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Coordination of the Cell Cycle in Trypanosomes

Richard J. Wheeler,[1,*] Keith Gull,[2] and Jack D. Sunter[3,*]

[1]Nuffield Department of Medicine, University of Oxford, Oxford OX1 3SY, United Kingdom; email: richard.wheeler@ndm.ox.ac.uk

[2]Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, United Kingdom; email: keith.gull@path.ox.ac.uk

[3]Department of Biological and Medical Sciences, Oxford Brookes University, Oxford OX3 0BP, United Kingdom; email: jsunter@brookes.ac.uk

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*These authors contributed equally to this article

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Abstract

Trypanosomes have complex life cycles within which there are both proliferative and differentiation cell divisions. The coordination of the cell cycle to achieve these different divisions is critical for the parasite to infect both host and vector. From studying the regulation of the proliferative cell cycle of the *Trypanosoma brucei* procyclic life cycle stage, three subcycles emerge that control the duplication and segregation of (*a*) the nucleus, (*b*) the kinetoplast, and (*c*) a set of cytoskeletal structures. We discuss how the clear dependency relationships within these subcycles, and the potential for cross talk between them, are likely required for overall cell cycle coordination. Finally, we look at the implications this interdependence has for proliferative and differentiation divisions through the *T. brucei* life cycle and in related parasitic trypanosomatid species.

Contents

1. INTRODUCTION—CELL CYCLES IN THE LIFE CYCLE

Cell cycles can be classified as proliferative, where both progeny have the same characteristics as the parent, or differentiation, where one or both of the resulting daughters has a different cell form or function. Animal stem cells are often characterized as having the potential for both proliferative symmetric divisions and asymmetric self-renewing divisions where one cell is differentiated and one retains proliferative capacity (114, 115). Here our main focus is on the coordination of the proliferative division of the procyclic form of *Trypanosoma brucei* that inhabits the tsetse vector gut.

Our insights into procyclic proliferative cell division have informed our understanding of the more complicated cell divisions—slender to stumpy, stumpy to procyclic, procyclic to epimastigote, and epimastigote to metacyclic—that must underpin successful transitions during the parasite life cycle (**Figure 1**). An alternation between proliferative and differentiation cell cycles underpins most, if not all, parasite life cycles. Hence, many differentiated microbial cells may have exited the cell cycle (in G_0) but are not terminally differentiated. Also, it has long been recognized that even proliferative divisions that result in two similar daughters may exhibit an element of asymmetry; here, we introduce the term nonequivalence to contrast the use of asymmetry for differentiation divisions. The yeast *Saccharomyces cerevisiae* provides an excellent example of nonequivalence, whereby during proliferative division the mother and daughter cell are defined by a bud and birth scar and a nonequivalence of organelle inheritance (72). This complexity raises three key points: First, understanding coordination of the cell cycle involves appreciating nonequivalence in proliferative divisions; second, asymmetry in differentiation divisions can be used for life cycle stage adaptations; and third, the ability to enter G_0 has implications for the control of the cell cycle.

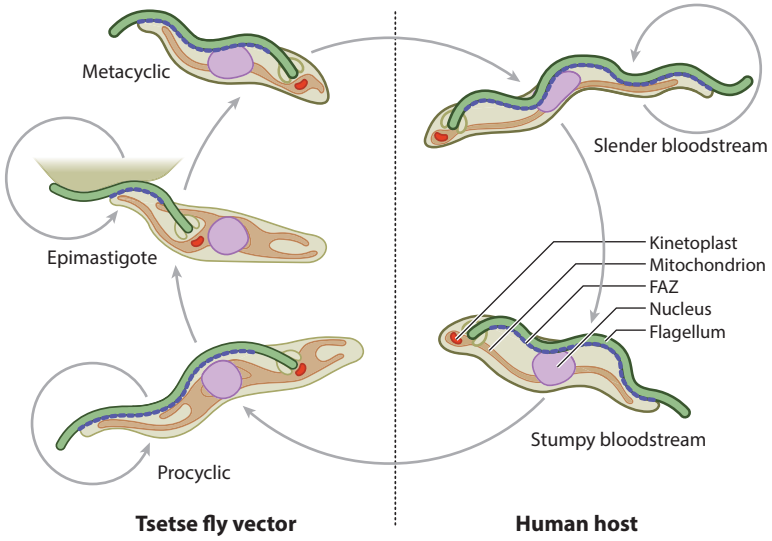

Figure 1

The major life cycle stages of *Trypanosoma brucei*. The life cycle can be broken down into at least five major stages (although intermediate stages have been described): The proliferative procyclic form in the tsetse fly vector gut; the proliferative epimastigote form, which adheres to surfaces in the tsetse salivary glands; the nonproliferative metacyclic form in the tsetse salivary glands, which is preadapted for transmission to the human; the proliferative slender bloodstream form in the human bloodstream (which also penetrates into other tissues); and the nonproliferative stumpy bloodstream form, which is preadapted for transmission to the tsetse fly. Transitions between these stages involve differentiation divisions. All stages have a trypomastigote morphology except for the epimastigote form, which has a characteristic morphology with a shorter flagellum attachment zone (FAZ) and nucleus posterior to the kinetoplast. The procyclic and epimastigote forms have an elaborated mitochondrial network relative to other life cycle stages, and the metacyclic and bloodstream forms feature a variant surface glycoprotein surface coat.

The *T. brucei* procyclic cell cycle is the best analyzed of any trypanosomatid, a group which also includes *Leishmania* species and *Trypanosoma cruzi*; its analysis requires an appreciation of the precisely defined cell organization that features single copies of many organelles (**Figure 2**). A G_1 procyclic trypanosome is described as 1K1N (1 kinetoplast, 1 nucleus). However, there is also a basal body (BB) from which extends the flagellum, which is laterally attached to the cell body by the flagellum attachment zone (FAZ). At the base of the flagellum is an invagination of the cell body membrane termed the flagellar pocket (FP). A rootlet quartet of microtubules (MtQ) nucleates near the BB, wrapping around the FP and then extending to the anterior cell tip (77). At the top of the FP is the flagellar pocket collar (FPC), which defines the overall shape of the FP (16). Distal to the FPC is the hook complex, a structure that hooks around the exit of the flagellum from the FP and has two arms that flank the FAZ and MtQ (37). Trypanosomatids have a cortical cytoskeleton made up of a regular single-layer array of subpellicular microtubules that run parallel with their minus ends oriented toward the anterior (111) and have uniform spacing over the entire cell. The FAZ is the key discontinuity in the array. The MtQ (and the FAZ filament) invades the subpellicular array at the FP (77) and creates a single seam of specialized microtubules antiparallel to the surrounding subpellicular microtubules (111).

Hence, in considering how a G_1 (1K1N) trypanosome progresses through division, three subcycles emerge: the duplication and segregation in an ordered and controlled manner of (*a*) the nucleus, (*b*) the kinetoplast, and (*c*) a set of complex cytoskeletal structures (**Figure 3**).

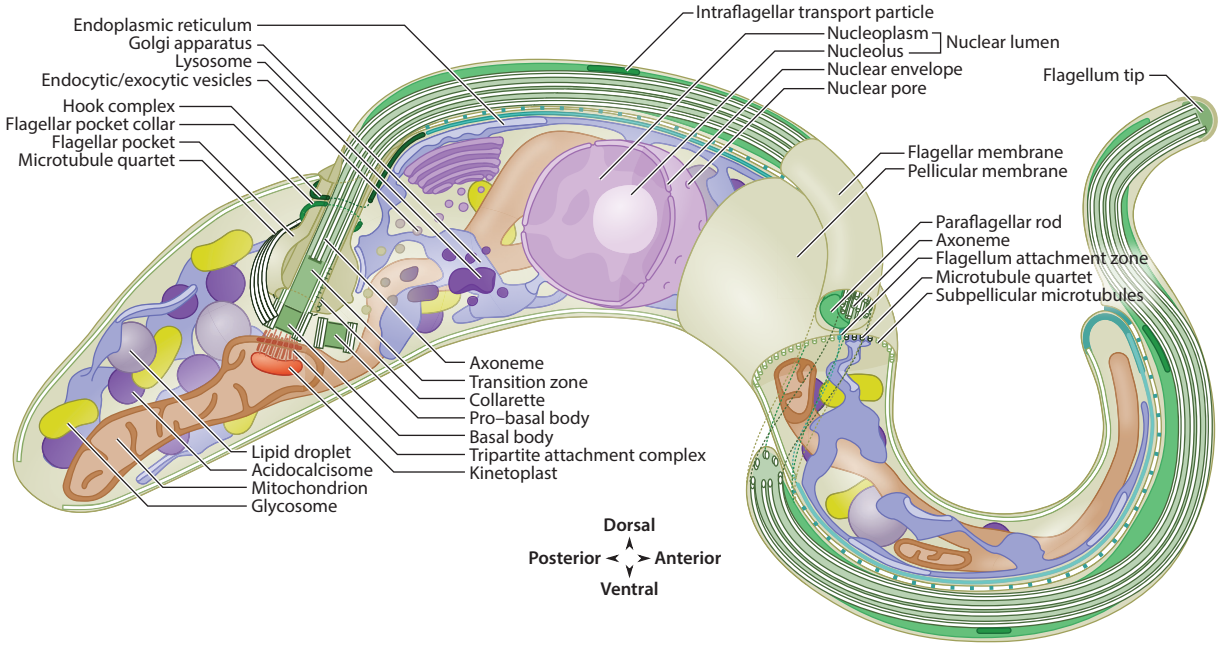

Figure 2

The morphology of the G_1 *Trypanosoma brucei* procyclic-form cell, drawn with reference to electron microscopy, electron tomography, and the localization of organelle components by fluorescence microscopy (both endogenous fluorescent protein tagging and immunofluorescence) in the literature.

2. INITIATION OF THE CELL CYCLE

T. brucei can enter a G_0 state, notably the formation of the stumpy form in the mammalian host, formally indicating that *T. brucei* can control whether or not to initiate the cell cycle. The proliferative slender bloodstream forms respond to the accumulation of oligopeptides generated by released peptidases (112). These signals act as a stumpy induction factor and signal the differentiation generating these specialized G_0 stumpy forms (124). The signaling pathway for exiting G_0 must presumably trigger nuclear S phase along with kinetoplast S phase and early cytoskeletal events—the first events of the three subcycles.

What signals entry from G_0 or G_1 into the cell cycle? The earliest cytoskeletal events in the *T. brucei* procyclic cell cycle are the nucleation of the new MtQ and the maturation of the pro-BB (see Section 5 below) (78). These events are nearly coincident, determined using BrdU labeling of newly synthesized DNA, with the onset of kinetoplast and nuclear S phases (145). However, BrdU has relatively low sensitivity compared to similar pyrimidine analog incorporation methods, specifically EdU (24), and there are some small inconsistencies with methods using quantitative cytometry from DAPI staining (123) or analysis of elutriation-synchronized populations (13). These differences may arise from the differing methods or from subtle differences in the cell lines or growth conditions. It consequently remains unclear whether there is precise synchrony and/or interdependent regulation of entry of the kinetoplast and nucleus into S phase. In *Leishmania mexicana* procyclic forms and *T. cruzi* epimastigotes, pro-BB maturation likely lags behind nuclear and kinetoplast S phase as new flagellum growth starts later in the cell cycle. Both also have near-synchronous kinetoplast and nuclear S phases (36, 139).

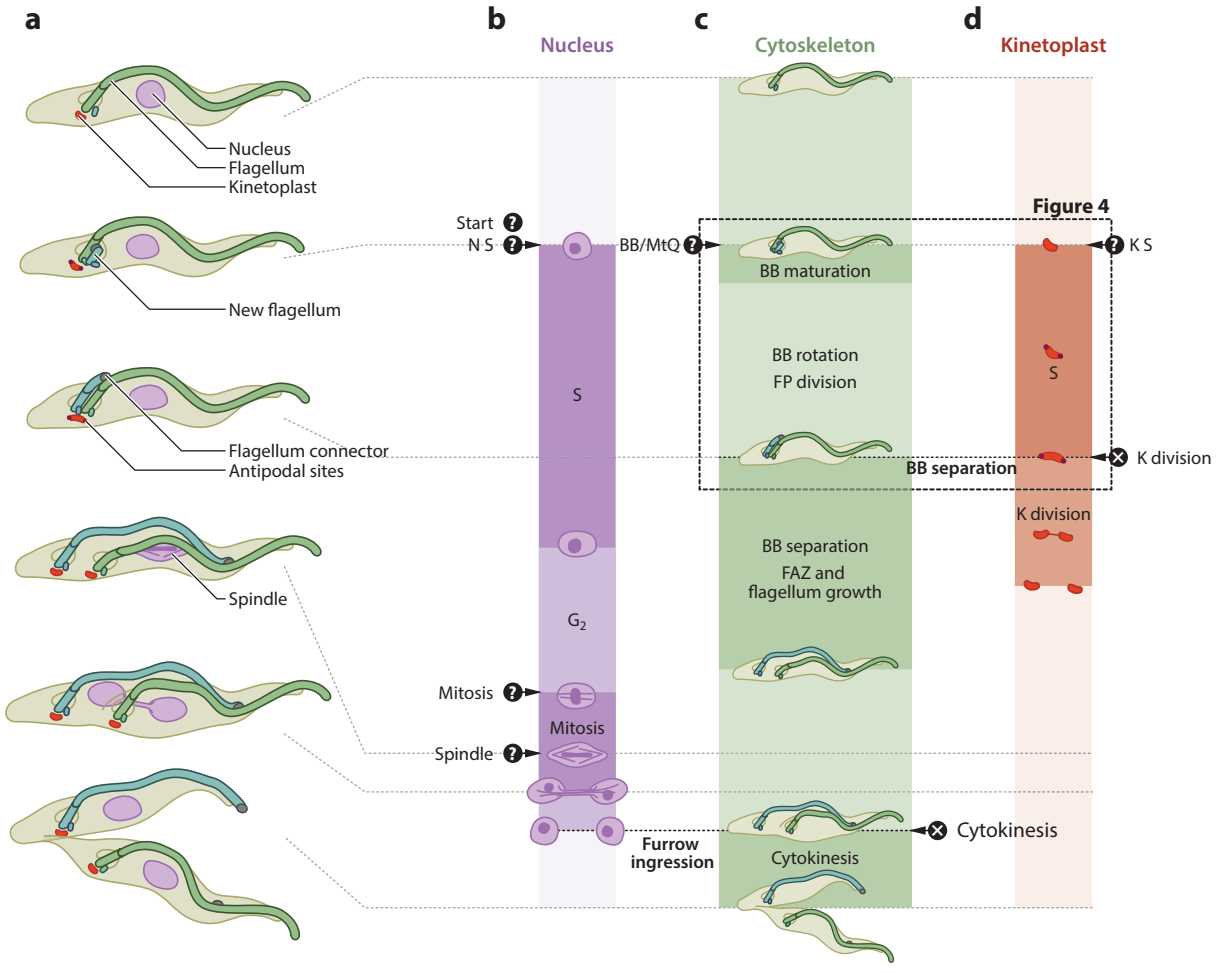

Figure 3

Key events in the *Trypanosoma brucei* procyclic-form cell cycle. The major cell cycle and cell division events and their approximate timing. (*a*) The morphology of the cell at different cell cycle stages, showing the G_1 cell, onset of new flagellum growth, emergence of the flagellum from the FP after BB rotation, anaphase, late mitosis, and cytokinesis. (*b–d*) The approximate timing of key cell cycle events within the three subcycles, on a vertical axis from the early (*top*) to late (*bottom*) events. The timings do not strictly copy any single reference and instead represent a synthesis of the literature and events known to be coincident. Potential checkpoints are indicated, along with the key cross-talk points where cytoskeletal events drive kinetoplast division and nucleus segregation. Crosses indicate good evidence for a lack of checkpoint and question marks indicate mixed or missing evidence. (*b*) Nuclear subcycle. (*c*) Cytoskeleton subcycle. (*d*) Kinetoplast subcycle. Abbreviations: BB, basal body; FAZ, flagellum attachment zone; FP, flagellar pocket; K, kinetoplast; MtQ, microtubule quartet; N, nucleus; S, synthesis.

In metazoa, a key decision point for starting the cell cycle is entry into nuclear S phase, which is irreversibly initiated by Cdk2 activation by cyclin E. Does the *T. brucei* cyclin analogous to cyclin E (CYC2) have an equivalent master regulator role in the cell cycle, coordinately initiating nuclear S phase, kinetoplast S phase, and cytoskeletal events? RNA interference (RNAi) knockdown of CYC2 does prevent nuclear S phase and mitosis, but according to some reports, cytoskeletal events can continue leading to cytokinesis generating zoids (anucleate cytoplasts) (47, 84). In *T. brucei*, further cyclins cooperate in the transition to nuclear S phase and their RNAi knockdown leads to

zoid generation to a greater or lesser extent (85). This variability may indicate poor penetrance of RNAi knockdown of cyclins involved in the G_1 to S transition allowing cytoskeletal cell division events to continue, or that absence of CYC2 does not prevent cytoskeletal events from occurring. Intriguingly, the kinase inhibitor AEE788 causes a partial block on new BB formation and inhibits nuclear and kinetoplast S phase (127), perhaps suggesting its target's (LBPK2) involvement in these early events. Newly available markers for BB duplication and maturation now enable greater temporal resolution; it will be interesting to revisit some of these cytoskeletal and DNA synthesis initiation events within the same mutational analyses.

3. THE NUCLEAR SUBCYCLE

Trypanosomes face the normal eukaryotic challenges in mitosis: checking for DNA damage prior to entering S phase, ensuring single firing replication origins, controlling the assembly of the spindle, chromosome capture, and organizing a bipolar spindle that accurately segregates the multiple chromosomes. Trypanosomes have many cyclins, cyclin-related kinases (CRKs), and mitogen-activated protein kinases (MAPKs). Some are characterized and have roles in controlling nuclear processes in the cell cycle, but many remain uncharacterized with no clear function. A few have unambiguous homology with characterized regulators of metazoan mitosis, pointing to key similarities but also divergence in the control of nuclear division (50, 81, 149).

3.1. Nuclear S Phase

Initiation of nuclear S phase involves cyclins CYC2, CYC4, CYC5, and CYC7 and associated Cdc2-related kinases (149) (**Figure 3*b***). This complexity is challenging to analyze as the proteins involved are poorly conserved at the sequence level even between yeast and metazoa (52, 71). Chromosome replication initiates from well-mapped origins of replication, of which there are relatively few (on the order of 1 per 100 kbp) and which do not have clear consensus sequences (20, 133). Their genomic location correlates with the start of polycistronic transcription units and particular histone modifications (133). *Leishmania major* has a comparable origin density, with differences in origin dynamics and mixed evidence for whether there is only one or multiple origins of replication per chromosome (89, 126). Licensing, then firing, of origins of replication involves recruitment of the origin recognition complex (ORC), the CMG complex (CDC45 and the MCM and GINS), and then DNA polymerase (26, 90, 132), indicating evolutionarily conserved but also divergent features (25, 89).

3.2. Mitosis

G_2 to the start of mitosis involves the cyclin B–like cyclins CYC6 and CYC8 (46, 53, 84) and the kinases CRK3 (CDK1-like) and CRK9 (33, 149) (**Figure 3*b***). Mitosis has a dependency on nuclear S phase but appears independent of kinetoplast S phase and cytoskeletal events, and its timing can vary: It occurs after kinetoplast division in *T. brucei* and *Trypanosoma cruzi* epimastigotes (36), during kinetoplast division in *Leishmania* (8, 139), and apparently at a variable time relative to kinetoplast division in *Trypanosoma abeli* (17). The closed mitotic spindle is a conventional microtubule-based structure (98). Outside of spindle kinesins and MLP2 few vital spindle proteins are known (61, 151). The chromosomes do not visibly condense despite trypanosomatids possessing potential condensins (58), and the nucleolus remains intact. Trypanosome chromosomes have well-mapped centromeres, with characteristic repetitive sequences (34). The centromeres are bound by kinetochores that are visible as electron-dense plaques (98) but have an unusual

molecular composition (5, 86, 97). The kinetochores bind to the microtubule-based spindle via an unconventional microtubule-binding site in KKT4 (86). Overall, the kinetochore components are either highly divergent (28) or evolved independently (5), a debate highlighting the limitations of bioinformatic sensitivity observed elsewhere in analyzing trypanosome cell cycle regulatory networks. Trypanosomes do not have clear orthologs for a spindle assembly complex (51), with the exception of MAD2, which, perhaps instructively, is localized at the BB (4). Trypanosomes do have orthologs of several anaphase-promoting complex (APC) components, of which APC1 and APC3 are known to be vital (51). The chromosomal passenger complex is somewhat divergent, with trypanosome-specific components (CPC1 and CPC2) (82), a Tousled-like kinase TLK1, and spindle kinesins (83) interacting with the Aurora B homolog AUK1.

While a canonical sequence of mitotic events occurs, evidence for the signaling networks controlling key events such as chromosome condensation (if it occurs), spindle assembly, metaphase, and APC/anaphase is limited. For example, expression of a noncleavable SCC1 prevents sister chromosome separation in mitosis (44), but the identity of securin and the mechanism of how separase is derepressed or activated remain unclear, although separase is present throughout the cell cycle with exclusion from the nucleus until the metaphase-anaphase transition (15). In summary, many molecular players are known but the actual signaling events and mechanisms less so, and there is insufficient evolutionary sequence conservation to make strong inferences.

Are there mitotic checkpoints? The G_2 to M DNA damage checkpoint in yeast requires, among others, Chk1, Wee1, and Cdc25, which do not have unambiguous homologs in trypanosomes. However, a Wee1-like kinase is required for trypanosome cell cycle progression (18). Therefore, we cannot exclude a checkpoint at the G_2 to M transition. The spindle checkpoint, which is responsible for the onset of anaphase, may be absent—many major spindle checkpoint proteins in yeast (Bub1, Mad1, Pds1) do not have clear trypanosome homologs or do not localize to the spindle (Mad2) (4). In contrast, cohesin (SCC1, Mcd1 in yeast)—the major mechanical component for chromosome cohesion—and separase are clearly present. The spindle checkpoint is challenging to analyze in *T. brucei* procyclic forms, as cytokinesis tends to occur even when mitosis fails. In itself this outcome suggests trypanosomes can delay anaphase, characteristic of a spindle checkpoint, but it is not a complete checkpoint as it does not prevent cytokinesis, allowing cytokinesis to overtake a delayed mitosis. This phenotype is reminiscent of the cell untimely torn (cut) phenotype seen in fission yeast cells, where cytokinesis proceeds despite failure to resolve mitosis, tearing a stalled mitotic nucleus between two daughters. Mutants in the cohesin/separase/securin machinery tend to generate this phenotype in humans and yeast (146, 153). However, in an analogous *T. brucei* mutant, overexpression of a dominant-negative noncleavable SCC1 tends to cause defective cytokinesis predominantly generating zoids (44) although it can give a torn nucleus (cut phenotype) more rarely (141). In humans and budding yeast the nocut cytokinesis checkpoint can prevent this defect (92). The tendency of *T. brucei* procyclics to generate zoids when nuclear division is disrupted is more an issue of lack of a cytokinesis checkpoint than evidence for lack of a spindle checkpoint. This phenotype is typical of the procyclic form but does not occur in the bloodstream form (106), where cytokinesis tends not to occur but the cell reenters the cell cycle, for example, when AUK1 is knocked down by RNAi (74). However, cytokinesis without mitosis is not a peculiarity of *T. brucei* procyclic forms and also occurs on conditional deletion of CRK3 in *L. mexicana*, leading to generation of zoids (33). The spindle checkpoint is a topic of recent interrogation primarily using CYC6 degradation as a marker of anaphase (53), and it will take careful analysis to ascertain whether the spindle checkpoint is truly absent or whether it is a partial checkpoint complicated by a missing cytokinesis checkpoint—it certainly has divergent features that challenge analysis.

Studies of trypanosome mitosis have recognized the many issues surrounding segregation of conventional (megabase) chromosomes plus the many smaller intermediate chromosomes and minichromosomes (144). The number of microtubules in the spindle indicates there are not enough for one microtubule plus end per chromosome (98). Minichromosome segregation is cohesin independent, indicating significant differences with megabase chromosomes (15). Several aspects of mitosis are poorly understood, leaving the mechanism for genome partition somewhat unclear.

3.3. Nuclear Positioning

Once the nucleus has progressed through mitosis, the nuclear envelope and attached endomembrane system must resolve. The organization of the cytoskeleton and the phenotypes of mutants suggest that cytokinesis is blind to the position of the nuclei (see Section 6), and daughter nuclei must therefore be positioned appropriately for inheritance following cytokinesis. The differences in nuclear positioning between life cycle stages and between different species with different cell morphologies suggest it may be complex and important yet poorly understood. In *Candida albicans* the mitotic spindle varies in length to allow correct positioning of the daughter nuclei between the yeast and hyphal states (11)—spindle length may also be a relevant mechanism in *T. brucei*.

4. THE KINETOPLAST SUBCYCLE

Trypanosomes have a single mitochondrion with a concatenated network of nonsupercoiled circular DNA molecules, comprising maxicircles (typically tens of identical copies of ~20–40 kb encoding the mitochondrial protein-coding genes) and minicircles (typically thousands of variable copies of ~1 kb, encoding guide RNAs for RNA editing) (108). Kinetoplast size and complexity likely necessitates a complex mitochondrial genome replication and segregation machinery (108, 117).

4.1. Kinetoplast S Phase

In kinetoplast S phase minicircles are detached from the network by a type II topoisomerase (TOP2mt), replicated (via a canonical theta intermediate with two replication forks), and then transported to the poles of the S phase kinetoplast—the antipodal sites (70, 108). Here they are connected back to the network, before any remaining nicks are repaired, restoring the concatenated network organization. *T. brucei* is unusual among kinetoplastids, as the minicircles are attached only to the poles of the kinetoplast while in most species the kinetoplast rotates under the antipodal sites, leading to minicircle attachment around the kinetoplast circumference. Replication of maxicircles is less well understood (70, 108).

While the process and machinery of kinetoplast DNA replication has been extensively analyzed, the regulation of kinetoplast S phase remains speculative. Expression level and localization of replication-associated machinery are cell cycle dependent (22), but it is not known what signal initiates kinetoplast S phase or regulates subsequent events (70, 108). There is some evidence for cross talk between the kinetoplast and nucleus (93). However, with caveats, a hard checkpoint is unlikely, as trypanosomes lacking mitochondrial DNA (*petite* mutants) exist (*T. equiperdum* and *T. evansi*) (80) and can easily be artificially generated (30, 137).

4.2. Kinetoplast Division

Division of the kinetoplast and segregation of the duplicated mitochondrial genome require the tripartite attachment complex (TAC) (117), which connects the BB and pro-BB to the kinetoplast

DNA through the mitochondrial double membrane (99) (**Figure 2**). Segregation appears to start during the latter stage of kinetoplast DNA replication (43) (**Figure 3*d***). Attachment of the kinetoplast DNA to the BB leads to daughter kinetoplast separation as the BBs move apart (43), with the long maxicircle molecules in the middle of the dividing kinetoplast left as a trailing structure—the Nabelschnur (43). Resolution of the Nabelschnur may require specific reorganization (i.e., topoisomerase activity), and overexpression of the only known Nabelschnur protein, LAP1, disrupts kinetoplast DNA segregation (105). The assembly of the TAC is not dependent on kinetoplast DNA, and it assembles de novo during kinetoplast S phase (60). The TAC is required for correct kinetoplast positioning and seems to act in kinetoplast segregation analogous to a kinetochore within mitosis (99). While the TAC is required for kinetoplast segregation, division of the kinetoplast also requires kinetoplast network deconcatenation. Control of kinetoplast segregation is not autonomous (117), indicating that a cellular decision to start forming a new TAC is key for correct kinetoplast division. However, while many of the TAC components and assembly order are now known (60) there is little evidence regarding signaling control.

4.3. Mitochondrion Division

The mitochondrion likely undergoes characteristic elaborations through the cell cycle, based on studies of the *T. brucei* bloodstream form (69) and *Crithidia fasciculata* (32). The plane of cytokinesis is defined by the FAZ, and as the mitochondrion is physically attached to the base of the flagellum via the TAC/kinetoplast, inheritance of the mitochondrion is assured. Notably, the mitochondrion always extends across the entire length of the trypanosome cell and is in the final cytoplasmic bridge prior to abscission (66, 69). A dynamin-like protein plays a role in this process (21).

5. THE CYTOSKELETAL SUBCYCLE

Cytoskeletal remodeling is a central part of the division process of any eukaryotic cell. However, for a trypanosome with a highly structured cytoskeleton and many single-copy organelles, an extreme level of regulation is unsurprising. Cytoskeletal elements such as the flagellum, FAZ, FP, and subpellicular microtubule array all need to be duplicated and segregated in a concerted manner integrated with the nuclear and kinetoplast cycles.

5.1. Basal Body (the Master Organizer)

Trypanosome BBs are a master organizer for the surrounding cytoskeleton, membranous structures, and organelles (135) (**Figure 2**). Regulation of pro-BB maturation, pro-BB biogenesis, and BB segregation and positioning is vital to ensure the shape and form of subsequent daughters (**Figures 3*a***, **4**).

The BB is a cylindrical set of nine microtubule triplets and acts as the template and foundation of the flagellum. The BBs exhibit conserved structure and core components, and a cell cycle–governed process of duplication and segregation that is conserved, albeit with variations, across eukaryotes (27, 59, 135). The G_1 cell possesses a mature BB with flagellum and a pro-BB (**Figure 4*a***). The latter will mature and dock with the FP membrane, forming the new flagellum (**Figure 4*c***), followed by formation of two new pro-BBs (**Figure 4*d***), which leads to a regular pattern of BB/pro-BB inheritance. Pro-BB maturation and new pro-BB biogenesis occur near synchronously with the start of S phase. Growth of the new flagellum is associated with a complete new cohort of cytoskeletal arrays of filaments, cell/flagellum membrane connections, and a MtQ. These new cytoskeletal structures eventually segregate from the old as part of the orchestration of cell architecture and polarity (77, 78).

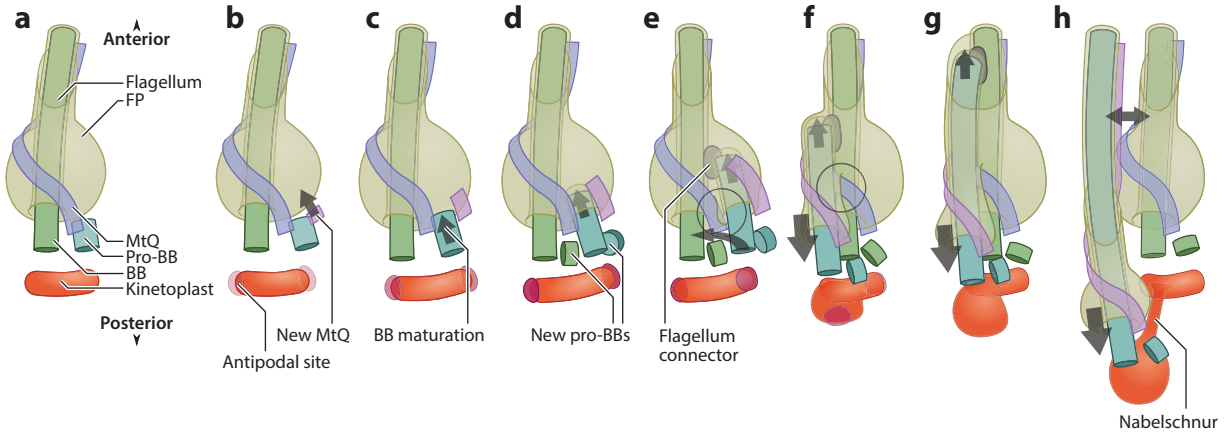

Figure 4

Spatial coordination of the events leading to flagellar pocket (FP) and kinetoplast division: the series of division events involving basal body (BB)-associated structures, based on electron tomography and serial section electron microscopy of the FP and kinetoplast during division. (*a*) The G_1 flagellar pocket. (*b–d*) The earliest events in division of the FP and associated structures are (*b*) onset of new microtubule quartet (MtQ) growth, (*c*) maturation and extension of the pro-BB, and (*d*) formation of new pro-BBs. These occur around the start of kinetoplast S phase. (*e*) As the flagellum and MtQ grow, the new BB and flagellum begin to rotate around the existing flagellum. This introduces a fold in the FP membrane (*circled*), around the old MtQ. The tip of the new flagellum is laterally attached to the side of the old by the flagellum connector. (*f*) Continued rotation forms the boundary between the new and old FPs, while the flagellum and MtQ growth continue. The new BB and flagellum start to move toward the posterior, contorting the kinetoplast into a bilobed shape. (*g*) Flagellum growth continues, leading to two flagella emerging from one FP neck as FP division nears completion. This occurs as the new pro-BBs approach the G_1 configuration and kinetoplast S phase comes to a close. (*h*) Posterior movement of the new BB and flagellum continues as lateral separation of the FPs begins and kinetoplast division nears completion.

What is the signal pathway for BB duplication? There is tight regulation of pro-BB maturation, biogenesis, and number control, but how these processes are regulated is still unclear. Experimentally it is hard to disentangle defects in pro-BB maturation or biogenesis; hence, they are often considered together as BB duplication. In metazoa, polo-like kinase 1 has an important role in this process, yet arguably the trypanosome PLK1 homolog appears not to be required for BB duplication (48, 68, 74, 87). PLK1 may be important for BB segregation, as depletion of SPBB1, a PLK1 substrate, inhibits BB segregation (64) (**Figure 3*c***). Unsurprisingly RNAi depletion of core structural components of the BB such as SAS6 inhibits BB duplication and flagellum elongation (62). Known BB regulatory proteins in kinetoplastids are LRTP, whose depletion leads to a dysregulation in BB duplication (95), and the NIMA-related kinase NRKC, whose overexpression causes supernumerary BBs (109). The strict cell cycle ontogeny of *T. brucei* BBs makes it a powerful and tractable system for understanding BB duplication, especially now that we are moving from component definition to understanding the regulatory mechanisms.

5.2. The Flagellum

The flagellum is a complex organelle that contains different substructures, including the transition zone, paraflagellar rod, and axoneme, each containing many proteins (19, 31) (**Figure 2**); recent work emphasized the asymmetries within individual structures (35). The intraflagellar transport (IFT) system, which transports molecules within the flagellum, is essential for flagellum assembly (29, 73). The majority of flagellum growth occurs within the first cell cycle (14, 38) and continues until the flagellum reaches a consistent length. The cell presumably preferentially

directs flagellum components to the new flagellum (14). In trypanosomes, the IFT system is not required to maintain the length of the assembled old flagellum; however, IFT trains move in both the old and the new flagellum and may have a role in maintenance (40). We postulate that maintenance of IFT machinery in old flagella may be important for flagellum structure modifications such as the generation of attachment plaques in the insect forms or disassembly of the *Leishmania* flagellum during amastigote differentiation (130, 140).

Flagellum assembly appears entirely dependent upon the BB. Hence, depletion of a variety of BB components leads to defects in flagellar assembly and concomitant division issues (27, 62, 95, 109). However, once flagellum assembly has begun, inhibition of BB segregation, and of FPC, hook complex, and FAZ duplication does not affect flagellum growth (16, 27, 87). During early new flagellum assembly the new BB rotates around the old BB (78) (**Figure 4*d–f***), ensuring that the new BB/flagellum are located closer to the cell posterior—critical for the new FAZ and cytokinetic furrow positioning. PLK1 is required for BB/flagellum rotation (87) (**Figure 3*c***). Rotational positioning of the BB in *Leishmania* spp. and *T. cruzi*, in which the proliferative forms have a promastigote, amastigote, or epimastigote form, may not be required.

5.3. Basal Body–Associated Structures

The MtQ is a specialized set of rootlet microtubules nucleated close to the BB that wrap around the FP, pass through the FPC and hook complex, and then form part of the FAZ (**Figure 2**). Together these BB-associated structures form a critical module for trypanosome morphogenesis, and regulation of their duplication both is interdependent and requires PLK1. The nucleation of a new MtQ precedes BB maturation (78). The new flagellum invades the existing FP (**Figure 4*d***), and cytoskeletal/membrane morphogenetic changes result in two separate pockets associated with individual flagella (78) (**Figure 4*e–h***). The proximal MtQ protein SPEF1 is required for MtQ, FPC, hook complex, and FAZ duplication and BB segregation but does not affect BB duplication or flagellum elongation (41). Many BB-associated structures are generated de novo (BB, flagellum, FAZ, and MtQ) and inherited coordinately. However the inheritance of the FPC and hook complex remains cryptic (16, 68). Given the close connections between the FPC and hook complex these two structures are likely to have a coordinated duplication (6, 37).

The FAZ is a complex of cytoskeletal structures and membrane junctions that connect the flagellar cytoskeleton to the cell body cytoskeleton through flagellar and cell body membranes (129) (**Figure 2**). The FAZ is assembled at its proximal end adjacent to the FP, whereas the flagellum is assembled from the distal end. Hence, these two interconnected structures have distinct and spatially separated assembly sites (131). Disruption of FAZ assembly by knockdown of FAZ proteins such as FLA1 or FAZ2 leads to flagellum detachment and cytokinesis defects with no effect on flagellum growth (79, 148). IFT protein depletion inhibits flagellum construction, leading to cells with a short new flagellum, short FAZ length, and cytokinesis defects (1, 29, 73). Therefore, flagellum growth is critical to coordinate FAZ-directed cytokinesis in procyclics. In *Leishmania* promastigote IFT null mutants have a near-normal FAZ length, are viable, and proliferate despite having a very short flagellum (2, 130). Therefore, the assembly of a long FAZ and flagellum is likely a necessity for trypomastigote morphologies.

There is clear coordination in the duplication of BB-associated structures. PLK1 locates to each before they duplicate (68) and has numerous potential targets (64, 91) including FAZ9, TOEFAZ1/CIF1, and SPBB1. The Cullin4-containing ubiquitin ligase complex $CRL4^{WDR1}$ has been shown to regulate TbPLK abundance, hence controlling PLK1 activity (63, 64, 91, 147).

The single Golgi apparatus is located between the endoplasmic reticulum exit site and the FP (**Figure 2**), which likely define its position, with circumstantial evidence for hook complex

involvement (56, 96). The new Golgi apparatus is assembled de novo adjacent to the existing Golgi apparatus (55). Other membrane-bound organelles, glycosomes, acidocalcisomes, and lipid droplets, are present in multiple copies, and segregation of these between the two daughter is likely random (94).

5.4. Cytokinesis Templated by the Flagellum Attachment Zone

Trypanosome shape is defined by the corset-like microtubule array (**Figure 2**) and associated microtubule-binding proteins. These microtubules are acetylated, glutamylated, and stable. Incorporation of new microtubules into the array is necessary for growth to maintain even spacing (121). Incorporation of new tubulin that is yet to be detyrosinated (142) or a pulse of YFP::α-tubulin (120) is predominantly toward the cell posterior. Growth of cell length and growth of cell width are associated with microtubule lengthening and/or new microtubule intercalation (122, 142). Generation of the correct cell shape therefore requires regulation of when and where microtubules extend or are incorporated. After the decision to enter the cell cycle, the cell undergoes a constant growth in length through the cell cycle (111). Mutational perturbations (perhaps cell cycle timings) can lead to an elongated posterior (a nozzle) phenotype (47, 102).

During mitosis, microtubule intercalation widens the central portion of the cell, in preparation for generation of the daughter cells by cytokinesis (122, 142). Trypanosomes have γ-tubulin and γ-tubulin ring complex proteins (GCP2, GCP3, GCP4), but unfortunately their localization or interrogation of function has not led to the definition of the sites of individual microtubule nucleation within the subpellicular array (118, 152). In terms of collective organization, most known pellicular microtubule-associated proteins cover the entire array (3, 10, 107), although some are restricted to particular regions; for example, CAP17 is concentrated toward the anterior (136). Similarly, at least one posttranslational tubulin modification (detyrosination) (122, 142) is enriched in particular areas. There are likely more subpellicular domains than just posterior and anterior; for example, PAVE1 is concentrated toward the posterior and ventral domains (57).

Forming the cytokinetic furrow involves organizing the microtubules of the highly anisotropic pellicular array. The first step for this event is the formation of the new FAZ, which invades along the line of the old FAZ (78, 121, 142). Separation of the old and new FPs along the anterior-posterior axis (**Figures 3*c***; **4*f*,*g***) plausibly occurs by sliding of the old/new FAZ and associated FP along a seam between microtubules in the subpellicular array—indeed any growth of the FAZ, microtubule quartet, and/or subpellicular microtubules requires some sliding due to their antiparallel organization. Next, widening the lateral separation between the old and new FAZ requires insertion of new microtubules (142) (**Figure 4*b***).

CIF1/TOEFAZ1, CIF2, and CIF3 are key regulators of the cytokinetic furrow (75, 91, 147, 150). These proteins are loaded onto the new FAZ tip and are carried to the site of cytokinesis furrow initiation; however, what determines the positioning of this site is unknown. Two key signaling proteins for metazoan cytokinesis, MOB1 and PLK1, are conserved in trypanosomes (81). MOB1 is required for accurate cytokinesis in procyclic trypanosomes (49), as are MOB1 kinases in bloodstream forms (BSFs) (88). In contrast, PLK1 appears not to be required for cytokinesis but is at the growing FAZ tip (125). It is conceivable that MOB1 and its associated kinases, perhaps along with further regulators, are responsible for licensing furrow ingression mediated by the TOEFAZ/CIF complex.

Cytokinesis proceeds from anterior to posterior (**Figure 3*a*,*c***), with the cytokinesis furrow starting at the distal tip of the new FAZ and proceeding along a fold in the cell (121, 142). The furrow placement relative to the old and new FAZ guarantees correct inheritance of the BBs, kinetoplasts, and FP complexes, but the nuclei must be positioned correctly. Conceptually, furrow

ingression must require some rearrangement of the microtubule array: For cytokinesis to occur without microtubule rearrangement, each side of the fold would have to be defined by a gap between neighboring microtubules in the subpellicular microtubule array, and these gaps would have to be brought together. This finding is not compatible with a cytokinesis furrow beginning partway along the parallel anterior-posterior-aligned microtubule array. Whether this rearrangement occurs before or during furrow ingression is not known, but two kinesins (57, 65) and katanin subunits (12) have been identified that may play roles in this process. Finally, the furrow resolves to a single point of connection between the posterior of one daughter cell and the side of the other daughter (**Figure 3*a***)—this narrow cytoplasmic bridge can persist while the daughter cells restart the cell cycle, although it is normally resolved (142). Abscission occurs without apparent use of actinomyosin. Flagellum-driven motility of both daughter cells may mechanically contribute to abscission (19, 110); however, it seems likely that there is also more conventional (but as yet undiscovered) membrane fission machinery involved (39).

After cytokinesis, the two daughter cells are nonequivalent (38, 142), and both must be remodeled during the early cell cycle. The capacity to remodel the cell posterior is arguably visible following failure of cytokinesis in procyclics caused by TOEFAZ1 or CIF2 RNAi (147, 150). Here, cytokinesis fails with cells persisting as 2F2K2N cells. These cells have two distinct posterior-like tips that presumably arise from remodeling the cytoskeleton. Some cells can reenter the cell cycle, giving 4F4K4N cells—these have four remodeled posteriors.

6. CROSS TALK BETWEEN THREE SUBCYCLES?

In procyclic forms, early evidence showed that following inhibition of nuclear S phase or mitosis, cytoskeletal cell cycle events such as flagellum formation and BB segregation progressed, leading to a cytokinesis event, albeit changed in outcome (106). This outcome indicated that there are differences with metazoa and yeast and suggested there are three somewhat independent subcycles within the cell cycle: nuclear, kinetoplast, and cytoskeletal (**Figure 3*b–d***). Given these three subcycles, is there evidence for cross talk between them? Perhaps one of the best indications that there is cross talk at a start/restriction checkpoint is the existence of a G_0 state (see Section 2).

The nuclear subcycle is conceptually very similar to the case of metazoa and yeast—controlled by cyclins, with the normal strict series of S phase and then mitosis. Dependency events within the kinetoplast subcycle such as between deconcatenation, minicircle/maxicircle replication, and reattachment are unclear. Within the cytoskeleton subcycle there are several known dependency relationships that are partly regulated by PLK1. A meta-analysis of many studies suggests a hierarchy involving the MtQ, BB/pro-BB, FPC, hook complex, flagellum, and FAZ leading to cytokinesis (16, 41, 87). However, we await a comprehensive analysis of these cytoskeleton dependencies within a single study.

At present, there is only limited evidence for concerted cross talk between these subcycles in procyclics. An overview of many studies reveals a lack of evidence for cross talk. In the nuclear subcycle, failure to progress through either nuclear S phase or mitosis does not disrupt BB, flagellum, FAZ formation, kinetoplast division, or cytokinesis, which generates zoids and 1K1N cells (e.g., 46, 132). In the kinetoplast subcycle, failure to duplicate the mitochondrial DNA does not disrupt BB separation, mitosis, or cytokinesis, leading to progressive loss of mitochondrial DNA (e.g., 137). In the cytoskeleton subcycle, disruption of BB duplication does not disrupt kinetoplast S phase or mitosis but blocks later cytoskeleton subcycle events including cytokinesis; this outcome gives (initially) 1K2Ns with duplicated K DNA (e.g., 62). One would imagine that the critical dialog points between the subcycles are BB separation and the completion of kinetoplast S phase, and cytokinesis and the completion of mitosis; however, there is a lack of evidence for any cross talk at these points (**Figure 3*b,d***).

Reverse genetics and candidate gene approaches have provided us with the dependencies within the subcycles. There is little insight into any cross talk, but there has been minimal experimental interrogation that would be likely to reveal such phenomena, and these may be revealed by the future use of intelligent forward genetic screens.

7. CELL CYCLE ADAPTATIONS

7.1. Other Life Cycle Stages

The BSF cell has differences in organelle positioning and concomitant variation in organelle segregation within the cell cycle (66, 67, 142). The major variation appears to be the orchestration of the cytoskeletal subcycle events rather than the kinetoplast and nuclear subcycles. These differences can give rise to different phenotypes following disruption of cytoskeletal components (19, 128).

Disruption of the nuclear subcycle can also lead to different phenotypes in BSFs in comparison to procyclics (46), suggesting there is a cytokinesis checkpoint in BSFs that is missing or unused in procyclics. Interestingly, variant surface glycoprotein (VSG) RNAi causes a 2K2N precytokinesis block (119). Does VSG level feed into a cytokinesis checkpoint? Perhaps disruption of nuclear S phase or mitosis reduces VSG expression acting as cross talk to cytokinesis.

7.2. Other Species and Their Morphologies

The order in which the first event of each subcycle occurs within the cell cycle varies in different kinetoplastids. There is no doubt that this variability has a relationship to their different morphologies. In *Leishmania* promastigotes (8, 139) and *T. cruzi* epimastigotes (36), the BB segregation distance is much reduced and the FAZ is much shorter (143), perhaps allowing BB duplication and kinetoplast S phase to occur later in the cell cycle, as discussed in Reference 145).

T. brucei and *Leishmania* spp. are unusual in that they do not possess an endocytic cytostome/cytopharynx and associated cytoskeletal structures, found in many other trypanosomatids including *T. cruzi*. The cytostome/cytopharynx complex disassembles in G_2 prior to FP division and is reassembled during late cytokinesis (7)—likely an ancestral feature of the cytoskeletal subcycle necessary, perhaps, to facilitate FP division.

7.3. Life Cycle Transitions

Asymmetric divisions are commonly seen at life cycle transitions to achieve a change in cell fate. This asymmetry is seen in *T. brucei* (113, 134), the closely related *T. vivax* and *T. congolense* (101, 104), and the more divergent *T. cruzi* and *Leishmania* spp. (76, 140). It is notable that metacyclic and stumpy cells in G_0 are the infective forms at points of transfer between host and vector—cell division processes are coordinated with life cycle progress and pathogenicity. *T. brucei* has many cyclins (50); perhaps some have a role in specialized asymmetric differentiation divisions.

A defining feature of different morphologies is the position of the FP complex. Depletion of ClpGM6, a FAZ protein, resulted in cells with a shorter FAZ and the repositioning of the FP complex (54), thus switching from trypomastigote to epimastigote-like form. Modulation of FAZ length therefore provides a simple route to alter the morphology of the trypanosome cell inheriting the new flagellum and is likely to be associated with transitions in cell shape during the trypanosome life cycle.

Cell divisions associated with life cycle transitions involve major changes in regulation of cell surface proteins, metabolic proteins, and cytoskeletal proteins important for cytokinesis.

Interestingly, there are several examples of paralogous pairs of cytoskeletal proteins, one expressed in the procyclic and one in the BSF (100, 107, 129, 136).

T. brucei forms gamete-like cells, but meiosis and fertilization remain cryptic. Gamete-like cells identified so far are flagellated and possess a haploid nucleus and one or two kinetoplasts with higher than usual DNA content (103). It is difficult to consider a process for formation of such cells or the restoration of the normal diploid state that does not require decoupling of the nuclear, kinetoplast, and cytoskeleton subcycles and possibly division to generate zoids.

8. CONCLUSIONS

Over a decade after the genome was sequenced we have a good overview of the trypanosome cell division process. We understand much of the order and timing of events and have detailed descriptions of molecular and cellular processes within each of three distinct subcycles within the overall cell cycle. The information that flowed from the genome has mainly been used to investigate the function of bioinformatically identified candidate proteins. Again, this type of analysis has been most informative within analyses of the subcycles. It seems likely that if we are to move to a more informed overview of the cell cycle and holistic cell division processes we will need new approaches. Firstly, we will need more informed use of cell synchronizing technologies and interrogation of asynchronous populations using global mRNA and protein analysis, as exemplified by References 9 and 23, which can of course also be applied to populations of differentiated cells such as the G_0 stumpy and metacyclic forms (116). Secondly, we will need more time-lapse single-cell studies or analysis of the cell cycle from asynchronous populations (138) using organelle landmarks (45) to unravel the assembly hierarchy of complex cellular structures. Finally, can we use forward genetic screens as a route for unbiased identification of proliferative and differentiation division regulators, as they have been used to unravel mechanisms of antigenic variation (42)?

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