

Chapter 16

Mechanism of Cell Division in *Entamoeba histolytica*

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Abstract The cell division cycle of *Entamoeba histolytica* shows important differences from that of unicellular and higher eukaryotes. We have observed that *E. histolytica* cultures are made up of a heterogeneous population of cells that contain one or many nuclei and varying DNA content in each nucleus. Chromosome segregation occurs on a variety of atypical microtubular assemblies, and daughter cells are formed from mechanical rupture of cytoplasmic extensions that may need “helper cells” to complete the separation. Our observations suggest that whole genome copies are lost when cells shift from axenic to xenic cultures or from trophozoites to cysts. Gain or loss of whole genome copies during changes in growth conditions is possibly sustained by the inherent plasticity of the amoeba genome. Molecular studies have shown that orthologues of conserved checkpoint proteins that regulate the eukaryotic cell cycle are absent in this organism. Absence of checkpoint control leads to unregulated DNA synthesis, asymmetrical chromosome segregation, and aberrant cytokinesis in eukaryotes. In spite of the perceived lack of control and atypical mode of genome multiplication and partitioning, these cells survive in a foreign host, to multiply and cause disease or remain dormant for long periods of time, followed by active growth. Absence of known regulatory mechanisms coupled to a unique form of cell division and propagation makes the events leading to formation of *Entamoeba* daughter cells an interesting and challenging study. This chapter summarizes our recent attempts in understanding the cell division process of *Entamoeba histolytica*.

16.1 Introduction

Most eukaryotic cells have stringent regulatory mechanisms to coordinate DNA duplication with chromosome segregation followed by cytokinesis so that daughter cells are formed with exact copies of their parental genetic material. In mitotic cells, the genome is copied just once during the S phase [1]. DNA-repair mechanisms

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ensure that errors generated during synthesis are corrected. The cell then prepares to segregate the two copies of the genome (M phase). Duplicate copies of the genome are aligned on a bipolar microtubular spindle and separated into two daughter cells [2].

Our studies during the past two decades showed that this paradigm was not followed in trophozoites of *Entamoeba* spp. We focused most of our studies on *Entamoeba histolytica* HMI:IMSS strain and also compared the data with other strains such as 200:NIH and more recent isolates that were maintained in axenic culture [3]. Analyzing the progression of the cell cycle in *E. histolytica* posed several challenges because routine microbiological methods and genetic manipulations could not be carried out in this organism. Thus, our understanding of the cell-cycle processes has largely relied on the use of multiparameter flow cytometry, immunofluorescence, scanning cytometry, and other molecular biological techniques.

16.1.1 The Axenic Cell Cycle of *E. histolytica*

In contrast to other eukaryotic cells, the different phases of the cell division cycle (G_1 , S, G_2/M) were not clearly demarcated in flow cytometric analysis of the axenic cultures of *E. histolytica* [4, 5]. Mathematical modeling analyzed the G_1 , S, and G_2/M phases of the *E. histolytica* cell cycle as a series of overlapping Gaussian curves, differing from the discrete peaks of these phases in other organisms (Fig. 16.1a, b) [4]. These results suggested that *E. histolytica* cultures were made up of cells containing heterogeneous amounts of DNA. Cells with polyploid nuclei were found in axenically growing populations of HMI:IMSS cells (Fig. 16.1c). Thus, heterogeneity of DNA content could be caused by differences in nuclear DNA content or the number of nuclei or both. The average DNA content of cells and the number of cells with DNA content $>2n$ was found to increase with time in axenic culture [6], which indicates that *E. histolytica* trophozoites possibly reduplicated their genome several times without nuclear division or cytokinesis. We confirmed that this heterogeneity is not specific to the laboratory strain *E. histolytica* HMI:IMSS and that other isolates of *E. histolytica* showed a similar phenotype [3]. Thus, typical checkpoint mechanisms that ensure the progression of chromosome segregation and cytokinesis (immediately after the completion of genome duplication) were either absent or altered in this parasite.

It has been difficult to synchronize *E. histolytica* cells in any one phase of the cell cycle. Mitotic blockers such as colchicine, nocodazole, and thiabendazole were not active against *E. histolytica* cells. Our best results were obtained by using serum starvation for 12 h followed by addition of serum [5, 6]. During serum starvation, cells were arrested at different phases. Using this method in our subsequent studies we could obtain synchronization for one mitotic cycle [7, 8]. Using BrdU (5'-bromo-2'-deoxyuridine) incorporation, we showed that after addition of serum, DNA synthesis was initiated after a lag phase of 2 h. More than 80 % of the cells showed a

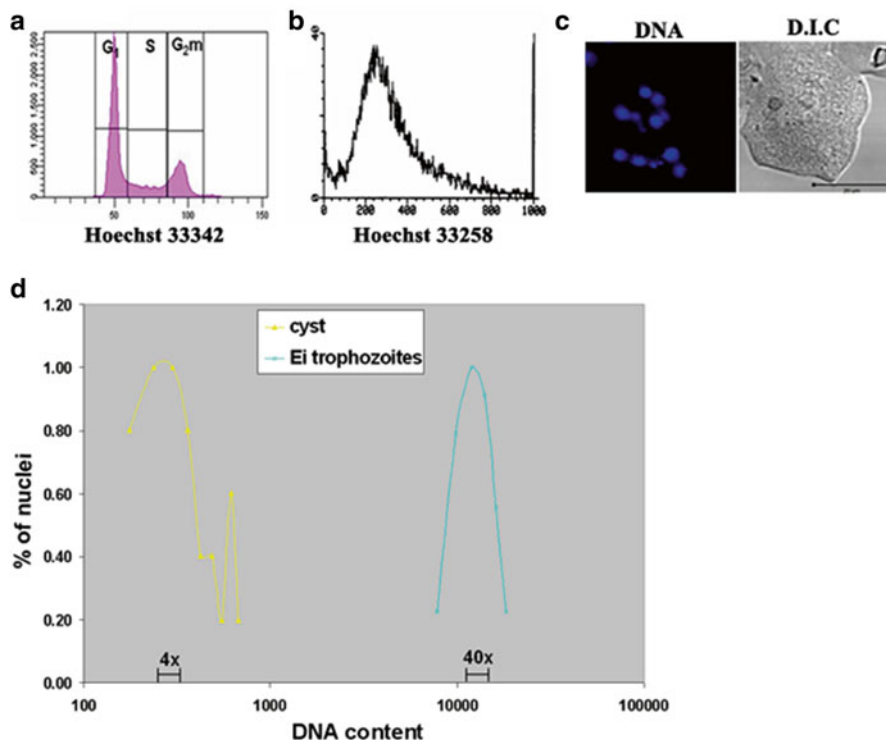


Fig. 16.1 Comparison of the cell-cycle phases as analyzed in a flow cytometer. **a** Flow cytometric analysis of a typical eukaryotic cell cycle (adapted from Louise Russel, Cutaneous Research, Institute of Cell and Molecular Science, Queens Mary College, London) showing distinct G₁, S, and G₂/M phases. **b** Flow cytometric analysis of the *Entamoeba histolytica* cell cycle (adapted from Gangopadhyay et al. [5]) shows the absence of discrete cell-cycle phases. **c** A multinucleated *E. histolytica* cell in an axenically growing culture. DNA has been stained with DAPI. Bar 20 μ M. **d** The DNA content of *Entamoeba invadens* cyst and trophozoites (~40 Mb) shown in the logarithmic scale. 4x and 40x indicate the average genome content corresponding to the major peaks in each cell type [3]

uniform DNA content at this time [6]. Cells continued to accumulate multiple genome contents subsequently in the next several hours [3, 6]. These data were confirmed both by BrdU incorporation and by scanning cytometry, clearly showing that nuclear and cell division are not temporally linked to genome duplication and segregation. In addition, the number of duplication cycles was not fixed as the nuclei accumulated heterogeneous amounts of DNA [3, 6]. Thus, genome segregation did not necessarily occur immediately after genome duplication. In serum synchronized cells, we observed that the number of microtubular assemblies increased 4 h after addition of serum and were highest after 8 h of serum addition [8]. The number of binucleated cells was highest at 10 h after addition of serum. These observations

suggested that the majority of cells in this synchronized population underwent several rounds of genome multiplication followed by chromosome segregation, possibly between 4 and 8 h after initiation of DNA synthesis, followed by nuclear division between 8 and 10 h [8].

16.2 The *E. histolytica* Genome Segregates on Atypical Microtubular Assemblies

Chromosome segregation is carried out on the mitotic spindle in eukaryotic cells. The sister chromatids are pulled apart on these spindle fibers: complete sets of chromosomes are moved to each pole of the cell where they are packaged into daughter nuclei. The mitotic spindle is composed of dynamic microtubular fibers that are polymers of α - and β -tubulin subunits. These subunits are nucleated at the microtubule organizing center (MTOC) [9]. One of the key proteins of the MTOC is the γ -tubulin that regulates the nucleation of microtubules in higher eukaryotes [10]. The homologues of α -, β -, and γ -proteins have been identified in *E. histolytica* [11–13]. The amino acid sequences of Eh $\alpha\beta\gamma$ -tubulins are significantly divergent from their eukaryotic homologues, and this difference has been attributed to their resistance to antimitotic drugs such as colchicines and benomyl [11, 13], although sensitivity to the microtubule stabilizing drug, taxol was predicted from the conserved taxol-binding site in the modeled tertiary structure of Eh γ tubulin [14].

Despite the presence of essential spindle and MTOC forming proteins i.e alpha, beta and gamma subunits [12, 15], metaphase-like equatorial alignment of condensed chromosomes could not be identified in *E. histolytica* cells [16, 17]. Anaphase and telophase were identified on the basis of nuclear shape [18]. Moreover, nuclear microtubular assemblies with fibers radiating from a central region in most *E. histolytica* cells were shown by indirect immunofluorescence [15].

In one of our studies, serum synchronized cells were fixed and stained with anti- β tubulin antibody, and a time-course study was done to observe the microtubular structures in *E. histolytica* cells. Several novel microtubular (MT) assemblies including monopolar, bipolar, and multipolar spindles for the segregation of chromosomal DNA were identified [8]. These unusual MT structures suggested that genome segregation occurred on different kinds of MT structures in *E. histolytica* cells, likely required for the heterogeneous number of genome copies in a single nucleus. A model was based on these observations (Fig. 16.2) from Mukherjee et al. [8]. This model highlights the possible modes of genome segregation on MT assemblies ranging from radial, bipolar, or fan-shaped structures to bundles of multiple MTs in assorted shapes. Real-time data with fluorescence-tagged MTs to validate this exciting hypothesis remains to be validated with live cell imaging using fluorescently labelled microtubules.

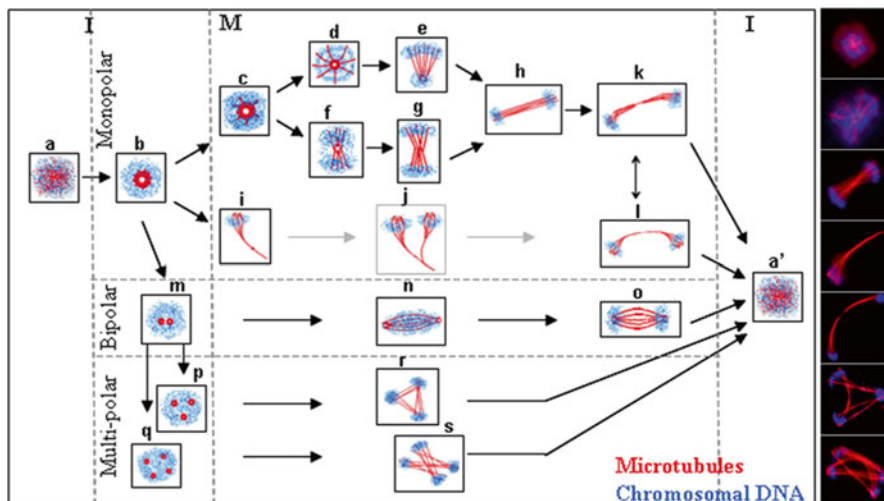
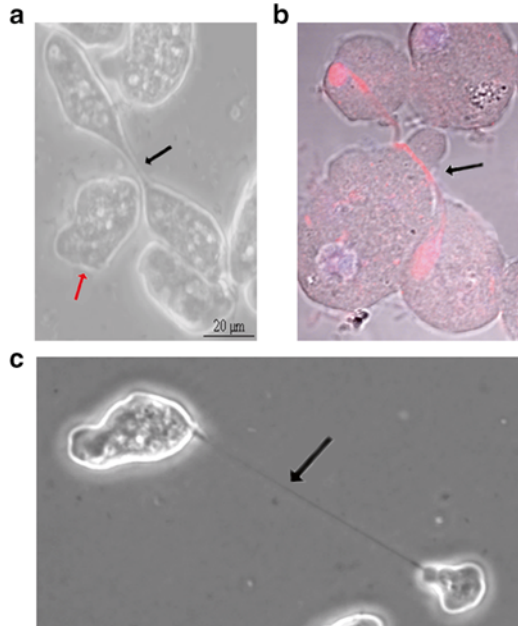


Fig. 16.2 Model showing chromosome segregation on different types of microtubular assemblies seen in *Entamoeba histolytica*. The schematic diagrams are adapted from the microscopic images shown alongside (on right) [8]. The cells were stained with anti-beta tubulin antibody and DAPI for staining the microtubules and DNA, respectively. Images were taken in a Zeiss LSM 510 META confocal microscope under 63× oil objective. The different MT assemblies suggest multiple modes of genome segregation

16.3 Cytokinesis in *Entamoeba histolytica*

Cytokinesis is the process of physical separation of a mother cell that gives rise to two daughter cells. One of the earliest modes of cell division used might have been the motility and consequent mechanical force driven by actin polymerization in a polymorphic cell [19]. Cytokinesis in *E. histolytica* can occur in three different ways. In the first mode, the intercellular bridge between dividing *E. histolytica* trophozoites is formed at random sites. The extension and rupture of this cytoplasmic bridge leads to formation of two daughter cells. In the second mode of cytokinesis the severing of the intercellular bridge is assisted by helper cells. In such cases the helper cells migrate to the intercellular bridge and rupture the bridge either mechanically or by unidentified mechanisms. Helper cell-assisted cytokinesis was estimated to occur in 45 % of the cases (Fig. 16.3a). Interestingly, the microtubular structure was found to extend through the entire length of the intercellular bridge (Fig. 16.3b). Finally, failure of cytokinesis is quite common where (Fig. 16.3c) the two halves of a cell are separated by an extremely long bridge that ultimately failed to separate and cytokinesis was aborted in approximately 20 % of the cells [8]. This event may well lead to cells with multiple nuclei in which the mitotic cycle continues for many rounds without successful cell division. Unequal or aberrant

Fig. 16.3 Various events leading to cytokinesis in *Entamoeba histolytica*. **a** Helper cell (red arrow) approaches the intercellular bridge (black arrow) between two daughter *E. histolytica* cells. **b** The microtubular assembly stretches across the intercellular bridge, a part of which was seen on top of a helper cell as the latter moves beneath the bridge. The cells were stained with anti- β -tubulin antibody and DAPI to visualize microtubules and DNA, respectively. **c** A still from live-cell imaging of cytokinesis in *E. histolytica* cells shows a long and thin intercellular bridge (black arrow) connecting the two daughter cells



division was frequent and gave rise to “anucleate” cells [8]. Altogether, the data suggested that the heterogeneous modes of cytokinesis contributed to the genetic heterogeneity in the population of *E. histolytica* cells.

16.4 Checkpoint Genes Are Absent in *Entamoeba histolytica*

The regulation of a typical eukaryotic cell division cycle depends upon a set of proteins known as the “checkpoint” proteins [20–22]. Cell-cycle progression is closely monitored by these checkpoint proteins and occurs only if the preceding phase has been completed correctly. Analysis of the *E. histolytica* genome shows that most of the conserved checkpoint proteins are absent. A large amount of information regarding the cell-cycle regulation in eukaryotes has been obtained from genetic and biochemical studies on the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* [23]. Additionally, it was seen that proteins controlling the cell cycle in yeasts were well conserved in other eukaryotes [24]. The complete genome sequence of *E. histolytica*, published in 2005, consists of about 8,201 genes with an average size of 1.17 kb. No homologues could be identified for one-third of the predicted proteins (32 %) from public databases. Even before the completion of the genome sequence few cell-cycle-related genes had been identified, such as p³⁴Cdc2 [25], Mcm2-3-5 [26], α -, β -, and γ -tubulin [11–13], and Diaphanous 1 [27] in *E. histolytica*. An in silico analysis of the *E. histolytica* genome for homologues of cell-cycle genes in *S. cerevisiae* was conducted for a better understanding of the amoeba cell cycle.

1. Genes involved in DNA replication initiation and entry into S phase.

Replication initiation in eukaryotes is characterized by the formation of a pre-replication complex (Pre-RC) with subsequent firing of the replication origins. In *S. cerevisiae* Orc1-6 (origin recognition complex) proteins bind at the pre-RC site and subsequently Cdc6, Cdt1, and Mcm2-7 are sequentially recruited at the site to form the pre-RC [28]. The helicase complex of Mcm2-7 remains inactive until it is activated by Cdc45 protein. The kinases Cdc7 and Cdk2 are involved in the loading of Cdc45 on origins. Cdc7 phosphorylates the subunits of Mcm2-7, which, through an unknown mechanism, changes the conformation of Mcm2-7 to facilitate the loading of subsequent factors such as Mcm10 and Cdc45. Our studies showed that sequence homologues of several proteins required for DNA replication initiation such as Mcm2-7, Cdc45, and subunits of GINS complex were present in the *E. histolytica* genome (Table 16.1). Except for a single protein that shared homology with both Cdc6 and Orc1p, none of the Orc2–6 proteins were identified in this organism. Studies with Eh Cdc6/Orc1 are currently in progress to understand the role of this protein in regulating DNA synthesis initiation.

2. G₁–S-phase checkpoint proteins in *E. histolytica*.

Regulation of DNA replication in *S. cerevisiae* involves four checkpoint proteins: Mec1, Mrc1, Tof1, and Dpb11. No homologs of these 4 proteins are found in *E. histolytica*. Several crucial genes required for the G₁–S transitions in

Table 16.1 *Entamoeba histolytica* genes encoding homologues of *Saccharomyces cerevisiae* genes involved in DNA replication initiation

Bait protein (length in aa)	Function	GenBank Accession number of <i>E. histolytica</i> homologue (length in aa)	AmoebaDB Accession number of <i>E. histolytica</i> homologue (length in aa)
MCM2 (868)	Replication helicase	XP_656059 (883)	EHI_117970
MCM3 (972)	Replication helicase	XP_653372 (601)	EHI_103600
MCM4 (934)	Replication helicase	XP_649080 (608)	EHI_187720
MCM5 (775)	Replication helicase	XP_648784 (639)	EHI_069980
MCM6 (1,018)	Replication helicase	XP_654108 (682)	EHI_118870
MCM7 (846)	Replication helicase	XP_650807 (690)	EHI_158110
Cdc28 (298)	Cdk2 like kinase	XP_652398 (291)	EHI_065280
Pol I (1,468)	DNA pol I	XP_657373 (1,132)	EHI_151520
Cdc45 (651)	Recruited to pre-RC	XP_657172 (543)	EHI_049900
Psf1 (208)	Subunit of GINS	XP_652581 (189)	EHI_136990
Psf2 (213)	Subunit of GINS	XP_655297 (199)	EHI_069340
Psf3 (194)	Subunit of GINS	XP_648782 (216)	EHI_069960

Genes sought but not found: MCM1, MCM10, Cdc7, DBF4, Orc2, Orc3, Orc4, Orc5, Orc6, Cdt1, geminin, DNA pol ϵ

Sequence homologues of cell-cycle proteins in *E. histolytica* genome were identified by BLAST using cell-cycle genes from *Saccharomyces* Genome Database (SGD) [<http://www.yeastgenome.org/>]. The amoeba sequence homologues identified in this search were reconfirmed in nr BLASTP searches (www.ncbi.nlm.gov/BLAST/). The hits were considered significant if the maximum “*e*” value obtained was 10⁻⁵ or less

Table 16.2 *E. histolytica* genes encoding homologues of *S. cerevisiae* checkpoint genes for G₁-S phase transition

Bait protein (length in aa)	Function	GenBank Accession number of <i>E. histolytica</i> homologue (length in aa)	AmoebaDB Accession number of <i>E. histolytica</i> homologue (length in aa)
Cdc28 (298)	CDK	XP_652398 (291)	EHI_065280
Skp1 (195)	Part of SCF ubiquitin ligase complex	XP_654128 (162)	EHI_118670
Cdc34 (295)	Ubiquitin-conjugating enzyme	XP_652735 (165)	EHI_048700
Cln1 (547)	G (sub)1 cyclin	XP_651788 (311)	EHI_121830
Cln2 (545)	Cyclin, role in START	XP_648667 (307)	EHI_030880
RecQL4 helicase (1,208)	Role in cell cycle START	XP_653505 (509)	EHI_023090

Sequence homologues of cell-cycle proteins in *E. histolytica* genome were identified by BLAST using cell-cycle genes from *Saccharomyces* Genome Database (SGD) [<http://www.yeastgenome.org/>]. The amoeba sequence homologues identified in this search were reconfirmed in nr BLASTP searches (www.ncbi.nlm.gov/BLAST/). The hits were considered significant if the maximum “*e*” value obtained was 10⁻⁵ or less

yeast and humans such as the homologues of p21, p27, p53, and retinoblastoma (RB) genes are absent in the genome of *E. histolytica*. The conserved intra-S-phase checkpoint proteins Chk1 and Chk2 (which act downstream of ATM and ATR kinases) are also absent in the *E. histolytica* genome; however, a homologue of human Chk2 has been identified in this organism [29] (Table 16.2).

3. Chromosome segregation and spindle checkpoint proteins in *E. histolytica*.

Chromosome segregation occurs in the M phase and is the process of separating the two sister chromatids formed as a result of DNA replication in the S phase. The key players regulating this process are the kinetochore proteins. An assembly of the kinetochore proteins is organized around the centromeric nucleosomes. The outer, central, and inner kinetochore assemblies form the bridge between DNA and microtubule in budding yeast [30]. Strikingly, except five kinesin-like proteins (Eh KlpA1–5), no other components of the kinetochore are found in *E. histolytica* (Table 16.3). One of these Eh Klps (Eh KlpA1/A2) is homologous to CENP-A. Eh Klp5, a BimC kinesin homologue, was localized with both radial and bipolar spindle assemblies and required for regulating genome content in *E. histolytica* [31]. However, the absence of other sequence homologues required for kinetochore formation suggests a typical kinetochore may not be present in *E. histolytica*.

4. Several G₂/M or spindle checkpoint proteins are absent from *E. histolytica*.

Spindle checkpoint proteins are activated when there is a perturbation in the alignment of chromosomes to the spindle. Monopolar, bipolar, and multipolar spindles have been observed in *E. histolytica* [8, 32]. However, the mechanism of chromosome alignment and partitioning on either of these spindles is not well

Table 16.3 *E. histolytica* genes encoding homologues of *S. cerevisiae* genes required for chromosome segregation

Bait protein (length in aa)	Function	GenBank Accession number of <i>E. histolytica</i> homologue (length in aa)	Amoeba DB Accession number of <i>E. histolytica</i> homologue (length in aa)
Cse4 (230)	Hs CENP-A homologue	XP_653386 (147)	EHI_051840
HRR25 (495)	Protein kinase	XP_657385 (335)	EHI_151950
Smc2 (1,171)	Component of the condensin complex	XP_657185 (1,151)	EHI_049770
Ipl1 (368)	Aurora kinase	XP_649507 (317)	EHI_193840
Tub1 (448)	α -tubulin	XP_653419 (455)	EHI_005950
Tub2 (458)	β -tubulin	XP_657170 (459)	EHI_049920
Mck1 (376)	Ser/Thr kinase	XP_657520 (370)	EHI_148220
Apc11 (166)	Catalytic core of APC	XP_651657 (87)	EHI_135110
Cin8 (1,001)	Kinesin motor protein	XP_649446 (863)	EHI_124890
Smc4 (1,449)	Condensin subunit	XP_654748 (1,226)	EHI_199700
Kin3 (436)	Protein kinase	XP_648115 (484)	EHI_048410
Cdc20 (611)	Cell-cycle regulated activator of APC	XP_657064 (377)	EHI_051010
Smc1 (1,226)	Chromosome segregation	XP_656581 (1,197)	EHI_050790
Cdc16 (840)	Component of APC	XP_656055 (497)	EHI_118010
Esp1 (1,631)	Separase, cysteine protease, sister chromatid separation	XP_651118 (1,450)	EHI_120330

Genes sought but not found: Bir1, Cbf1, Cbf2, Chl4, Ctf19, Ctf3, Ctf13, Cep3, Ctr9, Dsn1, Dam1, MCM16, MCM21, MCM22, MTw1, NNF1-2, APC1-9, Brn1, Bur2, Cik1, Cse2, Doc1, Dyn1, Pds1, Src1

Sequence homologues of cell-cycle proteins in *E. histolytica* genome were identified by BLAST using cell-cycle genes from *Saccharomyces* Genome Database (SGD) [<http://www.yeastgenome.org/>]. The amoeba sequence homologues identified in this search were reconfirmed in nr BLASTP searches (www.ncbi.nlm.gov/BLAST/). The hits were considered significant if the maximum “*e*” value obtained was 10^{-5} or less

understood. Sequence analysis shows absence of several spindle checkpoint proteins. Interestingly, homologues of two checkpoint proteins, Bub2, which blocks the mitotic exit by inhibiting Cdc14 in response to checkpoint activation, and Mps2, a dual specificity protein kinase required for spindle pole body duplication and spindle checkpoint activation, were found to be present in *E. histolytica* (Tables 16.4, 16.5, and 16.6). *E. histolytica* accumulates multinucleated cells, suggesting uncoupling of nuclear and cell division. Sequence analyses revealed that the homologues of all proteins required for cytokinesis in budding yeast are present in this organism (Table 16.7).

Table 16.4 *E. histolytica* genes encoding homologs of *S. cerevisiae* checkpoint genes required for spindle formation

Bait protein (length in aa)	Function	GenBank Accession number of <i>E. histolytica</i> homologue (length in aa)	AmoebaDB Accession number of <i>E. histolytica</i> homologue (length in aa)
Glc7 (312)	Mitosis	XP_651611 (302)	EHI_176170
Pph21 (370)	Catalytic subunit of protein phosphatase	XP_656214 (309)	EHI_011950
Cdc55 (527)	Protein phosphatase	XP_657525 (440)	EHI_148170
Mps1 (764)	Dual-specificity kinase, spindle pole body duplication	XP_653959 (352)	EHI_073650
Bub2 (307)	Spindle checkpoint protein	XP_649751 (289)	EHI_135170
Tpd3 (635)	Regulatory subunit A of the heterotrimeric protein phosphatase 2A, role in mitosis	XP_649444 (549)	EHI_124870

Genes sought but not found: Mad1-3, Bub1, Bub3, Cep3, Dma1, Bim1, Gac1

Sequence homologues of cell-cycle proteins in *E. histolytica* genome were identified by BLAST using cell-cycle genes from *Saccharomyces* Genome Database (SGD) [<http://www.yeastgenome.org/>]. The amoeba sequence homologues identified in this search were reconfirmed in nr BLASTP searches (www.ncbi.nlm.gov/BLAST/). The hits were considered significant if the maximum “*e*” value obtained was 10^{-5} or less

Table 16.5 *E. histolytica* genes encoding homologues of *S. cerevisiae* genes involved in mitosis

Bait protein (length in aa)	Function	GenBank Accession number of <i>E. histolytica</i> homologue (length in aa)	AmoebaDB Accession number of <i>E. histolytica</i> homologue (length in aa)
Kip3 (806)	Kinesin-related protein, mitotic spindle positioning	XP_656748 (629)	EHI_140230
HRR25 (495)	Protein kinase, DNA repair, chromosome segregation	XP_657385 (335)	EHI_151950
BNI1 (1,954)	Formin	XP_653884 (1,212)	EHI_192460
SAC3 (1,302)	Transcription, mRNA export from nucleus	XP_655223 (670)	EHI_136450
TOR1 (2,471)	Cell-cycle signaling, meiosis	XP_648644 (2,416)	EHI_104570
Ark1 (638)	Control of endocytosis	XP_652314 (519)	EHI_127410

Genes sought but not found: Cik1, Dma2, Dad3, Dad4, Dyn1

Sequence homologues of cell-cycle proteins in *E. histolytica* genome were identified by BLAST using cell-cycle genes from *Saccharomyces* Genome Database (SGD) [<http://www.yeastgenome.org/>]. The amoeba sequence homologues identified in this search were reconfirmed in nr BLASTP searches (www.ncbi.nlm.gov/BLAST/). The hits were considered significant if the maximum “*e*” value obtained was 10^{-5} or less

Table 16.6 *E. histolytica* genes encoding homologues of *S. cerevisiae* genes involved in exit from mitosis

Bait protein (length in aa)	Function	GenBank Accession number of <i>E. histolytica</i> homologue (length in aa)	AmoebaDB Accession number of <i>E. histolytica</i> homologue (length in aa)
Tem1 (246)	Termination of M-phase	XP_657549 (189)	EHI_027640
Cdc15 (975)	Promotes mitotic exit	XP_654672 (1,760)	EHI_009590
Mob1 (314)	Transcriptional regulator	XP_650241 (211)	EHI_159570
Lte1 (1,435)	Putative GDP/GTP exchange factor	XP_651306 (491)	EHI_139450

Genes sought but not found: Spo12, Net1, Cdc14, Rio1

Sequence homologues of cell-cycle proteins in *E. histolytica* genome were identified by BLAST using cell-cycle genes from *Saccharomyces* Genome Database (SGD) [<http://www.yeastgenome.org/>]. The amoeba sequence homologues identified in this search were reconfirmed in nr BLASTP searches (www.ncbi.nlm.gov/BLAST/). The hits were considered significant if the maximum “*e*” value obtained was 10^{-5} or less

Table 16.7 *E. histolytica* genes encoding homologues of *S. cerevisiae* genes involved in cytokinesis

Bait protein (length in aa)	Function	GenBank Accession number of <i>E. histolytica</i> homologue (length in aa)	AmoebaDB Accession number of <i>E. histolytica</i> homologue (length in aa)
Actin (375)	Cytoskeletal functions	XP_648054 (376)	EHI_107290
Ark1 (638)	Ser/Thr kinase	XP_652314 (519)	EHI_127410
Bir1 (954)	Baculovirus inhibitor of apoptosis repeat	XP_657286 (1,387)	EHI_093850
Cdc15 (975)	Protein kinase of the mitotic exit network	XP_654672 (1,760)	EHI_009590
Chs2 (964)	Chitin synthase II	XP_651026 (642)	EHI_170480
Cla4 (843)	Involved in localizing cell growth	XP_657512 (467)	EHI_148240
Exo70 (623)	70-kDa subunit of the exocyst complex	XP_650775 (765)	EHI_142040
Ipl1 (368)	Aurora kinase	XP_649507 (317)	EHI_193840

Sequence homologues of cell-cycle proteins in *E. histolytica* genome were identified by BLAST using cell-cycle genes from *Saccharomyces* Genome Database (SGD) [<http://www.yeastgenome.org/>]. The amoeba sequence homologues identified in this search were reconfirmed in nr BLASTP searches (www.ncbi.nlm.gov/BLAST/). The hits were considered significant if the maximum “*e*” value obtained was 10^{-5} or less

16.5 Novel Proteins That Regulate the Cell Cycle of *Entamoeba histolytica*

Analysis of the genome of *E. histolytica* showed that a large number of proteins involved in the cell division process of this parasite were either absent or significantly divergent. Given the fact that a large number of proteins encoded by the parasite are hypothetical opens up a possibility the *E. histolytica* may contain novel regulatory mechanisms to ensure that the cell cycle proceeds even in the absence of the conventional regulatory proteins. Functional analysis of a kinesin-like protein, *E. histolytica* Klp5 (EhKlp5), showed that increased expression of this protein, whereas promoting microtubular spindles leads to homogenization of the average DNA content in growing cells [31]. In addition, EhKlp2 was also found to alter the frequency of bipolar spindles and genome content in this parasite. EhKlp2-4 were found to associate with both microtubules and actin cytoskeletal networks although none of these proteins has any actin-binding domains [33]. Yeast two-hybrid analysis with the nonmotor domains of these proteins identified several actin-binding proteins as interactors (Grewal and Lohia, unpublished observations). This observation suggests that these kinesin proteins might associate with the actin cytoskeleton with the help of these interactors and might be involved in regulating motility and cell division.

A novel group of formin proteins was also discovered in *E. histolytica* of which EhFormin-1 and -2 led to delay in cell division [34]. EhFormins-5 and -8 were phylogenetically distinct from their other eukaryotic counterparts [34]. The characteristic FH1 domain, which is crucial in initiating actin polymerization, is absent in these formin proteins. An InterProScan (EMBL-EBI) of EhFormin-5 and -8 identified a GTPase-binding domain at the N-terminal of these proteins, indicating that this region was unique and may respond to unknown signaling mechanisms or protein networks that subsequently may effect actin remodeling. EhFormin-5 was a nucleocytoplasmic protein, which localized as a ring inside the nucleus of *E. histolytica*. Furthermore, EhFormin-8 also colocalized on microtubular structures, suggesting that both these proteins might be involved in the regulation of essential nuclear function such as assembly and disassembly of microtubules. Ectopically expressed EhFormin-5 and -8 decreased the genome content in the stable transformants, suggesting that both these proteins are involved in the process of chromosome segregation. To identify the downstream targets of EhFormin-5 and -8, the N-terminal regions of these two formins used as a bait to screen the Eh cDNA library by yeast-two-hybrid assay. EhFormin-5 interacted with a p21 Ras family GTPase, EhRas family GTPase, and five hypothetical proteins. The identification of EhRas family GTPase as an interactor of EhFormin-5 was a significant and novel finding, because, among the members of the p21 Ras family GTPases, only Rho and Rac proteins have been implicated in binding to formin proteins and assisting actin polymerization in other eukaryotic systems. EhFormin-8 interacted with a zinc-finger domain-containing protein, a helicase domain-containing protein, and two hypothetical proteins. Our data suggest that EhFormin proteins have evolved to perform cell cycle-specific functions in addition to their role as actin nucleators Grewal and

Lohia unpublished observations. Previous studies have shown that expression of a constitutively active mutant of RacG_{Eh} (RacG^{Gly12Val}_{Eh}), a dominant-negative mutant of RabA, and the kinase domain of PAK2_{Eh} led to cytokinetic defects and consequently to the accumulation of multinucleated cells [35–37]. Taken together, these observations suggest that cell division in *E. histolytica* requires the combined activity of several signaling molecules and proteins.

Calcium signaling plays a major role in the cell cycle of eukaryotes. *E. histolytica* encodes a large repertoire of novel multi-EF hand CaBPs [38]. The calcium-binding proteins characterized thus far have been implicated in having a crucial role in phagocytosis and cell proliferation [39, 40]. EhCaBP6 on the other hand was found to localize at the end of microtubular structures and on the intracellular bridge during cytokinesis [41]. Immunolocalization data suggest that EhCaBP6 is functionally similar to mitotic CaM proteins in other organisms. A large number of cellular processes are regulated by protein–protein interactions. Most proteins require physical interactions with other proteins to execute their biological function. In most eukaryotic cells, calmodulin initiates various signaling cascades by binding to target proteins. Immunofluorescence studies show that EhCaBP6 in mitotic cells may be interacting with the microtubules or with microtubule-associated proteins. In an effort to identify the proteins interacting with EhCaBP6, the latter was used as the bait in a yeast two-hybrid genetic screen against the *E. histolytica* cDNA library. Among the interactors obtained from the screen EhCaBP6 was also found to interact with a zinc-finger domain protein and ribosomal protein P2. The zinc-finger protein obtained in this screen was the same as obtained as an interactor for EhFormin-8. The interaction of both EhFormin-8 and CaBP6 with a zinc domain protein suggests that EhFormin-8 and EhCaBP6 are part of the same protein complex that affects genome segregation in *E. histolytica*. Thus, novel proteins such as EhFormins, kinesins, and EhCaBP6 have evolved to regulate crucial processes of microtubule assembly and chromosome segregation in the protozoan parasite *E. histolytica*.

16.6 Genome Content of Cysts and Trophozoites

Axenic cultures of *E. histolytica* have been used since 1961 after the introduction of the TYI-S-33 medium and are indispensable for molecular biology and cell biology studies of this parasite. It was observed that heterogeneity of DNA content was a common feature of all the *E. histolytica* strains growing under axenic conditions. *E. histolytica* normally grows in the presence of the microbial flora of the large intestine. We compared the DNA content of two recent isolates, *E. histolytica* 2592100 and DS4-868, that could be cultured both under xenic and axenic growth conditions. *E. histolytica* HM1:IMSS has been cultured axenically for more than 40 years and is difficult to revert to xenic culture. The nuclear DNA content of both strains was found to be tenfold lower when grown under xenic conditions compared the corresponding axenically grown cultures. Furthermore, a comparison of the size

of the nuclei of these two populations of *E. histolytica* revealed that the nuclear size in the xenically growing *E. histolytica* cells was less than that of axenically growing cells. Additionally, the number of multinucleated cells was also greater in the axenic cultures [3]. These results were corroborated by histological sections obtained from patient's large intestine [3]. In addition to xenic and axenic growth we compared the DNA content of trophozoites and cysts. *Entamoeba* cells are found in two major forms in nature, that is, cysts and trophozoites. Because in vitro encystation is difficult for *E. histolytica*, we analyzed the genome content of the cyst and trophozoites in *E. invadens*. During excystation a cyst gives rise to single amoeba. Scanning cytometry showed that excysted trophozoites had 40 times more DNA content than the cyst nucleus (Fig. 16.1d). Molecular data [3] supported our interpretation that several rounds of whole genome duplication occur during the conversion of a cyst into a viable trophozoite.

16.7 Concluding Remarks

Taken together, the data suggest that the *Entamoeba* genome is able to lose or gain multiple copies that are partitioned into cysts or daughter cells which are actively multiplying. Dynamic plasticity of the genome allows the cells to adapt to different growth conditions both inside and outside the human host. Absence of conserved regulatory mechanisms is therefore a likely necessity for these parasites to constantly adapt to challenging environmental changes. Over and above all these mechanistic differences, genome duplication, segregation, cell division, and conversion to different forms of the protist are unique among all eukaryotes, reminding us again that there is always a deviation from paradigms and rules.

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