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New Insights into the Molecular Mechanisms of Mitosis and Cytokinesis in Trypanosomes

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Abstract

Trypanosoma brucei, a unicellular eukaryote and the causative agent of human sleeping sickness, possesses multiple single-copy organelles that all need to be duplicated and segregated during cell division. Trypanosomes undergo a closed mitosis in which the mitotic spindle is anchored on the nuclear envelope and connects the kinetochores made of novel protein components. Cytokinesis in trypanosomes is initiated from the anterior tip of the new flagellum attachment zone, and proceeds along the longitudinal axis without the involvement of the actomyosin contractile ring, the well-recognized cytokinesis machinery conserved from yeast to humans. Trypanosome appears to employ both evolutionarily conserved and trypanosome-specific proteins to regulate its cell cycle, and has evolved certain cell cycle regulatory pathways that are either distinct between its life cycle stages or different from its human host. Understanding the mechanisms of mitosis and cytokinesis in trypanosomes not only would shed novel light on the evolution of cell cycle control, but also could provide new drug targets for chemotherapy.

1. Introduction

Trypanosoma brucei spp. (comprising *Trypanosoma brucei brucei* and the human infective forms *T. b. rhodesiense* and *T. b. gambiense*) are early branching, flagellated protozoan parasites causing sleeping sickness in human and nagana in cattle in 36 countries of sub-Saharan Africa, where they impose devastating impacts on human health and agriculture. The current WHO estimate indicates that about 30,000 people are infected per year and over 70 million people are at risk of infection (<http://www.who.int/mediacentre/factsheets/fs259/en/>). This disease places a heavy financial burden on the people living in Africa. It can be cured with medication if diagnosed early; however, it is fatal if left untreated. Unfortunately, the currently available drugs all have severe side effects; therefore, development of new drugs is urgently needed for combating this dreadful pathogen.

Trypanosomes are transmitted between mammalian hosts by the tsetse fly, *Glossina* spp. Within the insect host, the parasite is initially established in the midgut of the fly after a healthy fly takes a bloodmeal from an infected mammalian host, and subsequently it migrates to the salivary glands of the fly, where it prepares for transmission to the new

mammalian host. Within the mammalian host, the parasite lives freely and proliferates by binary fission in the bloodstream since it is capable of escaping the host immune responses through antigenic variation (McCulloch, 2004). Antigenic variation involves the sequential expression of antigenically distinct variable surface glycoproteins (VSGs) that are linked to the membrane of the cell by a glycosyl phosphatidylinositol (GPI) anchor. Upon uptake by the tsetse fly, bloodstream trypanosomes lose their VSG coat but instead express a different surface coat composed of procyclins, which are also GPI anchored on the plasma membrane (Roditi and Liniger, 2002). Due to the expression of the procyclin surface proteins, the parasite that lives in the insect host is generally referred to as the procyclic form, which proliferates by binary fission in the midgut of the insect, similar to the bloodstream form of the parasite.

Most studies on trypanosome cell cycle control have been carried out in the procyclic and bloodstream forms of the parasite because these two forms can be easily cultured *in vitro* and many genetic tools are available for gene manipulation. For example, gene knockouts, gene replacements, and in situ epitope tagging can be readily generated through homologous recombination. Tetracycline-inducible ectopic overexpression and RNA interference (RNAi) have been routinely practiced in almost all the laboratories that use trypanosomes as the model system. Moreover, forward genetic approaches through the use of genomic RNAi library (Morris et al., 2002) and the mariner-based transposon mutagenesis (Leal et al., 2004) offer great prospects for screening numerous mutants. Finally, with the development of efficient tandem affinity purification (Schimanski et al., 2005), high-throughput genome-wide RNAi analysis (Alsford et al., 2011), and proteome-wide quantitative mass spectrometry (Urbaniak et al., 2012), trypanosome has become an excellent model organism for carrying out basic science research, including the understanding of the molecular mechanisms of mitosis and cytokinesis, which would potentially reveal novel regulatory pathways that could be good drug targets for chemotherapy.

2. Cell Structure Of *T. brucei*

It has been well established that the shape of a trypanosome cell is maintained by a subpellicular array of microtubules, which are cross-linked to the plasma membrane and to each other, forming a cage-like structure with all the single-copy organelles positioned at distinct locations within the cell (Fig. 4.1A). The single flagellum, which possesses an axoneme consisting of the canonical 9 + 2 array of microtubules and a large lattice-like structure termed paraflagellar rod (PFR), exits from the flagellar pocket at the posterior portion of the cell, attaches to the cell body along most of its length via a specialized cytoskeletal structure termed flagellum attachment zone (FAZ), and extends to the anterior tip of the cell (Fig. 4.1). The subpellicular microtubules all have an intrinsic polarity, with their plus (+) ends located at the posterior end of the cell, which is the opposite orientation to that of the flagellar axoneme microtubules, which have their plus (+) ends located at the anterior tip of the cell. During cell division, the subpellicular microtubules do not break down, and newly synthesized microtubules are integrated into the old microtubule array. Therefore, the microtubule corset appears to be inherited by the two daughter cells in a semiconservative manner (Sherwin and Gull, 1989).

The FAZ is composed of a single protein filament and a specialized set of four microtubules that associate with the smooth endoplasmic reticulum (Kohl et al., 1999). The complete protein composition of the FAZ filament is still not known, but a number of proteins have been localized to the FAZ filament, and RNAi of some of these FAZ proteins impairs the assembly of the new FAZ filament and causes defective cytokinesis (Morriswood et al., 2013; Vaughan et al., 2008; Zhou et al., 2011). The flagellar basal body, which functions as the microtubule organizing center (MTOC) in trypanosomes and nucleates the flagellar axoneme microtubules, is located at the posterior portion of the cell, where it is physically connected, through a so-called tripartite attachment complex (TAC) across the mitochondrial membrane (Ogbadoyi et al., 2003), with the kinetoplast, the cell's unusual mitochondrial DNA complex that is composed of several dozen maxicircles and several thousand minicircles topologically interlocked and condensed into a disk-shaped structure. Unlike fungi and animals, a trypanosome cell possesses a single mitochondrion, which extends throughout the cell body and whose fission is tightly coupled with cell abscission. Trypanosome also contains a single Golgi apparatus, which undergoes *de novo* synthesis and is segregated during the cell cycle (He et al., 2004). The Golgi apparatus associates tightly with an ER exit site (ERES), which also undergoes duplication and segregation during the cell cycle. Between the old Golgi apparatus and the newly assembled Golgi apparatus, there is a bilobed cytoskeletal structure marked by TbCentrin2, one of the five centrin proteins in trypanosomes (He et al., 2005), with one of its lobe associating with the old Golgi and the other lobe with the new, growing Golgi apparatus. The precise function of the bilobe structure is still not clear, but it appears to be essential for Golgi duplication. The relative locations of the single-copy organelles and cytoskeletal structures are depicted in Fig. 4.1B.

3. Cell Cycle Of *T. brucei*

3.1. Duplication and segregation of organelles and cytoskeletal structures

3.1.1 Basal body—To ensure successful cell division, all the single-copy organelles must be precisely replicated, maintained at specific locations, and faithfully segregated to the daughter cells. During trypanosome cell cycle, basal body is the first organelle to be duplicated, which constitutes the first cytoskeletal event of the cell cycle. Although the basal body structure has been well described, the existence of a second premature basal body (probasal body) is often overlooked, and due to the lack of a good basal body marker, the probasal body is frequently not identified experimentally. The most commonly used basal body marker, YL 1/2, is a monoclonal antibody raised against the tyrosinated α -tubulin in the basal body, which only labels the mature basal body (Fig. 4.2). Despite its limitation to stain only the mature basal body, YL 1/2 can be readily used to monitor the duplication and segregation of the basal bodies during the cell cycle. At the G1 phase, there is a single mature basal body per cell, which sits near the base of the old flagellum and associates with the single kinetoplast. At the S phase, the basal body duplicates, and the duplicated basal bodies are slightly separated but remain associated with the duplicated but not separated kinetoplasts. During G2 and the subsequent cell cycle phases, the duplicated basal bodies are well separated, leading to the segregation of the duplicated kinetoplasts (Fig. 4.2). Through electron microscopy and electron tomography techniques, Lacomble et al. (2010) described in detail the duplication and rotation of the probasal body and mature basal body. At the start

of the cell cycle, the probasal body is positioned anterior to the mature basal body of the old flagellum. During the transition from G1 to S phase, the probasal body becomes mature and elongates to form the transition zone and then the axoneme of the new flagellum. In the meantime, two new probasal bodies are formed in the vicinity of the two mature basal bodies. The new basal body then undergoes an anticlockwise rotation around the old basal body and repositions from the anterior to the posterior side of the old flagellum. The rotation and the subsequent migration of the new basal body toward the posterior portion of the cell result in the division of the flagellar pockets and appear to be required for cell morphogenesis (Lacomble et al., 2010).

As the only visible MTOC in trypanosomes, basal body is structurally similar to the centrosome in animals. Three key components of the centrosome, SAS-4/CPAP, SAS-6, and BLD10/CEP135, constitute the evolutionarily conserved and ancestral module (Carvalho-Santos et al., 2010). Trypanosomes appear to express the well-conserved SAS-4/CPAP and SAS-6 homologs and a divergent BLD10/CEP135 homolog (Carvalho-Santos et al., 2010), but whether these homologs are *bona fide* basal body proteins and whether they play any roles in basal body biogenesis remain to be determined. Other than these putative basal body components, a number of proteins have been localized to the basal body and are involved in basal body duplication and/or segregation. γ -Tubulin, a key component of the γ -tubulin complex located at the spindle pole body (SPB) of the budding yeast, was the first protein to be localized to the basal body in trypanosomes (Scott et al., 1997). The major function of γ -tubulin, however, is to nucleate the flagellum axoneme microtubules and is not involved in regulating basal body duplication or segregation (McKean et al., 2003). TBBC, which stands for *T. brucei* Basal Body Component, is a large protein consisting almost entirely of the coiled coil structure and is mainly localized to the mature basal body (Dilbeck et al., 1999). RNAi of TBBC compromises the connection of the new flagellum to the old flagellum (Absalon et al., 2007), although its potential role in basal body duplication/segregation remains to be explored. The mammalian ortholog of TBBC is not known, but anti-TBBC antibody detects a protein in HeLa cell extract and immunostaining of HeLa cells are confined to the centrosomes, suggesting the presence of a close homolog of TBBC in the mammalian centrosomes. A NIMA (Never-In-Mitosis gene A)-like kinase, TbNRKC, is localized to both the premature and mature basal bodies, and RNAi-mediated silencing of TbNRKC in procyclic trypanosomes results in overproduction of basal bodies that fail to segregate, suggesting a role of TbNRKC in basal body segregation (Pradel et al., 2006). In mammals, the Polo-like kinase 4 or SAK (Snk/Plk-akin kinase) regulates centrosome duplication (Habedanck et al., 2005). Trypanosomes express a single PLK homolog, TbPLK, which is initially enriched at the basal body and, surprisingly, is required for basal body segregation but not duplication, likely by regulating the rotation of the duplicated basal bodies (Ikeda and de Graffenried, 2012; Lozano-Nunez et al., 2013). Centrin, an EF hand-containing calcium-binding protein, constitutes another conserved component of the centrosome in animals. Trypanosomes express five centrin-like proteins, an unusually large number of such proteins in a unicellular organism (He et al., 2005). At least three of the five centrin homologs, TbCentrin1, TbCentrin2, and TbCentrin4, are found at the mature basal body, and RNAi-mediated knockdown of TbCentrin1 clearly inhibits basal body duplication (He et al., 2005). However, a role for TbCentrin2 and TbCentrin4 in

basal body duplication and/or segregation remains to be clarified due to the controversial observations made by different research groups (He et al., 2005; Selvapandiyan et al., 2007, 2012; Shi et al., 2008). The precise mechanism underlying basal body segregation is still not well understood, but it was suggested that posterior migration of the newly formed basal body is controlled by the elongation of the new flagellum and the new FAZ filament (Absalon et al., 2007).

3.1.2 Kinetoplast—Due to its tight association with the basal body (Fig. 4.2), kinetoplast segregation likely is controlled by the movement of the basal body toward the posterior portion (Robinson and Gull, 1991). Kinetoplast replication occurs prior to nuclear DNA replication and is well coordinated with the latter. It apparently occurs concurrently with the repositioning of the newly formed basal body (Gluenz et al., 2011). Regulation of kinetoplast replication is under tight control in a manner that is reminiscent of the nuclear DNA replication licensing system (Klingbeil and Shapiro, 2009; Li et al., 2008b; Liu et al., 2009). A mitochondrial HslVU protease, a eubacterial counterpart of the eukaryotic 26S proteasome, plays an essential role in preventing kinetoplast rereplication, thus maintaining the copy number of both the minicircle and maxicircle DNA in the kinetoplast (Li et al., 2008b). TbHslVU regulates a key mitochondrial helicase, TbPIF2, which maintains the copy number of the maxicircle DNA (Liu et al., 2009), but the TbHslVU substrate(s) involved in controlling minicircle DNA replication remains to be identified. Once kinetoplast replication is completed, the kinetoplast undergoes symmetrical division by an unknown mechanism, resulting in the production of two equal-sized kinetoplasts in cells that are generally at the G2 phase of the cell cycle (Fig. 4.2). Segregation of the duplicated kinetoplasts is mediated by the separation of the duplicated basal bodies (Robinson and Gull, 1991), due to their tight physical association mediated by the TAC (Ogbadoyi et al., 2003). Three proteins have been identified as components of the TAC, p166 (Zhao et al., 2008), AEP-1 (Ochsenreiter et al., 2008), and p197 (Gheiratmand et al., 2013), and depletion of these proteins by RNAi all causes defects in kinetoplast segregation, suggesting the essential requirement of the TAC for kinetoplast segregation.

3.1.3 Flagellum and FAZ—The new flagellum is assembled when the new basal body is matured (Fig. 4.2, S phase). The short, newly formed flagellum is located in the existing flagellar pocket, and the tip of the new flagellum is attached to the old flagellum through the flagellum connector, an unusual motile transmembrane junction (Briggs et al., 2004). When the new flagellum outgrows the flagellar pocket, the PFR forms, and the flagellum is physically attached to cell body through FAZ. Following cell cycle progression, the new flagellum elongates and the FAZ also elongates (Fig. 4.2). However, the assembly of the flagellar axoneme and the assembly of the FAZ filament do not appear to be interdependent. In trypanosome cells deficient in intraflagellar transport, the flagellar axoneme is not assembled, but a short flagellar membrane still forms, which extends out of the flagellar pocket and is attached to the cell body via a short FAZ (Absalon et al., 2008; Davidge et al., 2006; Kohl et al., 2003). Conversely, trypanosome mutants that fail to assemble the FAZ filament can still assemble a full-length flagellum with normal axoneme and PFR (Vaughan et al., 2008; Zhou et al., 2011). Despite the essential role of FAZ in flagellum attachment, precisely how the FAZ filament is assembled remains poorly understood. To date, at least

nine proteins have been found to localize to the FAZ filament, and RNAi knockdown of some FAZ proteins each results in defects in new FAZ assembly (Lacomble et al., 2012; LaCount et al., 2002; Morriswood et al., 2013; Oberholzer et al., 2011; Vaughan et al., 2008; Woods et al., 2013; Zhou et al., 2011), suggesting the complexity of the FAZ filament and the requirement of multiple proteins for FAZ filament assembly.

3.1.4 Bilobe structure—The bilobe is a cytoskeletal structure originally identified as an unknown structure labeled by the 20H5 antibody raised against the *Chlamydomonas reinhardtii* Centrin and by anti-trypanosome TbCentrin2 antibody (He et al., 2005). Like other single-copy organelles in trypanosomes, the bilobe structure also undergoes duplication and segregation during the cell cycle (Fig. 4.3). At the G1 phase, 20H5 marks a single bilobe structure (Fig. 4.3, arrow) in addition to the basal body (Fig. 4.3, arrowhead). At the S phase, the bilobe structure is replicated, but the duplicated bilobe structures are not separated. At later cell cycle stages during which the basal bodies are separated and the nuclei are segregated, the bilobe structures are also segregated (Fig. 4.3). More than 10 proteins have been shown to localize to the bilobe structure (He et al., 2005; Morriswood et al., 2009, 2013; Shi et al., 2008; Zhou et al., 2010), and by immuno-EM the ultrastructure of the bilobe structure was revealed, which appears like a hairpin structure connecting the flagellar pocket collar and the FAZ filament (Esson et al., 2012). The exact function of the bilobe structure is still not clear, but it appears to be required for Golgi duplication (He et al., 2005). The old Golgi associates with the posterior lobe of the bilobe structure and the new Golgi is associated with the anterior lobe of the bilobe structure (He et al., 2004, 2005). In TbCentrin2 RNAi cells, duplication of the bilobe structure is impaired, resulting in defective duplication of the Golgi apparatus (He et al., 2005).

3.1.5 Golgi apparatus—Golgi duplication in trypanosomes likely is through *de novo* biogenesis, in which new Golgi is assembled next to the new ERES (He et al., 2004). During the G1 phase of the cell cycle, the trypanosome cell possesses a single Golgi apparatus, labeled by anti-GRASP (Golgi reassembly and stacking protein) antibody, which closely associates with the ERES, marked by EYFP (enhanced yellow fluorescent protein)-tagged Sec31 (Fig. 4.4). At the S phase, a small new Golgi appears, and both the new and old Golgi still associate with the duplicated ERESs, and during later stages of the cell cycle, such as G2 phase, mitotic, and postmitotic phases, the duplicated Golgi are further separated and the associated ERESs also undergo separation (Fig. 4.4). The mechanisms underlying Golgi duplication and segregation are still poorly defined, but presumably the segregation of Golgi could be dependent on the elongation of the flagellum and the FAZ filament and the posterior migration of the new basal body. Such a mechanism appears to be involved in the segregation of bilobe structure, ERES, and kinetoplast as well, but this has not been experimentally confirmed.

3.1.6 Mitochondrion—Trypanosomes contain a single mitochondrion that extends throughout the cell body. Unlike the mitochondria in yeasts and animals which undergo frequent fission and fusion throughout the cell cycle, trypanosome mitochondrion only undergoes a single fission event during cytokinesis. Mitochondrial fission and fusion in yeast involves three factors, Fis1p, Dnm1p, and Mdv1p (Shaw and Nunnari, 2002). The

trypanosome genome encodes homologs of Fis1p and Dnm1p (Chanez et al., 2006; Morgan et al., 2004), but no homolog of Mdv1p was found, likely due to the divergence of the sequence. Ablation of the dynamin homolog by RNAi in the procyclic form of *T. brucei* blocks mitochondrial fission and results in precise cytokinesis arrest (Chanez et al., 2006; Morgan et al., 2004), suggesting the conserved role of dynamin in regulating mitochondrial fission in trypanosomes. The result also highlights the requirement of mitochondrial division for cell division. It also suggests that mitochondrion division in trypanosomes is well coordinated with cell division, but the underlying mechanism remains to be explored.

3.2. Repertoire of cyclins and cyclin-dependent kinases

Cyclins and cyclin-dependent kinases (CDKs) are the fundamental regulators of the eukaryotic cell cycle. Through periodic synthesis and degradation of the cyclin partner, the CDK is sequentially activated and inactivated (Johnson and Walker, 1999). In the budding yeast *Saccharomyces cerevisiae*, nine cyclins are identified, with three of them, Cln1–Cln3, regulating the G1/S transition and the rest of them, Clb1–Clb6, driving the cell cycle through S phase to mitosis. Strikingly, all the nine cyclins bind to the same CDK, Cdc28, and due to the cell cycle stage-specific expression of cyclins, Cdc28 is activated at different cell cycle stages (Kuntzel et al., 1996). In striking contrast, in the fission yeast *Schizosaccharomyces pombe* only a single cyclin, Cdc13, and a single CDK, Cdc2, are required for controlling cell cycle progression across the G1/S and G2/M transitions, respectively (Humphrey and Pearce, 2005). The cyclin and CDK system in animals is more complex than that in the budding and fission yeasts. To date, at least 8 cyclin families, consisting of a total of 15 cyclins, and at least 9 CDKs are directly involved in cell cycle control in animals (Harper and Brooks, 2005). These cyclins and CDKs form distinct complexes and are involved in different cell cycle stages. Control of the G1/S transition is regulated by Cdk4, Cdk6, and the D-type cyclins. Regulation of S-phase progression requires Cdk2 and the E-type cyclin, and regulation of the G2/M transition requires Cdk1, Cdk2, and their A-type and B-type cyclin partners (Harper and Brooks, 2005; Malumbres and Barbacid, 2005).

In trypanosomes, at least 10 cyclins (CYC2–CYC11) and 11 Cdc2-related kinases (CRK1–CRK4 and CRK6–CRK12) were identified (Hammarton, 2007). This large number of cyclins and CDKs found in the trypanosome genome appears to suggest a rather complex cyclin–CDK system than one would expect for a unicellular eukaryote. The function of some of these cyclins and CRKs has been characterized and demonstrated to be involved in cell cycle control. The CYC2–CRK1 pair and the CYC6–CRK3 pair are likely the primary cyclin–CRK complexes for promoting the G1/S and G2/M transitions, respectively (Hammarton et al., 2003, 2004; Li and Wang, 2003; Tu and Wang, 2004). CYC2 is homologous to the PHO80 cyclins in yeast and CYC6 is a homolog of the B-type cyclin found in plants (Li and Wang, 2003). However, CYC4 and CYC8, which are related to CYC2 and CYC6, respectively, are also involved in the G1/S transition and the G2/M transition, respectively, but their role(s) in cell cycle control appears to be rather limited since RNAi of CYC4 and CYC8 only generates minor cell cycle defects (Li and Wang, 2003). Strikingly, CYC2 also interacts with CRK3 and likely is also involved in the G2/M transition (Gourguechon et al., 2007; Van Hellemond et al., 2000). CYC2 also interacts with

CRK2, which is also required for the G1/S transition (Gourguechon et al., 2007; Tu and Wang, 2005). Recently, CRK1 was found to interact with at least four PHO80-like cyclins, CYC2, CYC4, CYC5, and CYC7, and RNAi of each of the four cyclins resulted in defects in G1/S transition (Liu et al., 2013). CRK9, an extraordinarily large CDK homolog with a large sequence insertion in its catalytic domain, appears to regulate mRNA trans-splicing by phosphorylating an RNA polymerase II subunit, RPB1 (Badjatia et al., 2013a). This discovery questions whether CRK9 is involved in cell cycle control. Biochemical purification of CRK9-associated proteins led to the identification of a novel cyclin, CYC12, and RNAi-mediated silencing of CYC12 also disrupted mRNA trans-splicing (Badjatia et al., 2013b). These results suggest that CRK9 does not play any role in cell cycle regulation but rather is involved in mRNA splicing. Altogether, these findings suggest that at least two CRKs (CRK1 and CRK2) and four cyclins (CYC2, CYC4, CYC5, and CYC7) are important for promoting the cell cycle transition from G1 to S phase, whereas CRK3 and three cyclins (CYC2, CYC6, and CYC8) are involved in the G2/M transition. No cyclin and CRK have been found to play a role in the S phase. Additionally, homologs of the CDK inhibitors are also not identified, presumably due to their divergent sequence that has escaped the homology-based search against the trypanosome proteome. Nevertheless, the presence of an unexpected large number of cyclins and CDKs in an early branching unicellular eukaryote suggests the complexity of the cell cycle control system, which may reflect the complex life cycle of trypanosomes during which the parasite has to deal with different living environments, such as in the mammalian host and the tsetse fly host, for successful cell proliferation.

3.3. Life cycle stage-specific differences in cell cycle regulation

The life cycle of trypanosomes involves the transmission between mammalian hosts and the insect vector, tsetse fly. During the cyclic transmission, trypanosome cells undergo differentiation, which is characterized by morphological restructuring, surface coat switching, and biochemical adaptation (McKean, 2003). The most distinct morphological difference between the different life cycle forms of trypanosomes is the relative position of the kinetoplast. In the procyclic form, the kinetoplast is posterior and when it replicates and segregates, one kinetoplast remains posterior, but the other kinetoplast is positioned between the two segregating nuclei, resulting in the KNKN configuration (from posterior to anterior) of cells before cytokinesis initiation. In the bloodstream form, however, the kinetoplast replicates and segregates in the posterior portion of the cell, and both daughter kinetoplasts remain in the posterior region throughout the cell cycle, thus resulting in the KKNN configuration of cells before cytokinesis. In addition to the morphological difference between the life cycle forms, life cycle-specific differences in many other cellular processes, including chromatin structure (Schlimme et al., 1993), glucose transport (Munoz-Antonia et al., 1991), maintenance of plasma membrane potential (Van der Heyden and Docampo, 2002), cell motility (Broadhead et al., 2006), and cell cycle regulation (Hammarton et al., 2003; Li and Wang, 2006; Tu and Wang, 2004) have also been discovered. Nevertheless, the molecular mechanisms underlying these distinctions between the two life cycle forms remain poorly understood.

The fundamental architecture of the organelles, the cytoskeletal structures, and the overall cell shape between the life cycle forms appears to be of no significant difference. However, there apparently are fundamental differences in the cell cycle checkpoint systems between the two life cycle stages. When mitosis is impaired by chemical intervention, such as Rhizoxin-induced malformation of the mitotic spindle (Ploubidou et al., 1999; Robinson et al., 1995), or by genetic interference, such as RNAi-mediated knockdown of mitotic cyclin or CRK which causes mitotic defects (Hammarton et al., 2003; Li and Wang, 2003; Tu and Wang, 2004), the procyclic form of trypanosomes is still capable of completing its cytokinesis, leading to the production of anucleate cells. This suggests that either the procyclic trypanosomes lack the mitosis to cytokinesis checkpoint or the mitotically defective procyclic trypanosomes are able to bypass the mitosis–cytokinesis checkpoint. In the bloodstream form, however, cells with defective mitosis, which is caused by similar knockdown of the mitotic cyclin and CRK (Hammarton et al., 2003; Tu and Wang, 2004), are apparently unable to initiate cytokinesis, suggesting that the mitosis–cytokinesis checkpoint is operational in the bloodstream trypanosomes or that the bloodstream cells with mitotic defects are not capable of bypassing the checkpoint. In striking contrast to the procyclic trypanosomes, these mitotically defective and cytokinesis-arrested bloodstream trypanosomes are able to enter the next round of cell cycle repeatedly, albeit still mitotically defective, resulting in the production of giant cells with polyploid DNA that are not able to be segregated (Hammarton et al., 2003; Li and Wang, 2006; Tu and Wang, 2004). These interesting observations argue that bloodstream trypanosomes appear to lack the checkpoint system that controls the entry to the next G1 phase of the cell cycle in the absence of cytokinesis. Overall, it is clear that the two life cycle forms of trypanosomes employ distinct cell cycle checkpoint control systems.

4. Regulation of Mitosis

4.1. Closed mitosis in *T. brucei*

Eukaryotic cells must faithfully segregate their genetic material to the daughter cells during mitosis. It has long been established that lower and higher eukaryotes achieve this in strikingly distinct ways. Higher eukaryotes undergo an open mitosis, with the nuclear envelope completely disassembled at the G2/M transition and then reassembled upon the completion of mitosis. In contrast, many lower eukaryotes undergo a closed mitosis in which the nuclear envelope remains intact throughout mitosis and mitosis occurs within the nucleus. In the closed mitosis, proteins and molecules involved in mitosis are transported in and out of the nucleus through the nuclear pore complexes that are embedded in the nuclear envelope and serve as molecular sieves. Like many other lower eukaryotes, trypanosome also undergoes a closed mitosis. Throughout mitosis the nuclear envelope does not break down, which was easily detected by immunofluorescence microscopy with an antibody against a nuclear envelope protein and by electron microscopy (Ogbadoyi et al., 2000). Through biochemical purification followed by mass spectrometry, the trypanosome nuclear pore complex was characterized, which shares a similar architecture to that of fungi and animals (DeGrasse et al., 2009).

4.2. Spindle structure and assembly

It has been well recognized that trypanosome cells do not possess centrioles, but instead have the flagellar basal body as the cell's MTOC, which is involved in nucleating the flagellar axoneme microtubules and has little role in spindle assembly. Since mitosis is closed, the mitotic spindle is formed within the nucleus, with the two poles of the spindle located at the opposite ends of the nucleus, which are potentially the spindle poles in trypanosomes (Ogbadoyi et al., 2000). However, it is not clear whether trypanosomes possess a spindle pole structure similar to the SPB found in fungi. A search for the homologs of yeast SPB proteins in the trypanosome genome only identified the components of the γ -tubulin complex, which is known to localize to the flagellar basal body, the cell's only visible MTOC. Intriguingly, two proteins, the Tousled-like kinase TbTLK1 and a nuclear pore complex component, TbNup92, are found to localize to the spindle poles during mitosis in trypanosomes (DeGrasse et al., 2009; Li et al., 2007). RNAi of TbTLK1 results in defects in spindle formation in trypanosomes, suggesting that TbTLK1 is likely involved in spindle assembly. The potential role of TbNup92 in spindle assembly remains to be explored.

Using a monoclonal antibody against β -tubulin, KMX-1, the mitotic spindle can be readily detected during early to late mitotic phases in trypanosomes. Here, we report the morphological and conformational changes of the spindle during mitotic progression, which represents the first detailed illustration of the various shapes of the mitotic spindle in trypanosomes (Fig. 4.5). The spindle structure becomes clearly visible at metaphase, during which the spindle is short in length and appears like a diamond in shape, with the kinetochores of the chromosomes all aligned in the equator of the spindle (Fig. 4.5A). The spindle then gradually elongates, presumably through the sliding of the overlapping interpolar spindle microtubules which in general is mediated by kinesin motor proteins and/or through the separation of the spindle poles. This elongation of spindle microtubules correlates with the gradual segregation of chromosomes, which is often referred to as the anaphase of the cell cycle (Fig. 4.5B–G). The spindle structure in trypanosomes appears to contain less microtubule fibers than that in fungi and animals, and as a result the metaphase spindle and anaphase spindle appear to comprise only two lines of microtubules (more spindle microtubules may exist, but they are not visible), with both ends of the two lines merged together to form the SPBs that associate with or are an integral part of the nuclear envelope (Fig. 4.5A–C) (Ogbadoyi et al., 2000). Strikingly, during early anaphase when the spindle is elongated, the two visible microtubule lines start to twist by an unknown mechanism, thus making the shape of the spindle resemble the Arabic number 8, with the SPB likely remaining intact (Fig. 4.5D, E). This “8”-shaped spindle then apparently undergoes some conformational change by transforming from a closed conformation to an open bifurcated conformation assuming an “X” shape (Fig. 4.5F, G), which likely involves the disassembly of the SPB complex, although this is still not clear and needs to be further investigated. At later stages of anaphase, the spindle is further elongated, resulting in farther segregation of the duplicated nuclei. The spindle microtubules at late anaphase appear to bundle vigorously, leading to the transformation of the “X”-shaped spindle to a single microtubule bundle that connects the two far segregated nuclei (Fig. 4.5H–J). Prior to cytokinesis, the spindle bundle disappears.

Little is known about how the bipolar mitotic spindle is assembled in trypanosomes. It is generally accepted that spindle microtubules in animals are nucleated either by centrosomes or through chromatin-mediated pathways and that spindle microtubules in fungi are nucleated by SPBs embedded in the nuclear envelope. Unlike the organisms containing centrosomes or SPBs, spindle assembly in acentrosomal cells rely exclusively on chromatin-mediated pathways in which microtubules are nucleated and stabilized near the kinetochores (Dumont and Desai, 2012). Once the bipolar spindle is assembled, its maintenance is dependent on the cooperative action of microtubule motors, such as the Kinesin-5 family, which are plus end-directed motors, the Kinesin-14 family, which are minus end-directed motors, and the Kinesin-13 family, which are micro tubule depolymerases (Gatlin and Bloom, 2010). Both the Kinesin-5 and Kinesin-13 kinesins subject to phosphorylation by Aurora A kinase and Aurora B kinase, respectively, and this phosphorylation is required for assembly of a bipolar spindle. Moreover, Aurora B kinase also plays an additional role in chromosome biorientation by correcting microtubule–kinetochore misattachments, which also helps establish the bipolar spindle (Carmena et al., 2009). Finally, the spindle assembly check point (SAC) monitors microtubule–kinetochore attachment errors, and when an attachment error is detected by the SAC, anaphase onset is delayed through inhibiting the activity of the anaphase promoting complex/cyclosome (APC/C) (Peters, 2006).

The lack of centrosomes or SPBs in trypanosome cells argues that establishment of a bipolar spindle is most likely dependent on chromatin mediated pathways. To support such a notion, the proteins that are known to be required for centrosome-directed spindle assembly, such as Aurora A kinase and Kinesin-5, appear to be missing in trypanosomes. In contrast, the proteins that are implicated in chromatin-based pathways, such as Aurora B kinase and Kinesin-13, are present in trypanosomes and are involved in regulating spindle formation and the dynamics of spindle microtubules (Chan et al., 2010; Li and Wang, 2006; Tu et al., 2006; Wickstead et al., 2010). Intriguingly, despite the lack of a Kinesin-5 homolog in trypanosomes, two highly divergent orphan kinesins, TbKIN-A and TbKIN-B, cooperate with the Aurora B kinase homolog, TbAUK1, to regulate spindle assembly (Li et al., 2008a,c). Both TbKIN-A and TbKIN-B associate with nuclear DNA and the central spindle and interact with TbAUK1, but how the two kinesins promote spindle assembly is still not known. Unlike any other eukaryotes, trypanosomes express a greatly expanded repertoire of the Kinesin-13 family, and one of this family, TbKif13-1, is involved in regulating the dynamics of spindle microtubules (Chan et al., 2010; Wickstead et al., 2010). Nevertheless, it remains unclear whether TbKif13-1 plays any role in maintaining kinetochore–microtubule interactions as its mammalian counterpart. Further in-depth investigation is necessary to explore the mechanistic roles of the mitotic kinesins in controlling spindle assembly and spindle microtubule dynamics.

Homologs for most of the APC/C components have been identified in trypanosomes, which apparently form a functional ubiquitin E3 ligase that is required for metaphase–anaphase transition and is also involved in controlling the stability of the mitotic cyclin, CYC6 (Bessat et al., 2013; Kumar and Wang, 2005). The latter function of the APC/C in trypanosomes may suggest another role of the complex in promoting mitotic exit. However, homologs for most of the SAC complex except Mad2 are missing in the trypanosome genome (Berriman et al., 2005). Surprisingly, TbMad2 is localized to the basal body

(Akiyoshi and Gull, 2013a), arguing against its involvement in monitoring kinetochore–microtubule interaction errors during bipolar spindle assembly. Therefore, the lack of a SAC complex and the safeguard control mechanism in trypanosomes cannot be ruled out, which may highlight the unusual mechanism underlying spindle assembly and kinetochore–microtubule interactions in trypanosomes.

4.3. Kinetochore and its unusual protein composition

The kinetochore is a complex structure that establishes the attachment of spindle microtubules to chromosomes and is thus essential for faithful chromosome segregation. Under the transmission electron microscope, the kinetochore in vertebrate animals appears as a trilaminar stack of plates situated on opposite sides of the centromere of mitotic chromosomes (Brinkley and Stubblefield, 1966). The outer plate contains microtubule interacting proteins (such as CENP-E, etc.) and the SAC proteins, whereas the inner plate is immediately adjacent to the centromere and is organized on a chromatin structure containing nucleosomes with a specialized histone named CENP-A, which substitutes histone H3 in the centromere region, auxiliary proteins, and centromere DNA (Chan et al., 2005). Electron microscopic studies show that the mitotic chromosomes of trypanosomes also possess an electron-dense structure typical of the canonical kinetochore found in other eukaryotic organisms (Ogbadoyi et al., 2000). However, the observed number of kinetochore appears not to exceed 8 (Solari, 1995), but there are more than 100 chromosomes. This suggests either a non-conventional function of the kinetochore or the existence of alternative chromosome segregation mechanism(s) (Ogbadoyi et al., 2000). It is also very likely that those chromosomes without detectable kinetochores may contain smaller kinetochores whose ultrastructure is not readily visible by electron microscopy.

Despite the presence of canonical kinetochore structure in trypanosomes, the protein components of the kinetochore were largely unknown (Berriman et al., 2005). Only a few kinetochore protein homologs were found based on sequence homology (Berriman et al., 2005), which include the inner kinetochore protein MCAK (Kinesin-13 family kinesins) and the outer kinetochore protein TOG/MOR1. Surprisingly, the TOG/MOR1 homolog was found to be localized to the cytosol without enrichment in the nucleus in procyclic trypanosomes (our unpublished data), suggesting that it is either not a kinetochore component in trypanosomes or not a *bona fide* homolog of TOG/MOR1. Recently, 19 candidate kinetochore proteins were identified in trypanosomes by using localization-based screening and proteomics approach, and RNAi-mediated knockdown of these kinetochore proteins resulted in severe chromosome mis-segregation (Akiyoshi and Gull, 2013b). All of the 19 kinetochore proteins bear no detectable sequence homology to the kinetochore proteins in other eukaryotes, suggesting that trypanosomes employ a chromosome segregation mechanism involving novel kinetochore components (Akiyoshi and Gull, 2013b).

4.4. Novel chromosomal passenger complex and its role in mitosis

Chromosomal passengers are proteins that move from the centromere to the midzone of the spindle during mitosis. The first passenger protein identified is an inner centromere protein called INCENP (Cooke et al., 1987), which was later found to interact with an essential

mitotic kinase, the Aurora B kinase (Adams et al., 2000; Kaitna et al., 2000). It is now well known that the chromosomal passenger complex (CPC) is composed of the enzymatic subunit Aurora B and three regulatory components, INCENP, Survivin, and Borealin (Ruchaud et al., 2007). The three regulatory CPC proteins form a localization module, forming a three-helix bundle among the INCENP amino terminus, Survivin and Borealin (Jeyaprakash et al., 2007). This bundle is required for CPC localization to the centromere of chromosomes, which is mediated by the baculovirus IAP repeat domain of Survivin and the C-terminus of Borealin (Jeyaprakash et al., 2007). This bundle is also required for CPC localization to the spindle midzone and the anaphase midbody that is involved in cytokinesis initiation, but the precise mechanism remains a mystery. The CPC is present in all the eukaryotes examined so far, and all the four components are evolutionarily conserved from fungi to humans (Carmena et al., 2012).

Trypanosomes, however, express a unique CPC that only consists of three components (Li et al., 2008a). This novel CPC is composed of the well-conserved Aurora B kinase homolog, TbAUK1 (Li and Wang, 2006; Tu et al., 2006), and two hypothetical protein, TbCPC1 and TbCPC2. TbCPC1 appears to contain a so-called IN-box motif that is originally found in INCENP and is involved in binding to Aurora B kinase. However, TbCPC1 is relatively small, with an observed molecular mass of around 30 kDa, whereas the INCENP homologs found in many other organisms from yeast to human have a molecular mass of over 100 kDa. Nevertheless, the presence of the IN box suggests that TbCPC1 is a structural and likely also functional homolog of INCENP. TbCPC2 is extremely divergent and exhibits no significant sequence homology to the well-conserved CPC proteins found in fungi and animals (Li et al., 2008a,c). TbCPC2 could act as a functional homolog of Borealin or Survivin, but this still needs to be investigated.

Despite the structure distinction, the trypanosome CPC still exhibits a dynamic subcellular localization pattern typical of the canonical CPC found in animals. It is concentrated in the nucleus at G2 phase, and then migrates from the chromosome centromeres to the central portion of the mitotic spindle during metaphase to anaphase transition. Subsequently, it moves from the central spindle to the anterior tip of the new FAZ during the transition from mitosis to cytokinesis. Since trypanosome cells undergo a closed mitosis without breaking down the nuclear envelope during mitosis, this suggests that the CPC has to be exported out of the nucleus across the nuclear membrane. The detailed mechanism behind the nuclear export of the CPC is not clear. Finally, the complex travels along the cleavage furrow which ingresses longitudinally from the anterior tip of the new FAZ toward the posterior end of the cell (Li et al., 2008a, 2009) (also see Fig. 4.6 and below for detailed description). Since the three noncatalytic CPC proteins in animals are known to function as a single structural unit to target Aurora B kinase to various subcellular locations during mitosis (Jeyaprakash et al., 2007), it is not clear whether the two noncatalytic CPC proteins in trypanosomes play similar roles like the animal CPC components. Additionally, INCENP has an additional function by acting as a substrate activator of Aurora B kinase (Bishop and Schumacher, 2002). Since TbCPC1 is a structural homolog of INCENP, it will be necessary to determine whether TbCPC1 also has a role in activating TbAUK1. Further investigation of the function of the two CPC proteins would help understand the regulation of TbAUK1 and the mechanistic roles of TbAUK1 in mitosis and cytokinesis.

5. Mechanisms of Cytokinesis

5.1. Coordination of mitosis with cytokinesis

Eukaryotes employ a number of cell cycle checkpoint systems to ensure the fidelity of cell division. For example, DNA damage checkpoint monitors the DNA damages generated during the cell cycle, and when DNA damages are detected, cell cycle progression is slowed down, allowing the cell to repair the DNA damage before continuation of the cell cycle. The abscission checkpoint control system, which is triggered by the Aurora B kinase through phosphorylating a subunit of the endosomal sorting complex required for transport-III (Carlton et al., 2012), delays abscission until the chromatin bridge stuck in the cell division plane is resolved (Carmena et al., 2012). In all the eukaryotes studied to date, inhibition of mitotic progression unanimously blocks cytokinesis, resulting in the production of polyploid cells. Intriguingly, transition from mitosis to cytokinesis in the procyclic form of trypanosomes appears to be poorly coordinated. This interesting phenotype was originally observed when procyclic trypanosome cells were treated with Rhizoxin, a microtubule poison that disrupts the assembly of spindle microtubules and hence inhibits mitosis, anucleate cells, termed zoids, were produced (Ploubidou et al., 1999; Robinson et al., 1995), suggesting that cytokinesis can occur in the absence of mitosis in procyclic trypanosomes. Such observations were further confirmed by genetic interference with mitosis through RNAi-mediated silencing of mitotic genes, such as the mitotic cyclin, CYC6, and its partner, CRK3 (Hammarton et al., 2003; Li and Wang, 2003; Tu and Wang, 2004). These studies suggest that procyclic trypanosomes likely lack a checkpoint system for controlling mitosis–cytokinesis transition. Since basal body duplication and segregation appears to constitute the first cytoskeletal event in trypanosome cell cycle and inhibition of basal body duplication or segregation completely arrests cytokinesis (Das et al., 1994), it suggests that posterior migration of the basal body, which is believed to be controlled by the elongation of the new flagellum and the FAZ filament (Absalon et al., 2007), likely functions as the primary driving force for cytokinesis in pro cyclic trypanosomes, although the roles of the CPC, TbPLK, and other unidentified cytokinesis regulators should not be undermined.

5.2. Unusual mode of cytokinesis

Cytokinesis is the final step of the cell cycle during which two daughter cells physically separate after the duplication and segregation of chromosomes and organelles. Cytokinesis begins when the site of the future cleavage furrow is defined, and in fungi and animals it can be generally divided into the following steps: selection of the cell division plane, assembly of the contractile ring, constriction and disassembly of the contractile ring, and separation of daughter cells by membrane fusion (Pollard and Wu, 2010). Positioning the cleavage plane is critical for symmetrical cytokinesis, and at the site of cell division plane a contractile ring, which is composed of the myosin motor protein, actin filaments, and many other proteins, is assembled and attached to the plasma membrane (Barr and Gruneberg, 2007). The mechanism of cytokinesis through the actomyosin contractile ring structure likely appeared in the common ancestor of amoebas, fungi, and animals, which share most of the genes used for cytokinesis (Pollard and Wu, 2010). Plants use a different cytokinesis mechanism, the membrane fusion machinery, in which the plant cells fuse membrane vesicles to build new membrane between the daughter cells (Otegui et al., 2005). The mechanism of cytokinesis in

the organisms that branched earlier than algae and plants, such as the protozoan parasites trypanosomes and *Giardia*, also appears to be different from that in fungi and animals because these parasites apparently lack myosin II. Further investigation of the mechanism of cytokinesis in early branching protozoa would help understand the evolution of the cytokinesis regulatory machinery.

Cytokinesis in trypanosomes appears to be different from that in fungi and animals, not only because there is no evidence to support the existence of the actomyosin contractile ring-based cytokinesis machinery (Garcia-Salcedo et al., 2004) but also because cytokinesis cleavage furrow ingression occurs along the longitudinal axis from the anterior tip of the new FAZ toward the posterior tip of the cell between the two flagella (Li et al., 2008a,c, 2009; Vaughan and Gull, 2003, 2008). The precise selection site of the cytokinesis cleavage plane in trypanosomes is still not defined, but several lines of evidence strongly suggest that the anterior tip of the new FAZ is implicated (Robinson et al., 1995). As further support to this hypothesis, trypanosome mutants defective in intraflagellar transport, which generate a short new flagellum and a short new FAZ, undergo asymmetrical cell division to produce a small daughter cell with a short flagellum and a short FAZ (Kohl et al., 2003). The trypanosome mutant defective in new FAZ assembly due to the depletion of a FAZ filament protein CC2D also undergoes unequal cell division, but this produces a small daughter cell with a short FAZ and a full-length flagellum (Zhou et al., 2011). The latter study further reinforces the notion that it is the new FAZ, but not the flagellum, that defines the cytokinesis cleavage plane in trypanosomes.

5.3. Regulation of cytokinesis by CPC

Initiation of cytokinesis in animals is known to be initiated by two evolutionarily conserved protein kinases, Aurora B kinase and Polo-like kinase (PLK), both of which are enriched at the central spindle and the anaphase midbody. The central spindle is critical for positioning and establishment of the actomyosin contractile ring. The two kinases act cooperatively to regulate, through phosphorylation, a common downstream factor, the so-called centralspindlin complex that is composed of a Kinesin-6 family member, MKLP1, and a Rho GTPase activating protein, MgcRacGAP, in mammals (Mishima et al., 2002). The centralspindlin complex then binds to Ect2, a Rho GEF (guanine nucleotide exchange factor), and recruits the latter to the midbody (Somers and Saint, 2003). Ect2 is required to activate RhoA, a small GTPase, leading to the concentration of the latter in the equatorial region of the cell membrane (Piekny et al., 2005). Activation of RhoA further promotes actin polymerization and myosin II activation, leading to the formation of the actomyosin contractile ring (Carmena, 2008; Glotzer, 2005). Interestingly, the fission yeast Aurora B kinase homolog Ark1 is not essential for cytokinesis (Petersen and Hagan, 2003), suggesting that regulation of actomyosin ring assembly in fission yeast likely involves a mechanism distinct from that in budding yeast and animals. Like in animals, the PLK in fungi is essential for cytokinesis by functioning as an upstream component of the SIN (septum initiation network) or MEN (mitotic exit network) comprising a number of proteins, such as MOB1, DBF2, and Cdc14, all of which are conserved between budding and fission yeasts (Gruneberg and Nigg, 2003). Some of these SIN/MEN pathway components also have their mammalian homologs, which are also essential for cytokinesis in mammals.

However, how they are regulated by PLK in mammals remains poorly understood (D'Amours and Amon, 2004).

Although trypanosomes display a different mode of cytokinesis and lack the actomyosin-based cytokinesis apparatus, the Aurora B kinase homolog, TbAUK1, and the PLK homolog, TbPLK, are both implicated in cytokinesis (Hammarton et al., 2007; Kumar and Wang, 2006; Li and Wang, 2006; Tu et al., 2006). TbAUK1 functions as a crucial chromosomal passenger protein and plays multiple roles in mitosis (Li and Wang, 2006; Li et al., 2008a,c, 2009; Tu et al., 2006). It is also required for cytokinesis initiation, furrow ingression, and abscission (Li and Wang, 2006; Li et al., 2008a,c, 2009; Tu et al., 2006); however, the precise mechanisms of TbAUK1 in cytokinesis are still a mystery, mainly because none of its downstream factors in the cytokinesis pathway has been identified. Like the CPC in animals, the trypanosome CPC is also enriched on the central spindle during late mitosis (Fig. 4.6, 0 min), but it is not clear whether the central spindle in trypanosomes defines the cytokinesis cleavage plane as it does in animals. However, unlike the animal CPC that stays on the central spindle region upon the completion of mitosis, the trypanosome CPC starts to relocate to the anterior tip of the new FAZ (Fig. 4.6, 60 min), which is known to constitute the initiation site of cytokinesis in trypanosomes. However, it remains to be determined whether the CPC proteins at the anterior tip of the new FAZ are newly synthesized proteins or are the old CPC proteins trans localized from the central spindle. When the CPC is only confined to the anterior tip of the new FAZ (Fig. 4.6, 90 min), cytokinesis is initiated, and when the cleavage furrow ingresses toward the posterior end of the cell, the CPC travels along the furrow (Fig. 4.6, 115 and 120 min). At the final stage of cytokinesis during which the two daughter cells are connected at the posterior ends, the CPC disappears, likely because they are degraded (our unpublished data). This dynamic pattern of CPC localization during mitosis–cytokinesis transition and during cytokinesis progression, together with the genetic evidence (Li and Wang, 2006; Tu et al., 2006), strongly suggests a role for the CPC in cytokinesis initiation, progression, and completion.

5.4. Regulation of cytokinesis by other proteins

A highly divergent Rho-like small GTPase, TbrHP, and its RhoGAP, TbOCRL, were identified in trypanosomes (Abbasi et al., 2011). RNAi mediated silencing of both proteins each leads to defective cytokinesis, and the phenotype resembles that of the TbAUK1 RNAi mutant (Abbasi et al., 2011), implying that TbrHP and TbOCRL might function in TbAUK1-mediated pathway. Unlike in humans where active RhoA is concentrated at the cytokinesis initiation site (Piekny et al., 2005), however, TbrHP is diffusely localized throughout the cytosol (Abbasi et al., 2011). Nevertheless, the identification of a RhoA-like small GTPase and its activation partner as crucial cytokinesis regulators suggests that a RhoA mediated cytokinesis pathway is indeed operating in trypanosomes. However, it is not clear whether they function downstream of TbAUK1 and whether TbAUK1 can activate TbrHP. Intriguingly, TbrHP appears to regulate cytokinesis through its interaction with TRACK (Abbasi et al., 2011), which is the trypanosome homolog of the receptor for activated C kinase 1 (RACK1) and is involved in regulating cytokinesis onset and progression (Rothberg et al., 2006). Given the absence of the actomyosin contractile ring in trypanosomes, the mechanistic role of TbrHP in cytokinesis remains to be explored.

Like in animals, the trypanosome genome also encodes a few SIN/MEN pathway proteins, such as the homologs of MOB1 and DBF2 kinase, both of which are essential for cytokinesis in trypanosomes (Hammarton et al., 2005; Ma et al., 2010). Strikingly, both the MOB1 homolog and the DBF2 homolog in trypanosomes appear to play distinct roles in different life cycle forms. MOB1 appears to regulate cytokinesis completion in the bloodstream form and the positioning of cleavage furrow in the procyclic form (Hammarton et al., 2005). DBF2 homolog is essential for cytokinesis initiation in the bloodstream form, but is not required for cytokinesis in the pro cyclic form (Ma et al., 2010 and our unpublished observation). Surprisingly, unlike in yeast where MOB1 and DBF2 interact with each other, the two homologs do not interact in vivo in trypanosomes (Ma et al., 2010), which argues whether they act in the same pathway as their yeast counterparts. Nevertheless, these observations suggest that the cytokinesis regulatory path ways found in fungi and animals are likely also operating in trypanosomes.

It has been well accepted that regulation of cytokinesis involves more than 100 proteins in the cleavage furrow in fungi and animals (Pollard and Wu, 2010; Skop et al., 2004), and a conserved core of about 20 cytokinesis proteins are found in most animal cells (Glotzer, 2005). However, additional cytokinesis proteins are not conserved among animal cells. The number of proteins known to be involved in cytokinesis in trypanosomes is rather limited, with less than a dozen of proteins identified as putative cytokinesis regulators, including a type III phosphatidylinositol 4-kinase (Rodgers et al., 2007), KMP-11 (Li and Wang, 2008), a protein arginine methyltransferase (Fisk et al., 2010), a small GTPase (ARL2) (Price et al., 2010), Katanin and Spastin (Benz et al., 2012), an F-box protein (CFB2) (Benz and Clayton, 2007), a microtubule-associated protein (AIR9) (May et al., 2012), adenylyl cyclases (Salmon et al., 2012), and a kinetoplastid specific kinesin, TbKIN-C, and its partner kinesin, TbKIN-D (Hu et al., 2012a,b). However, the precise mechanistic roles of these proteins in cytokinesis are not well established, and none of these proteins was found concentrated at the cytokinesis cleavage furrow or at the anterior tip of the new FAZ, thus ruling out their direct participation in cytokinesis.

5.5. Unusual roles of PLK in organelle duplication and cytokinesis

The PLKs are evolutionarily conserved serine/threonine protein kinases and are localized to various mitotic structures, such as centrosomes, nucleus, central spindle, and anaphase midbody, where they play multiple essential roles in mitotic entry, bipolar spindle assembly, chromosome segregation, mitotic exit, and cytokinesis (Archambault and Glover, 2009; Barr et al., 2004). All the PLKs possess a canonical catalytic domain in the N-terminus and a C-terminal Polo-box domain consisting of one or two Polo-box motifs, which plays a regulatory role in controlling PLK localization and substrate binding (Barr et al., 2004).

The PLK homolog in *T. brucei*, TbPLK, exhibits a characteristic PLK structure, with a catalytic kinase domain in its N-terminus and two Polo box motifs in its C-terminus (Graham et al., 1998). TbPLK exhibits a dynamic localization pattern during the cell cycle. It is initially expressed during the S phase and is found in the basal body (de Graffenried et al., 2008; Umeyama and Wang, 2008). When the cell cycle proceeds, TbPLK is enriched in the bilobe structure that is undergoing duplication. After the bilobe is replicated and the

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duplicated bilobe structures start to segregate, TbPLK is found at the growing tip of new FAZ filament during late S phase, G2 phase, and early mitotic phases, such as metaphase and early anaphase (de Graffenried et al., 2008). However, at late anaphase and telophase during which the CPC is detected at the anterior tip of the new FAZ, TbPLK disappears from the anterior tip of the new FAZ (Li et al., 2010). Strikingly, unlike the PLK homologs found in any other organisms, TbPLK is not localized to the nucleus throughout the cell cycle (de Graffenried et al., 2008; Kumar and Wang, 2006) despite the presence of a nuclear localization signal located between the kinase domain and the Polo-box motif (Sun and Wang, 2011; Yu et al., 2012). Importantly, consistent with its absence in the nucleus, TbPLK apparently does not play an essential role in mitosis since RNAi of TbPLK in both the procyclic and bloodstream forms does not cause any detectable mitotic defects (Hammarton et al., 2007; Kumar and Wang, 2006).

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The function of TbPLK appears to correlate with its subcellular localization. Depletion of TbPLK by RNAi causes defective segregation of the duplicated basal bodies (Hammarton et al., 2007; Ikeda and de Graffenried, 2012), presumably by inhibiting the rotation of the new basal body (Lozano-Nunez et al., 2013). RNAi of TbPLK also causes defective bilobe duplication, leading to defects in assembling the duplicated Golgi apparatus (de Graffenried et al., 2008). Moreover, TbPLK deficiency also compromises the assembly of the new FAZ filament, resulting in detachment of the new flagellum (Ikeda and de Graffenried, 2012; Lozano-Nunez et al., 2013). Since TbPLK is required for basal body segregation and inhibition of basal body segregation inhibits cell division, it is still not clear whether TbPLK is directly involved in cytokinesis initiation. TbPLK appears to exert its effect primarily during the S phase because it is highly expressed at S phase during which basal body duplicates and rotation of the new basal body occurs (Lozano-Nunez et al., 2013; Umeyama and Wang, 2008). However, since TbPLK remains associated with the FAZ filament until early anaphase and cytokinesis initiation is known to initiate at anaphase in other eukaryotes, it is likely that TbPLK also plays a direct role in cytokinesis. In this regard, TbPLK presumably regulates certain cytokinesis regulatory proteins at the anterior tip of the FAZ filament. Such proteins are, unfortunately, still not identified, which hinders our understanding of the mechanistic roles of TbPLK in regulating cytokinesis.

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5.6. Roles of flagellum and FAZ in furrow positioning and cytokinesis initiation

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Defining the cytokinesis cleavage plane is fundamental to cells for symmetrical cell division to generate two identical daughter cells. In animals, the division plane is defined by the position of the central spindle, which is critical for the establishment of the actomyosin contractile ring. In trypanosomes, however, the cleavage plane is placed between the two flagella along the longitudinal axis, which appears to be in parallel with the mitotic spindle, and is initiated from the anterior tip of the new FAZ filament (Robinson et al., 1995; Vaughan and Gull, 2003). Growing evidence suggests that the new FAZ may provide structural information for positioning the cleavage plane (Robinson et al., 1995). Trypanosome mutants with a short new FAZ filament and an old full-length FAZ are capable of undergoing an asymmetrical cell division, producing a small-sized daughter cell with the short new FAZ and a normal-sized daughter cell containing the old full-length FAZ (Zhou et al., 2011). On the other hand, since positioning and elongation of the FAZ filament

is defined by the flagellum (Kohl et al., 1999), the growth of the new flagellum thus also plays a critical role in positioning the cleavage plane. This hypothesis is supported by the observation that trypanosome mutants with defective flagellum assembly can undergo an asymmetrical cytokinesis by producing a small-sized daughter cell with a short flagellum and a normal-sized daughter cell with a full-length flagellum (Kohl et al., 2003). The proposed role of the anterior tip of the FAZ as the initiation site of cytokinesis is further confirmed by the discovery that the CPC, a crucial regulator of cytokinesis, is localized to the anterior tip of the new FAZ before cytokinesis initiation (Li et al., 2008a,c, 2009).

6. Conclusions and Perspectives

During the past decade, significant advances in the understanding of the molecular mechanism of mitosis and cytokinesis in trypanosomes have been made, and it is now well accepted that most cell cycle regulatory pathways conserved from fungi to humans are also present in trypanosomes. However, trypanosomes have evolved some unusual cell cycle checkpoint systems and employ many trypanosome-specific cell cycle regulators to compensate for the absence of the regulators that are conserved in other eukaryotes. It is also well accepted that significant differences in cell cycle control exist between different life cycle stages.

Many questions with regard to the cell cycle control in trypanosomes remain unanswered. For example, what are the components of the cytokinesis cleavage furrow? What are the cell cycle checkpoints and how do they control cell cycle progression? How does the CPC travel across the nuclear envelope and how does CPC promote cytokinesis? What is the abscission machinery and how does the machinery cleave the membrane and the microtubule cytoskeleton? Future work should apply a combination of biochemical, genetic, and proteomics approaches for further exploration of trypanosome-specific cell cycle regulatory pathways and for identification of novel cell cycle regulators. There is no doubt that such endeavors will significantly advance our fundamental understanding of the mechanisms of mitosis and cytokinesis in trypanosomes and would also provide novel drug targets for chemotherapeutic intervention.

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Figure 4.1.

The cell structure of *Trypanosoma brucei*. (A) Cross-section of a trypanosome cell visualized under the transmission EM. The flagellar axoneme, paraflagellar rod (PFR), flagellum attachment zone (FAZ) filament, and the subpellicular microtubule corset are indicated. (B) Schematic illustration of a trypanosome cell showing the relative locations of its single-copied organelles.

Figure 4.2.

Duplication and segregation of basal body and flagellum during the cell cycle in trypanosomes. Procyclic trypanosomes were immunostained with YL 1/2 antibody and L8C4 antibody to label the basal body and flagellum, respectively, and then counterstained with DAPI to stain the nuclear and kinetoplast DNA. Bar: 5 μ m.

Figure 4.3.

Duplication and segregation of bilobe, a novel cytoskeletal structure during the cell cycle. Procyclic trypanosome cells were fixed in methanol, immunostained with the 20H5 antibody, which stains the basal body (arrowheads) and the bilobe structure (arrows), and counterstained with DAPI for nuclear and kinetoplast DNA. Bar: 5 μ m.

Figure 4.4.

Duplication and segregation of Golgi and the ER exit site during the cell cycle. Procyclic trypanosome cells expressing EYFP-tagged Sec31, a marker of the ER exit site, were immunostained with anti-GRASP antibody, a marker of the Golgi, and counterstained with DAPI for nuclear and kinetoplast DNA. Bar: 5 μ m.

Figure 4.5.

Morphology of the mitotic spindle during various stages of mitosis in the procyclic trypanosomes. Cells were immunostained with KMX-1, which stains the β -tubulin in the spindle in trypanosomes, and counterstained with DAPI for nuclear and kinetoplast DNA. Bar: 5 μ m.

Figure 4.6.

Cytokinesis initiation, progression, and completion in procyclic trypanosomes and the dynamic localization of the CPC during cytokinesis. A procyclic cell expressing EYFP-tagged TbCPC1 was visualized under the fluorescence microscope and the time-course live image was taken at various times to monitor the progress of cytokinesis and the localization of TbCPC1. Live cells were stained with Hoechst dye to label the nuclear and kinetoplast DNA. Arrowheads point to the TbCPC1 fluorescence signal at the central spindle, whereas the arrows indicate the TbCPC1 fluorescence signal at the anterior tip of the new FAZ and the cleavage furrow. Bars: 5 μ m.