Ancestral centriole and flagella proteins identified by analysis of *Naegleria* differentiation

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Summary

Naegleria gruberi is a single-celled eukaryote best known for its remarkable ability to form an entire microtubule cytoskeleton de novo during its metamorphosis from an amoeba into a flagellate, including basal bodies (equivalent to centrioles), flagella and a cytoplasmic microtubule array. Our publicly available full-genome transcriptional analysis, performed at 20-minute intervals throughout *Naegleria* differentiation, reveals vast transcriptional changes, including the differential expression of genes involved in metabolism, signaling and the stress response. Cluster analysis of the transcriptional profiles of predicted cytoskeletal genes reveals a set of 55 genes enriched in centriole components (induced early) and a set of 82 genes enriched in flagella proteins (induced late). The early set includes genes encoding nearly every known conserved centriole component, as well as eight previously uncharacterized, highly conserved genes. The human orthologs of at least five genes localize to the centrosomes of human cells, one of which (here named Friggin) localizes specifically to mother centrioles.

Key words: Naegleria, Assembly, Centriole, Evolution, Flagella

Introduction

Naegleria gruberi grows as an amoeba without flagella, centrioles or even cytoplasmic microtubules; it relies on an actin-based cytoplasmic cytoskeleton for chemotaxis and motility, and its mitotic spindle is contained within an intact nuclear envelope (Fulton, 1970; Fulton, 1977; Fulton and Dingle, 1971). However, when exposed to stressors such as changes in temperature or nutrient availability, *Naegleria* rapidly differentiates into a flagellate, forming a complete cytoplasmic microtubule cytoskeleton from scratch (Fig. 1) (Fulton and Dingle, 1967). This differentiation occurs synchronously – approximately 90% of cells assemble basal bodies (structures equivalent to centrioles) within a 15-minute window, followed by flagella approximately 10 minutes later (Fig. 1) (Fulton and Dingle, 1971). Although *Naegleria* assembles basal bodies de novo, protein incorporation occurs in the same order as that occurring during assembly of human centrioles (Fritz-Laylin et al., 2010a). The evolutionary distance of *Naegleria* from animals means that genes shared between *Naegleria* and humans were probably present in the ancestor of all eukaryotes (Cavalier-Smith, 2002; Ciccarelli et al., 2006) (for a review, see Fritz-Laylin et al., 2010b). Thus, *Naegleria* differentiation affords a unique opportunity to study ancestral features of centriole and flagellum assembly.

Interphase animal cells contain numerous microtubules emanating from microtubule organizing centers (MTOCs) called centrosomes. Centrosomes contain centrioles that are primarily composed of nine microtubule triplets, and the surrounding amorphous pericentriolar material (PCM) that anchors cytoplasmic microtubules. Centrioles are called basal bodies when they are used to organize axonemes, the microtubule core of eukaryotic cilia and flagella. These whip-like structures propel single-celled organisms and move fluids within multicellular organisms. Metazoan cells also have nonmotile cilia that function as 'cellular antennae' by gathering information about the surrounding environment using their varied signaling receptors (Marshall and Nonaka, 2006).

Proteomic analyses indicate that centrosomes and basal bodies contain many of the same proteins, a large number of which are thought to be functional components of centrioles (Andersen et al., 2003; Keller et al., 2005; Kilburn et al., 2007). However, only a handful of these proteins have been characterized functionally (Strnad and Gönczy, 2008). This is in part due to the technical difficulties associated with studying centriole assembly in most organisms. First, new centrioles usually assemble in association with a mature centriole, hindering proteomic characterization of assembly intermediates. Second, centriole assembly is usually tied to the cell cycle, rendering it difficult to distinguish centriole-specific genes from other induced cell cycle genes. And finally, de novo assembly (where centrioles are built in the absence of preexisting ones) usually occurs in a single cell or embryo, making proteomic or microarray-based approaches unfeasible. Here, we used the synchronous de novo basal body assembly pathway of *Naegleria* to overcome these technical roadblocks and identify genes used specifically for building basal bodies and flagella.

Results and Discussion

Flagella and basal body gene transcripts are induced with different kinetics

We isolated total RNA at 20-minute intervals during *Naegleria* differentiation (at 0, 20, 40, 60 and 80 minutes; Fig. 1) from three biological replicates (supplementary material Fig. S1A). The relative abundance of transcripts from each time-point was quantified using custom full-genome *Naegleria* DNA microarrays. Approximately 24% of *Naegleria* genes are induced at least twofold, and an additional 39% are reduced by at least 50% during the amoeba-to-flagellate transition (4065 and 6484 genes, respectively; $P<0.01$, after correction for multiple testing). Differentially regulated genes include those involved in stress

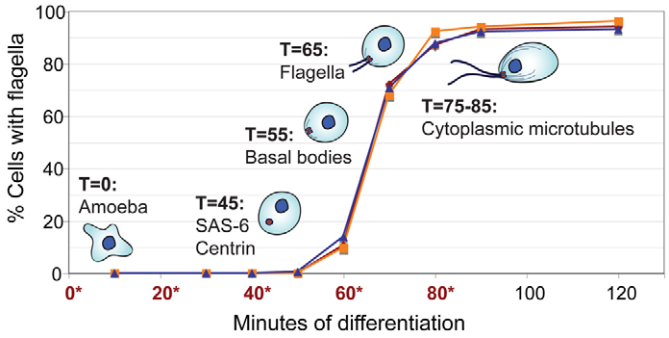

Fig. 1. Selected events during differentiation of *Naegleria*. Each of the three curves represents the percentage of cells with visible flagella during one differentiation replicate used for microarray analysis; the time-points collected are indicated with an asterisk (*). Important events during assembly of the *Naegleria* basal body are indicated.

responses (including Hsp20 and Hsp 90; data not shown) and core metabolism (including glycolysis, Krebs cycle and pyruvate–acetate metabolism; data not shown), as well as cytoskeletal components.

Only a fraction of the thousands of induced genes are likely to be microtubule related. To aid in our search for uncharacterized and evolutionarily conserved centriole proteins, we focused on genes found in *Naegleria* and other flagellates but missing in non-flagellated organisms [the flagellar motility (FM) gene set (Fritz-Laylin et al., 2010b)]. FM members include genes specific to basal bodies and flagella but exclude genes such as that encoding α-tubulin that are also used by organisms without flagella. To permit analysis of general microtubule proteins involved in basal body and flagella formation, we added *Naegleria* homologs of known microtubule cytoskeleton proteins (Fritz-Laylin et al., 2010b). Finally, we also added 63 genes conserved in organisms that undergo amoeboid movement and missing in organisms that do not undergo amoeboid locomotion [the amoeboid motility (AM) gene set] (Fritz-Laylin et al., 2010b), to serve as a specificity control.

Overall, 78% of the FMs and 60% of the AMs have at least twofold induction or repression, respectively ($P<0.01$, after correction for multiple testing), providing large-scale confirmation of previous evidence that *Naegleria* differentiation is controlled at the transcriptional level (Lai et al., 1988; Levy et al., 1998). We next investigated whether the timing of gene expression was linked to function.

Cluster analysis of the expression data for these 310 genes (the AM and FM gene sets, and *Naegleria* homologs of known microtubule genes) resulted in five major gene clusters (A–E; Fig. 2). Clusters A and C consist primarily of genes found in the FM gene set and have increased expression during differentiation. However, the genes in cluster A reach peak expression levels by 20 minutes and begin decreasing in expression by 40 minutes, whereas the expression of genes in cluster C peaks by 40 minutes and remains high through to 80 minutes. Manual inspection revealed that the cluster with earlier expression contains many known centriole genes (Table 1), whereas the later expression cluster contains flagella genes (supplementary material Table S1). The general induction of basal body genes before flagella genes agrees with the fact that *Naegleria* assembles its basal bodies before it assembles its flagella (t=55 and t=65 minutes, respectively) (Fig. 1) (Fritz-Laylin et al., 2010a; Fulton and Dingle, 1971).

Centriole-enriched gene cluster

The 55 genes found in the centriole gene cluster include *Naegleria* homologs of seven genes whose products are thought to be required for assembly of the centriole or basal body: ε-, δ- and η-tubulin, SAS-4 (CPAP), SAS-6, centrin (Cen2) and POC1 (for references, see Table 1). This set represents the majority of components shown to be required specifically for centriole assembly that are conserved outside animals (Carvalho-Santos et al., 2010; Hodges et al., 2010; Strnad and Gönczy, 2008). Other core centriole genes not found in the cluster either have not been identified in the *Naegleria* genome (*PLK4*) or were not included in the microarray (*BLD10*).

The centriole-enriched gene cluster also encodes homologs of microtubule nucleation factors [γ-tubulin, GCP3 and GCP6 (Raynaud-Messina and Merdes, 2007)], as well as proteins required for general microtubule functions, such as the microtubule-severing protein katanin p60, which is known to localize to centrosomes (Hartman et al., 1998). This gene set also includes several genes encoding centrosome-localized proteins of unknown function, and eight completely uncharacterized genes (Table 1).

Axonemal dyneins are large protein complexes containing light, intermediate and heavy chains that slide microtubules past each other to produce flagellar movement. Surprisingly, the centriole-enriched cluster includes nine axonemal dynein light and intermediate chain homologs, as well as a homolog of kintoun (PF13) that is required for assembly of dynein arm complexes (Omran et al., 2008). By contrast, *Naegleria* dynein heavy chain genes are expressed later along with other flagella-specific genes. Assembly of dynein light and intermediate chain complexes can be genetically uncoupled from assembly of the dynein heavy chain in *Chlamydomonas* (Omran et al., 2008). Although there are many possible reasons for the early expression of dynein light and intermediate chains, but not heavy chains, it is possible that *Naegleria* pre-assembles flagellar intermediate and light chain dyneins before incorporating heavy chains, before flagellar outgrowth.

Flagella-enriched gene cluster

The flagella-enriched gene cluster contains 82 genes (supplementary material Table S1), including genes encoding proteins used for transporting proteins to the base of the growing flagellum (BBS components BBS1–BBS5 and BBS7–BBS9) and within the flagellum to its growing tip (FLA3, kinesin 2, IFT20, IFT52, IFT57, IFT80, IFT88, IFT122 and IFT140), as well as structural components of the flagellum itself [including PF20 and PF16, RSP4 and Rib72 (Pazour et al., 2005)]. This gene set also includes 23 FM genes with homologs found in the *Chlamydomonas* flagella proteome (Pazour et al., 2005) but which are otherwise uncharacterized. Together, these data suggest that these proteins are probably core components of eukaryotic flagella and therefore prime candidates for future functional analyses.

To validate the putative flagellar components, we conducted a proteomic analysis of *Naegleria* flagella. We purified the flagella of ~4×10^8 flagellate cells using low-speed centrifugation followed by a sucrose step-gradient. The resulting sample contained flagella and no visible cell bodies and comprised largely two proteins of a size similar to that of α- and β-tubulin (supplementary material Fig. S1, panel B), as is typical for clean flagellar preparations (e.g. Kowit and Fulton, 1974). MUDPIT mass spectrometry analysis of the sample identified 415 proteins (supplementary material Table S2).

Of the 82 genes in the flagellar-enriched gene cluster, 23 were also identified in our proteomics analysis (supplementary material

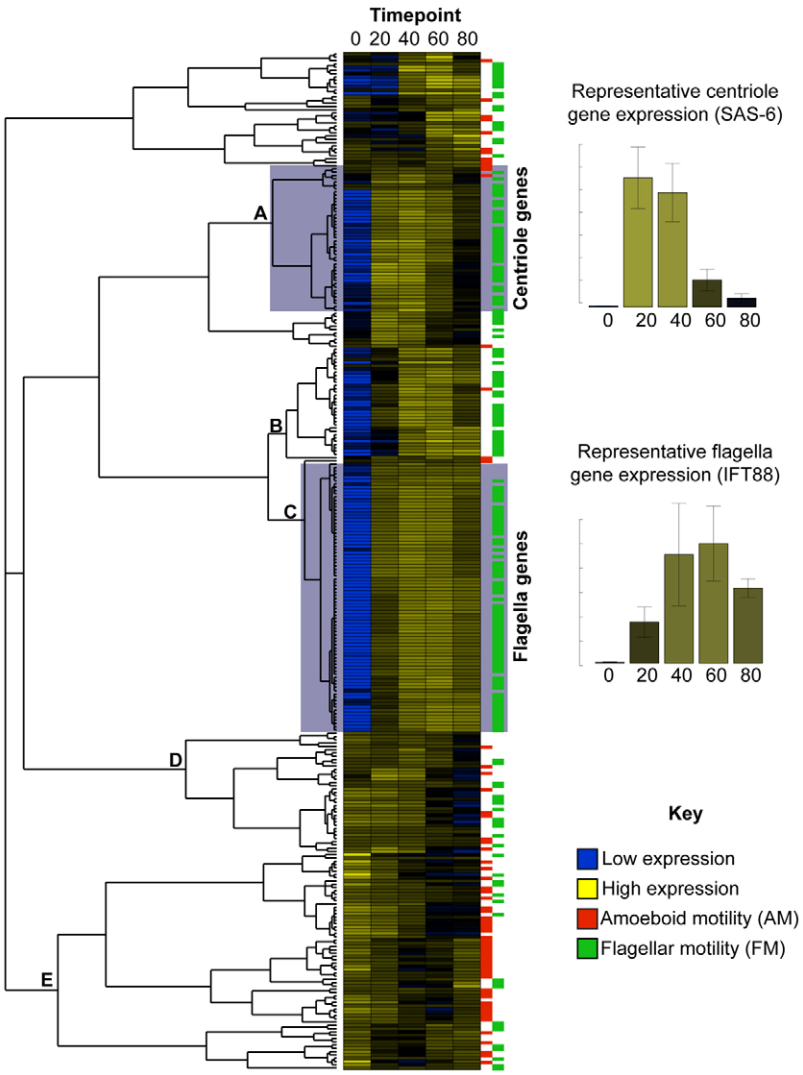

Fig. 2. Centriole- and flagella-enriched gene clusters. Each row represents one gene, with columns representing expression at the indicated time-point. Blue represents low expression, and yellow represents high expression. The cladogram groups genes based on similarity in expression across the five time-points; five major clusters are indicated (**A–E**). Red and green boxes on the right-hand side indicate membership of the AM (amoeboid motility) and FM (flagellar motility) gene sets, respectively. The graphs indicate relative gene expression – using as examples centriole-enriched cluster gene (*SAS-6*) and flagella-enriched cluster gene (*IFT88*). The data are plotted as the means ± s.d. (n=3).

Table S1), indicating that they are likely to be structural components of the flagellum itself (in contrast to proteins that might be required for flagellar function but are located within the cell body). Included in this overlap are seven flagellar-associated proteins (FAPs), which were identified in the proteomic analysis of *Chlamydomonas* flagella (Pazour et al., 2005) but remain otherwise uncharacterized. These proteins are therefore likely to be ancestral structural flagella components. The *Naegleria* flagellar proteome also includes a number of previously undescribed proteins (supplementary material Table S2), some of which might represent uncharacterized flagellar proteins.

Verification of putative centriole genes

The centriole-enriched gene cluster includes eight genes that have not previously been localized or otherwise characterized, which we refer to as 'putative conserved centriole components' (pCCCs; Table 1). Because orthologs of all centrosome-localized pCCCs can be found in a wide diversity of eukaryotes (supplementary material Table S3), they were probably present in the eukaryotic ancestor. To determine whether the pCCCs are likely to be centriole components, we transiently expressed N- and C-terminally GFP-tagged human orthologs of each pCCC in U2OS and HeLa human cell lines and used antibodies recognizing γ-tubulin to highlight centrosomes. To the eight unknown gene products, we added one whose homolog localizes to the base of the cilia of *Caenorhabditis elegans* (B9D2) and one that has only very recently been characterized in human cells (MOT52, also known as FOR20 or BBC20) (Sedjai et al., 2010). Five of the ten tagged proteins showed either diffuse cytoplasmic GFP or bright foci likely to be inclusion bodies (data not shown). This nonspecific localization neither confirms nor denies a possible centriole function. However, the remaining five localized within or near centrosomes using both N- and C-terminal GFP tags (Fig. 3A) and are described below.

First, MOT52 is found only in organisms with motile flagella (Merchant et al., 2007), and its homolog (BBC20) was found in the *Tetrahymena* basal body proteome (Kilburn et al., 2007). Recently, the human ortholog (FOR20) was reported to localize to pericentriolar satellites and to be involved in ciliary assembly (Sedjai et al., 2010). FOR20 is predicted to have a FOP dimerization domain (Pfam domain PF09398), required for the centrosomal localization of the FOP protein (Mikolajka et al., 2006). In transient transfections of both U2OS (Fig. 3A) and HeLa cells (data not shown), GFP-tagged FOR20 localized to multiple foci near centrosomes. A similar localization was reported using an antibody

Table 1. Centriole-enriched gene cluster

Class	Ortholog	Protein ID	Function	References
BB, centriole assembly	ε-Tubulin	44774	BB, centriole assembly	(Dutcher et al., 2002)
	δ-Tubulin	69007	BB, centriole assembly	(Dutcher and Trabuco, 1998; Garreau de Loubresse et al., 2001; O'Toole et al., 2003)
	η-Tubulin	65724	BB, centriole assembly	(Basto et al., 2006; Kirkham et al., 2003; Kleylein-Sohn et al., 2007; Leidel and Gonczy, 2003; Pelletier et al., 2006)
	SAS-6	68996	BB, centriole assembly	(Culver et al., 2009; Dammermann et al., 2004; Leidel et al., 2005; Nakazawa et al., 2007; Peel et al., 2007; Pelletier et al., 2006; Rodrigues-Martins et al., 2007; Strnad et al., 2007)
	SAS-4 (CPAP)	61107	BB, centriole assembly	(Basto et al., 2006; Kirkham et al., 2003; Kleylein-Sohn et al., 2007; Leidel and Gonczy, 2003; Pelletier et al., 2006)
	Centrin	44488 56351	BB, centriole assembly	(Baum et al., 1986; Koblenz et al., 2003; Kuchka and Jarvik, 1982; Salisbury et al., 2002; Taillon et al., 1992; Winey et al., 1991)
	POC1	33676	BB, centriole assembly	(Keller et al., 2009; Pearson et al., 2009)
MT-specific centrosome function	γ-Tubulin	56069	MT nucleation	(Raynaud-Messina and Merdes, 2007)
	GCP6	61337	MT nucleation	(Raynaud-Messina and Merdes, 2007)
	GCP3	434	MT nucleation	(Raynaud-Messina and Merdes, 2007)
	SSA11 (TTL13)	80835	Tubulin tyrosine ligase	(Hammond et al., 2008)
	Katanin P60	63871	MT severing	(Hartman et al., 1998)
	α-Tubulin	71268	MTs	(Dutcher, 2001)
		51830	MTs	(Dutcher, 2001)
	TBCE	81169	Putative tubulin chaperone	
BB, centriole, centrosome localization	FOR20 (MOT52)	52938	Pericentriolar satellites, centrosome	(Sedjai et al., 2010)
	POC12	29577	Unknown	(Keller et al., 2005)
	PIBF	73664	Unknown	(Lachmann et al., 2004)
	TTL1	33283	Tubulin tyrosine ligase	(Wloga et al., 2008)
BB-specific function	MKS6 (CC2D2A)	77673	Cilium, BB function	(Gorden et al., 2008)
	MKS3 (meckelin)	62841	BB migration, membrane docking	(Dawe et al., 2007)
	B9D1	52666	Transition zone	(Williams et al., 2008)
	B9D2	30379	Transition zone	(Williams et al., 2008)
Flagellar dynein complex	MBO2	62959	Maintain direction of motility	(Tam and Lefebvre, 2002)
	ODA6	60431	Dynein intermediate chain 2, axonemal	(Pazour et al., 2005)
	IDA7	57343	Dynein intermediate chain, axonemal	(Pazour et al., 2005)
	ODA6	79232	Dynein intermediate chain, axonemal	(Pazour et al., 2005)
	DLC1	74922	Dynein light chain, axonemal	(Pazour et al., 2005)
	IDA4 (P28)	82719	Dynein light intermediate chain, axonemal	(Pazour et al., 2005)
	BOP5	63304	Dynein light intermediate chain, axonemal	(Pazour et al., 2005)
	TCTEXT1	29177	Dynein light chain, axonemal	(Harrison et al., 1998; Kagami et al., 1998)
	DLC1	54720	Dynein light chain, axonemal	(Pazour et al., 2005)
	MOT24	32555	Dynein light chain, axonemal	(Merchant et al., 2007)
	MOT45 (kintoun, PF13)	80717	Axonemal dynein complex assembly	(Omran et al., 2008)
Putative flagellar function	FAP184	2066	Unknown	(Pazour et al., 2005)
	FAP215	66643	Nucleotidase	(Pazour et al., 2005)
	FAP127	44967	Unknown	(Pazour et al., 2005)
	FAP57	61313	Unknown	(Pazour et al., 2005)
Previously uncharacterized (pCCCs)	Friggin (MOT37)	61232	Mother centriole localization	This study
	POC11	70454	Centrosome localization	This study
	SSA3	68814	Centrosome localization	This study
	Q6PH85	49668	Unknown	
	POC16	62107	Unknown	
	LRR6	31069	Unknown	
	TECT3	65759	Unknown	
	MOT39	72718	Unknown	
Other	?	73058	Dual-specificity phosphatase	
	PSK1	78247	Nucleotide kinase	
	?	82958	High-mobility-group protein	
	?	48624	Lipid synthesis	
	?	29264	HUS-1-like protein	
	?	81040	BTG-domain protein	
	MOT50	54684	Chymotrypsin-like protein	
		71996	Chymotrypsin-like protein	

The 55 genes in the centrosome-enriched cluster are organized by function (or predicted function), followed by ortholog gene and/or protein names, with appropriate references in the last column. ?, no identifiable ortholog; BB, basal body; MT, microtubule. Sequence information for *Naegleria* protein IDs is available at www.jgi.doe.gov/naegleria.

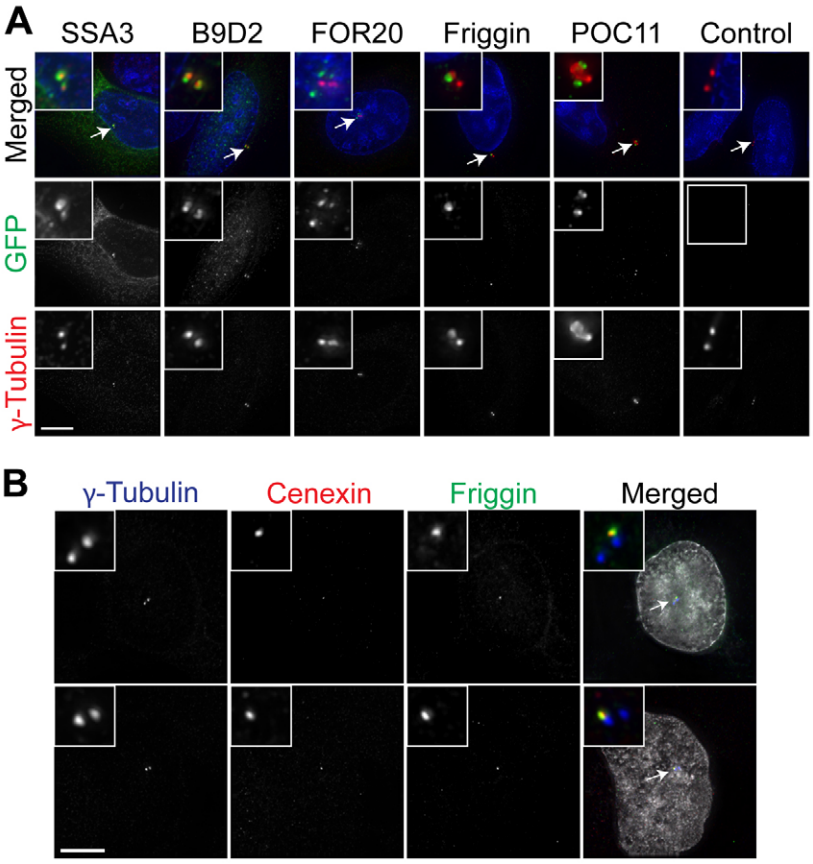

Fig. 3. Human orthologs of pCCCs localize to centrosomes, including the mother centriole protein Friggin. GFP-tagged human orthologs of the indicated pCCC are shown in green after transient transfection of U2OS cells. (**A**) Centrosomes (arrows) are stained using an antibody against γ-tubulin (red), and DNA is shown in blue. (**B**) Centrosomes (arrows) are stained with an antibody against γ-tubulin (blue), mother centrioles with an antibody against cenexin (red), and DNA is shown in white. Scale bars: 5 μm.

against FOR20 (Sedjai et al., 2010), thus validating our GFP tagging approach.

Second, B9D2 contains a B9 domain. The *C. elegans* homolog, TZA-1, localizes to the transition zone at the base of the cilium (Williams et al., 2008). Although the B9 domain has no known function, it is found in several proteins known to be localized to the centriole and/or basal body, including MKS1 (Dawe et al., 2007). Human B9D2 localized to centrosomes of U2OS cells, along with scattered foci throughout the cytoplasm (Fig. 3A).

Third, POC11 shows good conservation in many eukaryotes but has no identifiable domains other than a coiled-coil region. Localization of the human homolog (CCDC77) resulted in bright punctate spots within centrosomes of both U2OS and HeLa cells (Fig. 3A and data not shown, respectively), suggesting that POC11 represents a new family of centrosome proteins.

Fourth, SSA3 was so named for its predicted function in both motile and nonmotile flagella [SSA stands for 'sensory, structural and assembly' (Merchant et al., 2007)]. SSA3 has a conserved central region containing an 'ELMO/CED12' domain (Pfam domain PF04727), found in proteins that facilitate cytoskeletal rearrangements (Gumienny et al., 2001). SSA3–GFP-expressing cells contained diffuse cytoplasmic GFP, as well as centrosomal GFP in a small percentage (4%) of transfected cells that also displayed relatively small γ-tubulin foci (Fig. 3A). As γ-tubulin foci vary in size during the mammalian cell cycle (with small foci at G1- and early S-phases), SSA3 might localize to centrosomes in a cell-cycle-dependent manner.

Fifth, Friggin was originally named MOT37 for its predicted function in motile flagella (Merchant et al., 2007) and contains leucine-rich repeats (LRRs), which typically mediate protein–protein interactions [Pfam clan CL0022 (Kobe and Deisenhofer, 1994)]. Unexpectedly, the 542-residue human ortholog of MOT37 localized to only one of two γ-tubulin foci in both U2OS (Fig. 3A) and HeLa cells (data not shown), suggesting that it might be a component specific to either mature or immature centrioles.

Centrioles develop over two cell cycles, acquiring the basic nine-triplet pinwheel structure during the first cell cycle and various appendages that allow it to function as a basal body for axonemal assembly during the second cycle. Several gene products have been shown to be involved in the assembly of appendages (Chang et al., 2003; Gromley et al., 2003; Lange and Gull, 1995; Mogensen et al., 2000; Ou et al., 2002), only one of which, ε-tubulin (Chang et al., 2003), is conserved outside animals and likely to be ancestral to all extant eukaryotes.

To investigate whether MOT37 is a component of either mother or daughter centrioles, we expressed GFP–MOT37 and stained cells with an antibody recognizing the mother centriole component cenexin (Lange and Gull, 1995). GFP–MOT37 consistently colocalized with cenexin (Fig. 3B), indicating that MOT37 is a mother-centriole-specific protein that we predict is involved in the developmental transition from immature to mature centrioles. Because MOT37 probably represents a second ancestral mother centriole protein, we have named this eukaryotic protein family 'Friggin' after Frigg, the Norse goddess of motherhood.

Concluding remarks

Understanding how centrioles and flagella assemble and function requires a full inventory of components. Previous studies have

used proteomic approaches to attempt to identify a complete parts list for centrioles (Andersen et al., 2003; Keller et al., 2005; Kilburn et al., 2007) or flagella (for a review, see Inglis et al., 2006). Theoretically, proteomic analyses can identify all stably localized proteins, including those that are species specific or required for unrelated biological functions. By contrast, our analysis has identified comprehensive sets of genes required specifically for centriole and flagellar function, independent of their localization. Of eight previously uncharacterized genes that we predict are involved in centriole assembly, at least three have human orthologs with a centrosome-related localization. The conservation of these proteins in both *Naegleria* and human, probably spanning over a billion years of eukaryotic evolution (Brinkmann and Philippe, 2007), indicates that these proteins are important for centriole function. Our study adds significantly to the list of conserved centriole components, including an additional protein (Friggin) that is apparently specific to mature centrioles.

Our analyses extend previous observations (Fulton et al., 1995; Levy et al., 1998) of two major programs of transcription during *Naegleria* differentiation: an early round of transcription of basal body genes and a later round of flagellar genes (Fig. 2), a timing that mirrors the assembly of basal bodies before flagella (Fig. 1). Although we have limited our analysis to centriole and flagella genes, this represents only one aspect of the *Naegleria* amoeba-to-flagellate transition. A cursory analysis indicates that genes from other core pathways, including basic metabolism and the stress response, are also regulated differentially. We have deposited our microarray data in the NCBI Gene Expression Omnibus (Edgar et al., 2002) under GEO Series accession number GSE21527 and encourage other scientists to take advantage of this rich data set.

Materials and Methods

Naegleria differentiation and RNA isolation

N. gruberi strain NEG grown on *Klebsiella* was differentiated three separate times using standard protocols (Fulton, 1970). Synchrony was estimated by counting the percentage of flagellates after fixing in Lugol's iodine (Fulton and Dingle, 1967), using a phase-contrast microscope with a ×40 objective (n>100 for each time-point). 10^7 cells were harvested at each time-point, and RNA extracted using Trizol reagent (Invitrogen), purified using RNAeasy (Qiagen), treated with Turbo DNAse (Ambion), and repurified with RNAeasy (Qiagen), according to the manufacturers' instructions. RNA purity was verified by means of gel electrophoresis (supplementary material Fig. S1) and spectrophotometry.

NimbleGen expression oligoarrays

The *N. gruberi* whole-genome expression oligoarray version 1.0 (NimbleGen Systems) comprises 182,813 probe sets corresponding to 15,777 gene models predicted on the *N. gruberi* genome sequence version 1.0 (Fritz-Laylin et al., 2010a), and an additional 963 open reading frames (ORFs) identified in intergenic regions. For each gene, 11 unique 60-mer oligonucleotide probes were designed by NimbleGen Systems. The *Naegleria* V1.0 oligoarray is fully described at the Gene Expression Omnibus (GEO) (Edgar et al., 2002) under accession number GSE21527.

Preparation of samples, hybridization and scanning were performed by NimbleGen Systems (Madison, WI), following their standard operating protocol. The raw data were subjected to robust multi-array analysis (RMA) (Irizarry et al., 2003), quantile normalization (Bolstad et al., 2003) and background correction, as implemented in the NimbleScan software package, version 2.4.27 (Roche NimbleGen). Reproducibility between biological replicates was inspected using MA [log-intensity ratios (M) versus log-intensity averages (A)] and scatter plots of log intensities (supplementary material Fig. S1), and P-values were calculated in a simple paired-data comparison model and were corrected for multiple testing using the BH (false discovery rate controlled) procedure, all within the R statistical package (http://www.r-project.org/).

Expression clustering

The log-transformed expression data for the 310 cytoskeleton-related genes were subjected to gene normalization followed by hierarchical clustering, with centered correlation and complete linkage in the Cluster program (Eisen et al., 1998).

Proteomics of *Naegleria* flagella

Flagella were isolated using published methods (Kowit and Fulton, 1974) and mass spectrometry performed by the Vincent J. Coates Proteomics/Mass Spectrometry Laboratory at UC Berkeley, CA. A nano LC column was packed in a glass capillary, of 100 µm inner diameter, with an emitter tip. The column comprised 10 cm of Polaris c18 5-µm packing material (Varian), followed by 4 cm of Partisphere 5 SCX (Whatman). The column was loaded using a pressure bomb and washed extensively with buffer A [5% acetonitrile, 0.02% heptaflurobutyric acid (HBFA)], then directly coupled to an electrospray ionization source mounted on a Thermo-Fisher LTQ XL linear ion-trap mass spectrometer. An Agilent 1200 HPLC delivering a flow rate of 30 nl/min was used for chromatography. Peptides were eluted using a 14-step MudPIT procedure (Washburn et al., 2001), using buffer A, buffer B (80% acetonitrile, 0.02% HBFA), buffer C (250 mM ammonium acetate, 5% acetonitrile, 0.02% HBFA) and buffer D (250 mM ammonium acetate, 5% acetonitrile, 0.02% HBFA, 500 mM ammonium acetate). The programs SEQUEST and DTASELECT were used to identify peptides and proteins from the *Naegleria* genome (Eng et al., 1994; Tabb et al., 2002).

Localization of pCCCs

The following mammalian cDNAs from the human ORF collection in the form of Gateway entry vectors were purchased from Open Biosystems (Rual et al., 2004): B9D2 (CV025994), MOT52 (FOR20; EL735575), POC16 (EL737049), FM14 (EL735863), SSA3 (EL735155), MOT39 (CV023936) and TECT3 (EL736819), and these were verified by sequencing. The following cDNAs (and corresponding accession numbers) were obtained from Open Biosystems: POC11 (BC006444), MOT37 (BC016439) and LRRC6 (BC047286). Each ORF was amplified (primer sequences available upon request), transferred into a Gateway donor vector (pDONR221) and verified by sequencing. All ORFs were then transferred into the C-terminal (pCDNA-DEST47) and N-terminal (pCDNA-DEST53) GFP-tagged Gateway Vectors according to the manufacturers' protocols.

A total of 20,000 U2OS cells (ATCC catalog number HTB-96**)** was inoculated in 0.5 ml medium [DMEM (GIBCO catalog number 10569) supplemented with 10% FBS, 1% nonessential amino acids, and 1% sodium pyruvate] in 24-well plates containing coverslips. Cells were transfected the next day with lipofectamine 2000, and 14 hours later fixed for three minutes with methanol and prepared for immunofluorescence using standard methods (http://mitchison.med.harvard.edu/protocols/gen1.html), and the following antibodies: monoclonal antibody 20H5 against centrin (antibody 20H5) (Sanders and Salisbury, 1994), used at 1:400, antibody CD1B4 against cenexin (antibody CD1B4) (Lange and Gull, 1995) used at 1:3, and mouse monoclonal antibody against GFP (catalog number 11814460001, Roche) at a 1:500 dilution. Alexa-Fluor-conjugated secondary antibodies were sourced from Invitrogen (Carlsbad, CA) and used at a 1:500 dilution.

Fluorescence deconvolution microscopy

Images were collected with SoftWorX image acquisition software (Applied Precision, Issaquah, WA) on an Olympus IX70 wide-field inverted fluorescence microscope with an Olympus PlanApo ×100, (NA 1.35), oil-immersion objective and Photometrics CCD CH350 camera (Roper Scientific, Tuscon, AZ). Image stacks were deconvolved with the SoftWorX deconvolution software and flattened as maximum projections (Applied Precision, Issaquah, WA).

Sequence analysis

Domain predictions were performed using default parameters and version 24.0 of the Pfam database (Finn et al., 2008). pCCC orthologs were collected from the nonredundant ('nr') database at NCBI (Benson et al., 2009) with BLAST (Altschul et al., 1990).

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