates (Fulton, 1993), the only *Naegleria* species with a fully sequenced genome is *Naegleria gruberi* strain NEG-M (Fritz-Laylin et al., 2010). Although *N. gruberi* is a harmless bacterial predator, another *Naegleria* species, *Naegleria fowleri*, is an opportunistic human pathogen that can cause usually fatal meningoencephalitis (Visvesvara et al., 2007). However, even this species is not an obligate parasite, and usually relies on bacteria for its food source.

Although *Naegleria* has been most commonly used as a model for de novo basal body and flagellar assembly, our recent description of the *Naegleria* genome offered a new perspective on early eukaryotic evolution and opened up unexpected avenues for biological study (Fritz-Laylin et al., 2010). In addition to the vast amount of information gleaned from analyzing the estimated 15,727 *Naegleria* gene sequences, the unique genomic organization and nuclear behavior of *Naegleria* inform both mechanistic and evolutionary studies of eukaryotic biology. The *Naegleria* genome is composed of a 14 kb extrachromosomal plasmid (NCBI accession AB298288.1) and a 50 kb mitochondrial genome (Genbank accession AF288092.1), as well as the linear nuclear chromosomes. Pulsed-field gel electrophoretic analyses indicate that *Naegleria* has at least 12 chromosomes with sizes ranging from 0.7 to 6.5 MB, totaling approximately 42 MB (Fig. 2A). (Clark et al., 1990; Fritz-Laylin et al., 2010). In this review we will summarize the current knowledge of *Naegleria* genome biology, including its structure, content and behavior.

2. The sequenced *N. gruberi* is one of many species

The non-pathogenic *N. gruberi* was the only *Naegleria* species known until the discovery that a *Naegleria* species—named *N. fowleri* (Carter, 1970)— could cause fatal “primary amoebic meningoencephalitis” (PAM). More recently, two types of molecular systematic studies have led to a proliferation of “molecular species.” The first, the comparison of isoenzyme mobility patterns in many different isolates, demonstrated that *Naegleria* isolates can be grouped into a series of discontinuous clusters (Robinson et al., 1992). Sequence comparisons, particularly between ribosomal sequences, have also been utilized to differentiate *Naegleria* species. The rRNA genes are transcribed together in the following order: small subunit (18S) rDNA, an internal transcribed spacer (ITS1), 5.8S rDNA, a second ITS (ITS2), and the large subunit (28S) rDNA. In most species, the ITS1 is about 33–41 nucleotides in length and the ITS2 about 100–115. De Jonckheere and others used rapidly evolving ITS sequences to classify over 40 species of *Naegleria* (De Jonckheere, 2002, 2004; De Jonckheere and Brown, 1997). While there is no universally accepted definition of species boundaries among primarily asexual organisms or by using ITS regions, some clear discontinuities, whether morphological or molecular, are required. In this context, several species designated by De Jonckheere and others appear robust, but other species are defined based on as little as a single base change (De Jonckheere, 2004). Thus, while it is clear that there exist multiple species of *Naegleria*, the precise number and the evolutionary relationships among them warrant further study. In the meantime, the extensively utilized strain NEG was chosen to represent the species *N. gruberi* (De Jonckheere, 2002).

3. The *Naegleria* genome offers a new perspective on early eukaryotic evolution

All eukaryotes shared a common ancestor over a billion years ago (Brinkmann and Philippe, 2007), and the descendants of that ancestral eukaryote diverged into at least six major groups (although the evolutionary relationships between the groups remains contentious) (Roger and Simpson, 2009) (Fig. 3). *Naegleria* belongs to the eukaryotic group known as “JEH” for Jakobids, Euglenoids and Heterolobosea that also includes the distantly related *Euglena* and *Trypanosoma* species (Fig. 3). (Rodriguez-Ezpeleta et al., 2007). As the initial genome sequenced from a non-parasitic member of the JEH lineage, the *Naegleria* genome allowed us to compare, for the first time, complete sequences of non-reduced genomes from six major eukaryotic groups (Fritz-Laylin et al., 2010). Gene families present in the majority of lineages are inferred to have also been present in the common ancestor. Our analyses indicated that the common ancestor of extant eukaryotes had over four thousand genes that are still represented in extant eukaryotes, 40% of which are novel to the eukaryotic lineage, as they have no identifiable homolog in Archaea or Bacteria (Fritz-Laylin et al., 2010). Many of the eukaryotic-specific ancestral gene families have no characterized function to date. These genome comparisons highlighted the early evolution and deep conservation of eukaryotic features that include numerous introns, complex DNA and RNA metabolism, extensive metabolic and signaling capabilities, and amoeboid and flagellar motility (Fritz-Laylin et al., 2010).

4. *Naegleria* genes reveal a complete eukaryotic repertoire, plus a few surprises

Although much has been learned about the *Naegleria* cytoskeleton and amoeba-to-flagellate transition (Fulton, 1977, 1993), until recently, our understanding of most basic *Naegleria* biology was extremely limited. Analysis of the complete *Naegleria* genome sequence therefore revealed many capabilities and features previously undetected. For example, although cytological evidence of Golgi has not been observed, an extensive membrane trafficking gene repertoire indicates that *Naegleria* has the relevant genes (Dacks et al., 2003; Fritz-Laylin et al., 2010).

Similarly, the *Naegleria* genome sequence indicates that this amoeboflagellate retains complete actin and microtubule cytoskeletons, extensive mitotic and meiotic machinery, calcium/calmodulin-mediated regulation and various transcription factors (Fritz-Laylin et al., 2010; Iyer et al., 2008). The genome also encodes an extensive array of intracellular signaling machinery that presumably coordinates the environmental sensing and the morphological changes necessary for differentiating between three morphological states. This repertoire includes entire pathways not found thus far in parasitic protists (G-protein-coupled receptor signaling and histidine kinases), as well as 265 predicted protein kinases, 32 protein phosphatases, and 182 monomeric Ras-like GTPases. *Naegleria* also has 108 adenylate/guanylate cyclases, the highest proportion of cyclase genes encoded in any of 17 genomes examined (except for *T. brucei*, where there is large expansion of a single cyclase gene family of which members are predominantly found within the same genomic loci as the

variable surface glycoprotein (or VSG) surface coat genes that underpin antigenic variation in this organism (El-Sayed et al., 2005b). While some *Naegleria* cyclases are similar to those in trypanosomes (with a single transmembrane pass), others are more similar to human sequences (with multiple regions containing multiple transmembrane helices). Although the abundance of cyclases in *Naegleria* remains puzzling, many of these cyclases also contain other protein domains that may lead to an understanding of their function.

Similarly, the metabolism encoded by the *Naegleria* genome is also more diverse than anticipated (Ginger et al., 2010). Our analysis of the *Naegleria* genome indicates that *Naegleria* can oxidize glucose, various amino acids and fatty acids using a Krebs cycle and branched mitochondrial respiratory chain. However, the *Naegleria* genome also held a metabolic surprise: an Fe–Fe hydrogenase, and the enzymes required for its maturation. The presence of predicted mitochondrial targeting signals in all four of these genes suggests that *Naegleria* mitochondria are capable of using protons as an electron acceptor in anaerobic conditions to produce hydrogen. This observation is particularly interesting when compared to the mitochondria of other anaerobic microbial eukaryotes. Many lineages of anaerobic eukaryotes have organelles that are derived from mitochondria (Embley, 2006). In some cases, these organelles have lost proteins used for oxidative phosphorylation by typical mitochondria, but have the additional capacity for anaerobic hydrogen production. There are documented examples of organisms with such mitochondria in several major eukaryotic groups, including anaerobic heteroloboseans (Barbera et al., 2010; de Graaf et al., 2009). Although we do not know its origin, the presence of a mitochondrial Fe–Fe hydrogenase, associated maturation factors, and a complete aerobic respiration system suggests that *Naegleria* mitochondria resemble the evolutionary intermediate postulated to have occurred within the ancestor of all extant eukaryotes (Mentel and Martin, 2008) or in the lineages of the many extant anaerobic eukaryotes that have lost aerobic respiration (Hackstein et al., 2006).

5. The mitochondrial genome of *Naegleria*

Comparing the *N. gruberi* mitochondrial genome (Genbank accession AF288092.1) to the mitochondrial genome sequences of other JEH protists reveals interesting variations in genome organization and content (Table 1). For example, the 69 kb circular mitochondrial genome of *Naegleria*'s 'near' jakobid relation *Reclinomonas americana* (Fig. 3), although it encodes only 67 proteins, is the most eubacterial-like and gene-rich characterized to date (Gray et al., 2004). In contrast, species from the Euglenozoa (Fig. 3) each possess a single mitochondrion per cell whose genome is assembled from many, often small (<10 kb) circular DNA molecules and contains many fewer genes (Lukes et al., 2002; Roy et al., 2007). Mitochondrial gene expression in euglenozoans is also unusual in that U-insertion and U-deletion RNA editing or trans-splicing is necessary to decode or stitch together protein-coding transcripts (Lukes et al., 2009; Vitek et al., 2010).

N. gruberi, like *R. americana*, carries its entire mitochondrial genome (49,843 bases) on a single circular chromosome. The *Naegleria* mitochondrial genome is the most gene-rich outside of the jakobids and contains several features that have been lost from the mitochondrial genomes of other JEH lineages (Table 1). Such elements include genes

encoding more subunits of mitochondrial complex I than is seen in many protists, a subunit from mitochondrial complex II, and a *COX11* homolog, which is a complex IV assembly factor encoded only in the mitochondrial DNA of jakobids and *Naegleria*. The *Naegleria* mitochondrial genome also encodes some components of a mitochondrial cytochrome *c* and *c₁* maturation system. This was presumably the ancestral pathway used for maturation of mitochondrial *c*-type cytochromes, and is also retained in ciliate and land plant mitochondria (Allen et al., 2008). Like *Reclinomonas*, but in contrast to trypanosomatids, *Naegleria* encodes a complete set of tRNA within its mitochondrial genome, suggesting that tRNA import is not required to sustain mitochondrial gene expression (Lithgow and Schneider, 2010). However, despite the retention of mitochondrial-encoded genes and tRNAs, many *Naegleria* mitochondrial gene sequences appear to be evolving much faster than those of *Reclinomonas* (Gray et al., 2004). Finally, although analysis of the mitochondrial genome sequence gives no indication that insertion-deletion editing or *trans*-splicing of mitochondrial transcripts is necessary for mitochondrial gene expression, *Naegleria* was recently reported as the first organism outside of the plant kingdom to contain homologs of the DYW-type pentatricopeptide repeat (PPR) proteins involved in plant organellar RNA editing (Knoop and Rudinger, 2010). Three sites for C-U RNA editing of the type seen in some mitochondria (e.g. some plants and dinoflagellates) were also predicted. Similar to trypanosomatids (Pusnik et al., 2007), *Naegleria* also contains an elevated number of non-DYW-type PPR proteins that we predict, based on similarity to other eukaryotes (e.g. Pusnik et al., 2007), to be involved in organellar gene expression. Therefore, the mitochondrial genome sequence and the predicted mitochondrial biochemistry of *N. gruberi* combines elements of ‘textbook’ metabolism with traits that are either unusual or novel, and overall paints a complex picture of mitochondrial evolution in the JEH lineage.

6. The *Naegleria* genome includes novel repetitive elements

The *Naegleria* nuclear genome is quite gene-rich; the 15,727 predicted genes occupy approximately 60% of the chromosome sequence (Fritz-Laylin et al., 2010). Intergenic regions are short, typically less than 500 nucleotides. Given the compact nature of the *Naegleria* genome, and its evolutionary distance from other fully sequenced organisms, it is not surprising that algorithms such as RepeatMasker (Smit et al., 1996–2004), that use similarity to libraries of known repetitive elements to identify repeats, are able to identify only a tiny percentage (1.7%) of the *Naegleria* genome as representing a repetitive sequence. Even using de novo repeat identification algorithms, which could identify *Naegleria*-specific repetitive elements, only identified 3.4% additional genome sequence as repetitive. These *Naegleria*-specific repetitive elements include a few with similarity to previously described repetitive elements in other genomes (copia- and gypsy-like putative retrotransposons), elements with predicted domains associated with transposable elements, as well as novel sequences. Although the function of these repetitive elements remains unknown, it is possible that some may represent centromeres and/or novel transposable elements. As genomes of other free-living protists become available, it will be interesting to see if they contain repetitive elements like those identified in the *Naegleria* genome. It is quite possible that the commonly studied repetitive elements of animal and flowering plant genomes are

only the tip of the iceberg and there are many other conserved repetitive elements waiting to be discovered.

7. The *Naegleria* genome highlights early intron evolution

Intron content and size vary widely between eukaryotic genomes. For example, *Giardia lamblia* has only four small introns in total (Morrison et al., 2007), while some animals and plants average more than seven per gene (reviewed in (Roy and Gilbert, 2006)). Nearly 36% of *Naegleria* genes are predicted to contain at least one intron (median size 61 nucleotides) and 17% contain multiple introns. In fact, 22 genes have at least five introns with all splice junctions confirmed via ESTs (Fritz-Laylin et al., 2010). This finding was surprising given the paucity of *Naegleria* introns previously observed (Remillard et al., 1995) and the near absence of introns from the genomes of the related trypanosome genomes (Berriman et al., 2005; El-Sayed et al., 2005a). Furthermore, the positions of many of the *Naegleria* introns are also conserved, indicating that they are ancient. Although *Naegleria* has far fewer introns than found in a typical animal or flowering plant genome, it adds to the growing evidence that the ancestor of extant eukaryotes likely contained a fair number of introns, and genomes with few or no introns are derived (Archibald et al., 2002; Slamovits and Keeling, 2006).

A minority of *Naegleria* isolates, including strain NB-1, contain a parasitic intron called Nae.S516 within the 18S rDNA (Fig. 2B). This extensively-studied intron contains two self-splicing ribozymes and represents one of the most complex group-I introns ever described (Einvik et al., 1997; Einvik et al., 1998; Wikmark et al., 2006). The only known comparable twin-ribozyme group I intron is found in a single isolate of the very distantly related myxomycete *Didymium iridis* (Johansen et al., 2002).

For those *Naegleria* isolates that carry Nae.S516, proper self-removal is essential to generate functional SSU rRNA. The 1.3–1.4 kb intron has three elements arranged like Russian dolls (Fig. 2B). It is delimited on both ends by GIR2, a large group-I self-splicing intron. After being transcribed as part of the SSU rRNA, GIR2 is then spliced out by its self-contained catalytic ribozyme, releasing an intron circle. Within the GIR2 intron there is a smaller group-I-like intron, GIR1, which in turn is closely associated with an open reading frame encoding a 245-amino-acid homing endonuclease. In order to form a functional homing endonuclease mRNA, GIR1 cleaves the RNA just upstream of the coding region (Decatur et al., 2000). In *Didymium*, this cleavage event results in a short lariat cap on the resulting homing endonuclease mRNA (Nielsen et al., 2005), and a similar product is likely formed by *Naegleria* GIR1 (Elde et al., 1999).

During mating in the *Didymium iridis* strain that contains the twin ribozyme intron, the inner intron cleaves the circle internally to create an mRNA that expresses the homing endonuclease. This enzyme in turn cleaves a recognition site in an intronless copy of the SSU rDNA sequence in the mating pair. When this DNA break is repaired by gene conversion, the twin ribozyme is inserted into the invaded DNA (Johansen et al., 1997; Muscarella and Vogt, 1989). Although *Naegleria* homing endonuclease has been shown to retain function outside of *Naegleria* (Decatur et al., 2000), so far no activity of the homing endonuclease has been seen in *Naegleria*, and strains that have lost it are phenotypically

unaffected. It is puzzling that these introns retain functional homing endonuclease, since long-term retention of function implies some selective advantage. Perhaps, like *Didymium*, *Naegleria* expresses the homing endonuclease during a sexual event that has not yet been observed (see below). As Wikmark and others have noted, “Why the apparent asexual *Naegleria* amoebae harbors active intron homing endonucleases, dependent on sexual reproduction for its function, remains a puzzle” (Wikmark et al., 2006).

The GIR1 ribozyme is one of the smallest “stripped down” ribozymes described. *Naegleria* GIR1 can be reduced to 178 nucleotides (Jabri et al., 1997), and in vitro selection was used to increase its activity up to 300-fold, with as few as three base changes increasing activity 50-fold (Jabri and Cech, 1998). Because of this, and the fact that the homing endonuclease generates an unusual cleavage within its 19 bp DNA recognition site (Elde et al., 1999; Elde et al., 2000), both have potential for use in genetic engineering (Einvik et al., 1998).

The entire twin-ribozyme intron found in isolates of certain *Naegleria* species is always at the same position in the SSU rDNA, and is overall of similar sequence. Its patchy distribution among *Naegleria* species indicates that Nae.S516 entered or evolved within a common ancestor very early in the evolution of the genus (De Jonckheere, 1994; Wikmark et al., 2006). Since then, the intron has been periodically lost, and now most *Naegleria* isolates, including strain NEG, lack the intron. One isolate has been found that has retained the GIR2 intron but has lost the segment containing GIR1 and homing endonuclease (De Jonckheere and Brown, 1994).

Rare group I introns have also been found in the large subunit (28S) rDNA of a few *Naegleria* isolates (De Jonckheere and Brown, 1998; Haugen et al., 2002). Unlike the vertical inheritance of Nae.S516, these large subunit introns apparently arose by horizontal gene transfer, and so far have been less studied.

Their level of complexity and apparently ancient acquisition have made the *Naegleria* group I introns a popular model with which to study the evolution of selfish genetic elements, as well as ribozyme molecular mechanisms. Although sequences with high similarity to the ribosomal plasmid were not found on the chromosomal genome scaffolds, it is possible that additional complex group I introns await discovery in other parts of the *Naegleria* genome.

8. *Naegleria* rRNA genes are carried on an extrachromosomal nucleolar plasmid

The multiple copies of ribosomal RNA genes (rRNA, encoded by rDNA) found in eukaryotes are organized and maintained in a variety of different ways. For example, the *Dictyostelium* nucleus contains extrachromosomal arrays of hundreds of palindromic copies of rRNA genes (Cockburn et al., 1978), thought to be copied from a chromosomal “master copy” (Eichinger et al., 2005). However, a distant relative of *Dictyostelium*, *Entamoeba histolytica*, harbors its rRNA genes on self-replicating extrachromosomal circles (Bhattacharya et al., 1998). Similarly, the small and large rRNA genes of *Naegleria* are found exclusively on a 14 kb circular plasmid present in about 4000 copies in each cell of strain NEG-M (Clark and Cross, 1987). The plasmid has been completely sequenced in

strain NEG-M (Maruyama and Nozaki, 2007), and carries a single copy of each of the 18S, 5.8S, and 28S rRNA genes (Fig. 2A). Because no similar sequence has been found on linear chromosomes from which it could be “copied”, it is likely that the plasmid can also self-replicate (Clark and Cross, 1987). In strain NEG-M, the plasmid also contains two predicted open reading frames outside of the rRNA regions (Fig. 2A); one of 173 aa with no similarity to any other known protein sequence, and a second, transcriptionally silent ORF of 376 aa with similarity to the intron-contained homing endonucleases of other *Naegleria* species described above.

The rRNA plasmid localizes to the single large nucleolus (Fig. 2A). The *Naegleria* nucleolus, also called the karyosome or endosome in earlier studies, is a large, dense, roughly spherical mass 4–5 μm in diameter that occupies about 20% of the nuclear volume. The nature of the nucleolus has been a subject of interest both because of its size and its behavior during mitosis. The nucleolus does not disperse during mitosis, but develops a central furrow occupied by the chromosomes during metaphase. As mitosis proceeds, the nucleolar masses move toward the poles with the chromosomes (reviewed in (Fulton, 1970)).

Nucleoli are readily isolated in large quantities (Trimbur and Walsh, 1993), and can be studied using three monoclonal antibodies against different components of nucleoli (Trimbur and Walsh, 1993, antibodies available from the Developmental Studies Hybridoma Bank at the University of Iowa). Isolated nucleoli can be dispersed in high salt and later reassociated into NLPs (nucleolus-like particles) by dialysis. About 40% of the nucleolar DNA, RNA and protein are recovered in the NLPs. The process of solubilization and reassociation can be repeated multiple times with a consistent recovery of the components. The core of NLPs resembles the dense fibrillar component of nucleoli, while the cortex resembles the granular component of nucleoli (Trimbur and Walsh, 1993). This procedure has opened up an in vitro system with which the synthesis and processing of rRNA precursors, the reassociation of nucleolar proteins and the replication of the nucleolar plasmids may be studied. This system should also aid in understanding the role of the nucleolus in anchoring and/or assembling *Naegleria*'s unusual mitotic spindle.

9. *Naegleria* uses an unusual mitotic spindle likely made of divergent tubulin

In addition to having a unique set of genes and introns, the *Naegleria* genome divides using an atypical mitotic spindle. *Naegleria* amoeba undergo mitosis without nuclear envelope breakdown; the mitotic spindle forms within the intact nucleus (Fulton, 1970; Schuster, 1975). (This is often referred to as a “closed mitosis”, but see (De Souza and Osmani, 2007)). Furthermore, there are no centriole-like structures in *Naegleria* amebae (Fulton and Dingle, 1971), and the spindle microtubules run to the nuclear envelope without focusing on any potential associated microtubule organizing center (MTOC) (Fulton, 1970). The spindle formed is broad, and mitosis proceeds through the typical stages: metaphase, anaphase and telophase (Fig. 4A).

Although mitotic amoebae use tubulin for spindle microtubules, during its rapid differentiation to flagellates *Naegleria* synthesizes all flagellar tubulin, and the corresponding mRNAs, de novo (Kowit et al., 1974; Lai et al., 1988, 1994; Lai et al., 1979). This observation, coupled with evidence for *Naegleria* using different tubulins for mitosis and flagella, led to the multitubulin hypothesis that predicted eukaryotes contain multiple tubulin genes with distinct properties (Fulton and Simpson, 1976). Indeed, the *Naegleria* genome contains a superfamily of at least thirty tubulin genes spanning the subfamilies alpha through eta, with the alpha and beta tubulin genes each further separated into distinct clades (Fritz-Laylin et al., 2010). The largest alpha- and beta-tubulin clades include typical eukaryotic alpha and beta tubulins, as well as the previously characterized *Naegleria* flagellar tubulins (Lai et al., 1988, 1994). The alpha and beta tubulin clades also include tubulins distinct from those in other eukaryotes. In particular, one clade contains a divergent alpha-tubulin provocatively suggested to be mitosis-specific (Chung et al., 2002). The nature and role of the multiple tubulins are ripe for further study in *Naegleria*. Furthermore, although *Naegleria* encodes almost all well-conserved microtubule components, it remains to be seen which are used in the mitotic spindle that is potentially composed of divergent tubulin filaments.

Mitosis in *Naegleria* is also unusual due to the close association of the chromosomes with the large nucleolus (Fulton, 1970). During interphase, linear chromosomes are presumably found in the space between the nucleolus and the nuclear membrane (Fig. 2A), as indicated by DNA staining. At metaphase the condensed chromosomes form a dense band in the center of the nucleolus. As the chromosomes separate, the nucleolus becomes elongated to form a dumbbell shape. Further chromosome separation leads to extreme nucleolar elongation with the two bulbous ends connected by a thin band. The condensed chromosomes are embedded in the two separate nucleolar masses as the cell enters telophase. The connecting region, sometimes called the interzonal body, has been described as derived from the nucleolus (reviewed in (Fulton, 1970)).

Recent work using confocal microscopy combined with monoclonal antibodies against nucleolar proteins and microtubules has made it possible, for the first time, to define the 3D structure of the mitotic spindle and its relationship with the nucleolus (C. Walsh, in preparation). These studies demonstrate that during prophase, the earliest spindle microtubules develop in close association with the nucleolar surface. There is no evidence of an MTOC-like specific point of origin. Rather, the microtubules form a rough cage-like structure on the nucleolar surface. With the formation of more microtubules, the spindle, while still mostly within the nucleolar mass, begins to align into a broad barrel shape centered on the metaphase chromosomes. It is clear that as the spindle elongates, the spindle microtubules terminate within the separating nucleolar masses (Fig. 4B) (C. Walsh, in preparation). The association between the spindle microtubules and the nucleolar proteins is consistent with the previous observation that a nucleolar protein, BN46.51, is found associated with forming basal bodies as amoebae differentiate into flagellates (Trimbur and Walsh, 1992). This region seems to act as an MTOC for the cytoplasmic microtubules that form in flagellates (Walsh, 2007). It should be possible to use isolated nucleoli, or the in vitro assembly system described above, to seek an MTOC activity associated with the nucleolar surface.

10. *N. gruberi* strain NEG-M is heterozygous and tetraploid

Analysis of the full genome sequence has also helped determine the ploidy of *Naegleria*. Initial deductions of ploidy were first made by studying two clonal strains: NEG (and derivative NEG-M) from California, and NB-1 from England (Fulton, 1970). While no mutations could be isolated from NB-1, mutants are easily isolated from strain NEG, which has half the cell volume and half the DNA per cell. Therefore, NEG was defined as 1X, and NB-1 as 2X. Although the 2X state of NB-1 is stable, 1X NEG frequently become 2X. This change is almost certainly an asexual process of genome duplication, such as an error in chromosome separation during mitosis (endoreduplication), and can be selected for by more rapid migration of the amoebae on agar plates (Fulton, 1970, 1977). During the “evolution” that occurred as strain NEG adapted to growth in simple, soluble axenic media, the resulting strain, NEG-M, became a stable 2X (Fulton, 1974).

Early studies on the electrophoretic mobility of isoenzyme bands suggested that most, if not all, isolates of *Naegleria* are heterozygous for many enzymes, indicating that they are likely to be at least diploid (Adams et al., 1989; Cariou and Pernin, 1987). Even strain NEG appears heterozygous by this criterion (Robinson et al., 1992). However, the genes responsible for this result were not known, and the ploidy of *Naegleria* remained debatable until the sequencing of strain NEG-M. Much of the genome of NEG-M is heterozygous, with no more than 2 SNPs at any site (Fritz-Laylin et al., 2010). Since this 2X strain arose by simple doubling of NEG, the 1X strain also would have to be heterozygous. Thus it was confirmed that NEG is diploid, and NEG-M (and presumably NB-1) is tetraploid. Several other strains have been examined that are either 1X or 2X, but none that appear haploid (either 1/2X in DNA content or without multiple isoenzyme bands).

11. The *Naegleria* genome sequence provides hints of sexuality

When *Naegleria* was first used as a research organism, it seemed reasonable, by analogy to other eukaryotes, that the flagellates were gametes, and genetic analysis would be available once the conditions for mating were discovered (Fulton, 1970, 1977, 1993). Decades later, no one has ever induced mating, and the sexuality of *Naegleria* has become more puzzling.

Numerous observations suggest that *Naegleria* is at least primarily an asexual organism that reproduces by division of its amoebae to produce substantial clonal populations. Extensive laboratory studies, including many attempts to achieve mating (especially in strain NEG and related isolates) (Fulton, 1970 and C. Fulton, unpublished experiments), have revealed nothing but asexual reproduction. *Naegleria*, like many microbes, appears to reproduce predominantly asexually and forms “natural clones” that in some cases are distributed globally (Tibayrenc et al., 1990). Although this argues that *Naegleria* is asexual, the clonal theory of protozoa applies to several protists known to reproduce mainly asexually but that also have sexual cycles, such as the distantly related trypanosomes (Hide, 2008). In other words, these cosmopolitan natural clones establish asexual division as a major but not necessarily the sole means of reproduction.

However, several other lines of evidence strongly suggest sexuality in *Naegleria*. Perrin and his colleagues, through extensive isoenzyme studies, presented compelling evidence that one species, *N. lovaniensis*, does mate in nature, though there are no hints how this might occur (Cariou and Pernin, 1987; Pernin et al., 1992). A related amoeboflagellate, *Tetramitus rostratus*, which can reproduce as either an amoeba or a flagellate, appears to mate as flagellates (Fulton, 1970), and there are species of *Naegleria*, especially *N.* (formerly *Willertia*) *minor*, which have been observed to divide as flagellates (Dobson et al., 1993). The retention of functional homing endonucleases over long evolutionary periods in some isolates of *Naegleria* (above) is suggestive, since their only known function would be to invasively spread the twin-ribozyme intron during mating. Most striking, analysis of the genome of strain NEG-M revealed that it is a composite of two distinct haplotypes whose geometric distribution is consistent with the heterozygosity having arisen from an interbreeding population (Fritz-Laylin et al., 2010). In addition, the genome has homologs of apparently functional meiosis-specific genes (Fritz-Laylin et al., 2010). The maintenance of these genes in the genome suggests utility (Malik et al., 2008). All of these hints suggest that *Naegleria* is likely to be able to undergo genetic exchange, but leave mysterious the conditions or stage in the life cycle at which infrequent mating occurs. While the cosmopolitan success of natural clones similar to strain NEG may have occurred mainly through asexual reproduction, this strain is the heterozygous result of a past mating of two strains and it appears genetically equipped to mate again. If *Naegleria*, including *N. gruberi* NEG (De Jonckheere, 2004; Robinson et al., 1992), have the capacity for sexuality, then we currently are missing some essential knowledge or insight to be able to utilize this capacity.

12. Concluding remarks

Full sequencing of the genome has identified core biological features of *Naegleria*, including an extensive repertoire of cytoskeletal, signaling, and membrane trafficking elements, as well as the capacity to alternate between aerobic and anaerobic metabolism. In addition to *Naegleria*'s extensive gene content, the unique biology of its genome and nucleus may shed light on the origin, evolution and fundamental mechanisms of a variety of eukaryotic processes. Similar to using synchronous de novo basal body assembly to study conserved mechanisms of centriole and basal body assembly, the ease of isolation of the *Naegleria* nucleolus and rRNA plasmid hints at its potential as an in vitro system with which to study nucleolar behavior. More detailed study of the many distinctive features of *Naegleria* mitosis will likely provide insight into general mitotic features when compared to more canonical mitoses.

The *Naegleria* genome sequence has reignited efforts aimed at developing molecular tools to analyze the unique biology *Naegleria* offers. The additional evidence of meiotic activity revealed in the full genome sequence has revived the search for sexuality. Genetic transformation can presumably be developed for *Naegleria*, as it has for so many eukaryotes. There is one report of genetic transformation (Song et al., 2006), but it remains uncertain whether this approach will prove generally useful. In addition, the genome encodes the necessary components for RNA-mediated gene knockdown and we are conducting experiments to adapt this technology (as well as genetic transformation) for use in *Naegleria*. The unique features offered by *Naegleria* have already proven it an outstanding system for

exploratory biology, offering new insights into centriole assembly as well as laying the foundation for the multitubulin hypothesis. The knowledge base provided by the genome, along with the further development of molecular tools, will ensure that *Naegleria*'s unique biological capabilities will continue to yield new insights.

References

- Adams M, Andrews RH, Robinson B, Christy P, Baverstock PR, Dobson PJ, Blackler SJ. A genetic approach to species criteria in the amoeba genus *Naegleria* using allozyme electrophoresis. *Int J Parasitol.* 1989; 19:823–834. [PubMed: 2635158]
- Allen JW, Jackson AP, Rigden DJ, Willis AC, Ferguson SJ, Ginger ML. Order within a mosaic distribution of mitochondrial c-type cytochrome biogenesis systems? *FEBS J.* 2008; 275:2385–2402. [PubMed: 18393999]
- Archibald JM, O'Kelly CJ, Doolittle WF. The chaperonin genes of jakobid and jakobid-like flagellates: implications for eukaryotic evolution. *Mol Biol Evol.* 2002; 19:422–431. [PubMed: 11919283]
- Barbera MJ, Ruiz-Trillo I, Tufts JY, Bery A, Silberman JD, Roger AJ. *Sawyeria marylandensis* (Heterolobosea) has a hydrogenosome with novel metabolic properties. *Eukaryot Cell.* 2010; doi: 10.1128/EC.00122-10
- Barbrook AC, Howe CJ, Kurniawan DP, Tarr SJ. Organization and expression of organellar genomes. *Philos Trans R Soc Lond B Biol Sci.* 2010; 365:785–797. [PubMed: 20124345]
- Berriman M, Ghedin E, Hertz-Fowler C, Blandin G, Renauld H, Bartholomeu DC, Lennard NJ, et al. The genome of the African trypanosome *Trypanosoma brucei*. *Science.* 2005; 309:416–422. [PubMed: 16020726]
- Bhattacharya S, Som I, Bhattacharya A. The ribosomal DNA plasmids of *Entamoeba*. *Parasitol Today.* 1998; 14:181–185. [PubMed: 17040747]
- Brinkmann H, Philippe H. The diversity of eukaryotes and the root of the eukaryotic tree. *Adv Exp Med Biol.* 2007; 607:20–37. [PubMed: 17977456]
- Burki F, Shalchian-Tabrizi K, Pawlowski J. Phylogenomics reveals a new 'megagroup' including most photosynthetic eukaryotes. *Biol Lett.* 2008; 4:366. [PubMed: 18522922]
- Cariou ML, Pernin P. First evidence for diploidy and genetic recombination in free-living amoebae of the genus *Naegleria* on the basis of electrophoretic variation. *Genetics.* 1987; 115:265–270. [PubMed: 17246363]
- Carter RF. Description of a *Naegleria* sp. isolated from two cases of primary amoebic meningo-encephalitis, and of the experimental pathological changes induced by it. *J Pathol.* 1970; 100:217–244. [PubMed: 4989229]
- Chung S, Cho J, Cheon H, Paik S, Lee J. Cloning and characterization of a divergent alpha-tubulin that is expressed specifically in dividing amebae of *Naegleria gruberi*. *Gene.* 2002; 293:77–86. [PubMed: 12137945]
- Clark CG, Cross GA. rRNA genes of *Naegleria gruberi* are carried exclusively on a 14- kilobase-pair plasmid. *Mol Cell Biol.* 1987; 7:3027–3031. [PubMed: 2823115]
- Clark CG, Lai EY, Fulton C, Cross GAM. Electrophoretic karyotype and linkage groups of the amoeboflagellate *Naegleria gruberi*. *J Protozool.* 1990; 37:400–408. [PubMed: 2213653]
- Cockburn AF, Taylor WC, Firtel RA. *Dictyostelium* rDNA consists of non-chromosomal palindromic dimers containing 5S and 36S coding regions. *Chromosoma.* 1978; 70:19–29. [PubMed: 738167]
- Dacks JB, Davis LAM, Sjogren AM, Andersson JO, Roger AJ, Doolittle WF. Evidence for Golgi bodies in proposed 'Golgi-lacking' lineages. *Proc Biol Sci.* 2003; 270(Suppl 2):S168–S171. [PubMed: 14667372]
- de Graaf RM, Duarte I, van Alen TA, Kuiper JW, Schotanus K, Rosenberg J, Huynen MA, Hackstein JH. The hydrogenosomes of *Psalteriomonas lanterna*. *BMC Evol Biol.* 2009; 9:287. [PubMed: 20003182]
- De Jonckheere JF. Evidence for the ancestral origin of group I introns in the SSUrDNA of *Naegleria* spp. *J Eukaryot Microbiol.* 1994; 41:457–463. [PubMed: 7804245]

- De Jonckheere JF. A century of research on the amoeboflagellate genus *Naegleria*. *Acta Protozoologica*. 2002; 41:309–342.
- De Jonckheere JF. Molecular definition and the ubiquity of species in the genus *Naegleria*. *Protist*. 2004; 155:89–103. [PubMed: 15144061]
- De Jonckheere JF, Brown S. Loss of the ORF in the SSUrDNA group I intron of one *Naegleria* lineage. *Nucleic Acids Res*. 1994; 22:3925–3927. [PubMed: 7937113]
- De Jonckheere JF, Brown S. Defining new *Naegleria* spp. using ribosomal DNA sequences. *Acta Protozoologica*. 1997; 36:273–278.
- De Jonckheere JF, Brown S. Three different group I introns in the nuclear large subunit ribosomal DNA of the amoeboflagellate *Naegleria*. *Nucleic Acids Res*. 1998; 26:456–461. [PubMed: 9421500]
- De Souza CP, Osmani SA. Mitosis, not just open or closed. *Eukaryot Cell*. 2007; 6:1521–1527. [PubMed: 17660363]
- Decatur WA, Johansen S, Vogt VM. Expression of the *Naegleria* intron endonuclease is dependent on a functional group I self-cleaving ribozyme. *RNA*. 2000; 6:616–627. [PubMed: 10786852]
- Dobson PJ, Robinson BS, Christy P, Hayes SJ. Low genetic diversity in *Willartia magna* from wide geographical sources, and characterization of *Willartia minor* n.sp. (Heterolobosea, Vahlkampfiidae). *J Eukaryot Microbiol*. 1993; 40:298–304.
- Dykova I, Kyselova I, Peckova H, Obornik M, Lukes J. Identity of *Naegleria* strains isolated from organs of freshwater fishes. *Dis Aquat Organ*. 2001; 46:115–121. [PubMed: 11678226]
- Eichinger L, Pachebat JA, Glockner G, Rajandream MA, Sugang R, Berriman M, Song J, Olsen. The genome of the social amoeba *Dictyostelium discoideum*. *Nature*. 2005; 435:43–57. [PubMed: 15875012]
- Einvik C, Decatur WA, Embley TM, Vogt VM, Johansen S. *Naegleria* nucleolar introns contain two group I ribozymes with different functions in RNA splicing and processing. *RNA*. 1997; 3:710–720. [PubMed: 9214655]
- Einvik C, Elde M, Johansen S. Group I twintrons: genetic elements in myxomycete and schizopyrenid amoeboflagellate ribosomal DNAs. *J Biotechnol*. 1998; 64:63–74. [PubMed: 9823659]
- El-Sayed NM, Myler PJ, Bartholomeu DC, Nilsson D, Aggarwal G, Tran AN, Ghedin E, Worthey EA, et al. The genome sequence of *Trypanosoma cruzi*, etiologic agent of Chagas disease. *Science*. 2005a; 309:409–415. [PubMed: 16020725]
- El-Sayed NM, Myler PJ, Blandin G, Berriman M, Crabtree J, Aggarwal G, Caler E, Renauld H, et al. Comparative genomics of trypanosomatid parasitic protozoa. *Science*. 2005b; 309:404–409. [PubMed: 16020724]
- Elde M, Haugen P, Willassen NP, Johansen S. I-*Nja* I, a nuclear intron-encoded homing endonuclease from *Naegleria*, generates a pentanucleotide 3' cleavage-overhang within a 19 base-pair partially symmetric DNA recognition site. *Eur J Biochem*. 1999; 259:281–288. [PubMed: 9914504]
- Elde M, Willassen NP, Johansen S. Functional characterization of isoschizomeric His-Cys box homing endonucleases from *Naegleria*. *Eur J Biochem*. 2000; 267:7257–7265. [PubMed: 11106439]
- Embley TM. Multiple secondary origins of the anaerobic lifestyle in eukaryotes. *Philos Trans R Soc Lond B Biol Sci*. 2006; 361:1055–1067. [PubMed: 16754614]
- Fritz-Laylin LK, Prochnik SE, Ginger ML, Dacks JB, Carpenter ML, Field MC, Kuo A, Paredez A, et al. The genome of *Naegleria gruberi* illuminates early eukaryotic versatility. *Cell*. 2010; 140:631–642. [PubMed: 20211133]
- Fulton C. Amebo-flagellates as research partners: the laboratory biology of *Naegleria* and *Tetramitus*. *Methods Cell Physiol*. 1970; 4:341–476.
- Fulton C. Axenic cultivation of *Naegleria gruberi*. Requirement for methionine. *Exp Cell Res*. 1974; 88:365–370. [PubMed: 4609782]
- Fulton C. Cell differentiation in *Naegleria gruberi*. *Ann Rev Microbiol*. 1977; 31:597–629. [PubMed: 334046]
- Fulton C. *Naegleria*: a research partner for cell and developmental biology. *J Eukaryot Microbiol*. 1993; 40:520–532.

- Fulton C, Dingle AD. Basal bodies, but not centrioles, in *Naegleria*. *J Cell Biol.* 1971; 51:826–836. [PubMed: 4942778]
- Fulton, C.; Simpson, PA. Selective synthesis and utilization of flagellar tubulin. The multitubulin hypothesis. In: Goldman, R.; Pollard, T.; Rosenbaum, J., editors. *Cell Motility*. Cold Spring Harbor Laboratory; Cold Spring Harbor, NY: 1976. p. 987-1005.
- Ginger ML, Fritz-Laylin LK, Fulton C, Cande WZ, Dawson SC. Intermediary metabolism in protists: a sequence-based view of facultative anaerobic metabolism in evolutionarily diverse eukaryotes. *Protist.* 2010; 161:642–671. [PubMed: 21036663]
- Gray MW, Lang BF, Burger G. Mitochondria of protists. *Annu Rev Genet.* 2004; 38:477–524. [PubMed: 15568984]
- Gray MW, Lang BF, Cedergren R, Golding GB, Lemieux C, Sankoff D, Turmel M, Brossard, et al. Genome structure and gene content in protist mitochondrial DNAs. *Nucleic Acids Res.* 1998; 26:865–878. [PubMed: 9461442]
- Hackstein JH, Tjaden J, Huynen M. Mitochondria, hydrogenosomes and mitosomes: products of evolutionary tinkering! *Curr Genet.* 2006; 50:225–245. [PubMed: 16897087]
- Haugen P, De Jonckheere JF, Johansen S. Characterization of the self-splicing products of two complex *Naegleria* LSU rDNA group I introns containing homing endonuclease genes. *Eur J Biochem.* 2002; 269:1641–1649. [PubMed: 11895434]
- Hide G. Visualizing trypanosome sex. *Trends Parasitol.* 2008; 24:425–428. [PubMed: 18692442]
- Iyer LM, Anantharaman V, Wolf MY, Aravind L. Comparative genomics of transcription factors and chromatin proteins in parasitic protists and other eukaryotes. *Int J Parasitol.* 2008; 38:1–31. [PubMed: 17949725]
- Jabri E, Aigner S, Cech TR. Kinetic and secondary structure analysis of *Naegleria andersoni* GIR1, a group I ribozyme whose putative biological function is site-specific hydrolysis. *Biochemistry.* 1997; 36:16345–16354. [PubMed: 9405070]
- Jabri E, Cech TR. In vitro selection of the *Naegleria* GIR1 ribozyme identifies three base changes that dramatically improve activity. *RNA.* 1998; 4:1481–1492. [PubMed: 9848647]
- Johansen S, Einvik C, Nielsen H. DiGIR1 and NaGIR1: naturally occurring group I-like ribozymes with unique core organization and evolved biological role. *Biochimie.* 2002; 84:905–912. [PubMed: 12458083]
- Johansen S, Elde M, Vader A, Haugen P, Haugli K, Haugli F. In vivo mobility of a group I twintron in nuclear ribosomal DNA of the myxomycete *Didymium iridis*. *Mol Microbiol.* 1997; 24:737–745. [PubMed: 9194701]
- Knoop V, Rudinger M. DYW-type PPR proteins in a heterolobosean protist: plant RNA editing factors involved in an ancient horizontal gene transfer? *FEBS Lett.* 2010; 584:4287–4291. [PubMed: 20888816]
- Kowit JD, Fulton C. Programmed synthesis of tubulin for the flagella that develop during cell differentiation in *Naegleria gruberi*. *Proc Natl Acad Sci USA.* 1974; 71:2877–2881. [PubMed: 4604968]
- Lai EY, Remillard SP, Fulton C. The α -tubulin gene family expressed during cell differentiation in *Naegleria gruberi*. *J Cell Biol.* 1988; 106:2035–2046. [PubMed: 2838492]
- Lai EY, Remillard SP, Fulton C. A β -tubulin gene of *Naegleria* encodes a carboxy-terminal tyrosine. Aromatic amino acids are conserved at carboxy termini. *J Mol Biol.* 1994; 235:377–388. [PubMed: 8289261]
- Lai EY, Walsh C, Wardell D, Fulton C. Programmed appearance of translatable flagellar tubulin mRNA during cell differentiation in *Naegleria*. *Cell.* 1979; 17:867–878. [PubMed: 487433]
- Lithgow T, Schneider A. Evolution of macromolecular import pathways in mitochondria, hydrogenosomes and mitosomes. *Philos Trans R Soc Lond B Biol Sci.* 2010; 365:799–817. [PubMed: 20124346]
- Lukes J, Guilbride DL, Votycka J, Zikova A, Benne R, Englund PT. Kinetoplast DNA network: evolution of an improbable structure. *Eukaryot. Cell.* 2002; 1:495–502.
- Lukes J, Leander BS, Keeling PJ. Cascades of convergent evolution: the corresponding evolutionary histories of euglenozoans and dinoflagellates. *Proc Natl Acad Sci USA.* 2009; 106(Suppl 1):9963–9970. [PubMed: 19528647]

- Malik SB, Pightling AW, Stefaniak LM, Schurko AM, Logsdon JM Jr. An expanded inventory of conserved meiotic genes provides evidence for sex in *Trichomonas vaginalis*. PLoS ONE. 2008; 3:e2879. [PubMed: 18663385]
- Marande W, Lukes J, Burger G. Unique mitochondrial genome structure in diplomonads, the sister group of kinetoplastids. Eukaryot Cell. 2005; 4:1137–1146. [PubMed: 15947205]
- Maruyama S, Nozaki H. Sequence and intranuclear location of the extrachromosomal rDNA plasmid of the amoeba-flagellate *Naegleria gruberi*. J Eukaryot Microbiol. 2007; 54:333–337. [PubMed: 17669158]
- Maslov DA, Simpson L. RNA editing and mitochondrial genomic organization in the cryptobiid kinetoplastid protozoan *Trypanoplasma borreli*. Mol Cell Biol. 1994; 14:8174–8182. [PubMed: 7969154]
- Mentel M, Martin W. Energy metabolism among eukaryotic anaerobes in light of Proterozoic ocean chemistry. Philos Trans R Soc Lond B Biol Sci. 2008; 363:2717–2729. [PubMed: 18468979]
- Morrison HG, McArthur AG, Gillin FD, Aley SB, Adam RD, Olsen GJ, Best AA, Cande, et al. Genomic minimalism in the early diverging intestinal parasite *Giardia lamblia*. Science. 2007; 317:1921–1926. [PubMed: 17901334]
- Muscarella DE, Vogt VM. A mobile group I intron in the nuclear rDNA of *Physarum polycephalum*. Cell. 1989; 56:443–454. [PubMed: 2536594]
- Nielsen H, Westhof E, Johansen S. An mRNA is capped by a 2', 5' lariat catalyzed by a group I-like ribozyme. Science. 2005; 309:1584–1587. [PubMed: 16141078]
- Pernin P, Ataya A, Cariou ML. Genetic structure of natural populations of the free-living amoeba, *Naegleria lovaniensis*. Evidence for sexual reproduction. Heredity. 1992; 68:173–181.
- Pusnik M, Small I, Read LK, Fabbro T, Schneider A. Pentatricopeptide repeat proteins in *Trypanosoma brucei* function in mitochondrial ribosomes. Mol Cell Biol. 2007; 27:6876–6888. [PubMed: 17646387]
- Remillard SP, Lai EY, Levy YY, Fulton C. A calcineurin-B-encoding gene expressed during differentiation of the amoeboid flagellate *Naegleria gruberi* contains two introns. Gene. 1995; 154:39–45. [PubMed: 7867946]
- Robinson BS, Christy P, Hayes SJ, Dobson PJ. Discontinuous genetic variation among mesophilic *Naegleria* isolates: further evidence that *N. gruberi* is not a single species. J Protozool. 1992; 39:702–712. [PubMed: 1453360]
- Rodriguez-Ezpeleta N, Brinkmann H, Burger G, Roger AJ, Gray MW, Philippe H, Lang BF. Toward resolving the eukaryotic tree: the phylogenetic positions of jakobids and cercozoans. Curr Biol. 2007; 17:1420–1425. [PubMed: 17689961]
- Roger AJ, Simpson AG. Evolution: revisiting the root of the eukaryote tree. Curr Biol. 2009; 19:R165–R167. [PubMed: 19243692]
- Roy J, Faktorova D, Lukes J, Burger G. Unusual mitochondrial genome structures throughout the Euglenozoa. Protist. 2007; 158:385–396. [PubMed: 17499547]
- Roy SW, Gilbert W. The evolution of spliceosomal introns: patterns, puzzles and progress. Nat Rev Genet. 2006; 7:211–221. [PubMed: 16485020]
- Schuster FL. Ultrastructure of mitosis in the amoeboid flagellate *Naegleria gruberi*. Tissue Cell. 1975; 7:1–11. [PubMed: 1118856]
- Slamovits CH, Keeling PJ. A high density of ancient spliceosomal introns in oxymonad excavates. BMC Evol Biol. 2006; 6:34. [PubMed: 16638131]
- Smit, AFA.; Hubley, R.; Green, P. RepeatMasker Open-3.0. 1996–2004. <http://www.repeatmasker.org>
- Song KJ, Jeong SR, Park S, Kim K, Kwon MH, Im KI, Pak JH, Shin HJ. *Naegleria fowleri*: functional expression of the Nfa1 protein in transfected *Naegleria gruberi* by promoter modification. Exp Parasitol. 2006; 112:115–120. [PubMed: 16321386]
- Tibayrenc M, Kjellberg F, Ayala FJ. A clonal theory of parasitic protozoa: the population structures of *Entamoeba*, *Giardia*, *Leishmania*, *Naegleria*, *Plasmodium*, *Trichomonas*, and *Trypanosoma* and their medical and taxonomical consequences. Proc Natl Acad Sci USA. 1990; 87:2414–2418. [PubMed: 2320563]
- Trimbur GM, Walsh CJ. BN46/51, a new nucleolar protein, binds to the basal body region in *Naegleria gruberi* flagellates. J Cell Sci. 1992; 103:167–181.

- Trimbur GM, Walsh CJ. Nucleolus-like morphology produced during the in vitro reassociation of nucleolar components. *J Cell Biol.* 1993; 122:753–766. [PubMed: 7688750]
- Visvesvara GS, Moura H, Schuster FL. Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploidea*. *FEMS Immunol Med Microbiol.* 2007; 50:1–26. [PubMed: 17428307]
- Vlcek C, Marande W, Teijeiro S, Lukes J, Burger G. Systematically fragmented genes in a multipartite mitochondrial genome. *Nucleic Acids Res.* 2010; doi: 10.1093/nar/gkq883
- Walsh CJ. The role of actin, actomyosin and microtubules in defining cell shape during the differentiation of *Naegleria* amebae into flagellates. *Eur J Cell Biol.* 2007; 86:85–98. [PubMed: 17189659]
- Westenberger SJ, Cerqueira GC, El-Sayed NM, Zingales B, Campbell DA, Sturm NR. *Trypanosoma cruzi* mitochondrial maxicircles display species- and strain-specific variation and a conserved element in the non-coding region. *BMC Genomics.* 2006; 7:60. [PubMed: 16553959]
- Wikmark OG, Einvik C, De Jonckheere JF, Johansen SD. Short-term sequence evolution and vertical inheritance of the *Naegleria* twin-ribozyme group I intron. *BMC Evol Biol.* 2006; 6:39. [PubMed: 16670006]
- Yoon HS, Grant J, Tekle YI, Wu M, Chaon BC, Cole JC, Logsdon MJ, Patterson DJ, Bhattacharya D, Katz LA. Broadly sampled multigene trees of eukaryotes. *BMC Evol Biol.* 2008; 8:14. [PubMed: 18205932]

Fig. 1.

N. gruberi reproduces as amoebae, but can also form flagellates and dormant cysts. (Top) A live *N. gruberi* amoeba visualized using DIC microscopy is moving in the direction of the arrow. (Bottom right) A *Naegleria* flagellate stained with antibodies to alpha and beta tubulin. If alive, the flagellate would swim in the direction of the arrow. (Bottom left) Scanning electron micrograph of an empty *N. gruberi* cyst. Amoebae excyst through a pore like the one highlighted with an arrow.

Fig. 2.

N. gruberi (strain NEG-M) genome organization. A. (Top left) A transmission electron micrograph of a *N. gruberi* amoeba nucleus. The large, dark, nucleolus contains ~4000 copies of a plasmid encoding rRNA genes. The surrounding nuclear material harbors the linear chromosomes. (Bottom) A diagram representing the circular extrachromosomal plasmid carrying all three rDNA genes (28S, 18S, and 5.8S), and two predicted open reading frames. (Right): 12 linear chromosomes with indicated sizes (in Mb) are visualized by pulsed-field gel electrophoresis. Lanes contain increasing DNA content from left to right. B.

Schematic representation of Nae.S516, a parasitic twin-ribozyme intron found in some *Naegleria* isolates. This complex intron is found within the plasmid-localized 18S SSU rDNA, and contains two self-splicing group-1 introns: GIR2 initially removes the intron from the transcribed SSU rRNA to allow proper folding of the rRNA. Then, under certain specific conditions, GIR1 releases the homing endonuclease coding region from the remainder of the intron.

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Fig. 3. Relationship of *Naegleria* to other eukaryotes. A schematic of evolutionary relationships between seven major eukaryotic groups with widespread support in diverse molecular phylogenies (Burki et al., 2008; Rodriguez-Ezpeleta et al., 2007; Yoon et al., 2008). *Naegleria*'s position is highlighted in bold font. The disconnection between the groups indicates the uncertainty regarding their relationships and order of divergence.

Fig. 4. *Naegleria* nuclear division uses a broad microtubule spindle that terminates within nucleoli. A.) Mitotic *Naegleria gruberi* (strain NEG) cells of the indicated stages are shown, with microtubules highlighted by antibodies against alpha- and beta-tubulin (green) and DNA by DAPI (blue). B.) A *N. gruberi* cell in telophase with nucleoli (red) stained using antibody BN5.1 against the nucleolar protein BN46/51, and mitotic microtubules (green) stained using the anti-tubulin monoclonal antibody YOL 1/34 available from AbD Serotec. Note the termination of microtubules within the stained nucleoli.

Table 1

Mitochondrial genome organization and gene inventory in *N. gruberi* and other JEH protists. Data are summarized and adapted from Barbrook et al., 2010; Gray et al., 2004, 1998; Marande et al., 2005; Maslov and Simpson, 1994; Vlcek et al., 2010; Westenberger et al., 2006.

	Jakobids		Euglenozoans		Heteroloboseans	
	Reclinomonas americana	Malawimonas jakobiformis	Diplonema papillatum	Trypanosoma brucei	Naegleria gruberi	
Genome size	69,034	47,329	n.d. (maybe >300 kb)	23,016 kb (maxi-circle)	49,843	
Topology	Circular	Circular	Small circular chromosomes (6–7 kb) dispersed throughout the mitochondrion	Catenated network of >1000 mini- (1–2.5 kb) and 20–40 maxi-circles	Circular	
Protein-coding genes	67	49	d 5 ^c	18	46	
tRNA genes	26	36	n.d.	0	21	
Complex I subunits	12	8	d 1 ^d	7	11	
Complex II subunits	3	0	n.d.	0	1	
Complex III subunits ^a	1	1	d 1	1	1	
Complex IV subunits ^b	3	3	d 3	3	3	
ATP synthase subunits (complex V)	5	3	n.d. ^e	1	4	
c-Type cytochrome biogenesis subunits	4	3	n.d.	0	3	
Other protein-coding genes	39	31	n.d.	6	23	

^aIn each instance, it is *cytb* that is mitochondrially encoded (as in nearly all other mitochondrial genomes).

^bLike most other eukaryotes, it is cytochrome *c* oxidase subunits 1–3 that are mitochondrially encoded.

^cThe mitochondrial genome sequence of *D. papillatum* is not complete; the number and complete inventory of protein-coding, rRNA and tRNA genes present in the genome is therefore uncertain. n.d., not determined.

^dPartial sequences for an additional five mitochondrially encoded complex I subunits are described in Vlcek et al. (2010).

^ePartial sequence for one encoded complex V subunit is described in Vlcek et al. (2010).