Published in final edited form as:

Mol Microbiol. 2013 October ; 90(1): 130–146. doi:10.1111/mmi.12352.

The cooperative roles of PHO80-like cyclins in regulating the G1/S transition and posterior cytoskeletal morphogenesis in *Trypanosoma brucei*

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Abstract

Cyclins and cyclin-dependent kinases (CDKs) represent the fundamental, crucial regulators of the cell division cycle in eukaryotes. *Trypanosoma brucei* expresses a large number of cyclins and Cdc2-related kinases (CRKs). However, how these cyclins and CRKs cooperate to regulate cell cycle progression remains poorly understood. Here, we carry out directional yeast two-hybrid assays to identify the interactions between the 10 cyclins and the 11 CRKs and detect a total of 26 cyclin-CRK pairs, among which 20 pairs are new. Our current efforts are focused on four PHO80-like cyclins, CYC2, CYC4, CYC5, and CYC7, and their physical and functional interactions with CRK1. Silencing of the four cyclins and CRK1 leads to the increase of G1 cells and defective DNA replication, suggesting their important roles in promoting the G1/S transition. Additionally, CYC2-, CYC7-, and CRK1-deficient cells possess an elongated posterior that is filled with newly assembled microtubules. Further, we show that the four cyclins display distinct subcellular localizations and half-lives, suggesting that they likely undergo distinct regulation. Altogether, our results demonstrate the involvement of four CRK1-associated cyclins, CYC2, CYC4, CYC5, and CYC7, in promoting the G1/S transition and the requirement of CYC2 and CYC7 in maintaining posterior cytoskeletal morphogenesis during the G1/S transition.

Introduction

The eukaryotic cell cycle is governed by multiple regulatory proteins, such as cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors. By well-controlled periodic synthesis and destruction of cyclins, the corresponding CDK activities go through sequential activation and inactivation, which provides the primary means of cell cycle control (Johnson & Walker, 1999). In *Saccharomyces cerevisiae*, three cyclins (Cln1-Cln3) are involved in the control of the G1/S transition, whereas six related B-type cyclins (Clb1-Clb6) drive the cell cycle progression through S phase to mitosis. All these cyclins bind to the same CDK, Cdc28, in a sequential manner to activate Cdc28 (Kuntzel et al., 1996). In *Schizosaccharomyces pombe*, however, a single cyclin, Cdc13, and a single CDK, Cdc2, control both the G1/S and G2/M transitions (Humphrey & Pearce, 2005). Regulation of the cell cycle in animals is more complex, with multiple distinct families of cyclins and CDKs regulating the G1/S transition (Cyclin D and Cdk4 or Cdk6), S-phase progression (Cyclin E and Cdk2), S phase and mitosis (Cyclin A and Cdk2 or Cdk1), and mitosis only (Cyclin B and Cdk1) (Harper & Brooks, 2005).

The cell cycle control system in *Trypanosoma brucei*, an early branched microbial eukaryote and the causative agent of human sleeping sickness, appears to be different from that in

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yeasts and animals and is likely more complicated than previously thought. The genome of *T. brucei* encodes 10 cyclins (CYC2-CYC11) and 11 Cdc2-related kinases (CRK1-CRK4 and CRK6-CRK12) (Hammarton, 2007), among which the CYC2-CRK1 pair and the CYC6-CRK3 pair appear to be the primary cyclin-CRK complexes for promoting the G1/S and G2/M transitions, respectively (Li & Wang, 2003, Hammarton *et al.*, 2003, Tu & Wang, 2004, Hammarton *et al.*, 2004). Additionally, two cyclins, CYC4 and CYC8, which are related to CYC2 and CYC6, respectively, are also involved in promoting the G1/S transition and the G2/M transition, respectively (Li & Wang, 2003). Intriguingly, CYC2 also interacts with CRK3 to promote the G2/M transition (Van Hellemond *et al.*, 2000, Gourguechon *et al.*, 2007) and with CRK2 to control the G1/S transition (Gourguechon *et al.*, 2007). CYC6, an essential B-type cyclin involved in the G2/M transition in trypanosomes (Li & Wang, 2003, Hammarton *et al.*, 2003), also associates with CRK9, which is required for the G2/M transition, basal body segregation, and cytokinesis (Gourguechon & Wang, 2009). Recently, CRK9 was found to regulate trans-splicing by phosphorylating the RPB1 subunit of the RNA polymerase II (Badjatia *et al.*, 2013a), which raised the question of whether CRK9 is involved in cell cycle control. Further biochemical purification of CRK9 partners identified a novel cyclin, which was named CYC12, and RNAi of CYC12 also disrupted trans-splicing (Badjatia *et al.*, 2013b), indicating that CYC6 is not the cyclin partner of CRK9. It also suggests that CRK9 likely does not play a role in cell cycle regulation. Altogether, these findings suggest that at least two CRKs (CRK1 and CRK2) and two cyclins (CYC2 and CYC4) are important for promoting the transition from G1 to S-phase, whereas CRK3 and three cyclins (CYC2, CYC6, and CYC8) are required for driving the cell cycle transition from G2 to mitosis.

Despite the tremendous efforts leading to the identification of crucial cyclins and CRKs involved in the G1/S and G2/M transitions, the complete picture of the pairwise interactions among all the cyclins and CRKs is lacking and the potential function of the remaining cyclins and CRKs in cell cycle regulation remains to be defined. In this report, we undertook a systematic yeast two-hybrid assay attempting to detect all the interactions between the 10 cyclins and 11 CRKs. This approach allowed us to identify most of the known cyclin-CRK pairs and a large number of new cyclin-CRK pairs. Among these cyclin-CRK pairs, the association of CRK1 with four PHO80-like cyclins, CYC2, CYC4, CYC5, and CYC7, was of particular interest and was further characterized for their *in vivo* interactions in trypanosomes and their functional cooperation in cell cycle regulation and cell morphogenesis. Additionally, the subcellular localization and the stability of the four cyclins were also examined, which further revealed distinctions among these cyclins.

Results

A systematic yeast two-hybrid assay to map the pairwise interactions between the 10 cyclins and the 11 CRKs

The trypanosome genome encodes a surprisingly large number of cyclins and CRKs, but only a few cyclin-CRK pairs have been identified so far (Van Hellemond *et al.*, 2000, Hammarton *et al.*, 2003, Gourguechon *et al.*, 2007, Gourguechon & Wang, 2009, Monnerat *et al.*, 2013). To map all the potential interactions between the 10 cyclins and the 11 CRKs, we carried out a systematic yeast two-hybrid assay by pairing all the cyclins with all the CRKs. A total of 220 cyclin-CRK combinations were made by pairing the 10 cyclins fused to the Gal4 activation domain with the 11 CRKs fused to the Gal4 binding domain and by pairing the 10 cyclins fused to the Gal4 binding domain with the 11 CRKs fused to the Gal4 activation domain. Moreover, to distinguish false positive interaction due to protein self-activation of the Gal4 promoter in yeast, all the cyclins and CRKs fused to the Gal4 binding domain were also paired with the empty vector expressing only the Gal4 activation domain for examining the self-activation capability of the cyclins and CRKs. Those genes that

exhibit self-activation were further examined by spotting the yeast strains onto the quadruple drop-out plate to eliminate self-activation (see Material and Methods for details). Among all the cyclins and CRKs, only CYC9 and CRK12 exhibited self-activation and thus were further examined to eliminate false positive interactions (Supplemental Fig. 1). Positive interaction between cyclins and CRKs was scored based on the following criteria: growth of yeast from at least one of the three 10-fold serial dilutions on the selection plate was scored as positive. In the cases that reciprocal yeast two-hybrid assays between a cyclin and a CRK yielded inconsistent outcome, the interaction detected between them on any of the two orientations was also considered to be positive. Such interactions are, however, unidirectional in yeast two-hybrid assays.

Based on the criteria described above, a total of 26 cyclin-CRK pairs were identified (Table 1). Six cyclin-CRK pairs, CYC2-CRK1, CYC2-CRK2, CYC2-CRK3, CYC4-CRK1, CYC5-CRK1, and CYC9-CRK12, detected in this assay have been previously reported (Van Hellemond et al., 2000, Gourguechon et al., 2007, Monnerat et al., 2013); however, the interaction between CYC6 and CRK3, which was reported previously (Hammarton et al., 2003), was not detected in our assay for reasons that are not clear. It was also not detected in a previous study (Gourguechon et al., 2007). Surprisingly, CYC6, an essential B-type cyclin controlling the G2/M transition in trypanosomes (Li & Wang, 2003, Hammarton et al., 2003), did not interact with any CRKs in our assay (Table 1). CRK9, which is required for the G2/M transition and interacts with CYC6 *in vitro* in GST pull-down (Gourguechon & Wang, 2009), did not interact with any cyclins in our assay (Table 1). Like CRK9, CRK4 also did not associate with any cyclins (Table 1). However, CRK4 also appears to be essential for cell proliferation in both procyclic and bloodstream forms (Alsford et al., 2011). Western blot indicated that CRK4, CYC6, and CRK9 were expressed in yeast (Supplemental Fig. 2). The failure to identify the cyclin partners for CRK4 and CRK9 and to detect the interaction between CYC6 and CRK3 by yeast two-hybrid suggests that yeast two-hybrid did not work for them. It also suggests that biochemical approaches are needed for identifying their partners or for confirming the interactions. This was, however, not the focus of the current work and, therefore, was not pursued. As a support of this notion, through tandem affinity purification CRK9 was found to associate with a novel, highly diverged cyclin, named CYC12 (Badjatia *et al.*, 2013b), which explains why CRK9 did not interact with any of the 10 cyclins in our assay. This also validates the biochemical approach for confirming the cyclin-CRK pairs identified by yeast two-hybrid and for identifying the cyclin or CRK partners that were not detected by yeast two-hybrid.

A total of 20 new cyclin-CRK pairs are suggested based on yeast two-hybrid assays (Table 1), one of which is the CYC7-CRK1 pair (Fig. 1A). Altogether, CRK1 appears to interact with four cyclins, CYC2, CYC4, CYC5, and CYC7 (Fig. 1A), all of which belong to the PHO80-like cyclin family (Van Hellemond et al., 2000, Li & Wang, 2003, Hammarton, 2007), but only CYC2 was previously characterized in detail (Li & Wang, 2003, Hammarton et al., 2004). Strikingly, CYC2 appears to interact with at least seven CRKs, CRK1, CRK2, CRK3, CRK6, CRK8, CRK10, and CRK12 (Table 1). The functional cooperation between CYC2 and CRK1, CRK2, and CRK3 has been evaluated previously (Gourguechon et al., 2007), but the potential roles of CYC2-CRK6, CYC2-CRK8, CYC2-CRK10, and CYC2-CRK12 remain to be explored. Due to the essential involvement of CRK1 in the G1/S transition in trypanosomes (Tu & Wang, 2004), the identification of four cyclins as CRK1 partners argues that these cyclins likely all cooperate with CRK1 to promote the G1/S transition. Our current work was focused on the roles of the four cyclins and their functional interaction with CRK1.

In vitro and in vivo interactions of CRK1 with CYC2, CYC4, CYC5, and CYC7

To further confirm the interactions between CRK1 and the four PHO80-like cyclins, CYC2, CYC4, CYC5, and CYC7, we carried out GST pull-down assays and found that all four cyclins were capable of pulling down CRK1 from the trypanosome cell lysate (Fig. 1B), suggesting that they interact with CRK1 *in vitro*. To examine whether the four cyclins associate with CRK1 *in vivo* in trypanosomes, we performed co-immunoprecipitation, and the results shown in Figure 1C indicated that each of the four cyclins interacts with CRK1 *in vivo* in trypanosomes (Fig. 1C). In contrast, CYC6, a B-type cyclin required for the G2/M transition in trypanosomes (Li & Wang, 2003, Hammarton et al., 2003) and is known to interact with CRK3 but not CRK1 (Hammarton et al., 2003), was not precipitated with CRK1 by *in vitro* GST pull-down and *in vivo* immunoprecipitation (Fig. 1B,C).

RNAi of the four PHO80-like cyclins and CRK1 results in G1/S defects in the procyclic form

The identification of four cyclin partners of CRK1 (Table 1 and Fig. 1) led us to hypothesize that all four cyclins are important for the G1/S transition in trypanosomes. Previous studies have demonstrated the essential involvement of CYC2 and CRK1 in the G1/S transition (Li & Wang, 2003, Hammarton et al., 2004, Tu & Wang, 2004), but the function of CYC4, CYC5, and CYC7 was not investigated in detail. We therefore knocked down CYC4, CYC5, and CYC7 by RNAi in the procyclic form, and for a comparison we also carried out RNAi against CYC2 and CRK1. The RNAi appeared to be very potent, resulting in the knockdown of the mRNA level of CRK1 and all cyclins but CYC2 to less than 10% of that in the control cells, as measured by quantitative RT-PCR (Fig. 2A and Supplemental Fig. 3). CYC2 RNAi only led to the knockdown of CYC2 mRNA level to ~30% of that in the uninduced control (Fig. 2A and Supplemental Fig. 3). RNAi of CYC2, CYC4, CYC7, and CRK1 each caused significant growth defect, but CYC5 RNAi only slightly slowed down cell growth (Fig. 2B and Supplemental Fig. 3).

To further investigate the effect of cyclin and CRK1 RNAi on the nuclear division cycle, we stained the cells with DAPI for nuclear and kinetoplast DNA and counted the cells with different numbers of nuclei and kinetoplasts. In all the RNAi cell lines except the CYC5 RNAi cell line, the number of cells with one nucleus and one kinetoplast (1N1K) was gradually increased from ~80% to ~90% of the total cell population, which was accompanied by the decrease of 1N2K and 2N2K cells (Fig. 2C and Supplemental Fig. 3). RNAi of CYC5 also led to an increase of 1N1K cells from ~83% to ~90% (Fig. 2C). In trypanosomes, most of the 1N1K cells are at the G1 phase of the cell cycle and some 1N1K cells with an elongated kinetoplast are at the S phase. The 1N2K cells are generally at the G2 phase and early mitotic phases such as prometaphase and metaphase, whereas the 2N2K cells are always at late mitotic phases and telophase prior to cytokinesis. The increase of the number of 1N1K cells upon RNAi suggests that RNAi of the four cyclins and CRK1 either slowed down the cell cycle transition from G1 to S-phase or slowed down S-phase progression.

To distinguish between defective G1/S transition and S-phase delay, we carried out flow cytometry to measure the DNA content of the control and RNAi cells. RNAi of CRK1 and three cyclins, CYC2, CYC4, and CYC7, each led to a gradual increase of G1 cells from ~50% to ~70% and a gradual decrease of S-phase cells and G2/M cells after RNAi induction for 4 days (Fig. 3 and Supplemental Fig. 3). RNAi of CYC5 resulted in an increase of G1 cells from ~54% to ~65% and a corresponding decrease of S-phase cells from ~28% to ~17% after RNAi induction for 4 days (Fig. 3). These observations confirmed that RNAi of the four cyclins and CRK1 slowed down the G1/S transition but not S-phase progression.

RNAi of the four PHO80-like cyclins and CRK1 leads to defects in DNA replication

The accumulation of G1 cells upon RNAi of CRK1 and the four PHO80-like cyclins suggests that DNA replication was likely defective in these RNAi cells. To examine the effect of CRK1 and cyclin RNAi on DNA replication, BrdU incorporation assay was carried out. Control and the RNAi cells that have been induced for 4 days were incubated with BrdU for 16 hours and immunostained with anti-BrdU antibody. In the uninduced control cells, the majority (~98%) were BrdU positive (Fig. 4), and BrdU was found incorporated into both the nucleus and the kinetoplast (Fig. 4A). RNAi of CYC4, CYC7, and CRK1 all resulted in significant reduction of BrdU-positive cells to about 10% in CYC7 RNAi cells, ~12% in CYC4 RNAi cells, ~47% in CYC5 RNAi cells, ~27% in CRK1 RNAi cells, and ~41% in CYC2 RNAi cells (Fig. 4). However, RNAi of CYC2 and CYC5 caused less severe defects in DNA replication, resulting in the reduction of BrdU-positive cells to ~41% and ~47%, respectively (Fig. 4). These data further confirmed that transition to S-phase was compromised by RNAi against CRK1 and its cyclin partners.

RNAi of CYC2, CYC7, and CRK1 compromises posterior morphogenesis

In previous reports (Li & Wang, 2003, Hammarton et al., 2004), RNAi of CYC2 resulted in posterior elongation. Intriguingly, elongation of the posterior portion of the cell was also observed when CYC7 or CRK1 was knocked down (Fig. 5A). We define the posterior of the procyclic trypanosomes as the portion between the basal body and the posterior tip of the cell (Fig. 5A). The posterior length of the control 1N1K cells was calculated to be around 5-6 μm (Fig. 5A); however, the posterior of CYC7 and CRK1 RNAi cells became extensively elongated to more than 10 μm (Fig. 5A), similar to the CYC2 RNAi cells generated in this study (Fig. 5A) and reported previously (Li & Wang, 2003, Hammarton et al., 2004). In some extreme cases, the length of the posterior was increased up to 20 μm (data not shown). These cells with an elongated posterior constituted a significant percentage of the total cell population and increased gradually after RNAi induction (Fig. 5B). At day 6 of RNAi, the elongated cells (posterior length > 10 μm) constituted around 44-47% of CYC2, CYC7, and CRK1 RNAi cells (Fig. 5B). The posterior portion of CYC4 and CYC5 RNAi cells was not elongated (Fig. 5A), despite that RNAi of CYC4 also significantly slowed down the G1/S transition and RNAi of CYC5 slightly slowed down the G1/S transition (Figs. 2 and 3). These observations suggest that CYC4 and CYC5 are likely not involved in controlling posterior morphogenesis.

Previously, the elongated posterior of CYC2 RNAi cells was found to be intensively stained by YL1/2 antibody that specifically labels newly assembled tyrosinated microtubules (Hammarton et al., 2004). Since the microtubule corset in a trypanosome cell extends (or grows) toward the posterior tip of the cell during G1 and S phases (Sherwin et al., 1987), the elongation of the posterior portion could attribute to the excessive growth of the microtubule corset. To test whether the elongated posterior of CYC7 RNAi cells and CRK1 RNAi cells was also filled with newly assembled microtubules, cells were immunostained with YL 1/2 antibody. The results showed that the elongated posterior of both RNAi cells was intensively stained by YL 1/2, similar to the CYC2 RNAi cells (Fig. 5A), indicating that the elongated posterior was indeed filled with tyrosinated microtubules that are likely newly assembled to the microtubule corset. These data suggest that CYC2 and CYC7, by forming complexes with CRK1, likely function in the same pathway to control posterior morphogenesis during the G1/S transition in the procyclic form.

Another interesting phenotype caused by CYC7 and CRK1 RNAi was posterior branching (Fig. 6). The branched posteriors were also elongated and were also filled with newly assembled microtubules as detected by YL 1/2 antibody staining (Fig. 6A), but these cells only constituted about 4-5% of the total cell population (Fig. 6B). The occurrence of

branched posteriors in CYC7 and CRK1 RNAi cells further suggests the defect in posterior morphogenesis in these cells. However, it is not clear why CYC2 RNAi did not cause posterior branching.

Distinct subcellular localization and protein stability of the four PHO80-like cyclins

The fact that control of the G1/S transition in trypanosomes requires four cyclins to activate CRK1 suggests a more complicated regulatory scheme than previously thought (Li and Wang, 2003), which resembles the regulation of the G1/S transition by three G1 cyclins, Cln1, Cln2, and Cln3, in the budding yeast (Kuntzel et al., 1996). The association of four different cyclins with the same catalytic subunit CRK1 raised the question of how substrate specificity of CRK1 was achieved. One mechanism could be specific subcellular localization of specific cyclin-CRK1 complexes, as in the case of Cln2 and Cln3 (Edgington & Futcher, 2001). To examine whether the four PHO80-like cyclins are localized to distinct subcellular compartments, we performed immunofluorescence microscopic analysis of epitope-tagged cyclins and CRK1, which were expressed from their respective endogenous loci, in the procyclic form. To alleviate the concern whether tagging of cyclins and CRK1 affects their localization and, hence, their cellular functions, both alleles of the target genes were tagged, and the cells expressing the tagged proteins grew normally (data not shown), suggesting that the tagged proteins are functional. We found that three cyclins, CYC2, CYC5, and CYC7, were localized throughout the cytosol and a small amount of these proteins was detected in the nucleus (Fig. 7). Similarly, CRK1 was also distributed throughout the cytoplasm, with a small amount of CRK1 protein found in the nucleus (Fig. 7). In contrast, CYC4 was predominantly nuclear (Fig. 7). Further computational analysis of CYC4 sequence identified a putative nuclear localization signal (NLS) sequence in its C-terminal tail (data not shown), but no NLS sequence was found in CYC2, CYC5, CYC7, and CRK1. These observations suggest that the four cyclins have distinct subcellular locations in procyclic cells.

The level of cyclins is known to fluctuate during the cell cycle, which constitutes the primary means for CDK activation and inactivation. To investigate whether the four PHO80-like cyclins are ubiquitinated *in vivo* in trypanosomes, each of the four cyclins was overexpressed in *T. brucei* by induction with 0.1 µg/ml tetracycline for low level of overexpression, immunoprecipitated from the cell lysate, and immunoblotted with anti-ubiquitin antibody. All four cyclins were efficiently immunoprecipitated from the cells as monitored by western blot with anti-HA antibody (Fig. 8A), and ubiquitinated cyclins were detected as high molecular mass proteins immuno-labeled by anti-ubiquitin antibody in tetracycline-induced samples but not in the uninduced control samples (Fig. 8A). More ubiquitinated CYC2 and CYC7 were detected than ubiquitinated CYC4 and CYC5 (Fig. 8A, upper panel), despite that similar amount of the four cyclins was immunoprecipitated from the cell lysate (Fig. 8A, lower panel). This difference could be attributed to the various amounts of ubiquitinated cyclins present in the cell prior to the immunoprecipitation experiments. Nevertheless, these results suggest that all four cyclins likely are subject to ubiquitin/26S proteasome-mediated degradation.

Previously, CYC2 has been demonstrated to be degraded by the 26S proteasome, and its half-life has been estimated to be about 7.5 hours (Van Hellemond & Mottram, 2000). To investigate whether the other three cyclins are also short-lived proteins, we monitored their half-life. Cells expressing endogenously 3HA-tagged CYC4, CYC5, and CYC7 were treated with cycloheximide, and time-course samples were immunoblotted with anti-HA antibody to examine the degradation of 3HA-tagged cyclins. Additionally, to test whether the three cyclins are degraded by the 26S proteasome, MG-132, a potent inhibitor of the 26S proteasome, was added to cells in addition to cycloheximide. We found that all three cyclins were degraded, albeit at various rates (Fig. 8B). Within the 12-hr time course of

experiments, during which the cycloheximide-treated cells were still alive, CYC4 protein level went down gradually, but the protein was not completely degraded after 12 hours (Fig. 8B). The half-life of CYC4 was estimated to be about 2.5 hours (Fig. 8C). Strikingly, CYC5 and CYC7 appeared to have a much shorter half-life than CYC4. CYC5 level was reduced to ~14% of the original level after 80 minutes of cycloheximide treatment, whereas CYC7 level was reduced to ~10% of the original level after 40 minutes of cycloheximide treatment (Fig. 8B). Based on these experiments, the half-life of CYC5 and CYC7 was estimated to be about 18 minutes and 12 minutes, respectively (Fig. 8C). In the presence of MG-132, all three cyclins were stabilized (Fig. 8B), indicating that they are all degraded by the 26S proteasome.

Discussion

Trypanosomes possess an expanded repertoire of cyclins and cyclin-dependent kinases compared to the budding and fission yeasts (Hammarton, 2007), but only a few cyclin-CRK pairs have been identified and characterized, which significantly hindered our comprehensive understanding of the cooperative roles of cyclins and CRKs in trypanosome cell cycle control. In this paper, we report the pairwise interactions of the 10 cyclins and 11 CRKs by directional yeast two-hybrid assay. We identified all the known cyclin-CRK pairs except the CYC6-CRK3 pair, indicating the high efficiency of our assay. Moreover, we also identified 20 new cyclin-CRK pairs (Table 1). Among the 10 cyclins encoded by the trypanosome genome, six (CYC2, CYC4, CYC5, CYC7, CYC10, and CYC11) belong to the PHO80-like cyclin family, three (CYC3, CYC6, and CYC8) exhibit sequence homology to the B-type cyclins, and one (CYC9) exhibits sequence homology to the C-type cyclin (Li & Wang, 2003, Hammarton, 2007). Previous RNAi studies (Li & Wang, 2003, Hammarton et al., 2003, Hammarton et al., 2004, Monnerat et al., 2013) and a genome-wide RNAi assay (Alsford et al., 2011) showed that knockdown of all but two cyclins (CYC10 and CYC11) in the procyclic form and all but three cyclins (CYC7, CYC10, and CYC11) in the bloodstream form caused abnormal cell proliferation. For the 11 CRKs, except CRK6, CRK8, and CRK11, RNAi of seven CRKs (CRK1, CRK2, CRK3, CRK4, CRK7, CRK9, CRK10) all resulted in growth defects in both the procyclic and bloodstream forms, and knockdown of CRK12 caused growth inhibition only in the bloodstream form (Tu & Wang, 2004, Gourguechon & Wang, 2009, Alsford et al., 2011, Monnerat et al., 2013, Badjatia et al., 2013a). Based on these preliminary functional data and our current cyclin-CRK interaction data, as many as 17 cyclin-CRK pairs are formed by functionally important cyclins and CRKs, indicating a very complicated cyclin-CRK-mediated regulation scheme in trypanosomes, which appears to be more complicated than the cyclin-CDK system in the budding and fission yeasts. Although the *in vivo* interaction for the majority of the new cyclin-CRK pairs remains to be confirmed, our systematic yeast two-hybrid assay provided important references for future in-depth functional characterization of cyclin-CRK complexes in trypanosomes.

According to our yeast two-hybrid data, one cyclin can associate with multiple CRKs that are involved in different cell cycle stages. For example, CYC2 associates with as many as five important CRKs, CRK1, CRK2, CRK3, CRK10, and CRK12, and appears to regulate the G1/S transition with CRK1 and CRK2 (Gourguechon et al., 2007), the G2/M transition with CRK3 (Gourguechon et al., 2007), and likely other cell cycle stages with CRK10 and CRK12 that remain to be explored. Similarly, CYC8, a B-type cyclin involved in the G2/M transition (Li & Wang, 2003), appears to associate with three important CRKs, CRK2, CRK7, and CRK12, among which CRK2 is known to control the G1/S transition (Tu & Wang, 2004, Tu & Wang, 2005). The formation of CYC8-CRK2 complex suggests that CRK2 could also be involved in the G2/M transition, but this function was not revealed by RNAi of CRK2, which caused defects in the G1/S transition that could have masked any

potential defects in the later cell cycle stage(s), such as the G2/M transition. Additionally, the association of CYC8 with CRK7 and CRK12 suggests that CRK7 and CRK12 may be required for the G2/M transition, although this remains to be investigated. On the other hand, we found that one CRK is capable of interacting with multiple cyclins that likely control distinct cell cycle phases. For example, CRK12 associates with four PHO80-like cyclins (CYC2, CYC4, CYC5, and CYC7) and with a B-type cyclin, CYC8, as well as with the C-type cyclin CYC9 (Table 1). It suggests that CRK12 may play multiple roles, which likely include, but are not limited to, the control of G1/S and G2/M transitions. Again, further biochemical characterization of these cyclin-CRK complexes is needed to verify their *in vivo* interaction and functional analysis is necessary to ascertain their roles in cell cycle control.

Another CRK that associates with the same four PHO80-like cyclins as CRK12 is CRK1, which binds to all four cyclins both *in vitro* and *in vivo* (Table 1 and Fig. 1). There are two additional PHO80-like cyclins, CYC10 and CYC11, in the trypanosome genome, but neither is essential for cell proliferation (Alsford et al., 2011) and neither associates with CRK1 and CRK12 (Table 1). The fact that RNAi of all four CRK1-associated cyclins, CYC2, CYC4, CYC5, and CYC7, caused defects in cell proliferation and the G1/S transition in the procyclic form (Figs. 2, 3, 4) suggests that control of the G1/S transition requires four cyclins to cooperate with CRK1, in addition to the CYC2-CRK2 complex (Gourguechon et al., 2007). Moreover, given that the four cyclins also associate with CRK12, which appears to be only essential in the bloodstream form (Alsford et al., 2011, Monnerat et al., 2013), there may be life cycle stage-specific differences in the regulation of the G1/S transition. With the identification of multiple cyclin-CRK1 complexes that control the G1/S transition, a number of intriguing questions arose, which need to be addressed in the future for better understanding of the roles of these complexes. For instance, do the four PHO80-like cyclins act sequentially with CRK1 to promote the G1/S transition or do different cyclin-CRK1 complexes exert distinct functions that collectively drive the transition from G1 to S-phase, i.e. by regulating distinct downstream factors? It has been well established that substrate specificity of the CDK is generally determined by the intrinsic selectivity of the CDK active site as well as by substrate docking sites on its cyclin partner (Cross et al., 1999, Loog & Morgan, 2005, Ikui et al., 2007). A recent study on the dynamic CDK specificity during the cell cycle of the budding yeast further confirmed that cyclins do not merely activate CDK but also modulate the specificity of CDK active sites and through binding to different cyclins CDK shifts its specificity toward distinct substrates (Koivomagi et al., 2011). If trypanosomes have adopted the same mechanism as the budding yeast, the four cyclin partners of CRK1 could modulate the specificity of CRK1 toward different sets of substrates that are all necessary for the passage of cell cycle from G1 to S-phase in trypanosomes.

The essential involvement of CYC2 and CYC7, but not CYC4 and CYC5, in posterior morphogenesis in the procyclic form (Figs. 5 and 6) supports the notion that cyclins represent an important determinant of CRK1 specificity. It remains unclear whether CYC2- and CYC7-binding also alter CRK1 substrate specificity; however, the fact that only CYC7 RNAi produced cells with branched posteriors (Fig. 6) argues that CYC2-CRK1 and CYC7-CRK1 likely regulate distinct substrates. Posterior elongation in the procyclic trypanosomes was first observed in cells overexpressing a novel CCCH-type zinc finger protein, TbZFP2 (Hendriks et al., 2001). The molecular mechanism by which TbZFP2 modulates posterior microtubule extension remains elusive, but given that all the elongated cells overexpressing TbZFP2 are restricted to the G1 phase and early S phase (Hendriks et al., 2001), it suggests that posterior elongation upon TbZFP2 overexpression is likely attributed to defects in the G1/S transition. This notion was further supported by the production of elongated cells upon CYC2 RNAi (Li & Wang, 2003, Hammarton et al., 2004) and CRK1-CRK2 double RNAi (Tu & Wang, 2005), both of which compromised the G1/S transition. Our current finding,

however, argues that defects in the G1/S transition in the procyclic form likely are not the trigger for posterior elongation because CYC4-deficient cells, which exhibit similar G1/S defects as CYC2- and CYC7-deficient cells, were not elongated (Fig. 4). Rather, our results suggest that CYC2 and CYC7, together with CRK1, play additional roles in regulating posterior morphogenesis. Although we do not have enough information to speculate further on the detailed mechanism of CYC2-CRK1- and CYC7-CRK1-regulated posterior morphogenesis, we can conjecture about the potential role of the two cyclin-CRK1 pairs in controlling the process. Since the cytoskeletal microtubules at the posterior portion of a trypanosome cell undergo extensive growth toward the posterior end of the cell during late G1 phase and S phase (Sherwin et al., 1987), CYC2-CRK1 and CYC7-CRK1 likely are required to coordinate the growth of the posterior with the G1/S transition by restricting the excessive extension of the microtubule corset toward the posterior tip. In the absence of CYC2, CYC7, or CRK1, the restriction placed to limit excessive microtubule extension is lost, thus allowing microtubule extension to continue out of control, which results in an abnormally elongated posterior in cells arrested at the G1 phase.

The distinct functions of the four PHO80-like cyclins likely are attributed to their distinct subcellular locations, which may also contribute to CRK1 specificity. CYC4, which is predominantly enriched in the nucleus (Fig. 7), may regulate the nuclear processes that are necessary for the G1/S transition, whereas CYC2 and CYC7, both of which are distributed to the entire cytosol (Fig. 7), may be primarily responsible for regulating the cytoplasmic processes that are involved in both the G1/S transition and the polarized extension of cytoskeletal microtubules (see above). Further, the differential regulation of the stability of these cyclins (Fig. 8) may contribute to the temporal regulation of CRK1 activity. CYC2 and CYC4 are relatively stable, with an estimated half-life of about 7.5 hrs (Van Hellemond & Mottram, 2000) and 2.5 hrs (Fig. 8B, C), respectively, whereas CYC5 and CYC7 are extremely short-lived, with an estimated half-life of only 18 minutes and 12 minutes, respectively (Fig. 8B, C). The longer half-life of CYC2 appears to correlate with its additional role in the G2/M transition by activating CRK3 at the G2 phase (Gourguechon et al., 2007). In contrast, the shorter half-life of the other three cyclins argues that they likely only function during the G1/S transition.

In summation, we have identified, through directional yeast two-hybrid assay, 20 new cyclin-CRK pairs that are potentially involved in trypanosome cell cycle regulation, and we further demonstrated the essential involvement of four PHO80-like cyclins, CYC2, CYC4, CYC5, and CYC7, in the G1/S transition and the requirement of two PHO80-like cyclins, CYC2 and CYC7, in maintaining posterior morphology in the procyclic form of *T. brucei*. Future work will be directed to the identification of the substrates of CRK1 and the determinants of CRK1 specificity, which hopefully will further our fundamental understanding of the control of the G1/S transition and posterior morphogenesis in trypanosomes.

Materials and Methods

Trypanosome cell culture and RNAi

The procyclic 29-13 cell line (Wirtz et al., 1999) was cultured in SDM-79 medium supplemented with 10% fetal bovine serum, 50 µg/ml hygromycin, and 15 µg/ml G418 at 27°C. The procyclic 427 cell line was cultivated in SDM-79 medium containing 10% fetal bovine serum at 27°C.

For RNAi knockdown of cyclins and CRK1, a 400-500-bp DNA fragment corresponding to the N-terminal coding region of CYC2, CYC4, CYC7, and CRK1 was cloned into the pZJM vector. For CYC5 RNAi, a 544-bp fragment corresponding to the middle portion of the

coding sequence (nucleotides 1124-1667) was cloned into the pZJM vector. The resulting constructs were linearized by *Not*I digestion, and transfection of trypanosomes by electroporation was carried out according to our published procedures (Yu et al., 2012). Successful transfectants were cloned by limiting dilution in a 96-well plate. To induce RNAi, the clonal cell line was incubated with 1.0 µg/ml tetracycline.

Quantitative RT-PCR

Total RNA was purified with the TRIzol reagent (Invitrogen), treated with DNase I to remove any contaminated DNA, and used to synthesize first-strand cDNA with MMLV reverse transcriptase (Promega). Real-time PCR was carried out using the SYBR green PCR master mix (Applied Biosystems) on the CFX Real-Time System (Bio-Rad). Three replicates were run simultaneously in the Real-Time PCR machine. Actin gene was used as the control.

In situ epitope tagging of cyclins and CRK1 in the procyclic form of *T. brucei*

For subcellular localization of the four cyclins and CRK1 in the procyclic form, wild-type 427 cells were electroporated with pC-CYC::3HA-BSD or pN-3Myc::CRK1-PAC and selected under 10 µg/ml blasticidin or 1 µg/ml puromycin, respectively. Cells were cloned by limiting dilution in 96-well plates. To tag the second allele of the cyclins and CRK1, the clonal cell lines were further transfected with pCCYC::3HA-NEO and pN-3Myc::CRK1-NEO and selected under 40 µg/ml G418 in addition to blasticidin or puromycin. The transfectants were further cloned by limiting dilution.

To co-express PTP-tagged CRK1 and 3HA-tagged cyclins for co-immunoprecipitation, the 29-13 cell line was transfected with pC-CYC::3HA-BSD vectors, and then transfected with pN-PTP::CRK1-PAC for targeting to their respective endogenous locus. The double transfectants were selected with 1 µg/ml puromycin in addition to 10 µg/ml blasticidin. Cells were cloned by limiting dilution in 96-well plates.

In all the endogenous tagging experiments described above, correct *in situ* tagging of one of the two alleles or both alleles was confirmed by PCR and subsequent sequencing of the PCR fragment. Correct tagging of the proteins was also verified by western blot with anti-HA mAb, anti-Myc mAb, or anti-Protein A mAb (Sigma-Aldrich) for HA-, Myc-, and PTP-fusion proteins, respectively.

Yeast two-hybrid

Full-length coding sequences of the 10 cyclins and the 11 CRKs were each cloned into the pGADT7 vector to express proteins fused to the Gal4 activation domain (prey plasmid) and the pGBKT7 vector to express proteins fused to the Gal4 binding domain (bait plasmid). Yeast strains AH109 (mating type a) and Y187 (mating type α) were transformed with prey and bait plasmids, respectively, as well as empty vectors. Strains carrying different combinations of bait and prey plasmids were generated by mating the haploids in YPDA medium and then selected on SD-Leu-Trp plates. Each combination strain was then spotted in three ten-fold serial dilutions onto SD-Leu-Trp and SD-Leu-Trp-His plates. Growth of yeasts on SD-Leu-Trp-His plates indicated that the two proteins interact with each other. To rule out the false positive interaction due to protein self-activation of the Gal4 promoter, yeast strains harboring the bait plasmids were mated with yeast strains harboring the empty pGADT7 vector. For the self-activating gene(s), the strain(s) was spotted onto SD-Leu-Trp-His-Ade plates to eliminate self-activation.

In vitro GST pull-down

The full-length coding sequence of CRK1 was cloned into the pGEX-4T-3 vector for expressing GST-fused CRK1. Additionally, a hexa-Histidine tag was added to the C-terminus of CRK1 for The resulting plasmid was transformed into *E. coli* BL21 (DE3) strain, and expression of recombinant GST::CRK1-6xHis was induced by 0.5 mM IPTG at room temperature. GST::CRK1-6xHis fusion protein was first purified through a nickel-NTA column and then bound to glutathione sepharose beads and finally incubated with trypanosome cell lysate expressing 3HA-tagged CYC2, CYC4, CYC5, CYC6, and CYC7 at room temperature for 1 hr. Trypanosome cell lysate was prepared by incubating 5×10^7 cells in 0.5 ml co-immunoprecipitation buffer (25 mM Tris-Cl, pH7.6, 100 mM NaCl, 1 mM DTT, 1% Nonidet P-40, and protease inhibitor cocktail) on ice for 30 min and cleared by centrifugation at 14,000 rpm for 10 min. The glutathione sepharose beads were then washed six times with the immunoprecipitation buffer, and proteins were eluted by boiling the beads in 1× SDS-PAGE sampling buffer for 5 min. The eluate was separated on SDS-PAGE and immunoblotted with anti-HA mAb to detect HA-tagged cyclins. The PVDF membrane was stained with Coomassie blue to stain the recombinant GST::CRK1-6xHis. GST was used as the control.

Flow cytometry

Flow cytometry analysis of propidium iodide-stained trypanosome cells was carried out as previously described (Li et al., 2006). The DNA content of propidium iodide-stained cells was analyzed with a fluorescence-activated cell sorting scan (FACScan) analytical flow cytometer (BD Biosciences). The percentage of cells in each phase of the cell cycle (G1, S, and G2/M) was determined by the ModFit LT V3.0 software (BD Biosciences).

BrdU incorporation and detection

Control and RNAi cells after tetracycline induction for 4 days, which were at a density of 2×10^5 cells/ml, were incubated with 20 mM BrdU for 16 hrs, and the cells were fixed in ethanol. BrdU incorporation was detected by immunofluorescence microscopy with anti-BrdU mAb (1:40 dilution) (Sigma-Aldrich) and FITC-conjugated anti-mouse IgG (1: 400 dilution) (Sigma-Aldrich) according to our published procedures (Dang & Li, 2011).

Immunofluorescence microscopy

Cells were washed with PBS, fixed in 4% paraformaldehyde, and adhered to poly-L-Lysine treated coverslips. The cells on the coverslip were then incubated with primary antibodies for 1 hr at room temperature. The following antibodies were used in the present study: YL 1/2 mAb for tyrosinated α -tubulin (1:400 dilution) (Kilmartin et al., 1982); FITC-conjugated anti-HA antibody (1:400 dilution) (Sigma-Aldrich); anti-Myc mAb (1:400 dilution). After incubating with YL 1/2 antibody and anti-Myc antibody, cells were washed three times with wash buffer (0.1% Triton X-100 in PBS), and then incubated with FITC-conjugated anti-rat IgG or FITC-conjugated anti-mouse IgG (Sigma-Aldrich) at room temperature for 1 hr. All slides were mounted in VectaShield mounting medium (Vector Labs) containing DAPI and examined under an inverted microscope (Model IX71, Olympus) equipped with a cooled CCD camera (Model Orca-ER, Hamamatsu) and a PlanApo N 60× 1.42-NA DIC objective. Images were acquired and processed with the Slidebook5 software (Intelligent Imaging Innovations, Inc).

Co-immunoprecipitation and immunoblotting

Procytic cells co-expressing PTP::CRK1 and CYC2::3HA, CYC4::3HA, CYC5::3HA, CYC6::3HA, or CYC7::3HA, all of which were expressed from their respective endogenous locus by *in situ* epitope tagging (see above), were harvested, lysed in trypanosome co-

immunoprecipitation buffer, and incubated with 20 μ l IgG sepharose beads at 4°C for 2 hrs. The beads were washed six times with the immunoprecipitation buffer, re-suspended in 10% SDS to elute the proteins precipitated with IgG sepharose beads, and the supernatant were loaded onto a SDS-PAGE gel. Immunoblotting was performed with anti-HA mAb (Sigma-Aldrich) to detect co-precipitated 3HA-tagged cyclins and with anti-Protein A mAb (Sigma-Aldrich) to detect PTP::CRK1.

Protein ubiquitination assay

To detect ubiquitinated cyclins, 3HA-tagged CYC2, CYC4, CYC5, and CYC7 were overexpressed by induction with 0.1 μ g/ml tetracycline, immunoprecipitated from trypanosome cell lysate with EZview™ Red anti-HA affinity gel (Sigma-Aldrich), separated on SDS-PAGE, and immunoblotted with anti-ubiquitin mAb (Cell Signaling) for detecting ubiquitinated cyclins and with anti-HA mAb for detecting 3HA-tagged cyclins.

Protein stability assay

Cells expressing endogenously 3HA-tagged CYC4, CYC5, and CYC7 were treated with 100 μ g/ml cycloheximide for 12 hrs or 80 minutes. MG-132 (50 μ g/ml) was also added to one cell aliquot for 12 hrs or 80 minutes. Equal number of cells was collected every 3 hrs or 20 minutes. Cells were lysed in 1x SDS-PAGE sampling buffer, separated in SDS-PAGE, and immunoblotted with anti-HA mAb. The same blot was re-probed with anti-TbPSA6 pAb (Li *et al.*, 2002) as the loading control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Dr. George A. M. Cross of Rockefeller University for providing the procyclic 29-13 cell line and pLew100 vector and to Dr. Paul Englund of Johns Hopkins School of Medicine for providing pZJM vector. We also thank Dr. Arthur Günzl of the University of Connecticut Health Center for providing the pC-PTP-NEO vector. This work was supported by the startup funds from the University of Texas Medical School at Houston and by the NIH grants R56AI090070 and R01AI101437 to Z. L.

References

- Alsford S, Turner DJ, Obado SO, Sanchez-Flores A, Glover L, Berriman M, Hertz-Fowler C, Horn D. High-throughput phenotyping using parallel sequencing of RNA interference targets in the African trypanosome. *Genome Res.* 2011; 21:915–924. [PubMed: 21363968]
- Badjatia N, Ambrosio DL, Lee JH, Gunzl A. Trypanosome cdc2-related kinase 9 controls spliced leader RNA cap4 methylation and phosphorylation of RNA polymerase II subunit RPB1. *Mol Cell Biol.* 2013a; 33:1965–1975. [PubMed: 23478263]
- Badjatia, N.; Ambrosio, DL.; Nguyen, B.; Gunzl, A. Characterization of cdc2-related kinase 9 (CRK9), a key enzyme in trypanosome gene expression.. 5th Kinetoplastid Molecular Cell Biology Meeting; Woods Hole, MA. April 21-25, 2013; 2013b.
- Cross FR, Yuste-Rojas M, Gray S, Jacobson MD. Specialization and targeting of B-type cyclins. *Mol Cell.* 1999; 4:11–19. [PubMed: 10445023]
- Dang HQ, Li Z. The Cdc45.Mcm2-7.GINS protein complex in trypanosomes regulates DNA replication and interacts with two Orc1-like proteins in the origin recognition complex. *J Biol Chem.* 2011; 286:32424–32435. [PubMed: 21799014]
- Edgington NP, Futcher B. Relationship between the function and the location of G1 cyclins in *S. cerevisiae*. *J Cell Sci.* 2001; 114:4599–4611. [PubMed: 11792824]

- Gourguechon S, Savich JM, Wang CC. The multiple roles of cyclin E1 in controlling cell cycle progression and cellular morphology of *Trypanosoma brucei*. *J Mol Biol.* 2007; 368:939–950. [PubMed: 17376478]
- Gourguechon S, Wang CC. CRK9 contributes to regulation of mitosis and cytokinesis in the procyclic form of *Trypanosoma brucei*. *BMC Cell Biol.* 2009; 10:68. [PubMed: 19772588]
- Hammarton TC. Cell cycle regulation in *Trypanosoma brucei*. *Mol Biochem Parasitol.* 2007; 153:1–8. [PubMed: 17335918]
- Hammarton TC, Clark J, Douglas F, Boshart M, Mottram JC. Stage-specific differences in cell cycle control in *Trypanosoma brucei* revealed by RNA interference of a mitotic cyclin. *J Biol Chem.* 2003; 278:22877–22886. [PubMed: 12682070]
- Hammarton TC, Engstler M, Mottram JC. The *Trypanosoma brucei* cyclin, CYC2, is required for cell cycle progression through G1 phase and for maintenance of procyclic form cell morphology. *J Biol Chem.* 2004; 279:24757–24764. [PubMed: 15039435]
- Harper JV, Brooks G. The mammalian cell cycle: an overview. *Methods Mol Biol.* 2005; 296:113–153. [PubMed: 15576929]
- Hendriks EF, Robinson DR, Hinkins M, Matthews KR. A novel CCCH protein which modulates differentiation of *Trypanosoma brucei* to its procyclic form. *Embo J.* 2001; 20:6700–6711. [PubMed: 11726506]
- Humphrey T, Pearce A. Cell cycle molecules and mechanisms of the budding and fission yeasts. *Methods Mol Biol.* 2005; 296:3–29. [PubMed: 15576924]
- Ikui AE, Archambault V, Drapkin BJ, Campbell V, Cross FR. Cyclin and cyclin-dependent kinase substrate requirements for preventing rereplication reveal the need for concomitant activation and inhibition. *Genetics.* 2007; 175:1011–1022. [PubMed: 17194775]
- Johnson DG, Walker CL. Cyclins and cell cycle checkpoints. *Annu Rev Pharmacol Toxicol.* 1999; 39:295–312. [PubMed: 10331086]
- Kilmartin JV, Wright B, Milstein C. Rat monoclonal antitubulin antibodies derived by using a new nonsecreting rat cell line. *J Cell Biol.* 1982; 93:576–582. [PubMed: 6811596]
- Koivomagi M, Valk E, Venta R, Iofik A, Lepiku M, Morgan DO, Loog M. Dynamics of Cdk1 substrate specificity during the cell cycle. *Mol Cell.* 2011; 42:610–623. [PubMed: 21658602]
- Kuntzel H, Schulz A, Ehbrecht IM. Cell cycle control and initiation of DNA replication in *Saccharomyces cerevisiae*. *Biol Chem.* 1996; 377:481–487. [PubMed: 8922282]
- Li Z, Tu X, Wang CC. Okadaic acid overcomes the blocked cell cycle caused by depleting Cdc2-related kinases in *Trypanosoma brucei*. *Exp Cell Res.* 2006; 312:3504–3516. [PubMed: 16949574]
- Li Z, Wang CC. A PHO80-like cyclin and a B-type cyclin control the cell cycle of the procyclic form of *Trypanosoma brucei*. *J Biol Chem.* 2003; 278:20652–20658. [PubMed: 12665514]
- Li Z, Zou CB, Yao Y, Hoyt MA, McDonough S, Mackey ZB, Coffino P, Wang CC. An easily dissociated 26 S proteasome catalyzes an essential ubiquitin-mediated protein degradation pathway in *Trypanosoma brucei*. *J Biol Chem.* 2002; 277:15486–15498. [PubMed: 11854272]
- Loog M, Morgan DO. Cyclin specificity in the phosphorylation of cyclin-dependent kinase substrates. *Nature.* 2005; 434:104–108. [PubMed: 15744308]
- Monnerat S, Almeida Costa CI, Forkert AC, Benz C, Hamilton A, Tetley L, Burchmore R, Novo C, Mottram JC, Hammarton TC. Identification and Functional Characterisation of CRK12:CYC9, a Novel Cyclin-Dependent Kinase (CDK)-Cyclin Complex in *Trypanosoma brucei*. *PLoS ONE.* 2013; 8:e67327. [PubMed: 23805309]
- Sherwin T, Schneider A, Sasse R, Seebeck T, Gull K. Distinct localization and cell cycle dependence of COOH terminally tyrosinolated alpha-tubulin in the microtubules of *Trypanosoma brucei*. *J Cell Biol.* 1987; 104:439–446. [PubMed: 3546334]
- Tu X, Wang CC. The involvement of two cdc2-related kinases (CRKs) in *Trypanosoma brucei* cell cycle regulation and the distinctive stage-specific phenotypes caused by CRK3 depletion. *J Biol Chem.* 2004; 279:20519–20528. [PubMed: 15010459]
- Tu X, Wang CC. Coupling of posterior cytoskeletal morphogenesis to the G1/S transition in the *Trypanosoma brucei* cell cycle. *Mol Biol Cell.* 2005; 16:97–105. [PubMed: 15525678]
- Van Hellemond JJ, Mottram JC. The CYC3 gene of *trypanosoma brucei* encodes a cyclin with a short half-life. *Mol Biochem Parasitol.* 2000; 111:275–282. [PubMed: 11163436]

- Van Hellemond JJ, Neuville P, Schwarz RT, Matthews KR, Mottram JC. Isolation of *Trypanosoma brucei* CYC2 and CYC3 cyclin genes by rescue of a yeast G(1) cyclin mutant. Functional characterization of CYC2. J Biol Chem. 2000; 275:8315–8323. [PubMed: 10722661]
- Wirtz E, Leal S, Ochatt C, Cross GA. A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei*. Mol Biochem Parasitol. 1999; 99:89–101. [PubMed: 10215027]
- Yu Z, Liu Y, Li Z. Structure-function relationship of the Polo-like kinase in *Trypanosoma brucei*. J Cell Sci. 2012; 125:1519–1530. [PubMed: 22275435]

Figure 1. Interactions between CRK1 and CYC2, CYC4, CYC5, and CYC7

(A). Interactions between CRK1 and the four PHO80-like cyclins in yeast. Growth of yeast on -Leu-Trp-His plates indicates interaction between the two proteins. The asterisk shows the growth of pGBK-CYC9/pGADCRK1 strain on the -Leu-Trp-His plate due to self-activation and, therefore, is false positive. (B). GST pull-down to detect the *in vitro* interaction between CRK1 and the four cyclins. Recombinant GST::CRK1, expressed and purified from *E. coli*, was incubated with trypanosome cell lysate expressing 3HA-tagged CYC2, CYC4, CYC5, CYC6, and CYC7. Pull-down of CYCs was monitored by immunoblotting with anti-HA antibody. GST::CRK1 and GST were stained with

Coomassive blue. (C). Co-immunoprecipitation to detect *in vivo* interactions between CRK1 and the four cyclins. PTP::CRK1 and 3HA-tagged cyclins were co-expressed from their respective endogenous locus. Coimmunoprecipitation was carried out by incubating the cell lysate with IgG sepharose beads and subsequent immunoblotting of the immunoprecipitates with anti-HA mAb and anti-Protein A mAb, respectively.

Figure 2. RNAi of CYC4, CYC5, and CYC7 in the procyclic form of *T. brucei*

(A). Quantitative RTPCR to measure the mRNA level of the three cyclins before and after RNAi induction. (B). Effect of cyclin RNAi on cell proliferation. (C). Tabulation of cells with different number of nucleus (N) and kinetoplast (K) upon cyclin knockdown. Data are presented as the mean percent $\pm$ S.D. of total cells counted ($\sim$ 200) from three independent experiments.

Figure 3. Effect of CYC4, CYC5, and CYC7 RNAi on cell cycle progression

(A). Flow cytometry analysis of cyclin RNAi cells. (B). Quantitative analysis of the flow cytometry experiments from panel A, showing the percentage of G1, S-phase, and G2/M cells upon RNAi induction of the three cyclins.

Figure 4. Effect of cyclin and CRK1 RNAi on DNA replication in the procyclic form
(A). Immunofluorescence microscopy analysis of BrdU incorporation in cyclin and CRK1 RNAi cells. Bars: 5 μ m. **(B).** Tabulation of BrdU-positive cells before and after RNAi of cyclins and CRK1. Data are presented as the mean percent $\pm$ S.D. of total cells counted (~200) from three independent experiments.

Figure 5. Effect of cyclin and CRK1 RNAi on posterior morphology in the procyclic form
(A). Immunostaining of control and RNAi cells with YL 1/2 antibody to label the newly assembled microtubules at the posterior portion of the cell. The nucleus (N), kinetoplast (K), and basal bodies (BB) are indicated. The double arrowheads show the posterior portion of the cell, which is defined as the portion of cell between the basal body and the posterior tip of the cell. Bars: 5 μ m. **(B).** Tabulation of cells with an elongated posterior in control and CYC2, CYC7, and CRK1 RNAi cells. Data are presented as the mean percent $\pm$ S.D. of total cells counted (~200) from three independent experiments.

Figure 6. RNAi of CYC7 and CRK1 produces cells with branched posteriors

(A). Immunostaining of control and RNAi cells with YL 1/2 antibody to detect newly assembled microtubules at the posterior portion of the cell. The nucleus (N), kinetoplast (K), and basal bodies (BB) are indicated. Black arrows show the branched posteriors. Bars: 5 μ m. (B). Tabulation of cells with branched posteriors in control and CYC7 and CRK1 RNAi cells. Data are presented as the mean percent $\pm$ S.D. of total cells counted (~200) from three independent experiments.

Figure 7. Subcellular localization of the four PHO80-like cyclins and CRK1 in the procyclic form Cells expressing 3HA-tagged CYC2, CYC4, CYC5, and CYC7 and 3Myc-tagged CRK1, all of which were expressed from their respective endogenous loci, were immunostained with FITC-conjugated anti-HA antibody or with anti-Myc antibody and FITC-conjugated anti-mouse IgG and counterstained with DAPI for nuclear and kinetoplast DNA. Bars: 5 μ m.

Figure 8. *In vivo* ubiquitination and protein half-life of PHO80-like cyclins

(A). The four PHO80-like cyclins are ubiquitinated *in vivo* in trypanosomes. Expression of 3HA-tagged CYC2, CYC4, CYC5, and CYC7 were induced by tetracycline. The 3HA-tagged cyclins were immunoprecipitated from the cell lysate with anti-HA antibody and immunoblotted with anti-ubiquitin antibody and anti-HA antibody to detect ubiquitinated cyclins and 3HA-tagged cyclins, respectively. Black arrows indicate the 3HA-tagged cyclin proteins. (B). Half-life of CYC4, CYC5, and CYC7 determined by pulse-chase experiments. Cells expressing 3HA-tagged CYC4, CYC5, and CYC7 were treated with cycloheximide to inhibit protein synthesis, and time-course samples were collected for immunoblotting with

anti-HA antibody to detect 3HA-tagged cyclins and with anti-TbPSA6 antibody to detect the proteasome β subunit, which served as the loading control. To stabilize the cyclin proteins, MG-132 was added to the cells in addition to cycloheximide for 12 hrs or 80 min. (C). Quantitation of the level of CYC4, CYC5, and CYC7 shown in panel B. The signal intensity of protein bands was measured, normalized with the loading control, and plotted against the time of cycloheximide treatment.

Table 1

Interactions between cyclins and CRKs determined by yeast two-hybrid assays.

	CRK1	CRK2	CRK3	CRK4	CRK6	CRK7	CRK8	CRK9	CRK10	CRK11	CRK12
CYC2	+++	+++	+++		+++ [*]		+ [*]		+++		+++ [*]
CYC3											
CYC4	+++	[*] +			+++	+++ [*]	+ [*]			+	+++ [*]
CYC5	+++										+
CYC6											
CYC7	+++						+ [*]				+ [*]
CYC8		+++			+++	+++ [*]					+++
CYC9							+++ [*]				+++
CYC10										[*] +++	
CYC11											

+++; growth of yeast on all three 10-fold dilutions on the selective plate
+; growth of yeast on one of the three 10-fold dilutions on the selective plate
^{*} unidirectional interactions (see Supplemental Figure 3 for details)