Analysis of the Spatiotemporal Localization of Mitochondrial DNA Polymerases of *Trypanosoma brucei*

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ANALYSIS OF THE SPATIOTEMPORAL LOCALIZATION OF MITOCHONDRIAL DNA POLYMERASES IN TRYPANOSOMA BRUCEI

A Dissertation Presented

by

JENIFFER CONCEPCIÓN-ACEVEDO

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

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Department of Microbiology
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Department of Microbiology
DEDICATION

I dedicate this thesis to my loving grandmother Ramonita Gonzalez-Pérez. Mamá, thanks for teaching me that when it comes to learning from others I need to be humble because mentors are not necessarily those with an education but rather those that teaches you things in life to be a better person.

I also dedicate this thesis to the memory of my grandfather Emilio Concepción.

Le dedico esta tesis a mi amada abuela Ramonita González-Pérez. Mamá, gracias por enseñarme que cuando se trata de aprender de los demás tengo que ser humilde porque los mentores no son necesariamente los que tienen una educación, sino aquellos que te enseñan en la vida las cosas que te ayudará a ser una mejor persona.

También le dedico esta tesis a la memoria de mi abuelo Emilio Concepción.
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ABSTRACT
ANALYSIS OF THE SPATIOTEMPORAL LOCALIZATION OF MITOCHONDRIAL DNA POLYMERASES IN *TRYPANOSOMA BRUCEI*

FEBRUARY 2013

JENIFFER CONCEPCIÓN-ACEVEDO, B.A., UNIVERSITY OF PUERTO RICO Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Michele M. Klingbeil

The mitochondrion contains its own genome. Replication of the mitochondrial DNA (mtDNA) is an essential process that, in most organisms, occurs through the cell cycle with no known mechanism to ensure spatial or temporal constrain. Failures to maintain mtDNA copy number affects cellular functions causing several human disorders. However, it is not clear how the cells control the mtDNA copy number. The mtDNA of trypanosomes, known as kinetoplast DNA (kDNA), is a structurally complex network of topologically interlocked DNA molecules (minicircles and maxicircles). The replication mechanism of the kDNA differs greatly with all other eukaryotic systems. Key features of the kDNA replication mechanism include defined regions for main replication events, coordination of a large number of proteins to drive the replication process, and replication once per cell cycle in near synchrony with nuclear S phase. Two main regions known as the kinetoflagellar zone (KFZ) and the antipodal sites are where main kDNA replication events are known to occur (i.e, initiation, DNA synthesis and Okazaki fragment processing). So far, the localization of the proteins involved in kDNA replication is restricted to two main regions: the KFZ and the antipodal sites. Three mechanisms that directly regulate kDNA replication proteins and serve to control kDNA...
replication have been proposed: (1) Reduction and oxidation status of the universal minicircle sequence binding protein (UMSBP) controls its binding to the origin sequence, (2) Trans-acting factors regulate the stability of mRNA encoding mitochondrial Topoisomerase II during the cell cycle and, (3) Regulation of TbPIF2 helicase protein levels by a HslVU-like protease to control maxicircle copy number. These mechanisms seem to be protein specific and it appears that a combination rather than a single mechanism regulates kDNA replication.

In this study we used *Trypanosoma brucei* to understand how mitochondrial DNA replication is controlled. We investigated the mechanism of how proteins transiently localize to the sites of DNA synthesis during cell cycle stages. Our data provides a comprehensive analysis of the first two examples of *T. brucei* kDNA replication proteins that have a cell cycle dependent localization (Ch. 2 and 3). The localization of two of the three essential mitochondrial DNA polymerases (TbPOLIC and TbPOLID) is under tight cell cycle control and not regulated by proteolysis. TbPOLIC and TbPOLID localize to the antipodal sites during kDNA S phase, however, at other cell cycle stages TbPOLIC becomes undetectable by immunofluorescent analysis and TbPOLID disperses through the mitochondrial matrix. In agreement with this data, TbPOLIC and TbPOLID replication complexes were not detected using affinity purification presumably because only a fraction of these proteins are participating in replication at a given time (Ch. 4). We propose that spatial and temporal changes in the dynamic localization of essential kDNA replication proteins provide a novel mechanism to control kDNA replication.
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CHAPTER 1

1 TRYpanosomatid mitochondrial DNA replication: from
circles to complex networks

1.1 Global impact of trypanosomatid parasites

Kinetoplastid protozoa are flagellated organisms that comprise free-living as well
as parasitic species. They have the capacity to infect a broad range of hosts, including
humans, causing enormous medical and economic distress primarily in tropical and
subtropical countries (24, 55). These parasites undergo a biphasic and complex life cycle
that alternates between an insect vector and mammalian host. Each species is transmitted
by a different vector but each undergoes drastic morphological and metabolic changes
that are required for them to adapt to both environments (50). Trypanosomes and
leishmania are the two main pathogenic species that threat human health and cause
diseases ranging in severity from self-healing, cutaneous conditions to infections that are
fatal if left untreated (23, 55). These parasites are associated with diseases such as human
African trypanosomiasis (HAT), Chagas disease, and various forms of leishmaniasis (55).
All three infections are among the most neglected tropical diseases (NTDs) as they affect
the poorest populations most of which live in remote areas with limited surveillance and
health information systems (11, 22, 23). It is estimated that more than 20 million
individuals are infected with these pathogens, and together they claim 100,000 lives
every year; as such, they are considered the most lethal NTDs (10, 11, 55). They
represent a global public health problem; and collective efforts are necessary to develop
new tools and improve current strategies to control and eliminate kinetoplastid diseases.
1.2 Clinical manifestations and limitations of current treatments

1.2.1 Diseases

1.2.1.1 Leishmaniasis

Leishmania species are responsible for the spectrum of diseases referred to as leishmaniasis (i.e., cutaneous, CL; mucocutaneous, MCL; and visceral leishmaniasis, VL) (23, 55). All clinical manifestations arise after an infected sand fly injects parasites into the mammalian host. CL is characterized by ulcers in arms and legs that usually heal spontaneously but result in lifelong scars. The most disfiguring form is MCL, which invades the mucous membrane of the upper respiratory tract. VL is the most severe form of the disease and is characterized by substantial weight loss, swelling of the spleen and liver and irregular bouts of fever. It affects the internal organs and is generally fatal if left untreated. Cases of leishmaniasis have been reported in 98 countries within South Asia, East Africa and Latin America, and it is estimated that 12 million people are infected (10, 11). Chemotherapy remains an essential tool in the control of leishmaniasis infections. However, existing treatments (i.e., Pentavalent, antimonials, Amphotericin B, AmBiosome, Miltefosine and Paromomycin) have serious drawbacks (11). For instance, Amphotericin B, which is the preferred drug for VL, has a dose-limiting toxicity and requires 15-50 days of treatment. Overall, these drugs are characterized by their high cost, long treatment duration, cumbersome administration, and high toxicity. All of the above characteristics are common challenges that face current leishmania and trypanosome treatments and require immediate global attention.
1.2.1.2 Chagas disease

*T. cruzi* is the causative agent of Chagas disease, which is endemic to 19 countries in Latin America (55). It is transmitted primarily, but not exclusively, through the feces of the triatomine vector known as the “kissing bug.” Infection can also be acquired through other routes, including blood transfusion, organ transplant and congenitally (56). The disease manifests in two phases: acute and chronic (11). The acute phase lasts about two months and is characterized by high parasitemia in the blood. The first sign can be a skin lesion (chagoma) or inflammation of the eyelid (Romaña sign), but most cases are asymptomatic or present several non-specific symptoms such as fever, fatigue, body aches, and loss of appetite. During the chronic phase, the parasite targets two main tissues and manifests as two clinical forms: as a cardiac disease causing heart failure and as digestive track lesions, which result in the enlargement of the esophagus and colon.

Chagas disease has spread beyond its endemic presence in Latin America, and it is estimated that 8 million people are infected globally (11, 25). The Center for Disease Control (CDC) also predicts that due to migration from endemic areas, nearly 300,000 people in the United States are infected with chronic Chagas disease (25). Additionally, due to climate change, the range of vectors with the capacity to spread the disease has extended to North America (25). Benznidazole and nifurtimox are the available treatments effective against the acute phase of the infection (11). Drawnback of these drugs include: (1) low efficacy against the chronic phase, (2) prolonged regimen, (3) toxicity, and (4) multiple side effects (i.e. muscle pain, skin rashes, vomiting, weight loss and neuropathy) which result in large numbers of patients that fail to complete the treatment.
1.2.1.3 Human African trypanosomiasis

*T. brucei* subspecies *rhodesiense* and *gambiense* cause acute and chronic human African trypanosomiasis (HAT), respectively (51). When an infected tsetse fly takes a blood meal, parasites are injected into the bloodstream of the mammalian host and proliferate in the lymph and blood. This stage (stage 1) is known as the hemolymphatic stage and is characterized by headache, fever, general malaise, pain in the joint and inflammation of the lymph nodes (23). The parasites’ antigenic variation helps them to circumvent the immune system, and over time they will cross the blood brain barrier. The central nervous system (CNS) stage (stage 2) is characterized by neurological and psychiatric disorders such as confusion, hallucinations, sleep disturbance and coma that will lead to death if left untreated. HAT affects 36 countries in sub-Saharan Africa, and is estimated that 30,000 people are infected per year (51, 55). There are four registered treatments for HAT infection: Melarsoprol, Pentamidine, Eflornithine, and NECT (Nifurtimox-Eflornithine combination therapy) (4, 11). Melarsoprol is a highly toxic arsenic derivative that causes encephalopathy in up to 5% of patients. Pentamidine is not effective against stage 2, and Eflornithine monotherapy is difficult to administer because it requires intravenous administration every 6 hours for 14 days. In 2009, NECT was first available to treat stage 2 of HAT; it is considered a simplified therapy alternative that requires a shorter regimen of 14 injections of eflornithine for 7 days and 10 days of oral treatment with nifurtimox (42). This drug reduced the use of toxic drug and is now replacing the commonly used of Eflornithine and Melarsoprol monotherapies. However, it is not consider the best alternative to support elimination efforts.
1.2.2 Efforts to supplant current treatments

Currently there is no vaccine to prevent trypanosome or leishmania infections. Available treatments are limited, difficult to administer, often toxic and are becoming ineffective due to emergence of drug-resistant parasites (18, 29, 52). Efforts to develop new treatments are underway (10). A joint effort between international organizations, industry, academia and public-private partners (PPPs) have begun to improve kinetoplastid drug treatments. For example, nifurtimox (a Chagas disease treatment) and the four registered medicines for treating HAT have been donated to the World Health Organization (WHO) by Sanofi-Aventis and Bayer Schering Pharma through PPPs. Additionally, the WHO, in collaboration with Médecins Sans Frontières, distributes current treatments free of charge to countries in which these diseases are endemic. However, the available funds to produce these drugs are not sufficient to meet the quantities needed for disease control, and anti-trypanosomal drugs will be donated only until the end of 2012 (10). The development of new treatments to replace the use of toxic drugs with complex regimens should be a priority. Today, there is still an urgent need for safe, effective, and affordable drug treatments against kinetoplastid parasitic infections.

One avenue for the identification of new drug treatments is basic research, which provides profound understanding of the parasites’ cellular and biochemical processes. An organization that promotes research and development for the discovery of new treatments for kinetoplastid diseases is the Drugs for Neglected Diseases Initiative (DNDi). In 2011 they reported that in collaboration with their partners they are developing two drugs for HAT infections, Oxaborole SCY-7158 and Fexinidazole, which are in phase I and II of clinical studies, respectively (11, 30). They also reported the pediatric dose to treat
Chagas disease and two potential new treatments that are currently in the pipeline. They delivered one drug for VL (SSG & PM) and reported three other compounds for treating leishmaniasis infections, which are in pre-clinical studies.

Although improvements have been made, these diseases continue to expand and are still far from being eradicated within the next 10 years. Aside from their obvious clinical impact, these parasites are known for many fascinating biological features that could be exploited for drug target validation (47, 49). One of the most significant examples is their mitochondrion, which is characterized by containing many of the hallmarks known in other eukaryotic systems as well as multiple kinetoplastid-specific features (38).

1.3 The Intriguing Mitochondrion of Trypanosomes

The mitochondrion of *T. brucei* is considered a model system for studying many attractive aspects of this organelle biology. Special attention has been given to: the mechanisms in which they regulate mitochondrial activities in response to environmental changes, the complex mechanism of RNA editing and the replication and organization of the mitochondrial genome (38).

During its life cycle, *T. brucei* cycles between the insect gut and the bloodstream of the mammalian host (50). It experiences two very different environments that trigger drastic metabolic and morphological changes in the mitochondrion (38). The bloodstream form has a reduced mitochondrion, which lacks cytochromes and depends on glycolysis for ATP production. In contract, the mitochondrion of the procyclic form is active, and contains all classical complexes and generates ATP through oxidative phosphorylation
and substrate-level phosphorylation. Despite the fact that mitochondrial functions seem to be suppressed in the bloodstream form, RNA editing and mitochondrial DNA replication are both essential in the disease-causing form (bloodstream form) (2, 48). As such, essential proteins involved in both of these processes are being investigated as potential drug targets.

The mitochondrial DNA in trypanosomes is called kinetoplast DNA (kDNA) and its structure and replication has no counterpart in nature (26). In the cell’s single tubular mitochondrion the kDNA forms a disk-like structure, which is positioned in a particular region within the mitochondrial matrix near the basal body. The kDNA is a condensed, catenated-network composed of thousands of interlocked DNA circles known as maxicircles and minicircles. In *T. brucei*, maxicircles are ~ 23 kilobases (kb) and are composed of a variable region of ~8 kb (size varies between trypanosomatids) and a gene-coding region of 15 kb, where in the putative replication origin is located (38). Maxicircles are homologs to mitochondrial DNA in other eukaryotes as they encode subunits of the respiratory complex (e.g., cytochrome oxidase subunit I-III, ATPase subunit 6 and cytochrome reductase subunit b) as well as mitochondrial ribosomal RNAs (e.g., 9 S and 12 S). RNA editing is essential for the expression of some of these maxicircle-encoded genes. Maxicircle transcripts require insertion and/or deletion of uridine residues to generate functional open reading frames (ORF) (2). The sequence information for editing events is directed by guide RNA (gRNA). All these processes are accomplished by elaborate multiprotein RNA editing complexes in which a majority of the proteins are essential for parasite survival (2). Guide RNAs are encoded by minicircles and there are a few thousand per network. They are heterogeneous in
sequence but contain a conserved region of about 200 bp that contains an invariant sequence called the universal minicircle sequence (UMS) (1). The UMS is 12 nucleotides long (GGGGTTGGTGTA) and it presumably contains the origin of replication. Each minicircle is about 1 kb in size and is linked to an average of three neighbors (26). Both, maxicircles and minicircles, replicate unidirectionally by a theta mechanism. However, maxicircles remain attached to the network during replication as minicircles are released and replicated free from the network.

1.4 The replication mechanisms of kinetoplast DNA network

Replication of minicircles and maxicircles is essential in both life cycle forms as RNAi-mediated silencing of proteins involved in replication is lethal and results in loss of kDNA networks (8, 26). The African trypanosome is a popular organism for biological research and is considered the best-studied trypanosomatid species. The completion of its genome sequence in 2005, coupled with the characterization of the RNA interference (RNAi) pathway, turned them into a great model system for the analysis of gene function (5, 40). Importantly, the establishment of an RNA interferense inducible system allows studies of essential genes (59). This facilitated the development of genome-wide analysis for drug target validation and RNAi libraries for the identification and characterization of proteins involved in essential processes (3, 15, 40, 41, 61). RNAi has been a powerful tool for studying the function of proteins involved in kDNA replication (26). The RNAi phenotype of nearly twenty-two proteins, predicted to be involved in kDNA replication, have been reported over the last 10 years (Table 1.1). Fifteen of these were demonstrated
to play an essential role in kDNA replication and maintenance (Table 1.1). This approach led to significant progress in understanding the kDNA replication mechanism (26).

The organization and replication mechanism of the kDNA has remarkable differences relative to other eukaryotes, including (1) a catenated network composed of two forms of topologically interlocked circles, (2) an elaborate topoisomerase-mediated release and reattachment mechanism for minicircle replication, (3) a condensed kDNA network with a disk-shaped structure that is linked to the flagellar basal body, (4) a single round of mitochondrial DNA replication per cell cycle, and (5) the presence of multiple proteins with similar activities but different functions (26, 48). For the past 40 years all these data has been collectively acquired and integrated into the current model for kDNA replication (Fig. 1.1).
<table>
<thead>
<tr>
<th>kDNA replication protein</th>
<th>RNAi</th>
<th>*Essential</th>
<th>**Non-Essential</th>
<th>Not Determined</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>POLIC</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td>(31)</td>
</tr>
<tr>
<td>POLIA</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td>(31)</td>
</tr>
<tr>
<td>POLIB</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td>(6)</td>
</tr>
<tr>
<td>POLID</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td>(9)</td>
</tr>
<tr>
<td>Pol β</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>Pol β-PAK</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>PIF1</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td>(36)</td>
</tr>
<tr>
<td>PIF2</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td>(34)</td>
</tr>
<tr>
<td>PIF5</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td>(35)</td>
</tr>
<tr>
<td>PIF4</td>
<td>✓</td>
<td></td>
<td></td>
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<td>(34)</td>
</tr>
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<td>PIF7</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td>(34)</td>
</tr>
<tr>
<td>PIF8</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td>(57)</td>
</tr>
<tr>
<td>Ligase kβ</td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>Ligase ka</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td>(12)</td>
</tr>
<tr>
<td>PRI1</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td>(20)</td>
</tr>
<tr>
<td>PRI2</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td>(21)</td>
</tr>
<tr>
<td>SSE1</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td>(37)</td>
</tr>
<tr>
<td>TOPIA&lt;sub&gt;mt&lt;/sub&gt;</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td>(46)</td>
</tr>
<tr>
<td>TOPO II</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td>(58)</td>
</tr>
<tr>
<td>UMSBP</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td>(39)</td>
</tr>
<tr>
<td>p38</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td>(33)</td>
</tr>
<tr>
<td>p93</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td>(32)</td>
</tr>
</tbody>
</table>

* Growth phenotype and kDNA loss.
** No growth phenotype

Table 1.1 - Essential proteins for kDNA replication as determined by RNAi-mediated silencing
The kDNA replication process is initiated when covalently closed minicircles are released vectorially from the network into a specialized region between the kDNA disk and the basal body called the kinetoflagellar (KFZ) zone (Fig. 1.1) (17). Once in the KFZ, universal minicircle sequence binding protein (UMSBP) and p38 bind to the minicircle origin and trigger unidirectional replication via theta structure (1, 33). RNA primers for Okazaki fragments are not immediately removed at the KFZ (44). During later stages of kDNA replication, minicircle progeny containing gaps and RNA primers migrate to the antipodal site by a yet unknown mechanism. Nick/gapped minicircles at the antipodal sites undergo Okazaki fragment processing mediated by at least four proteins: structure-specific endonuclease 1 (SSE1), DNA polymerase β, DNA ligase kβ and TbPIF5 (Fig. 1.2) (12, 14, 35, 45). During the final stages of minicircles replication, topoisomerase II attaches minicircle progeny to the network and the final gap is repaired presumably by DNA polymerase β-PAK (Fig. 1.2) (45). Maxicircle replication is much less understood and so far only two proteins, DNA helicase (TbPIF2) and primase (PRI1), have been identified as essential for maxicircle replication (20, 34). It is clear that maxicircles remain linked to the network and they gradually accumulate at the center of a growing disk during later kDNA S phase (16, 19). Additionally, maxicircle segregation is the final step of the kDNA duplication cycle (19).
Figure 1.1 – kDNA Replication Model

Diagrammatic representation of kDNA replication events. (1) Early stages of minicircle replication include the release of individual minicircles into the KFZ where they initiate unidirectional theta structure replication. (2) Later stages are characterized by minicircle progeny re-attachment at the antipodal sites, still containing gaps, followed by Okazaki fragment processing. These gaps can be labeled with terminal deoxynucleotidyl transferase (TdT) and fluorescent dUTP (represented in green). Finally, replicated minicircles are re-attached to the network.
Figure 1.2 – Repertoire of Proteins Involved in kDNA replication

Localization of kDNA replication proteins to two main regions; the antipodal sites and the kinetoflagellar zone (KFZ). The arrows indicate the dynamic localization of two mitochondrial DNA polymerases at different stages of the kDNA duplication cycle. (1) Dynamic localization of POLID from the mitochondrial matrix to the antipodal sites. (2) Movement of POLIC from the KFZ to the antipodal sites.
1.5 Structural changes facilitate the description of the kinetoplast duplication cycle

Basal body (bb) maturation is one of the first cytological events during the trypanosome cell cycle (60). Its duplication process has emerged as a master indicator for the characterization of cell cycle stages. Replication of the kDNA occurs once per cell cycle and is intimately linked with basal body dynamics (43). Importantly, basal body movement drives the correct segregation and positioning of daughter kinetoplast and its perturbation causes failures in kDNA segregation (43). Gluenz and colleagues resolved the spatiotemporal relationship between kDNA replication and basal body dynamics (19).

They reported additional events occurring during early and later stages of the kDNA replication that were divided into five different stages (I-V). During stage I, cells contain a unit-sized kDNA disk, no visible antipodal sites and 1 bb/probb pair. Formation of the new flagellum and antipodal site detection occurs during stage IIa. In stage IIb, the new flagellum is positioned posterior to the mature flagellum, the kDNA disk has a domed shape and the antipodal sites are easily detected. In stage III the kDNA consist of two joined networks that are characterized for its bilobe or V shape structure. At this point two bb/probb pairs are detected, maxicircles accumulate at the center of the disk and antipodal sites are occasionally detected. During stage IV, the networks move apart and appear as two unit-sized disk that remain connected by a thread of maxicircles. As the cell progress from stage II to IV, the basal bodies move apart until they reach an approximately distance of 2 μm. During stage V, the networks are fully separated and they are morphologically the same as in stage I. As the cell goes from stage IV to V the
basal bodies reach an inter-basal body distance of ~ 4.5 μm followed by the segregation of maxicircle DNA and segregation of daughter networks.

1.6 Distribution of kDNA replication proteins

The kDNA replication process is highly dynamic and is predicted that some of the proteins that participate in this process are coordinated during the cell cycle (28, 32, 53). The characterization of the kinetoplast duplication cycle is a fundamental tool in our protein localization toolbox that allows us to precisely determine kDNA protein dynamics. Using basal body dynamics along with kDNA morphology changes we can provide additional information on the localization of proteins during the kDNA duplication cycle.

So far, the localization of the proteins involved in kDNA replication seems to be restricted to two main regions surrounding the kDNA disk: the KFZ and the antipodal sites (26). It was previously suggested that the localization of a subset of kDNA replication proteins was not static, but were most dynamic during *T. brucei* cell cycle (28, 32, 53). Initial attempts for describing protein dynamics were strictly based on kDNA morphology changes. In *Crithidia fasciculata* (a related kinetoplastid species), pol β and SSE1 were detected at the antipodal sites during kDNA S phase and become undetectable by immunofluorescence (IF) after segregation of the daughter network (14, 45) (Table 1.2). A similar pattern was reported for *T. brucei* SSE1. *C. fasciculata* Topo II localized to the antipodal sites during kDNA S phase and becomes diffuse near the disk during later stages of kDNA replication (Table 1.2) (27, 28). Additionally, *Trypanosoma cruzi* Tc38, a DNA binding protein, localized to the kDNA disk or antipodal sites during G1
and S phase. As cell cycle progression occurs Tc38 diffuses as a punctated signal covering regions far from the kDNA disk (13).

The large number of proteins required for kDNA replication, the previous observations on their dynamic localization, and our current knowledge on the multiple kDNA duplication stages suggest that the timing of kDNA replication stages is highly controlled. The dynamics of kDNA replication proteins with respect to kDNA duplication events have not been well defined. Understanding the dynamic changes of kDNA replication proteins will provide information on the mechanism how kDNA replication timing is controlled.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Organism</th>
<th>Localization during the cell cycle</th>
<th>Antibody</th>
<th>Protein levels</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UMSBP</td>
<td>*Crithidia</td>
<td>Adjacent to the face of the disk: During early S Phase UMSBP is located at two distinct sites. As kDNA replication progresses UMSBP is diffuse, and at final stages of cell division some cells have loss of UMSBP.</td>
<td>anti-UMSBP</td>
<td>Present at similar levels during cell cycle stages.</td>
<td>(1)</td>
</tr>
<tr>
<td>pol β</td>
<td>*Crithidia</td>
<td>Antipodal sites: Antipodal localization during S phase. When not localized in the antipodal site is not detected by immunofluorescence.</td>
<td>anti-pol β</td>
<td>Present at similar levels during cell cycle stages.</td>
<td>(28)</td>
</tr>
<tr>
<td>Topo II</td>
<td>*Crithidia</td>
<td>Antipodal sites: When not present at the antipodal sites is diffuse near the kDNA.</td>
<td>anti-topo II</td>
<td>Present at similar levels during cell cycle stages.</td>
<td>(28)</td>
</tr>
<tr>
<td>SSE1</td>
<td>*Crithidia</td>
<td>Kinetoplast disk: Present in cells that completed mitosis and contain either a dividing kinetoplast or two newly divided kinetoplasts.</td>
<td>anti-HA</td>
<td>Present at similar levels during cell cycle stages.</td>
<td>(14)</td>
</tr>
<tr>
<td>Ligase kα</td>
<td>*Crithidia</td>
<td>Antipodal sites: Present during initial S-phase and was minimum during G2/M. It was never present in cells undergoing division.</td>
<td>anti-HA</td>
<td>Not determine</td>
<td>(54)</td>
</tr>
</tbody>
</table>

Table 1.2 – Localization of *C. fasciculata* replication proteins during the cell cycle
1.7 Multiple mitochondrial DNA polymerases in *T. brucei*

The mitochondrion of *Trypanosoma brucei* harbors multiple mitochondrial DNA polymerases (31). Two of these are related to DNA Pol β (TbPol β and Pol β-PAK) and four are related to bacterial DNA polymerase I (Pol I); These are known as TbPOLIA, TbPOLIB, TBPOLIC and TbPOLID (31, 45). This is a unique feature of trypanosomes since other eukaryotes require a single mitochondrial DNA polymerase (Pol γ) for replication and repair processes. RNAi studies on the *T. brucei* pol I like enzymes demonstrated that three of these (TbPOLIB, IC and ID) are essential for kDNA replication in both cell cycle stages (6, 7, 9, 31). The later was established by monitoring cell growth, kinetics of kDNA loss, and effect on minicircle replication intermediates. TbPOLIB silencing resulted in growth inhibition, kDNA loss and the accumulation of a novel population of free minicircles that is mainly comprised of covalently closed minicircle dimers (6). It was also demonstrated to contribute to both leading and lagging strand synthesis. Using peptide antibodies against TbPOLIB C-terminal region this protein was reported to localize to the KFZ. Silencing of TBPOLIC and TbPOLID resulted in kDNA loss suggesting that each of the pol I-like DNA polymerases have a different contribution to the kDNA replication process (9, 31). Localization of TBPOLIC was mainly to the KFZ and TbPOLID was distributed throughout the mitochondrial matrix.

The localization of TbPOLID to the mitochondrial matrix suggested that this protein would require redistributing close to the kDNA in order to perform its role in kDNA replication. A detailed examination of the distribution of all pol I like
mitochondrial DNA polymerases becomes essential for understanding how these proteins are coordinated to perform their role in kDNA replication.

1.8 **Goal of this study**

It is not clear how multiple mitochondrial DNA polymerases are coordinated for replication of the kDNA network. The following dissertation research was conducted with the goal of understanding mitochondrial DNA polymerases’ spatial and temporal dynamics. This work answers important questions such as:

1. Do TbPOLIC and TbPOLID have a cell cycle dependent localization?
2. Where do TbPOLIC and TbPOLID localize during each cell cycle stages?
3. Do they colocalized during kDNA replication?
4. Is localization of these proteins regulated by the mitochondrial protease HslVU?

1.9 **Significance and contribution of this study**

Using a combination of fluorescent microscopy tools I provide a detailed and quantitative description of the movement of kDNA replication proteins during the cell cycle. I provide evidence to support that TbPOLIC and TbPOLID have a cell cycle dependent localization. Additionally, this study provides the first report of a kDNA replication protein that changes its localization through a mechanism that involves redistribution of the mitochondrial matrix fraction to the antipodal sites. This work represents the initial steps toward understanding mitochondrial DNA replication dynamics.
1.10 Thesis overview

Following this introduction, Chapter 2 defines the localization of mitochondrial DNA polymerase ID (TbPOLID) and establishes that this protein undergoes changes in localization during kDNA S phase. Using immunofluorescent microscopy it was demonstrated that the mitochondrial matrix fraction redistributes as foci that localize to the region where minicircles accumulate during later stages of replication. Experiments conducted in Chapter 3 demonstrate that the localization of a second mitochondrial DNA polymerase, TbPOLIC, is dynamic during kDNA replication. Using cell biology and genetic approaches my work shows that this protein localized to active mitochondrial DNA replication sites along with TbPOLID during the cell cycle is not due to proteolytic degradation and RNAi-mediated silencing of TbPOLID affects the localization of TbPOLIC during kDNA replication. Chapter 4, provides preliminary data that suggest that *T. brucei* mitochondrial DNA replication complexes are highly transient.

1.11 Bibliography


CHAPTER 2

2 DYNAMIC LOCALIZATION OF TRYPANOSOMA BRUCEI

MITOCHONDRIAL DNA POLYMERASE ID

2.1 Introduction

Mitochondrial DNA (mtDNA) is packaged into protein-DNA complexes, which by analogy to the bacterial chromosome are called nucleoids. The number of nucleoids per cell varies depending on cell type and each nucleoid contains several copies of mtDNA. Saccharomyces cerevisiae contains ~40 nucleoids (each containing 1-2 molecules of ~80 kb) (25) while as many as 800 can be found in mammalian cells (each containing 1-10 molecules of 16.5 kb) (20, 27). Nucleoids are dynamic macrocomplexes located in the mitochondrial matrix which act as units of mtDNA replication and inheritance; their composition can undergo remodeling in response to metabolic stresses (7, 26, 51). Yeast and mammalian nucleoids have been shown to be the sites of mtDNA replication using BrdU incorporation (39, 43). Also, not all nucleoids replicate concurrently; only a subset undergo replication at any given time. With no strict control related to cell cycle progression, segregation and inheritance of the nucleoid depends upon a membrane-associated apparatus that interacts with the fusion and fission machinery of the mitochondrial organelle network (2, 21, 35). Lastly, while the protein composition of nucleoids can vary among cell types and in response to metabolic conditions, the core of the nucleoid appears to remain constant. Core nucleoid proteins include transcription and replication factors such as mitochondrial transcription factor A, single stranded binding protein, Twinkle helicase and the sole mitochondrial DNA
polymerase, Pol γ (1, 23). In addition to nucleoid organization, the structure and mechanism of mtDNA replication shows remarkable variability among eukaryotes (43).

One of the most unusual and structurally complex mtDNA genomes is found in trypanosomatid parasites such as *Trypanosoma brucei*, the causative agent of African sleeping sickness. This mitochondrial genome, called kinetoplast DNA (kDNA), is a network composed of thousands of topologically interlocked minicircles and maxicircles that are condensed into a single disk-shaped nucleoid structure. Approximately 25 identical maxicircle copies (23 kb) encode a subset of respiratory chain subunits and rRNA, similar to other eukaryotic mtDNA. However, several cryptic maxicircle transcripts require post-transcriptional RNA editing (insertion and/or deletion of uridine residues) to generate functional open reading frames. RNA editing depends upon gRNAs encoded on the heterogeneous population of 5000 minicircles (1kb) (54). Therefore, the coordinated replication of both minicircles and maxicircles is essential for mitochondrial physiology and parasite survival.

Key features of the kDNA replication mechanism include replication once per cell cycle in near synchrony with nuclear S phase, a topoisomerase II-mediated release and reattachment of minicircles, and a multiplicity of DNA polymerases (six), helicases (six) and primases (two) for kDNA transactions (18, 19, 24, 31, 49). Briefly, covalently closed minicircles are released from the network into the Kinetoflagellar zone (KFZ), a specialized region between the kDNA disk and the mitochondrial membrane nearest the basal body (bb) (10). The free minicircles initiate unidirectional theta structure replication primarily through interactions with proteins such as universal minicircle sequence binding protein (UMSBP) and p38 (30, 37). Other proteins such as TbPOLIB,
TbPOLIC and primase also localize to this region. Minicircle progeny (still containing at least one gap) are subsequently reattached at two electron-dense areas positioned 180° apart at the network periphery called the antipodal sites. Several proteins associated with Okazaki fragment processing and reattachment to the network localize to the antipodal sites including SSE1, DNA pol β, DNA ligase kβ and topoisomerase II (Topo II mt) (9, 13, 22, 47, 55). As a result, there is spatial and temporal separation of replication events with early initiation occurring in KFZ followed by Okazaki fragment processing and reattachment at the antipodal sites. Reattachment occurs before all gaps have been filled. Once all minicircles have replicated, the gapped molecules undergo a final phase of gap filling presumably by Pol β-PAK and DNA ligase ka. Far less is known about maxicircle replication. In contrast to minicircles, the maxicircle remain catenated to the network during replication. Maxicircles accumulate at the center of a growing disk and will be the last ones to segregate (15). Currently, only two proteins have been shown to be essential for maxicircle replication, a DNA helicase (TbPIF2) and a primase (PRI1) (18, 31). Thus far, the main maxicircle replicase has not yet been described.

In the cell’s single mitochondrion, the kDNA forms a disk-like structure positioned in a particular region within the mitochondrial matrix near the flagellar bb. kDNA S phase occurs almost in synchrony with bb duplication. Additionally, kDNA segregation and positioning is dependent on bb movement and separation. Failure to segregate the bb results in networks that are unable to segregate, providing additional evidence for the link between bb segregation and kDNA division (17, 44). A filament system called the tripartite attachment complex (TAC) physically links the bb and the kDNA (42, 45). Depletion of a known TAC protein, p166, causes failure in kDNA
segregation (41, 57). The kinetoplast duplication cycle is characterized by morphological differences that can be divided into five distinct stages (15). At stage I, cells contain 1 kDNA disk no visible antipodal sites and 1 bb/pro-basal body (probb) pair. During stages II and III the kDNA transitions from a bilobed to a V-shape structure and both stages contain 2 bb/probb pairs as well as antipodal sites. Segregation of the double sized kDNA network initiates during stage IV where networks remain connected by a thread of maxicircles which is resolved in stage V when both networks are morphologically the same as in stage I. In both stages (IV and V) 2bb/probb are observed and antipodal sites are not detected.

The spatial and temporal localization of kDNA replication proteins is likely important for their participation in this highly coordinated and dynamic process. So far, the localization of the proteins involved in kDNA replication seems to be largely to the KFZ and the antipodal sites. Since the discovery of the first antipodal kDNA replication protein, topoisomerase II (36), it has been shown that many more proteins share a pattern of antipodal localization. It has been proposed that the composition of proteins at the antipodal sites is dynamic and the localization of some of these proteins to the antipodal sites seem to be periodic (22, 50). Initial localization studies of the three essential mitochondrial DNA pols indicated that POLIB and POLIC localized to the KFZ while POLID was distributed throughout the mitochondrial matrix (24). The localization of POLID throughout the mitochondrial matrix suggests that this protein could redistribute close to the kDNA network or is needed in a very low abundance to perform its role in kDNA replication. Using a POLID-PTP tagged single expressor cell line and immunofluorescence microscopy, we characterized in detail the dynamic localization of
POLID during the *T. brucei* cell cycle. Here we describe a detailed localization pattern for TbPOLID in which the protein accumulates as foci during stage II-III of kDNA S phase, and becomes dispersed throughout the mitochondrial matrix at all other cell cycle stages. We provide evidence that TbPOLID changes in localization occur through a mechanism that involves redistribution of the mitochondrial matrix fraction to the antipodal sites. Together these data demonstrate that TbPOLID is spatially and temporally available to perform its essential role in kDNA replication.

2.2 Materials and Methods

2.2.1 Chromosomal tagging and single allele deletion

2.2.1.1 pPOLID-PTP-NEO

POLID C-terminal coding sequence (1635 bp) was PCR amplified from *T. brucei* 927 genomic DNA using forward (5’-ATA ATA GGG CCC TGC TCG TCA AGA GGT GCG-3’) and reverse (5’-ATA ATA CGG CCG CAG TGT CTC CTC AAT GAC AAC G-3’) primers containing ApaI and EagI sites, respectively. The PCR amplified fragment was ligated into ApaI and NotI restriction sites of pC-PTP-NEO (Schimanski and Gunzl, 2005) to create pPOLID-PTP-NEO vector.

2.2.1.2 POLID knockout construct pKOPOLID\textsuperscript{Puro}

pKO\textsuperscript{Puro} is a derivative of pKO\textsuperscript{NEO/HYG} series (28), and was a gift from Paul Englund (38). A 629 bp TbPOLID 5’ UTR fragment was PCR amplified using forward (5’-CTC GAG CAG GGA AAG ATA GCG CCT-3’) and reverse (5’-ATC GAT AAA AAG AAG GAT GCG-3’) primers containing XhoI and ClaI sites respectively, and
ligated into the pKO<sup>Puro</sup>. Subsequently, a 483 bp TbPOLID 3’ UTR fragment was PCR amplified using forward (5’-ACT AGT GTG TCC TAT AGC AGT AAC G-3’) and reverse (5’-GCG GCC GCA GCA ATT TTC CGC AC-3’) primers containing SpeI and NotI sites respectively, and ligated into SpeI and NotI sites in the downstream polylinker portion of the pKO<sup>Puro</sup> vector to generate the pKOPOLID<sup>Puro</sup> construct. After digestion with XhoI and NotI, the 3359 bp fragment containing the puromycin resistance marker flanked by the POLID UTRs was used for transfection into parasites.

2.2.1.3 Myc tagging of TbPIF2

The original pPIF2-myc construct (31) (a generous gift from Paul Englund) was modified to create the pPIF2-Myc-BLA construct. Briefly, we modified this vector by replacing the neomycin resistance marker with the blasticidin resistance marker from the pMotag2H vector (40) using HindIII and BamHI digestion.

2.2.2 Trypanosome growth and transfection

Procyclic Trypanosoma brucei Lister 427 strain was cultured at 27°C in SDM-79 containing 15% heat inactivated serum and were transfected by electroporation with SnaBI linearized pPOLID-PTP-NEO (10 μg). A stable population was first selected with 50 μg/ml G418 followed by limiting dilution as described previously (4, 6) resulting in a plating efficiency of 70%. Clonal cell line TbID-PTP P2B7 was then transfected with XhoI/NotI digested pKOPOLID<sup>Puro</sup> vector (15 μg/ml) and the population was selected with 50 μg/ml G418 and 1 μg/ml Puromycin (Puro). Following limiting dilution cloning, a plating efficiency of 44% was obtained, and clonal cell lines were analyzed for POLID-PTP expression and proper chromosomal integration by Western Blot and Southern Blot.
analyses respectively. Three individual clones were analyzed for growth rate and potential defects in kDNA morphology. For each clone, doubling time was ~9 hours, similar to 427 parental cell line, and no detectable defects in kDNA morphology were observed following DAPI staining. The data presented in this study corresponds to clonal cell line POLID-PTP/IDKO\textsuperscript{Puro} P2H7 that we named TbID-PTP.

2.2.3 Fluorescent Microscopy

2.2.3.1 Immunofluorescence

TbID-PTP cells were harvested for 5 min at 1,000 x g, resuspended in cytomix and adhered to poly-L-lysine (1:10) coated slides for 5 min. Cells were then fixed for 5 min using 4% paraformaldehyde and washed three times (5 minutes each) in phosphate-buffered saline (PBS) containing 0.1 M glycine (pH 7.4) followed by methanol permeabilization (overnight, -20°C). Cells were then washed in PBS 3 times for 5 min, followed by incubation with anti-protein A serum (Sigma) and rat monoclonal antibody YL1/2 (Abcam) together for 90 min diluted 1:5000 and 1:400 respectively in PBS containing 1% BSA. Cells were then washed 3 times in PBS containing 0.1% Tween-20 and incubated with secondary antibodies (60 min), Alexa Fluor 594 goat anti-rabbit and Alexa Fluor 488 goat anti-rat, both diluted 1:250 in PBS containing 1% BSA. DNA was stained with 3 µg/ml 4′-6′-diamidino-2-phenylindole (DAPI), and slides were washed 3 times in PBS prior to mounting in Vectashield (Vector Laboratories). Immunolabeling of exclusion zone filaments using Mab 22 was performed as described in (3).
2.2.3.2 In situ TdT labeling

Cells were fixed in paraformaldehyde, permeabilized in methanol and labeled with TdT as previously described (22, 30). Briefly, cells were rehydrated in PBS and incubated for 20 min at room temperature in 25 μl of 1X TdT reaction buffer (Roche Applied Science) containing 2 mM CoCl$_2$. Cells were then labeled for 60 min at room temperature in 25 μl reaction solution (1X TdT reaction buffer, 2 mM CoCl$_2$, 10 μM dATP, 5 μM Alexa Fluor 488-dUTP and 40 U of TdT). The reaction was stopped by the addition of 2X SSC. Slides were then processed for immunolocalization of PTP tagged protein as described above.

2.2.3.3 Immunofluorescence microscopy of detergent extracted and DNase treated cells

2.2.3.3.1 Detergent Extraction

Cells were adhered to poly-L-lysine coated slides (5 min), extracted for 5 min in NP-40 Buffer (0.25% NP-40, 10 mM Tris-HCl, 1 mM MgCl$_2$, pH 7.4), fixed in 4% paraformaldehyde and then processed with our standard TdT labeling and immunofluorescence protocol described above. To confirm POLID-PTP foci resistance to detergent extraction we also performed extractions using 1% NP-40 (5 min) and incubated for 60 min with 10 Units of DNase I (NEB), washed 3 times in 1X PBS and fixed in 4% paraformaldehyde. Slides were then processed for immunolocalization of PTP tagged protein as described above. For RNase treatment, detergent extracted cells were incubated with 60 μg of RNase A (Invitrogen) for 20 min prior to fixation and immunodetection.
2.2.4 Image analysis and quantification

2.2.4.1 Microscope and software

Images were acquired with a Nikon Eclipse E600 microscope using a cooled CCD Spot-RT digital camera (Diagnostic Instruments) and a 100X Plan Fluor 1.30 (oil) objective. Brightness and contrast was adjusted for all images using Adobe Photoshop CS4.

2.2.4.2 Measurements of basal body distance

Cells were labeled with YL1/2 and anti-Protein A for bb and POLID-PTP detection respectively. The distance between bb was measured in 149 cells from randomly selected fields using the Spot Imaging Solution software (Diagnostic Instruments). These cells were classified based on their kDNA morphology and the presence or absence of POLID-PTP foci.

2.2.4.3 Fluorescence intensity calculation

Fluorescence intensity (FI) was determined using Image J software (http://imagej.nih.gov/ij/). The freehand selection tool was used to determine the total FI of whole cells that were foci positive (WC). The oval selection tool was used to measure the FI of independent foci. To determine the net fluorescence intensity of the mitochondrial matrix (MM), we subtracted the FI of the foci from the FI of WC (See equation 2-1, numerator). The same principle was used to determine the net area of the mitochondrial matrix (See equation 2-1, denominator). Therefore, equation 2-1 shows the net fluorescence intensity of the MM.
To obtain the fold increase within an individual focus, we calculated the ratio of focus FI to net FI of the MM. Background subtraction was performed on all images using the Spot Imaging Solution software (Diagnostic Instruments). Standard error of the mean was determined by analyzing the FI from 10 different cells. Analysis of standard error of the mean (SEM) was performed using GraphPad Prism version 5.00 for Mac OS X (GraphPad Software, San Diego California USA).

2.2.5 TdT labeling quantification

Only intact cells by differential interference contrast (DIC) were included in the analysis. More than 300 cells were scored from three separated experiments (~1000 total cells). Early and Late TdT positive cells were classified as 1N1K_{div} cells and TdT negative cells were classified based on kDNA morphology observed by DAPI staining. The numbers of POLID-PTP foci were distinguished by focusing up and down through several focal planes. Cells with three foci were hard to distinguish from 2 foci cells due the distance between each focus, and therefore may be under-represented in this analysis.

2.2.6 Western Blotting

Parasites were harvested at 3,500 x g for 10 min (4°C) and cell pellets were washed once in PBS supplemented with protease inhibitor cocktail Set III (1:100) (CalBioChem). Cells were lysed in 4X SDS sample buffer containing 5% beta-
mercaptoethanol and incubated at 94°C for 4 minutes. Proteins were separated by SDS-PAGE and transferred to PVDF membrane overnight at 90 mA in transfer buffer containing 0.1% methanol. Membrane was incubated in 1% Roche blocking reagent (60 min) followed by incubation with antibodies (60 minutes) diluted in 0.5% blocking reagent (60 min). PTP (ProteinC-TEV-ProteinA) tagged protein was detected with 1:2000 Peroxidase-Anti-Peroxidase soluble complex (PAP) reagent (Sigma), which recognize the protein A domain of the PTP tag. For additional antibody detections, membrane was stripped for 15 min with 0.1 M glycine (pH 2.5), washed in TBS with 0.1 % Tween-20, blocked and re-probed with C. fasciculata specific Hsp70 antibody (1:10,000) (12) followed by secondary chicken anti-rabbit IgG-HRP (1:10,000) or T. brucei anti-Pol β (1:1000) (47) follow by anti-rat (1:5000). Detection of PIF2-Myc was done using anti-Myc (1:1000) from Santa Cruz followed by secondary goat anti-mouse (1:1000). BM Chemiluminescence Western Blotting Substrate (POD) from Roche was used for protein detection.

### 2.2.7 Cycloheximide treatment

Cell lines expressing POLID-PTP and PIF2-Myc were incubated for 6 hours with 100 μg/ml cycloheximide. Cells were harvested every hour and processed for Western Blot analysis or processed for immunolocalization of PTP tagged protein as described above.

### 2.2.8 Trypanosoma brucei 427 synchronization

Synchronization of Trypanosoma brucei procyclic cells was adapted from (8). Briefly, POLID-PTP cells with a doubling time of ~9 hours were cultured in SDM-79
containing 50 µg/ml G418 and 1 µg/ml puromycin. Cells were incubated in medium containing 0.2 mM hydroxyurea (HU) for 8 hours followed by HU release. Following HU washout, cells were fixed every hour for 10 hours and stained with DAPI. Cells were quantified at each time point (n=200) and classified based on kDNA morphology. To determine TbPOLID-PTP protein levels during synchronization, cells were harvested every hour after HU washout, and whole cell extracts were analyzed by Western blot.

2.3 Results

2.3.1 POLID is detected as discrete foci

The majority of kDNA replication proteins studied localize to specific regions surrounding the kDNA, mainly the antipodal sites and the KFZ (18, 19, 32, 33, 49). Previously, we demonstrated that TbPOLID is one of three mitochondrial DNA polymerases essential for parasite survival and maintenance of the kDNA network (6). However, initial localization of TbPOLID using a peptide antibody showed that it was distributed throughout the mitochondrial matrix (24) suggesting that POLID would need to redistribute to the kDNA disk to perform its essential role in kDNA replication. To investigate the localization of TbPOLID in detail we generated an exclusive expresser cell line (TbID-PTP) in which one TbPOLID allele was deleted and the remaining allele was fused to the PTP sequence by targeted integration of the construct pPOLID-PTP-NEO (Fig. 2.1 and 2.3A). PTP integration was confirmed in parental cell line P2B7 by Southern Blot analysis (Fig. 2.2). This cell line was later transfected with pKOPOLID_{Puro} for single allele deletion. The expected size product of POLID-PTP (~ 200 kDa) was detected in all 17 clonal cell lines by Western Blot using Peroxidase-Anti-Peroxidase
(PAP) antibody (data not shown). The PTP tag was only detected in TbID-PTP whole cell extracts with no cross reactivity observed in 427 WT whole cell extracts (Fig. 2.3B). Growth rate was analyzed in three TbID-PTP clones and each cell line had a doubling time of ~9 hours, similar to 427 parental cell line, and had no detectable defects in kDNA morphology as shown by DAPI staining (Fig. 2.4). TbPOLID is essential for cell viability and kDNA replication, thus our data indicate that the PTP tag does not impair the essential function of this protein. Fluorescence microscopy of TbID-PTP clonal cell lines revealed localization throughout the mitochondrial matrix as previously described. In addition, we detected a new localization pattern in a subpopulation of the cells, where POLID-PTP was present as discrete fluorescent spots at regular positions, always in close proximity with the kDNA disk (Fig. 2.4B). Cells containing POLID-PTP foci displayed decreased mitochondrial matrix fluorescent signal. The results obtained were very similar for all three clones; clonal cell line P2H7 was selected in this study (Fig. 2.4).
Figure 2.1 – Circular map of pC–POLID-PTP-NEO genome integration vector

The pC-PTP-NEO vector is derived from pBluescript SK (+) and it contains a PTP (Protein A-TEV-Protein C) coding sequence (grey) and a neomycin (orange) resistance gene. This construct is designed for stable integration of the PTP into the genome. POLID C-terminal coding sequence (pink) (1635 bp) was cloned into the vector to allow homologous recombination. The vector was linearized within POLID sequence and transfected by electroporation.
Figure 2.2 – Integration of pC-POLID-PTP-NEO in P2B7 clonal cell line

(A) 427 wild type (WT) and POLID-PTP P2B7 genomic DNA was isolated and digested with NruI and XhoI for Southern Blot analysis. (B) Schematic representation of WT and POLID ORF with flanking restriction enzyme sites; PTP allele: integrated pC-POLID-PTP construct at the 3’ end of polymerase ORFs with flanking restriction enzyme sites.
Figure 2.3 – TbID-PTP exclusive expresser cell line

(A) Diagrammatic representation (not to scale) of the TbPOLID gene locus in clonal cell line TbID-PTP. The TbPOLID coding region (green) was replaced in one allele by a puromycin resistance gene (PURO), and in the second allele the PTP sequence was fused to the 3’ end of the coding region by the targeted insertion of the pPOLID-PTP-NEO construct. Coding regions of selectable marker genes are indicated by a blue box (PURO) and red box (NEO). The PTP sequence is indicated by a black box and introduced gene-flanking regions by small orange boxes. (B) Immunoblot analysis of whole-cell extracts of wild-type (WT) and TbID-PTP cells. A total of $5 \times 10^6$ cells were loaded per lane, and tagged protein was detected with PAP reagent (top panel). The same blot was stripped and reprobed with Hsp70 antibody as a loading control.
Figure 2.4 – Characterization of TbID-PTP clonal cells

(A) The growth of three clonal cell lines was monitored every 24 hours for 6 days. The doubling times of the cell line selected for this study (P2H7) was about 9 hours as with WT cells. (B) Localization of POLID-PTP in clonal cells. POLID-PTP was detected using anti-protein A (red), and DNA was stained with DAPI (blue).
The appearance of POLID-PTP foci in a subpopulation of the cells suggests that POLID undergoes redistribution from the mitochondrial matrix to the kDNA disk. Alternatively, changes in protein abundance could also account for this variation in POLID-PTP localization. The only mitochondrial protease that has been shown to regulate kDNA replication is TbHslVU (29). This bacterial-like protease regulates maxicircle replication through degradation of the helicase, TbPIF2 (31). To determine if proteolytic degradation plays a role in the regulation of POLID-PTP localization pattern, we inhibited protein synthesis using 100 µg/ml of cycloheximide (CHX) over a time course of six hours. For this experiment we generated a cell line expressing POLID-PTP and PIF2-Myc and we monitored protein levels by immunoblot.

Additionally, POLID-PTP foci formation was monitored by immunofluorescence. As expected, PIF2-Myc levels decreased to undetectable levels after 2 hours of CHX treatment (Fig. 2.5A). However, POLID-PTP protein levels remained unchanged during CHX treatment similar to Pol β, which is not regulated by proteolytic degradation (Fig. 2.5A). This data suggests that POLID is a stable protein and is not regulated by proteolytic degradation. We next investigated whether the accumulation of POLID-PTP foci was dependent upon new protein synthesis. After 2, 4 and 6 hours of CHX treatment cells were fixed and analyzed for POLID-PTP localization (Fig 2.5B). At each time point, we detected POLID-PTP foci in a subpopulation of the cells demonstrating that the foci are stable and their formation was not dependent on newly synthesized protein.
Figure 2.5 – POLID-PTP protein levels and foci formation after CHX treatment

(A) Western blot detection of POLID-PTP, PIF2-Myc, Pol β and Hsp70 protein levels following CHX treatment. Cells were harvested every hour and $5 \times 10^6$ cells were loaded into each well. (B) Immunofluorescence detection of POLID-PTP foci following CHX treatment. Cells were fixed at 2, 4 and 6 hr intervals and stained/labeled with DAPI (blue) and anti-Protein A (red). POLID-PTP foci are indicated by arrows. Scale bar, 10 µm.
2.3.2 POLID-PTP has a dynamic localization during the cell cycle

BB duplication occurs almost simultaneously with the initiation of kDNA S phase (56). Importantly, multiple studies have demonstrated that bb duplication and positioning are tightly linked with kinetoplast replication and segregation (15, 45, 46). bb duplication events and inter-bb distance are easily monitored by light microscopy, providing a marker for early and late events of *T. brucei* kDNA S phase. To precisely determine if the appearance of POLID-PTP foci is coordinated with cell cycle progression, we monitored POLID-PTP localization in relationship with bb duplication events. Basal bodies were labeled using the monoclonal antibody YL1/2 that detects the tyrosinated form of the alpha-tubulin subunit. Cells with one kDNA network had a single bb/pro-bb pair and the localization of POLID-PTP was dispersed throughout the mitochondrial matrix (Fig. 2.6A, 1N1K cells). When cells transition from 1N1K to 1N1K$_{div}$ (cells undergoing kDNA replication) an additional signal corresponding to the new bb was detected and POLID-PTP is observed as discrete foci close to the kDNA disk in most, but not all cells (Fig 2.6A, 1N1K$_{div}$, Foci + and Foci -). The 1N1K$_{div}$ cells with bb positioning farther apart (indicating a later stage in cell cycle progression) had POLID-PTP localized throughout the mitochondrial matrix (Fig. 2.6A, 1N1K$_{div}$, Foci -). The matrix localization pattern persisted in cells with two bb signals associated with two newly segregated kDNA networks (1N2K) and cells that were undergoing cytokinesis (2N2K). These data demonstrate that POLID-PTP accumulates as foci during kDNA S phase and redistributes to the mitochondrial matrix at other cell cycle stages.
To determine if total POLID-PTP fluorescence intensity (FI) changed during cell cycle progression, we quantified and compared the fluorescent intensity (FI) from individual cells at different cell cycle stages. A small increase in fluorescent intensity was detected in 1N1K\textsubscript{div} foci + cells (Fig. 2.6B, red bar). However, the increase was not statistically significant when compared with 1N1K, 1N1K\textsubscript{div}, foci negative, 1N2K and 2N2K cells.

To confirm POLID-PTP redistribution during the cell cycle, we determined protein levels during HU synchronization of TbID-PTP cells. We subjected cells to 8 hour incubation with 0.2 mM HU and, after removal of HU, collected samples every hour for protein and immunofluorescence analysis. For immunofluorescence analysis, we stained cells with DAPI and quantified 200 cells per time point from three separate experiments. Cells with a large, elongated or V shape kDNA were classified as 1N1K\textsubscript{div} (dividing kDNA). Pre-HU washout, 62% of the population had already replicated and segregated their kDNA (1N2K), 17% had a single unit kDNA (1N1K) and 10% were undergoing kDNA replication (1N1K\textsubscript{div}), and 11% had completed kDNA segregation and mitosis (2N2K) as judged by DAPI staining (Fig. 2.7A). After 4 hours HU washout, 1N1K cells increased to 62% followed by an increase in 1N1K\textsubscript{div} cells between 5-7 hours (27-44%) (Fig. 2.7A). We monitored POLID-PTP matrix and foci localization during a separate HU synchronization experiment (Fig. 2.7B). Karyotypes from several time points were examined and compared with previous HU experiments demonstrating similar karyotypic distribution (data not shown). After 1-3 hours HU removal, more than 85% of the cells had POLID-PTP localized throughout the mitochondrial matrix (Fig. 2.7). As the percentage of cells with POLID PTP matrix localization decreased (4-8 hours...
post-HU release), cells with POLID-PTP foci increased during these time points (up to 31%, 6 hours post-HU release). These time points correspond to the increase in \(1N1K_{\text{div}}\) cells (Fig. 2.7A and B). This data further confirms that POLID has a cell cycle dependent localization. C. fasciculata Pol β protein level was previously shown to remain constant during synchronization, here we demonstrate that this is also the case for \(T. brucei\) Pol β, hence we used this protein as a control for assessing constant protein levels during HU synchronization (Fig. 2.7C, lower) (22). We detected POLID-PTP during HU synchronization using the PAP reagent (Fig. 2.7C, upper panel). No significant changes in POLID-PTP levels were detected for any of the enriched cell populations (Fig. 2.7C). These data support our previous analyses demonstrating that the total fluorescence intensity was consistent in cells at different cell cycle stages (Fig. 2.6B).

To confirm the accumulation of POLID-PTP fluorescence within a focus we quantified the signal ratio of the focus to the mitochondrial matrix from 10 independent cells. To obtain the net FI of the mitochondrial matrix we subtracted the fluorescent intensity of each focus from the FI of the whole cell (see equation 2.1, methods). The focus FI was then calculated and normalized to the mean FI of the mitochondrial matrix, which revealed that the FI within an individual fluorescent focus increased 4-fold compared to mitochondrial matrix signal (Fig. 2.8A). Together these data demonstrate that POLID-PTP foci accumulation results from a redistribution of matrix protein to the kDNA disk and not to a dramatic increase in POLID protein abundance.

The distance between the new and old basal bodies gradually increases through cell cycle progression and provides another indicator for cell cycle stages. Recently, Gull and colleagues provided a detailed examination that demonstrated the tight link between
kDNA morphogenesis and bb dynamics (16). They defined five stages (Stage I-V) of the kDNA duplication cycle that differ in kDNA shape, bb and flagellar pocket duplication status and the presence of antipodal sites. To determine the specific stage during kDNA S phase when POLID-PTP accumulates as foci we measured the inter-bb distance. Cells with discrete POLID-PTP foci had a minimum distance between the basal bodies of 0.62 μm and a maximum distance of 1.82 μm (Fig. 2.8B). The mean bb distance for foci positive cells was 1.1 μm (1.160 ± 0.037, N=77) and 1.3 μm (1.338 ± 0.056, N=47) for cells with no detectable foci (Fig. 2.8B). Once the basal bodies reached a distance of more than ~2 μm (Stage IV), POLID-PTP always localized throughout the mitochondrial matrix (Fig. 2.8B). These data indicate that POLID-PTP foci accumulate close to the disk only during stages II and III of the kDNA duplication cycle characterized by a domed or bilobed network shape and 2 bb/probb. Together these data demonstrate that the redistribution of POLID-PTP from the mitochondrial matrix to foci near the kDNA is tightly coordinated within the 1N1K_{div} T. brucei cell cycle stages.
Figure 2.6 – Localization of POLID-PTP during the cell cycle

(A) Representative cells from an unsynchronized population. TbID-PTP cells were dually labeled with anti-proteinA that detects POLID-PTP (red) and YL1/2 for the detection of bb (green). DNA was stained with DAPI (blue). Scale bar, 5 µm. (B) Mean fluorescent intensity of POLID-PTP during the cell cycle. The FI was determined in 43 cells at different stages of the cell cycle based on kDNA morphology and bb positioning. The red bar represents cells with POLID-PTP foci.
Figure 2.7 – Hydroxyurea synchronization in TbID-PTP cells.

(A) Quantification of synchronized cells in 60 min intervals post HU washout. Error bars correspond to the SEM from three separate experiments. Black solid bars represent 1N1K cells, light grey bars correspond to cells that classify by DAPI staining that were undergoing kDNA replication, dark grey bars are the percentage of 1N2K cells, stripe bars represent 2N2K cells and white bars are others (multiple kDNA or nuclei, and zoids). Only intact cells by DIC were included in the quantification. (B) Quantification of POLID-PTP distribution during HU synchronization. The percentage of cells with POLID-PTP localized to the mitochondrial matrix are represented by open circles and percentage of cells containing POLID-PTP fociby filled circles. (C) Western blot analysis of TbID-PTP whole cell extracts (5 x 10^6 cells/lane) detected with PAP reagent (upper panel) and anti-Pol β (lower panel).
Figure 2.8 – Redistribution of TbPOLID during basal body segregation

(A) Fold increase in FI of POLID-PTP foci. The net FI of the mitochondrial matrix (gray bar) was calculated and normalized to determine the focus FI fold increase (red bar) (SEM, 4.4±1.21; n=10). (B) Distance between bb during the cell cycle. Measurements are from randomly selected cells (n=149) containing 2 bb/pro-bb pairs with POLID-PTP foci (red) and without foci (blue).
2.3.3 POLID-PTP colocalized with replicating minicircles at the antipodal sites

To precisely define the spatial and temporal localization of POLID-PTP foci during kDNA replication we fluorescently labeled replicating minicircles and maxicircles using terminal deoxynucleotidyl transferase (TdT) and fluorescein conjugated dUTP. Replicating minicircles containing gaps will accumulate at the antipodal sites during kDNA S phase allowing us to distinguish the specific localization of POLID-PTP in relationship with replicating minicircles. Multiple TdT labeling patterns were observed, and each is characterized by the distribution of free gapped minicircles (8). During kDNA G1 cells contain one nucleus, a unit size kDNA network (1N1K) and unreplicated minicircles and maxicircles. These cells were TdT negative (TdT -) as minicircles (and maxicircles) were covalently closed with no gapped or nicked regions and POLID-PTP was mainly dispersed throughout the mitochondrial matrix (Fig. 2.9, 1N1K). During early stage of kDNA replication the antipodal sites are enriched with multiply gapped minicircles resulting in a strong TdT signal (Fig. 2.9, 1N1K\textsubscript{div}). At later stages of kDNA replication, the gapped minicircle progeny is attached to the network and these single unit networks become uniformly label with TdT. Once kDNA network have segregated (1N2K), TdT (TdT post) labels the entire network until all minicircles are repaired and can no longer be labeled. POLID-PTP was detected as foci in a subpopulation of TdT positive cells that contained a single kDNA network (Fig. 2.9, 1N1K\textsubscript{div}). In 1N2K cells POLID-PTP foci disappear and POLID-PTP became dispersed throughout the mitochondrial matrix (Fig. 2.9, fourth and fifth row). POLID foci were never detected in
post replicating networks that were TdT negative and the distribution to the mitochondrial matrix persisted during cytokinesis.

We were interested to determine the percentage TdT positive cells that exhibit POLID-PTP foci and confirmed by quantification that redistribution occurred strictly during kDNA replication. We examined ≥ 300 cells from three separate TdT labeling experiments and classified them by the presence (red bar) or absence of foci (blue bars) and kDNA morphology. On average, 29% of the total cells had a single unit kDNA (1N1K) with no TdT signal and no obvious POLID-PTP foci (Fig. 2.10A). Cells that were TdT positive and had a single kinetoplast (1N1K\textsubscript{div}) represented a 41% of the total population, 16 % of these had no POLID-PTP foci while 25% had discrete POLID-PTP foci (Fig. 2.10A). Cells that segregated their network (1N2K) represent 9% of the total population and in all of these we could not detect obvious accumulation of POLID-PTP foci (Fig. 2.10A). POLID-PTP foci were never detected in 2N2K cells, these represented 8% of the population.

We observed that cells undergoing kDNA replication display different numbers of POLID-PTP foci (Fig. 2.10B). When we investigated POLID foci in replicating cells (1N1K\textsubscript{div}), 39% of these had no evident foci in proximity with the kDNA disk; instead, POLID was detected throughout the mitochondrial matrix (Fig. 2.10B, i). A single discrete focus was observe in 11% of these cells and 44 %, had two independent POLIID-PTP focus that colocalized with replicating minicircles at the antipodal sites (Fig. 2.10B ,ii and C). One interesting observation was that 6 % of cells undergoing kDNA replication had a third focus that accumulated at the center of the network (Fig. 2.10B and C, arrowhead) and based on the kDNA morphology, most of these cells had a bilobed
kDNA. Together this data indicated that redistribution of POLID-PTP occurs specifically during kDNA S phase and it correlates with early and later stages of minicircle replication.
Figure 2.9 – Localization of TbPOLID in respect to TdT labeled minicircles

(A) POLID-PTP (red) was detected using anti-Protein A. Gapped/replicating minicircles were labeled with terminal deoxynucleotidyl transferase (TdT) (green). Represented images of different karyotypes are shown along with the corresponding POLID-PTP and TdT labeling patterns. Scale bar, 5 µm.
Figure 2.10 – Percentage of cell with POLID-PTP foci

(A) Distribution of POLID-PTP foci in a population of TdT-labeled cells. Cells were classified based on kDNA morphology and the presence (red bar) or absence (blue bars) of POLID foci. The 1N1K\textsubscript{div} category included early and late TdT-positive cells. Others (gray bar) included cells with abnormal karyotypes, including multinucleated cells and zoids. (B) Representative images of POLID-PTP foci in 1N1K\textsubscript{div} TdT-positive cells. (i) Early TdT cells with a single POLID-PTP focus. (ii) Early TdT cells with two distinct POLID-PTP foci. (iii) Early TdT cells with three foci (arrowhead). (iv and v) Late TdT cells with diffuse POLID-PTP signal. Scale bar, 1 µm. (C) The population of 1N1K\textsubscript{div} cells divided into subcategories based on the number of independent foci per cell.
2.3.4 POLID-PTP foci associate with cytoskeleton elements

Extraction of cells using a non-ionic detergent removes soluble and membrane proteins while retaining cytoskeleton elements and DNA associated proteins. Extraction followed by immunofluorescence has been extensively used to determine that multiple nuclear replication proteins such as DNA polymerase α and PCNA are tightly associated with DNA specifically during S phase (53). We used this approach to determine if TbPOLID antipodal localization results from transient binding to kDNA. Cells were extracted with 0.25% NP-40 prior to fixation and then analyzed for TdT labeling of newly replicated minicircles, POLID-PTP foci and bb staining. POLID-PTP foci were detected in 20% of the cells following detergent extraction and these foci colocalized with TdT positive minicircles at the antipodal sites (Fig. 2.11, merge); similar to the results obtained with unextracted cells (Fig. 2.11A). However, the portion of POLID-PTP that localized to the mitochondrial matrix was significantly decreased suggesting that this fraction of POLID-PTP was solubilized under the extraction conditions (Fig. 2.11A). After detergent extraction, cells with 3 foci were also detected (Fig. 2.11B - DNAse, arrow). POLID-PTP foci are retained along with cytoskeletal elements such as the basal bodies and the exclusion zone filaments even under robust extraction conditions (1% NP-40) (data not shown). To test if localization of POLID-PTP as discrete foci was dependent on DNA binding, we DNase treated the detergent extracted cells. After 60 minutes of DNase treatment, DAPI staining indicated no detectable nuclear DNA or kDNA (even after longer exposure times). Digestion with DNase did not eliminate the POLID-PTP foci demonstrating that TbPOLID antipodal localization was not result of
transient binding to kDNA (Fig. 2.11B, +DNase). Cytoskeleton components such as basal bodies and exclusion zone filaments remained intact after detergent extraction and DNase treatment. These data suggest that the specific localization of POLID-PTP foci to the antipodal sites during early stages of kDNA replication is not strictly dependent upon DNA association. Instead, the localization depends mainly upon interactions with cytoskeletal features that persist following extraction.
Figure 2.11 – Detection of POLID-PTP foci in extracted cells

Detection of POLID-PTP foci in extracted cells. (A) Detergent-extracted cells prior to formaldehyde fixation labeled with TdT (green) and anti-protein A (red). Colocalization of POLID-PTP foci with replicating minicircles at the antipodal sites is represented in yellow (merge; see enlargement). Scale bar, 10 μm. (B) Detergent-extracted cells labeled with anti-protein A (red), YL1/2 (green, upper panel), or MAb 22 (green, lower panel). POLID-PTP foci and cytoskeletal components such as basal bodies and exclusion zone filaments were detected in detergent-extracted cells with (+DNase) or without (-DNase) DNase treatment. Three foci were also detected after extraction (enlargement). Scale bar, 5 μm.
2.4 Discussion

Trypanosome kDNA is the most complex mitochondrial DNA in nature. Important properties include a catenated network composed of minicircles and maxicircles, a single disk-shaped nucleoid structurally linked to the flagellar bb, and an elaborate topoisomerase-mediated release and reattachment mechanism for minicircle replication. Two distinct regions, the antipodal sites and the KFZ, have emerged as important sites for the localization of kDNA replication proteins and minicircle replication intermediates. Previously, we demonstrated that three mitochondrial DNA polymerases (TbPOLIB, IC, ID) are essential for parasite survival and kDNA replication in both life cycle stages (4-6, 24). While TbPOLIB and IC were detected in the KFZ, TbPOLID did not specifically localize near the kDNA disk (24). Given its essential role in kDNA replication, the localization of TbPOLID to the mitochondrial matrix suggested major differences in the mechanism of how this protein engaged in kDNA replication. Here, we provide a detailed examination of TbPOLID localization during the T. brucei cell cycle, and demonstrate that TbPOLID localizes as discrete foci flanking the kDNA disk in a subpopulation of cells (Fig. 2.4B). Consistent with an essential role in minicircle replication, TbPOLID colocalizes with replicating/gapped minicircles at the antipodal sites during kDNA replication (Fig. 2.9). Importantly, we demonstrated that the major changes in TbPOLID localization occur by redistribution of this stable protein (Fig. 2.8A). Because the minicircle replication stages are spatially and temporally separated, redistribution of TbPOLID represents a mechanism for kDNA replication proteins to participate in this highly coordinated process. This study represents the first detailed
characterization of a *T. brucei* kDNA replication protein with dynamic localization during the cell cycle.

Originally, the Okazaki fragment processing proteins, pol β and SSE1, as well as TopoII<sub>mt</sub> were localized to the antipodal sites (13, 14, 36). More recently, a great majority of newly identified essential kDNA replication proteins with roles in origin binding, priming and processive replication also localize to the antipodal sites (p38, p93, PRI1, PRI2, PIF1, PIF5) suggesting that the role of the antipodal sites is not limited to Okazaki fragment processing. Interestingly, several antipodal site proteins appear to transiently associate with this region demonstrating that the protein composition is dynamic (22, 47, 50). Studies from the related trypanosome *Crithidia fasciculata* established that antipodal localization of Pol β and SSE1 correlated with kDNA synthesis and not other cell cycle stages (13, 22). Redistribution was proposed as a mechanism for the localization to the antipodal sites. However, these proteins were undetectable by IF when not present at the antipodal sites even though protein levels remained constant (13, 22). Here we provide strong evidence that TbPOLID antipodal localization is due to dynamic redistribution from the mitochondrial matrix in a time-dependent manner during kDNA synthesis. Then following kDNA replication, TbPOLID foci disperse from the antipodal sites back to the mitochondrial matrix. Analysis of total TbPOLID FI in individual cells or protein levels in a synchronized population show that TbPOLID levels remain constant throughout different stages of the cell cycle (Fig. 2.6B and 2.7C). Consistent with this data, we show that TbPOLID is highly stable (Fig. 2.5A) indicating that it is not regulated through proteolytic degradation by the mitochondrial protease HslVU (29). Although POLID levels remained essentially constant, we found a four-fold
increase in FI per TbPOLD focus and a corresponding decrease in FI within the mitochondrial matrix (Refer to Equation 2.1). While a majority of TbPOLD is recruited to the antipodal sites and a central focus, approximately 10% remained dispersed in the mitochondrial matrix. It is unclear why a small fraction of TbPOLD is not recruited to the antipodal sites.

Moreover, recruitment of TbPOLD to the center of the bilobed network was typically associated with stage III of kDNA replication. It was recently confirmed by FISH that replicating maxicircles accumulate at the midzone of the network during this stage of kDNA replication (15). It is tempting to speculate that the fraction of POLID concentrated at the midzone plays a role in maxicircle replication. Although our previous data on silencing TbPOLD revealed a role in minicircle replication, maxicircle copy number declined more rapidly and completely than minicircles. These data suggested that POLID might have a primary role in maxicircle replication and the effects on minicircles could be a secondary response to silencing (6). So far, only two proteins have definitive roles in maxicircle replication, the primase PRI1 and the helicase PIF2 (18, 31). Significant questions about maxicircle replication remain unanswered including what is the maxicircle replicase. Importantly, we have shown that TbPOLD is now the first essential mitochondrial DNA polymerase that localizes to the antipodal sites and to the kDNA midzone. However, further studies are required to determine if TbPOLD is indeed the maxicircle replicase or if the central focus represents other POLID functions such as replication restart or DNA repair.

The multiplicity and spatial separation of the T. brucei mitochondrial DNA polymerases suggests that several protein complexes assemble around the kDNA disk
(KFZ and antipodal sites). Others have suggested that the antipodal sites are organized into subdomains populated by different enzyme activities (9, 16). Multiple lines of evidence support this view. First, Okazaki fragments processing enzymes Pol β and SSE1 appear to colocalize (13) while TopoIImt and Ligk β do not precisely colocalize (9). Second, ethanolic-phosphotungstic acid staining showed regions that differ in staining intensity indicating variability of protein concentration in different regions of the antipodal sites (16). The spatial separation of proteins within the antipodal sites could facilitate the formation of functionally different protein complexes. Currently we do not know if POLID colocalizes with other kDNA replication proteins or if this polymerase occupies a unique antipodal subdomain. To further understand the dynamics among antipodal site subcomplexes it will be necessary to identify other components that colocalize with POLID. The existence of subdomains at the antipodal sites suggests that the molecular basis that governs antipodal localization could vary between kDNA replication proteins. So far, the antipodal localization signal is completely unknown.

In model eukaryotes, some replication proteins are dynamically recruited to nuclear DNA replication foci (RF) through transient association with the clamp loader PCNA (52). This protein appears to serve as a stationary platform for the recruitment of replication factors such as DNA ligase I, DNA methyltransferase and Fen1 via interactions with a short amino acid motif termed the PCNA binding domain (11, 52). A kDNA PCNA-like protein has not yet been identified but stable components of the antipodal sites might function as stationary loading platforms similar to PCNA. Alternatively, specific antipodal site targeting signals could be present in kDNA replication proteins. Upon entry into or exit from kDNA S phase, the signals could
mediate protein-protein interactions or act as sites of posttranslational modification. Currently, it is not known if any of the kDNA replication proteins undergo posttranslational modifications or even if these events are involved in assembly or disassembly of protein to the sites of kDNA replication.

In the absence of a kDNA PCNA-like protein, one possible mechanism for stable association of protein to the antipodal sites could be by a direct or indirect association with cytoskeletal elements. The KFZ is transversed by structural filaments that form part of the TAC and link the kDNA disk to the basal bodies to control network segregation. It is possible that similar structural elements transverse the antipodal sites to give stability to protein complexes that localize to subdomains in this region. While no structural elements have been reported for this region, EM analyses indicate there are structural differences at the antipodal sites compared to the proximal mitochondrial matrix (16). Alternatively, these could protect this highly specific region where replication intermediates are detected from other processes occurring at the MM. Intriguingly, we show that the dynamics of TbPOLID foci was not dependent on membranous structures or other soluble proteins following detergent extraction. TbPOLID and replicating/gapped minicircles colocalized at the antipodal sites even following detergent extraction with stringent conditions (Fig. 2.11A). These data suggest that cytoskeletal elements may play a role in the stable association of proteins and DNA to the antipodal sites. Consistent with these findings, we demonstrate that TbPOLID antipodal localization was not affected in the absence of DNA (Fig. 2.11C). A specialized mitochondrial nucleoid associated structure is also present in yeast mitochondria (35). Anchoring of yeast nucleoids to the cytoskeleton through Mgm1p and Mmm1p is
essential not only for segregation and inheritance, but also replication. Interestingly, yeast mitochondrial DNA polymerase, Mip1, is a stable component of this structure (35). Additionally, mammalian mtDNA foci are associated with cytoskeletal elements as they remained stable after membranes and soluble components were extracted (21). This association is maintained by KIF5B, the kinesin motor protein implicated in moving mitochondria along microtubules (21). The identification of the link between the antipodal sites and a cytoskeletal component will help us understand how minicircle replication intermediates and kDNA replication proteins assume such a defined localization pattern.

The coordination of proteins involved in early and later stages of kDNA replication is a fundamental yet poorly understood process. Intramitochondrial localization of kDNA replication proteins have provided a framework for the current model of kDNA replication and have been important for formulating new hypotheses. Our study represents a significant step towards understanding the spatial and temporal coordination of proteins during kDNA replication stages. All of the changes determined from images and quantification of the TbPOLID dynamic redistribution (Fig. 2.6 and Fig. 2.7) are summarized schematically in Fig. 2.12. During G1 phase, cells contain a single bb/probb pair, pre-replication kDNA networks with covalently closed minicircles and maxicircles that do not label with TdT, and POLID fluorescence that is detected throughout the mitochondrial matrix (Fig. 2.12A). Upon entering kDNA S phase, a small percentage of cells display POLID fluorescence throughout the kDNA disk (Fig. 2.12B), then POLID colocalizes at two independent foci with minicircle replication intermediates (labeled with TdT) as they accumulate mainly at the antipodal sites and the kinetoplast is
associated with two bb/probb pairs (Fig. 2.12C). As kDNA replication progresses and the network elongates to form a bilobed structure, POLID remains associated with the antipodal sites while a third focus accumulates at the center of the stage III kinetoplast (Fig. 2.12D). At this time point the gapped minicircle are still associated with the antipodal sites. Subsequently, the cell enters into a post-replicative stage where gapped minicircles are detected throughout the kDNA disk and the POLID fluorescence is no longer focused in spots but instead is diffuse throughout the kDNA disk and appears to redistribute to the mitochondrial matrix (Fig. 2.12E). The kinetoplast then enters stage IV where two unit size disks have moved apart (1.68 µm (15)) but are still connected by the nabelschnur (Fig. 2.12F). At this stage there are no longer any free minicircle replication intermediates detected, and POLID has nearly completed redistribution to the mitochondrial matrix. The kinetoplasts continue to separate as the cell completes mitosis and undergoes cell division. During these post-replicative stages POLID is always found distributed in the mitochondrial matrix (Fig. 2.12G, H) until the next round of kDNA synthesis begins.
Figure 2.12 – Schematic representation of POLID dynamic localization throughout the *T. brucei* cell cycle

(A) Cells in G1 (1N1K) with a single kDNA network (blue) with POLID localized throughout the mitochondrial matrix (MM; red). These cells contained a single bb/pro-bb pair (pp/ppb; green).

(B) Localization of POLID foci during kDNA duplication cycle (S<sub>k</sub>) POLID fluorescence throughout the kDNA disk in cells with a bb/pro-bb pair close together. (C) POLID foci concentrate at the antipodal sites (antipodal sites are shown in brown). (D) A third focus accumulates at the midzone of a bilobe network. (E) Antipodal sites are not visible, and POLID is now detected in the MM. (F) KDNA daughter networks held together by the nabelschnur. POLID is only detected through the MM. (G and H) In 1N2K and 2N2K cells POLID remains dispersed throughout the MM.
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2.6 Bibliography


3 TRYANOSOMA BRUCEI MITOCHONDRIAL DNA POLYMERASE IC UNDERGOES CHANGES IN LOCALIZATION THAT ARE COORDINATED WITH KDNA’S PHASE

3.1 Introduction

Trypanosoma brucei is a flagellated parasitic protist that has a number of remarkable biological features that distinguish it from other eukaryotes (2, 16, 20, 23, 24, 30). One of these features is their complex mitochondrial genome known as kinetoplast DNA (kDNA). The kDNA is composed of two circular DNA molecules known as minicircles (1 kb) and maxicircles (23 kb) that are topologically interlocked to form a DNA network (24). Each network contains about five thousand minicircles copies and twenty-five maxicircles (24). Maxicircles are homologs to mitochondrial DNA in other eukaryotes as they encode several subunits of the respiratory complex and mitochondrial ribosomal RNAs (35). Extensive RNA editing (insertion and/or deletion of uridine residues) of maxicircle transcripts is required to generate functional open reading frames (ORF) (2). The sequence information for editing is directed by minicircle-encoded guide RNA (gRNA). Therefore, the information encoded within minicircles and maxicircles is fundamental for mitochondrial functions, thus replication of both is essential for cell viability.

The mechanism of kDNA replication is characterized by an ordered series of discrete events (46). The current model suggest that kDNA replication initiates with the vectorial release of individual minicircles from the network into the kinetoflagellar zone (KFZ); a specialized region between the kDNA disk and the flagellar basal body (bb)
(11). In the KFZ, free minicircles initiate unidirectional theta structure replication mediated by universal minicircle sequence binding protein (UMSBP) and p38 (1, 32). Subsequently, they migrate to regions known as the antipodal sites that are situated at the network periphery, where they undergo Okazaki fragment processing (15, 41). Proteins involved in this process include structure specific endonuclease 1 (SSE1), DNA polymerase β (Pol β), DNA ligase kβ, and topoisomerase II (Topo II mt) (10, 14, 43, 47).

To ensure that all minicircles replicate only once, gapped minicircle progeny are reattached to the network and the final gaps are repaired once all minicircles are replicated. DNA ligase kα and Pol β-PAK have been implicated in this process (10, 43). Less is known about maxicircle replication. They are known to remain catenated to the network and undergo theta structure replication. Fluorescent in situ hybridization analysis demonstrated that they gradually concentrate in the middle of a growing network (18). Additionally, unlinking of maxicircles marks the final stage of kDNA segregation (18). So far, only a limited number of the proteins expected to be involve in maxicircle replication have been describe. A helicase (TbP1F2) regulated by the mitochondrial protease HslVU is known to be involved in regulating maxicircle synthesis (31, 33). A primase (PRI1) is also required for maxicircle replication since silencing of this protein resulted in preferential loss of maxicircles (21).

The mitochondrion of Trypanosomes harbors multiple enzymes with similar activities but non-redundant roles in kDNA replication. Among these are two primases (PRI1 and PRI2), both with a PriC-2 and RNA binding motif (21, 22), and two ligases (ligase kα and β) which are responsible for repairing gaps in the minicircle progeny (10). PRI1 and PRI2 were shown to be essential for maxicircle and minicircle replication,
respectively. In addition, six helicases and six DNA polymerases are known to localize to *T. brucei* mitochondrion (27, 33). The helicases (TbPIF1-6) are related to yeast ScPif1p mitochondrial/nuclear helicase (33). Three of the six helicases (TbPIF1, 2 and 8) are essential for cell viability and kDNA maintenance (33, 34, 50). They have multiple roles that range from minicircle (TbPIF1) and maxicircle replication (TbPIF2) to kDNA segregation (TbPIF8) (33, 34, 50). *T. brucei* mitochondrial DNA polymerases belong to family A and X DNA polymerases which contain replicative and repair enzymes. Within the family X, *T. brucei* has two Pol β-like enzymes (Pol β and Pol β-PAK) that are presumably involved in Okazaki fragment processing (43). None of the four family A DNA polymerases (TbPOLIA, B, C and D) of *T. brucei* mitochondrion are homologous to Pol γ; the sole mitochondrial DNA polymerase in other eukaryotes (26). Instead, they are similar to bacterial DNA polymerase I (Pol I). Three of these (TbPOLIB, IC and ID) are essential for cell growth and kDNA replication in both life cycle stages (insect and bloodstream form) (5–7, 26).

Initial localization analyses using peptide antibodies showed that TbPOLIB and TbPOLIC were detected in the KFZ region and TbPOLID was distributed throughout the mitochondrial matrix (27). Localization of TbPOLID suggested that this protein had to undergo changes in localization to perform its essential role in kDNA replication. We performed a detailed examination of TbPOLID localization using a combination of tools: changes in kDNA morphology linked to kDNA synthesis, basal body duplication events to distinguish cell cycle stages and TdT-labeled minicircles as a marker for antipodal sites (9). The tight link between basal body (bb) duplication and kDNA synthesis allowed Gluenz and colleagues to described five different stages (I-V) within kDNA replication
(18). Briefly, cells at stage I, contained 1 kDNA disk no visible antipodal sites and 1 bb/pro-basal (probb) pair. During stages II and III, the kDNA transitions from a domed to a bilobed shape network and both stages contain 2 bb/probb pairs as well as antipodal sites. The segregation of replicated kDNA network initiates during stage IV and as cells transition from stage IV to V a thread of maxicircles that maintains the networks connected is resolved. Resulting networks are morphologically the same as in stage I. In both stages (IV and V), 2bb/probb are observed and antipodal sites are not detected. We demonstrated that TbPOLID changes in localization were coordinated with stages II and III of the kDNA replication cycle (9). During these stages, TbPOLID concentrated as foci that colocalize with TdT-labeled replicating minicircles at the antipodal sites. These data demonstrated that TbPOLID is available to perform its role in replication as a result of spatial and temporal changes in its localization (9).

Studies in the related kinetoplastid *Crithidia fasciculata* indicate that several kDNA replication proteins (i.e., pol β, UMSBP, Topo II, SSE1 and ligase kα) also undergo changes in localization during the cell cycle (1, 14, 25, 49). These observations together with TbPOLID localization data suggest that a dynamic change in protein localization is a potential mechanism to control kDNA replication. So far, the following three mechanisms have been proposed to regulate kDNA replication proteins and as a result kDNA replication events: (1) Reduction and oxidation status control binding of the universal minicircle sequence binding protein (UMSBP) to the origin sequence (40), (2) Trans-acting factors regulate the mRNA stability of kDNA replication proteins during the cell cycle (37), and (3) Regulation of TbPIF2 helicase protein levels by a HslVU-like protease to control maxicircle copy number (31). In this study, we provide a
comprehensive analysis of TbPOLIC localization. We demonstrate that TbPOLIC foci localize to the antipodal sites during stage II of the kDNA replication cycle and remain below the levels of detection at other cell cycle stages. Proteolytic degradation is not involved in the regulation of TbPOLIC localization, as protein levels remain constant after inhibition of protein synthesis. Additionally, we demonstrate that TbPOLIC colocalize with active kDNA replication sites and TbPOLID foci at the antipodal sites. Taken together, these data demonstrate that a second kDNA replication protein of T. brucei accumulates to the antipodal sites in a cell cycle dependent manner.

3.2 Materials and Methods

3.2.1 Plasmid construction

3.2.1.1 POLIC knockout constructs pKOPOLIC\textsuperscript{Puro} and pKOPOLIC\textsuperscript{BSR}

For generating the pKOPOLIC\textsuperscript{Puro} construct a 430 bp \textit{TbPOLIC} 5’ UTR fragment was PCR-amplified and was ligated into the Xhol and HindIII sites in the upstream polylinker of the pKO\textsuperscript{Puro} vector (primers MK340 and MK341) \cite{29}. Subsequently, a 448 bp \textit{TbPOLIC} 3’ UTR fragment was PCR-amplified and was ligated into the SpeI and XbaI sites in the downstream polylinker portion of the pKO\textsuperscript{Puro} vector to generate the pKOPOLIC\textsuperscript{Puro} construct (primers MK342 and 343). After digestion with Xhol and XbaI, the 3097 bp fragment containing the puromycin resistance marker flanked by the POLIC UTRs was used for transfection into parasites. To generate pKOPOLIC\textsuperscript{BSR}, the puromycin resistance cassette from pKOPOLIC\textsuperscript{Puro} was replaced with blasticidin cassette. Briefly, the puromycin cassette was released from pKOPOLIC\textsuperscript{Puro} after AscI and PacI digestion and the blasticidin sequence from the pKO\textsuperscript{BSR} vector was ligated into
pKOPOLIC vector to generate the pKOPOLIC<sup>BSR</sup>. For primer sequences refer to Table 3.1.

### 3.2.1.2 PTP tag constructs

pPOLIC-PTP-PURO was generated as described (5). To generate the pPOLIC-PTP-NEO construct, *TbPOLIC* C-terminal coding sequence (2226 bp) was PCR-amplified from *T. brucei* 927 genomic DNA (primers MK249 and MK240). *TbPOLIC* PCR-amplified fragment was ligated into the ApaI and NotI restriction sites of the pC-PTP-NEO vector (45). The pPOLID-PTP-NEO was generated as previously described (9). For primer sequences refer to Table 3.1.

### 3.2.1.3 pMOPOLIC-HA-PURO

*TbPOLIC* C-terminal coding sequence (1279 bp) and 3’UTR (996 bp) region were PCR-amplified from *T. brucei* 927 genomic DNA using primers MK534 and MK535 and MK538 and MK539, respectively (Table 3.1). PCR-amplified fragments were ligated into the pMOTagHA vector (39) to generate the pMOPOLIC-HA-PURO.

### 3.2.2 Trypanosome growth

Procyclic *Trypanosoma brucei* Lister 427 strain was cultured in SDM-79 media with 15% heat-inactivated serum as described in (9) and 29-13 cells for RNAi were grown in 15 μg/ml G418 and 1μg/ml hygromycin.
3.2.3 Generation of cell lines

3.2.3.1 PTP and HA tag cell lines

**TbIC-PTP.** For POLIC-PTP tagged cells, 427 WT cells were transfected by electroporation with XhoI/XbaI digested pKOPOLIC<sup>Puro</sup> (15 µg) in 4-mm cuvettes by use of a BTX 630 electroporator at a peak discharge of 1.6 kV with a resistance of 25 Ω. A stable population of *TbPOLIC* single knockout cells was selected with 1 µg/ml Puromycin (Puro) followed by cells limiting dilution as described previously (9). Southern Blot analysis confirmed single allele deletion in clonal cells. Clonal cell P1A8, expressing a single *TbPOLIC* allele was then transfected with the pPOLIC-PTP-NEO after linearization with AatI. POLICKO<sup>Puro</sup>/IC-PTP cells were selected in media containing 1 µg/ml Puromycin (Puro) and 50 µg/ml G418. After limiting dilution POLICKO<sup>Puro</sup>/IC-PTP cells, POLIC-PTP localization and kDNA morphology was monitored in three individual clones (P2C2, P2A1 and P2C1). No detectable defects in kDNA morphology were observed following DAPI staining. The data presented in this study corresponds to clonal cell line POLICKO<sup>Puro</sup>/IC-PTP P2C1, which we named TbIC-PTP.

**TbID-PTP/ICHA.** To generate a cell line co-expressing POLIC-HA and POLID-PTP, POLID-PTP P2B7 (9) cells we transfected by electroporation with PstI/XbaI digested pMOPOLIC-HA-PURO. TbID-PTP/ICHA cells were selected in media containing 50 µg/ml G418 and 1 µg/ml puromycin. Following cells limiting dilution, Western Blot analysis of 8 clones demonstrated that all cells expressed POLID-PTP and POLIC-HA. Clone P2A5 was selected in our study and we named this cell line TbID-PTP/ICHA.
3.2.3.2 RNA interference cell lines

**TbIC-PTP/SLID.** The stem-loop vector pSLID for *TbPOLID* RNAi was generated as previously reported (7). The pPOLIC-PTP-PURO construct was stably integrated into a 29-13 cell line, by transfection, as previously described (5). This cell line was then transfected with the pSLID RNAi construct that was linearized with NotI. Cells expressing a POLC-PTP and the intramolecular stem-loop vector to target *TbPOLID* RNAi were subsequently transfected with pKOPOLIC\textsuperscript{BSR} for knockout of *TbPOLIC* wild type allele. Cells were then selected with 15 μg/ml G418, 50 μg/ml hygromycin, 2.5 μg/ml phleomycin, 1 μg/ml puromycin and 10 μg/ml blasticidin resulting in cell lines expressing a single PTP-tagged POLIC allele (POLIC-PTP/SLID/ICKO\textsuperscript{BSR}). After limiting dilution of POLIC-PTP/SLID/ICKO\textsuperscript{BSR} cells, 4 clonal cell lines were evaluated for PTP expression by western blotting, *TbPOLID* RNAi silencing phenotype and *TbPOLIC* single allele knockout. Single knockout was confirmed by PCR amplification of POLIC-PTP/SLID/ICKO\textsuperscript{BSR} gDNA using primers MK542 and MK666 that annealing to the 5' end of the Blasticidin cassette and the *TbPOLIC* downstream gene (Tb927.7.4000) to generate a 2.5 kb amplicon. Primer sequences are listed in Table 3.1. PCR product was sent for sequencing and confirmed proper integration of the Blasticidin cassette. Clonal cell line P1F3 was selected for this study. We named this cell line TbIC-PTP/SLID.

3.2.4 *TbPOLID* RNAi

*TbIC-PTP/SLID* cells were induced for RNAi by adding 1 μg/ml tetracycline, and cell growth was monitored daily using a Z2 model Coulter Counter (Beckman Coulter).
3.2.5 **BrdU metabolic labeling**

Mid-log phase cells were grown for 3 hours in the presence of 50 µM BrdU and 50 µM deoxycytidine. Cells were fixed and permeabilized in methanol as described above followed by three 5 min washes in 1X PBS and 0.1% Triton X-100 extraction for 15 min. Cells were washed three times (5 minutes each) in 1X PBS and incubated in 2N HCl for 20 mins at room temperature followed by three washes with 1X PBS (5 minutes each) and blocked for 15 minutes using 1X PBS containing 1% BSA. BrdU incorporation was detected by incubating cells with anti-BrdU (1:50), clone PR-1 488 conjugated from Millipore for 60 minutes. Cells were then washed 3 times in 1X PBS + 0.1% Tween-20 (5 min each) and incubated for 60 minutes with the secondary antibody Alexa Fluor® 488 goat anti-mouse (1:50). Cells were then washed 3 times in 1X PBS + 0.1% Tween-20 followed by POLIC-PTP immunofluorescence as described above.

3.2.6 **In situ TdT labeling and quantification**

Cells were fixed in 4% PFA, permeabilized in methanol and labeled *in situ* with TdT as previously describe (9). TdT-labeled cells were quantified from three separate experiments (~900 total cells) and only intact cells as viewed by differential interference contrast (DIC) were included in the analysis. Early and late TdT-positive cells were classified as 1N1K_{div} cells, and TdT-negative cells were classified based on kDNA morphology identified by DAPI staining.

3.2.7 **Immunofluorescence (IF)**

Cells were harvested by centrifugation for 5 min at 1,000 x g, resuspended in 1X phosphate-buffered saline (PBS) and adhered to poly-L-lysine (1:10) coated slides for 5
mins. Cells were then fixed for 5 minutes using 4% paraformaldehyde (PFA) and washed three times (5 minutes each) in 1X PBS containing 0.1 M glycine (pH 7.4). Cells were permeabilized with 0.1% Triton X-100 for 5 minutes and washed in 1X PBS 3 times for 5 minutes. PTP-tagged proteins were detected by incubating with anti-protein A serum (Sigma, 1:3000) for 60 mins followed by Alexa Fluor® 594 goat anti-rabbit (1:250) for 60 minutes. POLIC-HA was detected by incubating cells with anti-HA 3F10 (Roche, 1:100) for 60 minutes followed by a 60 minutes incubation with Alexa Fluor® 594 goat anti-rat (1:100). Detection of basal bodies and DNA was done using the YL1/2 antibody and DAPI staining respectively as described in (9). Slides were then washed 3 times in 1X PBS prior to mounting in Vectashield (Vector Laboratories).

3.2.8 Image acquisition and analysis.

Images were acquired with a Nikon Eclipse E600 microscope using a cooled CCD Spot-RT digital camera (Diagnostic Instruments) and a 100X Plan Fluor 1.30 (oil) objective. Brightness and contrast was adjusted using Adobe Photoshop CS4.

3.2.8.1 Measurements of inter bb (bb) distance

Cells were labeled with YL1/2 and anti-protein A for bb and POLID-PTP detection respectively. The distance between bb was measured in 122 cells from randomly selected fields using Image J software (http://imagej.nih.gov/ij/). These cells were classified based on their kDNA morphology and the presence or absence of POLIC-PTP foci.
3.2.8.2 Colocalization analysis

An overlay of individual images acquired with the FITC and TRITC channel was done using Image J software. The colocalization plugin (http://rsb.info.nih.gov/ij/plugins/collection-finder.html) was used to identify overlapping pixels. We evaluated 157 cells to determine the percentage of cell that exhibit colocalization of POLIC-HA and POLID-PTP foci.

3.2.9 Statistics

Analysis of standard error of the mean (SEM) was performed using GraphPad Prism version 5.00 for Mac OS X (GraphPad Software, San Diego California USA).

3.2.10 Western Blotting

Cells were harvested at 3,500 x g for 10 min (4°C) and pellets were washed once in PBS supplemented with protease inhibitor cocktail Set III (1:100) (CalBioChem). Cells were lysed in 4X SDS sample buffer (BioRad) containing 5% beta-mercaptoethanol and incubated at 90°C for 5 min. Proteins were separated by SDS-PAGE on a 8% acrylamide gel and transferred to a PVDF membrane overnight at 4°C at 90 mA in transfer buffer containing 0.1% methanol. Membranes were incubated in 1% Roche blocking reagent (60 minutes) followed by incubation with antibodies (60 minutes) diluted in 0.5% blocking reagent (60 minutes). PTP (ProteinC-TEV-ProteinA) tagged proteins were detected with 1:2000 Peroxidase-Anti-Peroxidase soluble complex (PAP) reagent (Sigma). POLIC-HA was detected with rat monoclonal anti-HA (1:1000, 3F10 clone from Roche) followed by secondary goat anti-rat (1:1000, Sigma). For subsequent detections, membranes were stripped for 15 minutes at 37 °C with 0.1 M glycine (pH
2.5), washed in TBS with 0.1 % Tween-20, blocked and re-probed with one the following primary/secondary antibody combinations: *C. fasciculata* specific anti-Hsp70 (1:10,000) (12) /chicken anti-rabbit (1:10,000, Roche), *T. brucei* anti-Pol β (1:1000) (47) /goat anti-rat (1:5000) and anti-TAO (*T. brucei* alternative oxidase; 1:100) (8) /goat anti-mouse (1:1000) and anti-tubulin (1:20,000, Sigma) /goat anti-mouse (1:1000). All secondary antibodies were HRP conjugated. Signal was detected with BM Chemiluminescence Western Blotting Substrate (POD) from Roche.

**3.2.11 Cycloheximide treatment**

TbIC-PTP cells and TbID-PTP/POLIC-HA co-expressing cells were incubated for 6 hours with 100µg/ml cycloheximide. Cells were harvested every two hours and processed for Western Blot analysis (9).

**3.2.12 Trypanosoma brucei 427 synchronization**

Synchronization of *Trypanosoma brucei* procyclic cells was performed as reported previously (9). Briefly, POLIC-PTP single expresser cells were cultured in SDM-79 containing 50 µg/ml G418 and 1 µg/ml puromycin. Cells were incubated in medium containing 0.2 mM hydroxyurea (HU) for 10 hours followed by HU release. Following HU washout, cells were fixed every two hours over a period of 10 hours and labeled with anti-Protein A and DAPI. Cells were quantified (200 cells per time point, n=2) at indicated time points and classified based on the presence or absence of POLIC discrete foci as described above. To determine TbPOLIC-PTP protein levels during synchronization, cells were harvested every two hours after HU washout, and whole cell extracts were analyzed by Western blot.
3.2.13 RNA isolation and quantitative PCR

TbIC-PTP/SLID uninduced and induced cells (0 and 2 days) were harvested at 4°C (3,500 rpm for 10 min) and pellets were washed with cytomix (51). Total RNA was extracted from $5 \times 10^7$ cells using the TRIsol reagent (Ambion) according to the manufacturer's protocol. 10 µg of RNA was treated with 10 units (30 minutes at 37°C) of RNase-free DNase I (BioRad) to remove any DNA contamination. Subsequently, RNA was clean using the RNA cleaned and concentrator kit (Zymo Research). The High Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Ambion) and the Multi-Scribe Reverse Transcriptase were used to convert 500 ng of total RNA to cDNA. RT-PCT was performed in a 10-µl reaction contained 1 µl cDNA template, 5 µl FastStart universal SYBR Green master (Rox) kit (Roche Diagnostics Corp., Indianapolis, IN), 300 nm forward and reverse primers each, and nuclease-free water. Primers used for this analysis are listed in Table 3.1. All data was normalized to GAPDH. The normalized values from induced samples were compared against uninduced controls for the relative expression levels of mRNA. Relative mRNA levels shown in Fig. 3.7 are represented as means of two experimental replicates and three separate RNAi induction experiments.
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</table>

**Table 3.1 – Primers used in this study**

List of primers used for epitope tagging, generating and confirming single allele knockout, and qPCR. Underline region within the primer sequence correspond to the position of the linker that is indicated in the last column.
3.3 Results

3.3.1 POLIC has a cell cycle dependent localization

Multiple DNA polymerases are involved in kDNA replication, but the mechanism by which these DNA polymerases are spatially and temporally coordinated during early and late kDNA replication remains largely unknown. Previously, we demonstrated that TbPOLID undergoes changes in localization that are coupled to the kDNA duplication cycle (9). We hypothesized that cell cycle-dependent dynamic localization of T. brucei mitochondrial DNA polymerases provides a mechanism for spatial and temporal regulation during kDNA replication stages. Using immunofluorescence microscopy we investigated the localization dynamics of TbPOLIC, one of three essential pol I-like mitochondrial DNA polymerases that was previously detected in the kinetoflagellar zone (KFZ) (26). Here, we examined in detail the localization of POLIC using an exclusive expresser cell line, TbIC-PTP, in which one TbPOLIC allele was deleted and the other allele was fused to the PTP tag sequence (44). TbIC-PTP clonal cells (P2C2, P2A1 and P1C2) were analyzed for POLIC-PTP tag expression (~200 kDa), proper chromosomal integrations, and kDNA morphology (data not shown). The data shown in our study correspond to clonal cell line P1C2. Using anti-Protein A sera we detected POLIC-PTP as discrete foci by immunofluorescence microscopy in a subpopulation of the cells (Fig.3.1). POLIC-PTP foci were always in close proximity with the kDNA disk and were mainly detected in cells that had initiated kDNA duplication (Fig. 3.1A). These cells were identified based on the size and shape of DAPI-stained networks (1N1K_{div} cells) (18, 48). POLIC-PTP was undetectable by IF in cells that segregated their networks (1N2K,
2N2K) and in those cells with a unit-sized kDNA disk. Additionally, weak POLIC-PTP signal was detected in 1N1K cells only after increasing image contrast (Fig. 3.1A arrowheads in higher contrast image and enlargement, i). In these cells, the signal is diffuse near the kDNA network rather than concentrated as distinct foci at the antipodal sites as seen in 1N1K_div cells (Fig. 3.1A enlargements i and ii). The diffuse staining accumulating near the kDNA network could be a region where POLIC begins to accumulate as it moves to the antipodal sites. However, this signal was barely detectable in 1N1K cells and difficult to characterize. POLIC-HA gave the same staining pattern detected in POLIC-PTP tagged cells. POLIC-HA foci were present in 1N1K_div cells and were only detected in 1N1K cells after increasing image contrast (Fig. 3.2A, arrowheads in higher contrast and enlargement i).

The T. brucei mitochondrial protease HslVU (31) could be responsible for regulating TbPOLIC protein levels. To determine if proteolytic degradation plays a role in POLIC localization, we monitored POLIC-PTP protein levels after inhibition of protein synthesis using cycloheximide (CHX). Cells were harvested every 2 hours to monitor protein levels during a 6 hr time course (Fig. 3.1B and 3.2B). POLIC protein levels remained unchanged during CHX treatment, as do those of Hsp70, Pol β and TbPOLID, which are not regulated by TbHslVU (Fig. 3.1B and 3.2B) (9). These data demonstrate that TbPOLIC is a stable protein that is not regulated by proteolysis (Fig. 3.1B and 3.2B).

We hypothesize that POLIC accumulates as discrete foci in a cell cycle dependent manner. To test this hypothesis we incubated TbIC-PTP cells for 10 hours with 0.2 mM hydroxyurea (HU) for synchronization. To determine POLIC-PTP protein levels, cells
were harvested at 2-hour intervals after HU released for a period of 10 hours and whole cell extracts were analyzed by Western Blot (Fig. 3.3B). Consistent with our CHX data, no significant changes in POLIC-PTP protein levels were detected upon HU release (time 0-10) (Fig. 3.3B, top panel). This data further supports that POLIC protein levels remain constant during the cell cycle. We next monitored individual cells for POLIC-PTP foci at several time points after HU released (Fig. 3.3C, black). For immunofluorescent microscopy we stained cells with DAPI and anti-protein A and scored 200 cells per time point from 2 separate experiments. We identified periodic changes in the population of POLIC-PTP foci positive cells at different time points post HU release (Fig. 3.3C). After HU release, more than 50% of the cells had already replicated and segregated their kDNA (1N2K and 2N2K cells) and 44% had a single unit kDNA (1N1K) as judged by DAPI staining (Fig. 3.3A). At this point, cells with POLIC-PTP foci represent 13% of the total population and are always associated with 1N1K cells (Fig. 3.3A). Following 4 and 6 hours of HU release, 1N1K cells represented 62% and 84% of the total population respectively (Fig. 3.3A). During these time points, cells that had completed kDNA segregation (1N2K, 2N2K) decreased to 36% and 15% respectively and the number of POLIC-PTP foci positive cells remained at about 35%. The number of cells with POLIC-PTP foci decreased to 15% after 10 hours of HU release (Fig. 3.3C). At this time 1N1K cells represented 77% of the population. Together, these data demonstrate that detection of POLIC-PTP foci is limited to a pool of 1N1K cells, suggesting that this protein has a cell cycle dependent localization.
Figure 3.1 – Localization of POLIC-PTP foci in a subset of the population

(A) Localization of POLIC-PTP in an unsynchronized population of cells. POLIC-PTP was detected using anti-protein A (red), and DNA was stained with DAPI (blue). Brightness and contrast of the POLIC-PTP image was adjusted in Adobe Photoshop to generate the higher contrast panel. Arrowheads indicate cells with undetectable POLIC prior adjustment. Rows i and ii are enlargements of the area indicated by the white square in the merge field. Scale bars, 10 μm in standard images and 2 μm in enlarged images. (B) Western blot detection of POLIC-PTP, Pol β, and Hsp70 protein levels following CHX treatment. Cells were harvested every 2 hours, and 5x10⁶ cells were loaded into each well.
Figure 3.2 – Localization of POLIC-HA in unsynchronized population

(A) Detection of kDNA and POLIC-HA using DAPI (blue) and anti-HA (red), respectively. Brightness and contrast of the POLIC-HA image was adjusted in photoshop to generate the higher contrast panel. Arrowheads indicate those cells with undetectable POLIC prior to adjustment. Rows i and ii are enlargements of the area indicated by the white square in the merge field. Scale bar sizes are 10 μm in the larger panels and 2 μm in the enlarged image (B) CHX experiment using a cell line that co-expressed POLID-PTP and POLIC-HA. Following CHX addition, cells were harvested every 2 hours and whole cell extracts were analyzed by Western blot analysis. Membrane was detected with anti-HA, PAP and anti-Hsp70 from 5x10⁶ total cells.
Figure 3.3 – Hydroxyurea synchronization

(A) Karyotypic analysis before (pre) and after HU release. Cells fixed every 2 hours after HU release. (B) Western blot detection of POLIC-PTP after hydroxyurea (HU) release (top panel). Membrane was stripped and re-probed with anti-Hsp70 (bottom panel). (C) The percentage of POLIC-PTP foci positive cells (red circles) and those with undetectable POLIC-PTP foci (black circles) after HU release.
3.3.2 Accumulation of TbPOLIC foci correlates with stage II of the kDNA duplication cycle

Bb duplication is one of the first cytological events that indicates progression through the cell cycle and occurs almost in synchrony with initiation of kDNA S phase (18). Separation and movement of the bbs are critical processes in the cell cycle since these are responsible for the segregation of the kDNA. Gluenz and colleagues used IF and transmission electron micrograph to monitored kDNA morphology and bb dynamics and define five stages (I to V) of the kDNA duplication cycle. During stage I and V, the kinetoplast networks are morphologically the same and contain a single bb/probb pair. During stages II to IV the replicated kDNA networks move apart but are still linked by a maxicircle thread or Nabelschnur that is finally resolved during the transition from stage IV to V. A critical point in the kDNA replication cycle occurs when the bbs move ~2 μm apart (42). An acceleration of bb separation occurs facilitating the completion of kDNA network segregation. We recently defined TbPOLID localization during the cell cycle using bb dynamics coupled to kDNA morphology changes (9).

To precisely determine the time within the trypanosome cell cycle when TbPOLIC accumulate as foci, we used bb duplication as a marker for cell cycle stages. Log phase cells from an asynchronous population were DAPI stained and bbs were labeled with YL1/2 antibody, which detects tyrosinated alpha tubulin, and anti-Protein A for POLIC-PTP detection (Fig. 3.4A, B and C). Cells with a unit-sized kinetoplast (1N1K) have 1 bb/pro-bb pair and TbPOLIC is almost below the level of detection (Fig. 3.4B top panel, column i). Weak POLIC-PTP signal near the kDNA disk is observed after increasing the image contrast (Fig. 3.4B and C bottom panel, i column). Different
events occur within stage II (IIa and IIb); during stage IIa, cells contain a single kinetoplast (1N1K\textsubscript{div}) associated with two close-positioned bb (Fig. 3.4A bottom panel, ii). At this stage, we detect a single POLIC-PTP focus (or two foci that cannot be resolved) located mainly between the kDNA disk and bb or kinetoflagellar zone (KFZ) (Fig. 3.4B and C, column ii). Cells in stage IIb have a domed-shaped kDNA (1N1K\textsubscript{div}), two bb/pro-bb pair (Fig. 3.4A, iii and iv) and two POLIC-PTP foci situated at opposite sites of the kDNA disk (Fig. 3.4B and C, iii, iv). During this stage we also detect some POLIC-PTP signal between foci suggesting that the protein is moving from the center to opposite sites of the disk (Fig. 3.4B, iii, iv). At stage III, the kDNA consists of two joined disks characterized by its bilobe shape (Fig. 3.4A, v), two pairs of bb/pro-bb and an almost undetectable POLIC-PTP (Fig. 3.4A, B and C, v). Low levels are detected in the KFZ when increasing image contrast (Fig. 3.4B and C, v bottom panel) but discrete POLIC-PTP foci were rarely detected. During stages IV and V, characterized by the presence of two kinetoplasts, each associated with one of the two bbs that have separated farther apart from each other, (Fig. 3.4A, vi and vii bottom panel) POLIC-PTP was not detected (Fig. 3.4B and C, vi,vii). These data demonstrate that POLIC-PTP signal accumulates near the kDNA disk during stage II of the kDNA replication cycle.

To quantitatively analyze the specific stages of the kDNA replication cycle when POLIC-PTP foci are detected, we measured the inter-bb distance in individual cells. The distance between basal bodies was measured in 122 randomly selected cells that were later grouped based on their karyotype and presence or absence of POLIC-PTP foci (Fig. 3.4D, red and blue respectively). Cells with strong discrete POLIC-PTP foci had a minimum inter-bb distance of 0.66 \( \mu \text{m} \) and a maximum distance of 1.75 \( \mu \text{m} \) (Fig. 3.4D).
The mean bb distance of POLIC-PTP foci-positive 1N1K<sub>div</sub> cells was 1.1 μm (1.05 ± 0.02; n=64) (Fig. 3.4D, red) and 1.3 μm (1.33 ± 0.10; n=25) for cells with undetectable foci (Fig. 3.4D, 1N1K<sub>div</sub>, blue). Accumulation of POLIC-PTP foci occurs in stage II of the kDNA duplication cycle. Defined and well-organized POLIC-PTP foci were never detected at and post 2 μm of inter-bb distance or in cells with a single bb. Together these data indicate that POLIC-PTP has a cell cycle dependent distribution and is linked to stage II of the kDNA duplication cycle.
Figure 3.4 – Localization of POLIC-PTP at different stages of the cell cycle stages

(A) Representative cells labeled with DAPI (blue) and YL1/2 (green). Rows from i-vii represent cells from different stages of the kDNA duplication cycle. Scale bar, 5 μm. (B) Cells labeled with anti-protein A for the detection of POLIC-PTP (red). Higher contrast images are shown in the bottom row. (C) Corresponding merge panels from DAPI, YL1/2 and anti-protein A. Enlargements are shown in the bottom row. (D) Bb distances measured in individual cells (n=122 cells) containing two bb/pro-bb pairs. POLIC-PTP foci (red) or undetectable POLIC-PTP (blue). Error bars represent the SEM.
3.3.3 POLIC foci colocalize with newly synthesized DNA at the antipodal sites

Free minicircles undergo theta structure replication in the KFZ and their progeny migrate to the antipodal sites for Okazaki fragment processing. Here, minicircle progeny containing at least one gap will accumulate during kDNA replication. Gapped minicircles at the antipodal sites can be detected by terminal deoxynucleotidyl transferase (TdT) in situ labeling; a method that provides a spatial marker for the antipodal sites. To further define the localization of POLIC-PTP foci in respect to the kDNA disk, we fluorescently labeled gapped/replicating minicircles using TdT and fluorescein conjugated dUTP. All different TdT labeling patterns were observed as indicated by Early, Late, and Post TdT labeled cell (Fig. 3.5A). kDNA networks that have not initiated replication are TdT negative (1N1K) and POLIC-PTP foci were under the level of detection (Fig. 3.5A, 1N1K). During early stages of kDNA replication the antipodal sites are enriched with multiply gapped minicircle replication products resulting in a strong TdT signal at the network poles (Fig. 3.5A, 1N1K/div, Early TdT). At this stage, POLIC-PTP foci were detected in a subpopulation of Early TdT-positive cells and colocalized with antipodal TdT signal (Fig. 3.5A, 1N1K/div, merge, enlarged inset). During later stages of kDNA replication (1N1K/div), the gapped minicircle progeny are reattached to the network and POLIC-PTP is no longer detected (Fig. 3.5A, 1N1K/div, Late TdT). A subset of these cells showed diffuse POLIC-PTP labeling in the kDNA disk after adjusting images for a higher contrast (data not shown). Strong signal corresponding to POLIC-PTP foci was never detected upon network segregation (Fig. 3.5A 1N2K post, 1N2K and 2N2K TdT-negative).
Next, we determined the percentage of TdT-positive cells that exhibited POLIC-PTP foci and examined ≥ 300 cells from three separate TdT labeling experiments. Individual cells were classified by the presence (red bar) or absence of POLIC-PTP foci (blue bars) and their karyotype (1N1K, 1N1K<sub>div</sub> 1N2K and 2N2K). Cells with a unit-sized kDNA (1N1K), no TdT signal and no obvious POLIC-PTP foci (Fig. 3.5B, 1N1K, blue bar) represented 34% of the total population (34.0 ± 3.05, N=3). TdT-positive cells with a single kinetoplast represented 44% of the total population (Fig. 3.5B, 1N1K<sub>div</sub>, red and blue bar). POLIC-PTP foci were detected in a subpopulation of 1N1K<sub>div</sub> TdT-positive cells (Fig. 3.5B, 1N1K<sub>div</sub>, red bar) and represented 26% (25.6 ± 2.33, N=3) of the total population. TdT-positive cells with no detectable POLIC-PTP foci (Fig. 3.5B, 1N1K<sub>div</sub>, blue bar) represented 18% (18.3 ± 2.33, N=3) of the total population. POLIC-PTP foci were never detected in 1N2K (11.6 ± 0.33, N=3) or 2N2K (7.0 ± 1.52, N=3) cells (Fig. 3.5B). Together these data demonstrate that POLIC-PTP colocalize with gapped/replicating minicircles at the antipodal sites specifically during early TdT labeling.

To confirm that POLIC-PTP foci are present at the antipodal sites during active kDNA replication we incubated cells with BrdU, a thymidine analogue that is incorporated into newly synthesized DNA. We used immunofluorescent microscopy to visualize the newly synthesized DNA. Only a subset of 1N1K cells are BrdU positive (Fig. 3.5C, i-iv, green) while 1N2K and 2N2K are always BrdU negative, as previously described (18). During early stages of kDNA S phase, newly synthesized DNA is detected at the two antipodal sites of the kDNA network (Fig. 3.5C, i and ii). In these cells, a fraction of POLIC-PTP foci (red) colocalize with newly replicated DNA at the
antipodal sites (Fig. 3.5C, i and ii). During later stages of kDNA replication, the newly replicated molecules are distributed through the kDNA network and the nuclear DNA becomes BrdU positive as it initiates S phase (Fig. 3.5C, iii and iv). Only subsets of these cells are positive for POLIC-foci and we differentiated them by monitoring the kDNA morphology. Cells with dome-shaped kDNA that had incorporated BrdU into kDNA and nuclear DNA, were positive for POLIC-PTP foci (Fig. 3.5C, iii). Cells positive for BrdU in the nucleus and the kDNA, had no detectable POLIC-PTP foci when the kinetoplast consisted of two joined disks (forming a bilobed shape) (Fig. 3.5C, iv). Cells that were BrdU positive only for the nucleus had completed kDNA replication and did not exhibit discrete POLIC-PTP foci (Fig. 3.5C, v). These data further support the hypothesis that POLIC-PTP has a cell cycle dependent distribution that is linked to early stages of kDNA S phase. Together our data demonstrates that the localization of POLIC-PTP foci is specific to the sites of newly synthesized DNA.
Figure 3.5 – Localization of POLIC-PTP in respect to TdT and BrdU labeling

(A) Localization of POLIC-PTP (red) after TdT *in situ* labeling (green). Representative images for TdT labeling patterns are shown (TdT -, Early, Late and Post). Enlargements in the merged row correspond to those cells that displayed POLIC-PTP and TdT colocalization (yellow) at the antipodal sites. Scale bar, 5 μm. (B) Distribution of POLIC-PTP foci in a population of TdT-labeled cells. Individual cells were classified based on kDNA morphology and the absence (blue) or presence (red) of POLIC-PTP foci. Labels on the top of the graph indicate the TdT labeling status for each karyotype (positive (+), negative (-), or both (+/-)). Others (grey bar) included cells with abnormal karyotypes including multinucleated cells and zoids. Error bars correspond to the SEM from three separate experiments. More that 300 cells were analyzed in each experiment. (C) POLIC-PTP localization (red) following metabolic labeling of newly synthesized DNA. Representative images for BrdU labeling patterns are shown (green). Enlargements in the merged column correspond to cells that displayed POLIC-PTP and BrdU colocalization (yellow) at the antipodal sites. Scale bar, 5 μm.
3.3.4 POLIC and POLID foci colocalize at the antipodal sites during kDNA S phase

The antipodal sites are two protein rich regions that are located at opposite sites of the kDNA disk (15). They contain a number of enzymes with different activities and functions. It has been suggested that the antipodal sites are organized into functionally distinct subdomains populated by various enzymes with different activities (19). It was previously suggested that Topo II and Ligase kβ could occupy different antipodal site subdomains since they did not precisely colocalize (10). However, little is known regarding the spatial and temporal coordination of proteins during kDNA replication stages.

Previously, we reported that POLID-PTP localization to the antipodal sites was cell cycle dependent (9). TbPOLID localizes to the antipodal sites only during kDNA S phase. Here, we demonstrate that a second mitochondrial DNA polymerase, TbPolic, localizes as strong discrete foci at the antipodal sites during early stages of kDNA replication. To determine the spatial pattern of TbPOLIC and TbPOLID foci during kDNA replication, we generated a cell line co-expressing POLIC-HA and POLID-PTP and monitored their localization by immunofluorescence. We determined the kDNA replication status (early and late kDNA S phase) based on the morphology of DAPI-stained networks. As previously reported, POLID-PTP was detected throughout the mitochondrial matrix as well as discrete foci at the antipodal sites (Fig. 3.6, green).

Accumulation of POLID-PTP (green) foci is detected very early in kDNA replication as determined by kDNA morphology (Fig. 3.6, POLID-PTP, arrow). In these cells, POLIC-HA foci (red) are undetectable (Fig. 3.6 POLIC-HA). As the kDNA
assumes a more domed-shaped structure (1N1K_{div} cells) POLID-PTP (green) and POLIC-HA (red) foci are detected and seem to precisely colocalize with each other (Fig. 3.6, merge and enlargement i). In later stages of kDNA replication, when the kDNA assumes a bilobe shape (Fig. 3.6, ii top panel), POLIC-HA and POLID-PTP partially colocalized (Fig. 3.6, ii bottom panel). At this stage, the signal corresponding to POLID-PTP foci was less organized and began to diffuse throughout the mitochondrial matrix. Cells that were in later stages of kDNA replication had no detectable POLIC but a fraction of POLID remained diffuse around the kDNA network (Fig. 3.6, POLID-PTP, arrowhead). In agreement with our previous data, these data demonstrated that both proteins localize to the antipodal sites during kDNA replication.

We analyzed 12 randomly selected fields and approximately 150 individual cells to determine the percentage of cells that exhibited colocalization (precise and partial) of POLIC-HA and POLID-PTP. Using the colocalization finder plugin from Image J we determine that these proteins colocalized in a subset of cells (17% of the cells analyzed). Together, these data demonstrate that two of the pol-I like DNA polymerases, TbPOLIC and TbPOLID, appear to coincide in time and space in a fraction of cells during early stages of kDNA replication.
Figure 3.6 – Colocalization of POLIC-HA and POLID-PTP

POLID-PTP (green) and POLIC-HA (red) were detected in a coexpressing cell line. Multiple karyotypes are indicated in DAPI stained cells (blue). Arrow and arrowhead in POLID-PTP field indicate those 1N1K_{div} cells that were positive for POLID-PTP foci and negative for POLIC-PTP detection. Columns i and ii are enlargements of the area indicated by the white square in the merge field. Scale bar, 10 µm.
3.3.5 Depletion of TbPOLID causes a reduction in POLIC-PTP foci positive cells

To explore if accumulation of POLIC foci to the antipodal sites during kDNA replication is affected by loss of POLID, we generated a single expresser POLIC-PTP tagged cell line and transfected it with a POLID stem-loop RNAi vector (TbIC-PTP/SLID clone P1F3). POLIC single allele knockout was confirmed by PCR and sequencing analysis (data not shown). To knockdown the expression of TbPOLID, we induced formation of TbPOLID-specific intramolecular stem-loop dsRNA by adding tetracycline to the cells. Loss of TbPOLID caused growth inhibition starting at day 4 (uninduced; $7.6 \pm 0.2$, N=4 and induced; $7.5 \pm 0.2$, N=4) and persisted through the course of an 8 days induction, in agreement with previous report on TbPOLID silencing (Fig. 3.7A) (7). At day two of the induction, the relative amount of TbPOLID mRNA decreased by 55%. No significant reduction in the mRNA levels for the two other essential mitochondrial pols (TbPOLIB and TbPOLIC) was detected by quantitative PCR analysis (Fig. 3.7B). To assess the effect of TbPOLID knockdown on kDNA networks we monitored progressive loss of kDNA at day 4, 6 and 8 of induction (Fig. 3.7C). We quantified 200 individual cells (uninduced and induced) from three separate RNAi inductions per time point for normal, small and loss of kDNA (Fig. 3.7C). DAPI-stained networks that exhibited normal-sized networks represented 98% in an uninduced population. At day 4 of the induction, the percentage of cells with normal-sized networks decreased to 31% as the percentage of cells with small kDNA increased to 62%. Only 7% of the cells contained normal-sized kDNA following 8 days of TbPOLID RNAi (Fig. 3.7C). At this time, there was a dramatic increase in cells with no detectable kDNA (72%) as the percentage of cells with small kDNA declined to 20%. Kinetics of kDNA
loss in TbIC-PTP/SLID cells was comparable to those previously reported (Fig. 3.7C) (7).

We evaluated the effect of *TbPOLID* RNAi on the accumulation of gapped/replicating minicircles at the antipodal sites using TdT labeling. Additionally, POLIC-PTP foci positive cells were monitored during *TbPOLID* RNAi. Uninduced and induced (day 4 and 8) cells were fixed and labeled with DAPI, anti-protein A and TdT. In an uninduced population, 30% of the cells are TdT-positive and have the same labeling patterns as described in Fig. 3.5 (Fig. 3.8A and B). In these cells POLIC-PTP foci colocalize with gapped/replicating minicircles at the antipodal sites (Fig. 3.8B, day 0). After 4 days of *TbPOLID* RNAi the number of TdT-positive cells decreased to 9% (Fig. 3.8A). Additionally, the population of cells with POLIC-PTP foci decreases to 12% after *TbPOLID* silencing (Fig. 3.8C). On day 4 of the induction, POLIC-PTP foci are present only in TdT-positive cells (Fig. 3.8B, day B). Nearly all cells are negative for TdT labeling and POLIC-foci on day 8 of the induction (Fig. 3.8A and B). These data suggest that accumulation and assembly of POLIC-PTP foci to the site of replication is dependent on POLID expression and kDNA replication.
Figure 3.7 – Effect of TbPOLID RNAi

(A) TbIC-PTP/SLID clonal cell line P1F3 was grown in the absence (open circles) or presence (filled squares) of tetracycline (1 μg/ml) to express the TbPOLID stem-loop dsRNA. Cell density was plotted as function of cumulative doublings. Values represent the mean of four independent RNAi induction experiments. (B) qRT-PCR analysis for the relative amounts of TbPOLIB, TbPOLIC and TbPOLID mRNA levels following two days (D2) of TbPOLID RNAi. Uninduced (day 0) GAPDH was used as our normalizer. Normalized values from induced samples were compared against uninduced controls for the relative expression levels of mRNA. Values represent the mean from three separate experiments. (C) Quantitation of kinetics of kDNA loss by microscopy. More than 200 cells per timepoint were scored for normal sized kDNA (open circles), small kDNA (filled squares) or no kDNA (open squares). Others (filled triangles) represent cells with abnormal karyotypes. Values represent the mean from three independent experiments. Error bars represent the SEM.
Figure 3.8 – Effect of TbPOLID RNAi induction on POLIC-PTP localization

(A) Quantification of TdT-positive cells after 4 and 8 days of TbPOLID silencing (200 cells per time point). (B) Detection of POLIC-PTP (red) and gapped/replicating minicircles after TdT labeling (green) during TbPOLID silencing. DAPI-stained DNA is shown in blue. Representative images are shown. Scale bar, 10 μm. (C) Quantification of TdT-positive (dark grey) and TdT-negative (lighter grey) in uninduced and POLID RNAi-induced cells (Day 4, 6 and 8). Values represent the mean of three separate experiments (200 cells per time point). Error bars represent the SEM.
3.3.6 POLID knockdown alters POLIC protein levels

We next asked if POLIC-PTP protein levels were affected by perturbation of *TbPOLID*. The protein levels of POLIC as well as other mitochondrial protein (alternative oxidase (TAO) and mtHsp70) were monitored at day 4, 6 and 8 of *TbPOLID RNAi* (Fig. 3.9A). Membranes corresponding to three separate experiments were probed with PAP, anti-TAO, anti-mtHsp70 and anti-tubulin (Fig. 3.9A). Detections from one representative experiment are shown on figure 3.9A. The intensities of each band were quantified using Image J and were normalized with the corresponding β tubulin control (Fig. 3.9B). POLIC-PTP protein levels decrease by 22% after 4 days of *TbPOLID* silencing while TAO and Hsp70 show a slight decrease of 7% and 12%, respectively. After 8 days of *TbPOLID* RNAi, mitochondrial proteins (TAO, Hsp70 and POLIC) are differentially affected and only POLIC-PTP protein levels seem to decrease (35% decrease) (Fig. 3.9A and B). At this point we detect a 20% increase in TAO and Hsp70 protein levels. We did not detect proteolytic processing POLIC at different time points of the induction (Fig. 3.9A). Here we demonstrate that POLIC-PTP protein levels are affected following *TbPOLID* silencing.
Figure 3.9 – POLIC-PTP protein levels following TbPOLID silencing

(A) Western blot detection of POLIC-PTP, alternative oxidase (TAO) and Hsp70, during TbPOLID RNAi. Cells were harvested 4, 6, and 8 days post-induction, and $5 \times 10^6$ cells were loaded into each lane. The membrane was probed with antibodies against each individual protein.

(B) Quantification of the relative protein levels during TbPOLID RNAi. Values were normalized against tubulin. Values represent the mean of three independent induction experiments. Error bars represent the SEM.
3.4 Discussion

There has been significant progress in understanding the basic mechanisms involved in the replication of minicircles and maxicircles. Nearly 30 proteins involved in the kDNA replication process have been characterized at the single protein level and implicated in specific steps of replication (24). However, a good understanding of how subsets of these proteins might interact or how they are spatially and temporally regulated during the various phases of kDNA replication remains largely unexplored. Recently, we characterized the cell cycle-dependent dynamic localization of the essential mitochondrial DNA polymerase POLID. This protein redistributes from the mitochondrial matrix to the antipodal sites where it is spatially and temporally available to perform its essential role in kDNA replication. Given the increasing number of proteins that are reported to localize to the antipodal sites, we hypothesized that dynamic localization might be a mechanism in which to coordinate the numerous proteins with the known temporal steps in kDNA replication. In this study we provide evidence that dynamic spatiotemporal localization provides tight cell cycle control for mitochondrial DNA polymerase IC. These data and our previous report (9) suggest that dynamic localization may be a common mechanism among a subset of kDNA replication proteins that regulates their participation in the different stages of kDNA replication.

Our localization studies of a second essential kDNA replication protein, TbPOLIC, indicate that this protein transiently accumulates at the antipodal sites in a subset of 1N1K cells that are actively replicating their kDNA. Similar to previously published data on POLID dynamic localization, POLIC-PTP foci were detected in 25%
of the cells in an unsynchronized population that corresponded to a subset of the 1N1Kdiv population that are considered early in the kDNA replication phase (TdT +, early). However in contrast to POLID-PTP, the POLIC-PTP signal was not detected by IF in the remainder of the population (Fig. 3.1A, 3.4B and 3.5A). Similar observations were reported for *T. brucei* mitochondrial DNA Pol β and *C. fasciculata* SSE1 and Pol β, and steady state protein levels did not change indicating that protein abundance was not the reason for the undetected IF signal (14, 43).

As proposed for Pol β localization, the periodic detection of POLIC-PTP by IF may be due to epitopes that are partially exposed in response to conformational changes induced by proceeding through the cell cycle. Alternatively, POLIC-PTP may be at an undetectable level when not concentrated at the antipodal sites and is only detected when local concentrations of the protein increase near the kDNA disk. In support of the latter possibility, we detected a low level POLIC-PTP signal in a small fraction of 1N1K cells when increasing image contrast (Fig. 3.1, arrowheads). This signal was not antipodal, but instead appeared to localize between the kDNA disk and the flagellar bb (possibly KFZ) or was concentrated in a single elongated zone, possibly as two foci that were barely resolvable (Fig. 3.4C i and ii merged). This low level signal pattern was present just prior to initiation of kDNA replication (single bb) or at very early stages (two closely spaced bb), and resembled the previously reported TbPOLIC localization (26). These observations were consistent between cells lines expressing TbPOLIC fused to a PTP of HA tag indicating that the localization patterns were not due to the tag. (Fig. 3.1A and 3.2A).
Using basal body duplication events and TdT labeled-minicircles, we determined that TbPOLIC localized as foci to the antipodal sites during stage II of the kDNA replication cycle (Fig. 3.4 and 3.5). During stages I and III, TbPOLIC was detected as a rather diffuse signal to the kDNA that it was visible only in higher contrast images (Fig. 3.4). Moreover, TbPOLIC foci were detected in cells with BrdU-positive kDNA (at the network poles). Consistent with TbPOLIC role in kDNA replication, a fraction of these foci colocalized with the sites of DNA synthesis (Fig. 3.5C). One could speculate that low levels of TbPOLIC are required at the KFZ during early stages of kDNA replication. As minicircles accumulate to the antipodal sites it is possible that all TbPOLIC molecules gradually accumulate as foci to the antipodal sites. Localization of TbPOLIC to both regions (KFZ and antipodal sites) in a cell cycle dependent manner could indicate that TbPOLIC have multiple roles in kDNA replication.

The antipodal sites contain a majority of the proteins that are required for kDNA replication transactions. With the demonstration of TbPOLIC at the antipodal sites, the number of DNA polymerases that localized to this region adds to three, two of them known to be essential proteins (TbPOLIC and TbPOLD). We generated a co-expressing cell line and demonstrated that POLIC-HA and POLID-PTP precisely colocalized during early stages of kDNA replication (Fig. 3.6i). However, at later stages of kDNA replication (domed shape kDNA disk - Fig. 3.6ii), POLIC-HA and POLID-PTP exhibited only partial colocalization. POLIC-HA was below the level of detection when the cells were not undergoing kDNA replication and was only visible in 1N1K cells after increasing image contrast as shown in figure 3.1A. In both stages, TbPOLID was clearly detected as a diffuse signal that localized to the kDNA disk region (Fig 3.6, arrow and
arrowhead). The stoichiometry of TbPOLIC and TbPOLID at the site of replication is not known. TbPOLID could be more abundant than TbPOLIC, thus accumulation of TbPOLID to the site of replication can be visualized even at the very early stages of kDNA replication. We propose that both proteins arrive and exit the antipodal sites at about the same time but there is a difference in the concentration of TbPOLIC and TbPOLID molecules recruited.

To evaluate if perturbing TbPOLID has an impact on TbPOLIC localization to the antipodal sites we silenced TbPOLID via RNAi. The localization of POLIC-PTP was evaluated in a single expresser cell line that expressed the stem-loop vector for TbPOLID RNAi silencing. Previously, we demonstrated that TbPOLID silencing resulted in growth inhibition, kDNA loss and a parallel decline in covalently closed (unreplicated) and nicked/gapped (replicated) minicircles (7). TbPOLID silencing in this new cell line resulted in growth inhibition after 4 days and kDNA loss consistent with TbPOLID RNAi phenotype (Fig. 3.7A and C). Moreover, in this study using TdT in situ labeling we demonstrated that TbPOLID silencing caused a rapid decline (Day 4) of gapped/replicating minicircles at the antipodal sites indicating that minicircle replication is impaired (Fig. 3.8A and B). POLIC-PTP localization to the antipodal sites was also affected during TbPOLID silencing (Fig. 3.8B). At day 4 of the induction, only cells in which replication was not yet inhibited (TdT positive) had POLIC-PTP foci (Fig. 3.8B). POLIC-PTP was never detected in cells with small kDNA and no kDNA. TbPOLIC localization to the antipodal sites seems to depend on TbPOLID. We do not discard that these proteins interact at a very defined stage of the cell cycle and that their interaction serve as a signal for localization. So far, we have not been able to identify TbPOLIC or
TbPOLID interacting partners through several approaches, which suggest that these interactions are transient, and cell cycle mediated.

In other eukaryotes, several processes such as mtDNA maintenance, transcription and translation are known to be under proteolytic control (28). For example, the Lon protease in *Drosophila* mitochondrial regulates transcription by degrading the mitochondrial transcription factor A (TFAM), which is essential for mitochondrial transcription and mitochondrial DNA packaging (36). RNAi-mediated silencing of Lon, resulted in increased mtDNA copy number and TFAM abundance. It has been proposed that Lon has also a role in mtDNA replication, however the levels of proteins involved in replication such as pol γ and mtSSB were not affected by Lon silencing (36). Lon protease has not been annotated in the *T. brucei* genome (4). However, a bacterial-like HslVU protease is known to control minicircle and maxicircle copy number by degrading the master regulators that participate in the replication process (31). Knockdown of HslVU caused an increase in TbPIF2 protein levels and an accumulation of maxicircles demonstrating that TbPIF2 helicase is a substrate of HslVU and controls maxicircle synthesis (33). A minicircle regulator has not yet been identified. HslVU does not appear to play a role in the transient accumulation of TbPOLIC foci as TbPOLIC protein levels do not change when protein synthesis is inhibited or during hydroxyurea synchronization (Fig 3.1B and 3.2B). Additionally, two other kDNA replication proteins with antipodal site localization (TbPOLID and Pol β) are not under proteolytic control (9, 25). Together these data imply that alternative mechanisms regulate cell cycle-dependent localization of kDNA replication proteins.
Thus far, the mechanism(s) that govern dynamic antipodal site localization have not been identified. The antipodal sites could represent structural features that act as a landing pad for proteins at the various stages of kDNA replication. Binding affinities and interactions with cytoskeletal elements or companion proteins could be regulated by posttranslational modifications like phosphorylation. However, phosphoproteome studies in *T. brucei* have not revealed that any of the mitochondrial DNA polymerases are phosphorylated (38). These studies were performed on unsynchronized populations in which only about 15-20% of the cells are at the kDNA synthesis stage, therefore the pool of phosphorylated replication proteins may be below the level of detection in those experiments. Additional posttranslational modifications (i.e., methylation, palmitoylation, acetylation), are also key elements for regulating protein function and localization. Protein arginine methylation has evolved as important regulatory factor for a number of cellular processes including RNA processing, transcription and subcellular localization (3). The *T. brucei* genome encodes five putative arginine methyltransferases (PRMT), one of which was identified in a mitochondrial fraction after subcellular fractionation studies (17). A proteomic analysis of arginine methylation identified over 850 arginine-methylated proteins, 200 of these were predicted to be localized to the *T. brucei* mitochondrion (J. Fisk and L. Read, personal communication). Interestingly, three methylated residues were identified within TbPOLIC. It is possible that TbPOLIC foci accumulate at the antipodal sites in response to an arginine-methylation event such as POLIC’s interaction with proteins that are involved on its recruitment to the antipodal sites. Alternatively, TbPOLIC’s enzymatic activity may be regulated by arginine methylation such that only methylated TbPOLIC participates in kDNA replication and
localizes to the antipodal sites. In mammalian systems, arginine methylation of DNA polymerase β enhances polymerase activity by increasing the binding affinity to DNA and also regulates binding to PCNA (12, 13). Further studies are necessary to determine the physiological significance of TbPOLIC methylation.

In conclusion, our findings strengthen the hypothesis that spatiotemporal localization of kDNA replication proteins is a mechanism that may be more used for regulating kDNA maintenance. Given the topological complexity of the catenated kDNA network and the predicted large number of proteins to maintain this structure, it is not surprising that trypanosomes have evolved multiple mechanisms for coordinating and regulating kDNA replication proteins.

### 3.5 Acknowledgements

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### 3.6 Bibliography


CHAPTER 4

4 TRYpanosoma BRUCEI MITOCHONDRIAL DNA REPLICATION COMPLEXES: TRANSIENT INTERACTIONS DURING KDNA REPLICATION

4.1 Introduction

Protein–protein interactions (PPI) are essential for a wide variety of biological processes such as gene expression, protein degradation, cell cycle control, and DNA replication. When proteins interact within the cell, they form dynamic molecular machines that are responsible for maintaining cellular homeostasis. DNA replication is a fundamental biological process that requires a multi-protein replication complex to form the replication machinery. DNA polymerases, which catalyze the polymerization of matched DNA bases into a DNA strand, are among the proteins at the core of this multi-protein complex. In addition to the DNA polymerase, other proteins that work in coordination to assist in the DNA replication process are helicas, primases, single stranded binding protein (SSE), and ligase. In eukaryotic cells, different replication complexes are assembled to replicate the mitochondrial and nuclear genomes. Each complex has its own specific collection of proteins, but they share similar activities. Additionally, there is some variability in complex composition between organisms. For example, the replication of mammalian mitochondrial DNA requires a single mitochondrial DNA polymerase, pol γ, for replication and repair processes. It has been demonstrated that a limited number of proteins, namely pol γ, the TWINKLE helicase, and ssDNA-binding protein are required to generate DNA products of about 16kb, which is the size of the mammalian mtDNA molecule. However, this is not the case for
replicating the mitochondrial genome of the parasitic protist *Trypanosoma brucei*. These organisms have at least six mitochondrial DNA polymerases for duplicating and repairing their mitochondrial DNA. Over time, researchers continued to identify additional enzymes, some of these with similar activities but non-redundant roles in kDNA replication. Not surprisingly, the structure and replication mechanism of the mitochondrial DNA of *T. brucei* differs significantly from the mammalian system. The distinctive mitochondrial genome of *T. brucei*, called kinetoplast DNA (kDNA), is organized in a catenated network of minicircles and maxicircles. The kDNA network is condensed into a disk-shaped structure within the mitochondrial matrix close to the flagellar basal body and surrounded by multiple proteins that play specific roles at different stages of the network replication. Information regarding the interactions of these proteins during the kDNA replication is limited. Uncovering protein-protein interactions during kDNA replication will help us understand how this process is coordinated.

Few binary interactions have been identified using immunoprecipitation (IP) analysis. Pol β and ligase kβ localize to the antipodal sites and are involved in the final stages of kDNA replication (9, 29). Using IP analysis Ray and colleagues demonstrated that these two proteins physically interact (34). Their data suggest that Pol β, together with ligase kβ, are responsible for repairing minicircle gaps. UMSBP, which has a role in the initiation of minicircle replication, was recently shown to interact with two kinetoplast-associated proteins (KAP3 and KAP4). This discovery resulted in the characterization of a new role for UMSBP in condensation and compaction of the kDNA (15). With the large number of proteins involved in replication it is thought that many unknown protein-protein interactions coordinate kDNA replication. However, no
mitochondrial DNA replication core complexes are well characterized in any kinetoplast protozoa or other eukaryotes. Isolation of mitochondrial DNA replication complexes from *T. brucei* will provide a better understanding of mitochondrial DNA replication.

Several methods have been developed for the purification of protein complexes. However, many researchers prefer methods which, (1) allows purification of a protein complex under nearly physiological conditions, (2) yields sufficient amount of protein for Mass Spectrometry (MS) analysis, (3) and results in a functional complex for activity assays. One such method for purifying protein complexes utilizes an affinity tag fused to the protein of interest that is expressed at, or close to its endogenous expression levels. This standard method is called tandem affinity purification (TAP) and it allows the purification of protein complexes from total cell lysate under mild conditions (26, 27). Protein complexes are isolated in two purification steps which utilize an affinity tag fused to the C or N terminal region of the protein of interest. The epitope-tagged protein is expressed at near endogenous levels, which decreases the potential of non-specific interacting partners due to over expression. The original TAP tag was developed in yeast and used to identify subunits of an RNA-protein complex (SnRNP) (27). The TAP tag consists of two IgG-binding domains of the *Staphylococcus aureus* protein A (ProtA) and a calmodulin-binding peptide (CBP) that interacts with calmodulin in the presence of calcium (27). A major disadvantage of ProtA is that elution of the tagged protein requires low pH and denaturing conditions. To overcome this problem a tobacco etch virus (TEV) protease recognition sequence was engineered between the ProtA and the CPB domains, allowing proteolytic release of the IgG-bound complex under native conditions. During the first affinity step, a total cell extract is applied to a column containing IgG-coupled
sepharose beads. The TAP-tagged protein and associated components remain bound to
the IgG matrix and are isolated from the cell extract. Following several washes to
remove contaminants, the target protein and interacting partners are released from the
IgG matrix using the TEV protease. Although TEV protease is a highly specific cystein
protease that recognizes a sequence of seven amino acids (ENLYFQ(G/S)), one should
ensure that there is no TEV protease recognition site within the protein of interest. After
TEV cleavage, the tagged protein retains only the CBP binding domain, which is utilized
in the second affinity step. The proteins in the TEV eluate are incubated in a column
containing a calmodulin affinity resin and calcium. The complex binds to the matrix via
the CPB binding motif that is present in the protein of interest. One advantage of this
second step is that the protein complex is released from the CBP matrix using mild
conditions and EDTA to chelate the calcium ions. In most cases the eluted protein
complex remains functionally active because of the low-stringency conditions used
throughout the purification. Following TAP, the composition of the protein complex is
determined by MS analysis. TAP in combination with MS has enabled rapid
identification of genome-wide interactions.

Complex networks of protein interactions in mammalian cells, bacteria, plants,
yeast, Drosophila and parasites have been identified using TAP (10, 24, 28, 33, 36, 39).
The first large-scale analysis of protein complexes using the classical TAP tag for TAP
was reported in yeast (11). In this study, researchers were able to group proteins with
multiple cellular functions (i.e, cell cycle regulation, RNA metabolism, protein synthesis,
signaling, and others) into 234 protein complexes (11). Additionally, they were able to
propose a cellular role for 231 proteins that had no previous functional annotation. This
study provided a genome-wide characterization of protein-protein interactions in yeast and also developed a tool that was transferred to a wide range of organisms.

However, the classical TAP tag is not optimal for isolating every protein complex and has been inefficient in some cases (10, 30). To overcome some of the problems associated with the TAP tag, several variants of the original TAP tag have been developed and optimized for various protein complexes and multiple organisms (19, 41). One of the most common modifications is the replacement of the CBP domain for a different affinity tag (e.g., a Streptavidin binding peptide, a Histidine tag, a Flag tag or a Protein C epitope) (19, 41). One of the main reasons to make this modification is that the free calmodulin in cell extracts can prevent binding of the tagged protein to the affinity matrix (10, 31). In many cases, the modified tag yields satisfactory results by improving the efficiency of the second affinity step (31). For example, Baserga and colleagues developed a biotinylation tag when purifying protein complexes from mammalian cells growing in monolayer cultures, which increased the yield of the fusion protein (10). In trypanosomes, initial attempts to TAP-purify the small nuclear RNA-activating protein complex (SNAPc) from crude trypanosome extracts were inefficient due to the calmodulin affinity purification step (31). In an effort to improve TAP for their applications, Gunzl and colleagues developed a new combination epitope tag by replacing the CBP domain with a protein C domain (ProtC) (31). The protein C is derived from human protein C and is specifically expressed in hepatocytes. The new tag, called the PTP tag (Protein C-TEV-Protein A) was proven to improve the efficiency of the second affinity step and allowed the isolation of low abundance protein complexes (Fig. 4.1). So far, the PTP tag has been successfully used to purify functionally different
complexes that localize to different cellular compartments (Table 4.1.). Additionally, it has been transferred to other systems such as *P. falciparum* for the purification of elongation factor subunits (Table 4.1.).

The purification of protein complexes using TAP offers several advantages over classical methods (i.e., glutathione S-transferase pulldown and immunoprecipitation; IP). First, the protein of interest is expressed at endogenous levels, which decreases the number of non-physiological interactions due to over expression. Second, the protein complex is isolated from native cells or tissues under mild conditions that are designed to preserve protein-protein interactions occurring in *vivo*. Third, the TAP procedure requires only two sequential steps of affinity purification, which makes it a simplified method for rapid isolation of protein complexes. Additionally, the two affinity steps increase the specificity of the purification since it decreases the complexity of the sample, leading to greater sample purity and reproducibility. In each affinity step non-specific proteins and contaminants are greatly reduced in comparison with single step affinity purification such as IP. The use of mild conditions (e.g., low detergent concentrations and salt) prevents the loss of interacting proteins and keep the protein complexes close to physiological conditions throughout the purification. Most importantly, the protein complex in most cases remains functionally active and can be used for in *vitro* assays. Additionally, the eluted protein complex can be analyzed by MS, which allows the study of protein complexes on a proteomic scale.

While TAP in combination with MS has proven to be essential for systematic identification of target-associated protein complexes, it has some limitations.
First of all, any effects of the epitope tag should always be determined as these can interfere with protein function and may prevent the protein from assembling into a complex. If the protein is essential, one approach to determine whether the tagged-protein is functional is to knock out the wild type allele and exclusively express the epitope-tagged allele in the cell. One should subsequently monitor the cells’ viability and confirm that the tagged protein localizes to the correct cellular compartment. During TAP, contaminants such as tubulin, IgG and keratin are not always removed and are usually detected in the MS analysis; this can mask the detection of low abundance proteins. In many cases, a lack of previous structural knowledge of the tagged protein makes the selection of an optimal tag for your protein of interest an empirical process since the tag can be masked by tertiary structure. Another technical problem to consider is that after cell lysis proteins from all cellular compartments are mixed together, which increases the possibility of non-specific interactions and proteolytic degradation. It has been shown that TAP is not always the best method for capturing transient and weak protein-protein interactions. Therefore, it sometimes needs to be coupled with in vivo cross-linking, which introduces additional limitations. One of the most significant limitations of TAP is that the isolated protein complex reflects the interactions happening at one specific time. Hence, dynamic interactions that are regulated during the cell cycle will not be identified.

Here, we systematically PTP-purify three essential mitochondrial DNA polymerases of *T. brucei* to explore interacting proteins within a putative replisome. MS analyses of final fractions of all PTP TAP purification experiments identified the tagged protein as a monomer. This data demonstrates that these interactions are highly transient and that PTP TAP is not effective for isolating kDNA replication complexes.
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**Table 4.1 – Complexes purified using the PTP tag**

PTP tag has been successfully used in *T. brucei*, *P. falciparum* and human cells for the purification of protein complexes with different biological functions and which are located in various cellular compartments.
Tandem affinity purification is an established tool for the purification of protein complexes using two affinity steps. (A) PTP tag developed for the purification of protein complexes in *Trypanosoma brucei*. The CBP binding domain from the TAP tag was replaced by a protein C domain (purple) which is fused to the C terminus of the protein of interest (Pol I-like, teal). The protein C domain is followed by the tobacco etch virus protease recognition sequence (dark grey rectangle). The protein A domains (pink) within the PTP tag will bind to IgG sepharose during the first affinity steps. Potential contaminants present in the cell extract are shown in burgundy and interacting partners are shown in orange. (B) TEV protease recognizes the TEV cleavage site and the immobilized protein complex is then released from IgG. (C) In the second affinity step, the protein complex will bind to the anti-protein C matrix via the protein C domain. (D) The target protein complex is eluted from the beads by chelating calcium ions using EDTA.
4.2 Materials and methods

4.2.1 Chromosomal tagging and single allele deletion

4.2.1.1 pPOLIB-PTP-NEO and pIBKO-Puro

The POLIB C-terminal coding sequence (2261 bp) (Fig. 2) was PCR-amplified from T. brucei 927 genomic DNA using forward primers (5'-TTG TGT GGG CCC GGC TAT CGA CAA GTC TCT CTC TC-3') and reverse (5'-- TGT TGT CGG CCG CAC CGT AAT TTC TAC ACT GTC-3') primers containing ApaI and EagI sites, respectively. The PCR-amplified fragment was ligated into ApaI and NotI restriction sites of pC-PTP-NEO to generate the pPOLIB-PTP-NEO vector. For pKOPOLIBPuro cloning a 518 bp TbPOLIB 5' UTR fragment was PCR amplified using forward (5'-- TAT AGA CTC GAG GTT GTT GTT TGC CCA CCG TTC G -3') and reverse (5'-- TAT AGA AAG CTT ATC ACT ATG CGG ACC ACC AG -3') primers containing XhoI and HindIII sites, respectively, and was ligated into pKOPuro. Subsequently, a 314 bp TbPOLIB 3' UTR fragment was PCR amplified using forward (5'-- TAT ATA ACT AGT GAC ATT CCC AGG TGT TAA GTT G -3') and reverse (5'-- TAT ATA TCT AGA CAC TTC TGC CCT CGC CC -3') primers containing SpeI and XbaI sites, respectively, and was ligated into the and XhoI and XbaI sites in the downstream polylinker portion of the pKOPuro vector to generate the pKOPOLIBPuro construct. After digestion with XhoI and XbaI, the 3047 bp fragment containing the puromycin resistance marker flanked by the POLIB UTRs was used for transfection into parasites.
4.2.1.2 pPOLIC-PTP-NEO and pICKO-Puro

The POLIC C-terminal coding sequence (2226 bp) was PCR-amplified from T. brucei 927 genomic DNA using forward (5’-TGT TGT GGG CCC GTT CGC TCT ACG CAG GAT ATC AGC-3’) and reverse (5’-TGT TGT CGG CCG CT GGA CA CTC CCC TAG TGA TG -3’) primers containing ApaI and EagI sites, respectively. The PCR amplified fragment was ligated into ApaI and NotI restriction sites of pC-PTP-NEO to generate the pPOLIC-PTP-NEO vector. For pKOPOLICPuro cloning a 430 bp TbPOLIC 5’ UTR fragment was PCR amplified using forward (5’-ATA ATA CTC GAG CAG GAG GAG ACG GCG GC-3’) and reverse (5’-ATA ATA AAG CTT CGG GCA ACT GAG CAG C-3’) primers containing XhoI and HindIII sites, respectively, and was ligated into pKOPuro. Subsequently, a 448 bp TbPOLIC 3’ UTR fragment was PCR amplified using forward (5’-TGT TGT ACT AGT CAG AGG CGT ATT GCT ATT G-3’) and reverse (5’-ATA ATA CTC GAG CAG GAG GAG ACG GCG GC-3’) primers containing SpeI and XbaI sites, respectively, and was ligated into the SpeI and XbaI sites in the downstream polylinker portion of the pKOPuro vector to generate the pKOPOLICPuro construct. After digestion with XhoI and XbaI, the 3097 bp fragment containing the puromycin resistance marker flanked by the POLIC UTRs was used for transfection into parasites.

4.2.1.3 pPOLID-PTP-NEO and pIDKO-Puro

POLID C-terminal coding sequence (1638 bp) was PCR amplified from T. brucei 927 genomic DNA using forward (5’-ATA ATA GGG CCC TGC TCG TCA AGA GGT GCG-3’) and reverse (5’-ATA ATA AAG CTT CGG GCA ACT GAG CAG C-3’) primers containing ApaI and EagI sites, respectively. The PCR amplified fragment
was ligated into ApaI and NotI restriction sites of pC-PTP-NEO to generate pPOLID-PTP-NEO vector. The POLID knockout construct pKOPOLIDPuro was generated by ligating the PCR-amplify TbPOLID 5’ UTR region (629 bp) that was amplified with forward (5’-CTC GAG CAG GGA AAG ATA GCG CCT-3’) and reverse (5’-ATC GAT AAA AAG AAG GAT GCG-3’) primers containing XhoI and ClaI sites respectively. Subsequently, the TbPOLID 3’ UTR fragment (483 bp) was PCR amplify using forward (5’-ACT AGT GTG TCC TAT AGC AGT AAC G-3’) and reverse (5’-GCG GCC GCA GCA ATT TTC CGC AC-3’) primers containing SpeI and NotI sites respectively, and ligated into SpeI and NotI sites in the downstream polylinker portion of the pKOPuro vector to generate the pKOPOLIDPuro construct. After digestion with XhoI and NotI, the 3359 bp fragment containing the puromycin resistance marker flanked by the POLID UTRs was used for transfection into parasites.

4.3 Trypanosomes’ growth and crude cell extract

Procyclic form Trypanosoma brucei was cultured in semi-defined media containing 15% heat-inactivated serum. For PTP purification, 3L of cells were grown in polycarbonate Erlenmeyer flasks with caps at room temperature with constant shaking to a density of ~1x10^7 cells/ml. Cell density was determined using a Coulter Counter (model Z2; Beckman Coulter). Cells were then harvested (3,500 rpm, 4°C for 15 min) using a Sorvall RC 6 centrifuge. The cell pellet was washed twice in 10 ml of ice-cold tryp buffer (20 mM Tris-HCl [pH 7.4], 100 mM NaCl, 3 mM MgCl₂, 1 mM EDTA). Subsequently, the cell pellet was re-suspended in ice-cold transcription buffer (150 mM sucrose, 20 mM potassium L-glutamate, 3 mM MgCl₂, 20 mM HEPES-KOH [pH 7.7], 2 mM dithiothreitol, leupeptin [10 µg/ml]) and incubated for 10 minutes on ice. Cells were
then lysed using a glass Dounce homogenizer with a type A pestle by applying rapid strokes continuously for about 10 minutes or until more than 70% of the cells were broken.

This cell suspension was aliquoted, shock-frozen in liquid nitrogen, and stored at −70°C. For a whole-cell extract preparation, a 900-μl aliquot was thawed, mixed with 100 μl of transcription buffer containing 1.5 M KCl, and incubated for 20 minutes on ice. Subsequently, the extract was spun at 21,000 × g for 10 min at 4°C. The supernatant was transferred to multiple 1.5 ml tubes and used as an input in the first affinity step of PTP purification.

### 4.4 PTP affinity purification

For IgG affinity chromatography, the extract was transferred to a 0.8- by 4-cm Poly-Prep chromatography column (Bio-Rad), where 200 μl settled bead volume of IgG Sepharose 6 Fast Flow beads (Amersham, Piscataway, NJ) had been equilibrated with PA-150 buffer (150 mM potassium chloride, 20 mM Tris-HCl [pH 7.7], 3 mM MgCl2, 0.5 mM dithiothreitol, 0.25% NP-40). PTP-tagged proteins were bound to IgG Sepharose by rotation for 2 hours at 4°C in the presence of protease inhibitors. Following flowthrough collection, beads were washed with 35 ml of PA-150 buffer and equilibrated with 15 ml of TEV protease buffer (PA-150 with 0.5 mM EDTA). Tagged proteins were eluted by re-suspending the beads in 2 ml of TEV protease buffer containing 300 units of AcTEV protease (Invitrogen, Carlsbad, CA) and rotating the closed column overnight at 4°C. The TEV protease eluate was diluted to 6.5 ml by a wash of the IgG Sepharose beads with PC-150 buffer (PA-150 buffer containing 1 mM calcium chloride) and protease inhibitor tablet. For anti-ProtC affinity purification, calcium chloride was added.
to the eluate to a final concentration of 2 mM. The eluate was combined in a new column with a 200 µl settled bead volume of anti-protein C affinity matrix (Roche, Indianapolis, IN) equilibrated in buffer PC-150 and rotated for 2 hours at 4°C. After the flowthrough was collected, the matrix was washed with 60 ml of PC-150 and ProtC-tagged proteins were eluted with EGTA elution buffer (5 mM Tris-HCl [pH 7.7], 10 mM EGTA, 5 mM EDTA, 10 µg/ml leupeptin). Subsequently, the proteins were bound to 10 µl of hydrophobic StrataClean resin (Stratagene, La Jolla, CA), released into sodium dodecyl sulfate (SDS) loading buffer at 80°C, separated on SDS-polyacrylamide gel electrophoresis (PAGE) gels, and stained with Gel Code blue.

4.5 Sedimentation analysis

Sedimentation analysis was carried out in 3.5 ml 10–40% linear sucrose gradients (20 mM HEPES–KOH, pH 7.7, 150 mM potassium chloride, 20 mM potassium L-glutamate, 3 mM MgCl₂, 0.1% NP-40), which were centrifuged in a Beckman SW50 rotor for 19 hours at 49,000 rpm and 4°C. Fractions were collected (200 µl each) on ice from top to bottom and protein was precipitated using StrataClean. Proteins were separated on 8% SDS-polyacrylamide gels and stained with SYPRO Ruby (Invitrogen) according to the manufacturer's protocol.

4.6 Mass Spectrometry

Individual protein bands were excised from polyacrylamide gels, digested with trypsin, and analyzed by liquid chromatography-tandem MS. The resulting spectra information was used to identify T. brucei proteins through the Mascot search engines.
4.7 Western Blot

For Western blot analysis of PTP-tagged mitochondrial DNA polymerases, cells were harvested at 3,500 x g for 10 minutes (4°C) and pellets were washed once in 1X PBS. Cells were lysed in 4X SDS sample buffer containing 5% beta-mercaptoethanol and incubated at 90°C for 5 minutes. Proteins were separated by SDS-PAGE on an 8% acrylamide gel and transferred to a PVDF membrane overnight at 4°C at 90 mA in 1X transfer buffer containing 0.1% methanol. Membranes were incubated in 1% blocking reagent from Roche (60 minutes) followed by incubation with antibodies diluted in 0.5% blocking reagent (60 minutes). PTP (ProteinC-TEV-ProteinA) tagged proteins were detected with 1:2000 Peroxidase-Anti-Peroxidase soluble complex (PAP) reagent (Sigma). *T. brucei* lipoamide dehydrogenase (LipDH) was detected with anti-LipDH (1:10:000) and secondary chicken anti-rabbit (1:10:000). Relative protein levels were quantified using the Image J software and normalized against LipDH. PTP purification fractions were separated in a 4-12% gradient SDS-PAGE gel. For Western Blot, proteins were transfer to PVDF membranes, which were subsequently incubated in 1% blocking with 1mM CaCl$_2$ over night. Detection of tagged-proteins after TEV protease cleavage was done with anti-protein C (1:2000) and secondary goat anti-mouse (1:2000). All antibody steps were performed in the presence of 0.5% blocking and 1mM CaCl$_2$. For SDS-PAGE, proteins were detected by Gel Code blue and SYPRO Ruby according to the manufacturer’s protocol (Invitrogen).
Figure 4.2 – Schematic of PTP fusion to the C-terminus of target genes

The C-terminal protein-coding region of POLIB (blue), IC (yellow) and ID (green) mitochondrial DNA polymerase were cloned into the pC-PTP-NEO vector (not to scale). In each case, more than 1000 bp were ligated into the vector for homologous recombination. For genomic integration, each vector was linearized within the target sequence using unique restriction sites indicated by the arrowheads (AatI and SnaBI). The resistance marker cassette containing the neomycin phosphotransferase gene (NEO<sup>R</sup>) is indicated in red. Gene flanks providing RNA processing signals for PTP fusion and resistance marker are the 3′ flank of TbRPA1, HSP70 genes 2 and 3 intergenic region (H23) and the β-α tubulin intergenic region (aqua).
4.8 Results

4.8.1 Three mitochondrial DNA polymerases expressed the PTP tag

To investigate mitochondrial DNA polymerases replication complexes in *T. brucei* the 18.2 kDa PTP (Protein A-TEV-Protein C) tag was fused to the C-terminal region of each protein. The PTP tag is a modified TAP tag originally developed for the purification of transcription factors of *T. brucei*. This tag was proven to be efficient for the purification of protein complexes with roles in tRNA import, protein synthesis and nuclear DNA replication (Table 4.1). To detect PTP-tagged proteins, we separated POLIB-PTP, POLIC-PTP and POLID-PTP whole cell lysate by SDS-PAGE and probed with the PAP reagent. No cross-reactivity was observed in the wild type cell lysate (Fig. 4.3B). Bands corresponding to the predicted sizes of POLIB-PTP, POLIC-PTP and POLID-PTP (177, 200 and 198 kDa, respectively) were detected by Western Blot analysis (Fig. 4.3A and B). The mitochondrial matrix protein lipoamide dehydrogenase (LipDH) was used to monitor protein loading (Fig. 4.3B, lower panel). Bands corresponding to each polymerase had different intensities suggesting that these proteins are expressed at different levels in an unsynchronized population (Fig. 4.3B). We estimated the expression levels of each protein by measuring the intensity of each band using the Image J software and normalized each sample to its corresponding LipDH signal to determine their relative level. Values are represented as a function of fold difference relative to POLIB levels (POLIB levels were set to 1). The expression of POLIC-PTP was 3-fold lower that POLIB as shown in Fig. 4.3C, grey bar. However, POLID seems to be expressed at higher levels, at about 2-fold higher than POLIB (Fig.
4.3C, white bar). This data demonstrates that we had generated cell lines that express PTP tag fusion proteins. Additionally, no lower molecular weight bands were detected, suggesting that the different levels in protein abundance were not caused by protein degradation.
Figure 4.3 – PTP-tagged mitochondrial DNA polymerases

(A) Predicted sizes of PTP-tagged mitochondrial DNA polymerases (not to scale). (B) Western blot analysis of whole cell lysates from wild type (WT) cells and from three independent cell lines expressing mitochondrial DNA polymerase IB, IC or ID fused to the PTP. Tagged proteins were detected using PAP reagent (Peroxidase anti-peroxidase soluble antigen-antibody complex), which detects the protein A portion of the PTP tag. The mitochondrial matrix protein lipoamide dehydrogenase (LipDH) was used as a loading control. Each lane contains $5 \times 10^6$ cell equivalents. Molecular weight markers are shown on the left side. (C) POLIB and POLID fold difference relative to POLIB protein levels. LipDH was used as a normalizer.
4.8.2 PTP purification of T. brucei mitochondrial DNA polymerase IB

To identify POLIB interacting proteins we used Tandem Affinity Purification (TAP) in combination with MS. We collected samples from each purification step and separated proteins by SDS-PAGE. Anti-protein C detection demonstrated that the IgG affinity step was highly efficient, as no POLIB-PTP was detected in the IgG Flowthrough (IgG FT) (Fig. 4.4A). Followed TEV protease cleavage, POLIB-P was detected in the TEV eluate fraction (TEV Elu). The TEV Eluate was applied to an anti-protein C affinity matrix. No POLIB-P was detected in the protein C Flowthrough (Prot C FT). Bound POLIB-P was eluted from the anti-Protein C affinity matrix using EGTA as shown in Fig. 4.4A (last two lanes). To detect any proteins that co-purify with POLIB-P, we separated these by SDS-PAGE and used Gel Code blue as well as SYPRO Ruby, a more sensitive fluorescence-based method of protein detection. Upper molecular weight proteins were seen after both staining methods (Fig. 4.4B, Elu, bands 1-2). Three additional bands between 100 and 30 kDa were detected with SYPRO Ruby (Fig. 4.4B, Elu, bands 3-6). Bands 3 to 5 were excised from the gel and sent for MS analysis. For band 3, we identified 28 peptides that covered 28% of the POLIB amino acid sequence. This allowed clear identification of POLIB by a Mascot search of the GeneDB database. Band 3 corresponded to truncated POLIB. Additional peptides detected corresponded to contaminants such as keratin, albumin and α and β tubulin. Tb927.3.5590 has no predicted function, and is annotated as a conserved hypothetical protein and has been detected before in the mitochondrial proteome (25). POLIB could be part of a high molecular weight complex. However, further studies are required to determine if TbPOLIB participate in a high molecular weight protein complex.
Figure 4.4  PTP purification of mitochondrial DNA polymerase IB

(A) Western Blot analysis of POLIB-PTP tandem affinity purification fractions; Input, IgG Flowthrough (FT), TEV eluate, protein C Flowthrough and EGTA Eluate. Tagged protein was detected using anti-Protein C sera. (B) Input, TEV eluate and final eluate fractions separated on SDS-polyacrylamide gel electrophoresis (PAGE) gels and stained with SYPRO Ruby and Gel Code blue stains. Final fraction from POLIB-PTP purification contained 7 bands ranging from 30 to 280 kDa in size which were identified post gel staining. Molecular weight markers are shown on the left side. (C) Peptides identified in the final eluate from POLIB PTP purification using Mass Spectrometry.

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<tr>
<th>Proteins identified by Mass Spectrometry</th>
<th>Total of Peptides Identified</th>
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<tbody>
<tr>
<td>Keratin 1</td>
<td>25</td>
</tr>
<tr>
<td>Albumin</td>
<td>3</td>
</tr>
<tr>
<td>Tb927.3.5590</td>
<td>5</td>
</tr>
<tr>
<td>α and β Tubulin</td>
<td>7</td>
</tr>
<tr>
<td>POLIB</td>
<td>28</td>
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4.8.3 POLIB-P sedimentation profile

We next wanted to confirm that high molecular weight proteins sediment with POLIB-P. Proteins purified via PTP purification were subjected to a linear 10-40% sucrose gradient and separated by centrifugation (Fig. 4.5). Samples from individual fractions were taken from top to bottom and separated by SDS-PAGE. SYPRO Ruby was used to visualize proteins. A fraction of POLIB-P sediments as a monomeric form, based on typical migration of IgG in a sucrose gradient (Fig. 4.5, fraction 11-12). The majority of POLIB-P sediments with a high molecular weight complex at the bottom of the gradient (Fig. 4.5, fraction 20). These data further confirm that POLIB is part of a high molecular weight complex. Additionally, it suggests that a fraction of POLIB is part or a transient or unstable complex that cannot be isolated using standard TAP.
Figure 4.5 – Sucrose gradient

POLIB-P final eluate. Typical sedimentation of TEV (29 kDA), Taq (95 kDA), IgG (150 kDA) and TST (230 kDA) are indicated on top of the gel (arrow head). Molecular weight markers are shown on the left side.
4.8.4 POLIB-PTP purification after formaldehyde cross-linking

To detect transient POLIB-PTP PPI, we used formaldehyde cross-linking in combination with PTP TAP. We selected formaldehyde because it has a short length arm (~2.2 Å) allowing only closely associated proteins to covalently cross-link, which minimizes the formation of unspecific cross-links. To determine the optimal formaldehyde concentration, we incubated POLIB-PTP tagged cells for 5 minutes with multiple formaldehyde concentrations (Fig. 4.6A). We found that treatment with increasing concentrations of formaldehyde resulted in an increasing amount of POLIB-PTP appearing as a smear that migrated at higher molecular weights than non-cross-linked POLIB-PTP (Fig. 4.6A). Along with the increase of POLIB-PTP high molecular weight complex (red), there was a decrease in the non-cross-linked POLIB-PTP (Fig. 4.6B, blue). We selected 0.5 % formaldehyde for further PTP TAP experiments because at this concentration about 30% of POLIB-PTP protein was in a high molecular weight complex. Cells were incubated with 0.5% formaldehyde for 5 minutes and cell extract was incubated on IgG affinity column followed by Protein C matrix. Western Blot analysis of each individual fraction was performed to determine the efficiency of the purification (Fig. 4.6C). POLIB-PTP was detected in the IgG Flowthrough demonstrating that the IgG affinity step was highly inefficient. This data suggest that formaldehyde was able to cross-linked POLIB-PTP to other proteins, but that the chemical cross-linker inhibited binding of the PTP-tagged protein to the IgG beads during the first affinity step.
**Figure 4.6 – In vivo formaldehyde cross-linking and PTP purification**

(A) POLIB-PTP detected after cross-linking with different formaldehyde concentrations. 5x10^6 cells were loaded per lane and tagged protein was detected with the PAP reagent. (B) The intensity of monomeric POLIB-PTP (blue) and POLIB-PTP (red) complexes was measured using Image J and plotted. Intensities were compared to POLIB-PTP that was not cross-linked. (C) PTP purification after 0.5% formaldehyde cross-linking.
4.8.5 PTP purification of *T. brucei* mitochondrial DNA polymerase IC

We then isolated POLIC-PTP using TAP and identified proteins in the final eluate by MS. Samples were collected at each purification step and analyzed by Western Blot. The mixture of proteins in the final elute was digested in solution and analyzed by MS. Western Blot analysis with anti-protein C and CaCl$_2$ demonstrated that both affinity steps (IgG and protein C) were highly efficient, as POLIC was not detected in either fraction (IgG FT and Prot-C FT) (Fig. 4.7A). POLIC-P was detected after TEV protease cleavage (TEV Elu) and EGTA elution (ProtC FT). The final protein C flowthrough was dialyzed and sent for MS analysis. A lower molecular weight band of about 100 kDa was detected after dialysis and corresponded to protease degradation of the tagged protein (Fig. 4.7A). MS analysis identified 29 peptides that covered 25% of POLIB amino acid sequence (Fig. 4.7B). This allowed clear identification of POLIC by a Mascot search of the GeneDB database. Additional peptides corresponded to contaminants such as keratin, albumin and α and β tubulin were detected (Fig. 4.7B). This data suggest that TbPOLIC is not associated with a protein complex. However, if TbPOLIC participate in a protein complex we speculate that these PPI are unstable and cannot be detected by classical TAP and MS.
Figure 4.7 – PTP purification of mitochondrial DNA polymerase IC

(A) Efficiency of the purification was monitored by immunoblot via anti-protein C antibody detection of the different fractions. (B) Proteins identified by Mass Spectrometry.

<table>
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<td>16</td>
</tr>
<tr>
<td>POLIC</td>
<td>29</td>
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4.8.6  PTP purification of \textit{T. brucei} mitochondrial DNA polymerase ID

We then investigated whether the third essential mitochondrial DNA polymerase, POLID, associates with a stable complex that can be isolated by PTP and identified by MS. POLID-PTP was purified from a crude trypanosome cell extract by two consecutive affinity steps: IgG and anti-protein C affinity purification. MS analysis was performed to identify proteins from a mixture (EGTA final) and the origin of each peptide was determined by Mascot search engine. POLID-PTP was proteolytically processed while generating a cell extract and the truncated POLID-PTP protein was about 150 kDa (Fig. 4.8, 1X input). This truncated form was also detected in the EGTA final (Fig. 4.8B, asterisk). All proteins identified by SDS-PAGE and SYPRO Ruby staining had molecular weights above 97 kDa (Fig. 4.8B, final Elu). MS analysis identified 31 peptides that covered 25\% of the POLID amino acid sequence allowing clear identification of POLID by a Mascot search of the GeneDB database (Fig. 4.8C). As with previous purifications, we detected peptides corresponding to contaminants such as keratin, albumin and \(\alpha\) and \(\beta\) tubulin (Fig. 4.8C). Tb927.10.10590, which was detected in the mitochondrial proteome, was also detected in POLID final elution. However, this protein is predicted to be a nucleosome core histone H2B protein. These data suggest that TbPOLID is not directly associated with other proteins or it participate in transient/weak complexes that cannot be isolated with standard PTP TAP methods.
Figure 4.8 – PTP purification of mitochondrial DNA polymerase ID

(A) Efficiency of the purification was monitored by immunoblot. Anti-protein C antibody detected the tagged protein in the different PTP purification fractions. A lower molecular weight band was observed that corresponded to protease degradation of the tagged protein. (B) SYPRO ruby staining SDS-PAGE gel. (C) Proteins identified by Mass Spectrometry.
4.9 Discussion

Kinetoplast DNA duplication involves the release, replication and reattachment of thousands of minicircles (14). Maxicircles remain attached to the network but must be replicated almost concurrently with minicircles. So far, about 30 proteins have been demonstrated to participate in kDNA replication (14). Although there is some overlap in the activities of these proteins, they seem to have different functions in replication and maintenance of the kDNA network. Some of these proteins have restricted activity to either minicircle or maxicircle DNA templates. For example, TbPIF2 and PRI2 have important roles specifically in maxicircle replication; TbPIF2 regulates maxicircle copy number and PRI2 is expected to prime the replication of maxicircle DNA (13, 21).

Proteins involved in the different stages (i.e., initiation, primer removal and segregation) of minicircle replication include UMSBP, p38, PRI2, SSE1, LIG ka and TbPIF1 (14). Regardless the stages of the kDNA replication process in which they participate, these proteins must be coordinated within the cell cycle to ensure complete and accurate kDNA replication.

Protein-protein interactions (PPI) are responsible for coordinating the transitions between the sequential steps required for accurate kDNA replication. However, information regarding PPI during kDNA replication is limited. To provide information regarding kDNA replication complexes we tagged three of the four Pol I-like mitochondrial DNA polymerases (POLIB, IC and ID) with a PTP epitope tag for TAP (Fig. 4.3). We have previously shown that these three DNA polymerases are essential for kDNA replication and cell viability (5, 6, 16). The PTP tag is not detrimental for the
function of any of the tagged proteins; cell growth and kDNA replication are not impaired in single expresser cell lines. Additionally, we have demonstrated that two of these polymerases (TbPOLIC and TbPOLID) are imported into the mitochondrion and localize to the region of kDNA replication (Chapter 3) and (7).

Using TAP in combination with MS we attempted to characterize mitochondrial DNA polymerases replication complexes in T. brucei. Each tagged protein was able to efficiently bind during the IgG and Protein C affinity steps as demonstrated by Western blot analysis (Fig. 4.4, 4.7 and 4.8). The final eluates were evaluated by MS and the majority of the peptides that were identified corresponded to PTP-tagged DNA polymerases (Fig. 4.4, 4.7 and 4.8). Despite our efforts to minimize human keratin contamination, it remained the second most abundant peptide identified on each purification. However, keratin contamination seems like a common problem during TAP purification and has been reported by others (23). Additionally, albumin, IgG heavy chain and α and β-tubulin were consistently detected. Following POLIB-PTP purification we only detected peptides corresponding to one other trypanosomal protein, which is annotated as a conserved hypothetical protein (Tb927.3.5590). This protein has no predicted domains but was predicted to localize to the mitochondrion because it was detected in the mitochondrion proteome of procyclic form trypanosomes (25). However, only two peptides corresponding to this protein were detected in the mitochondrial proteome study. Further analyses are necessary to determine if this protein is in the mitochondrion, is involved in kDNA replication and physically interacts with POLIB. Additional peptides were not detected after POLIC PTP TAP, suggesting that this protein is not part of a complex or PPI are transient/weak that are not detected with standard PTP
TAP methods. A putative histone H2B was detected in the final eluate of POLID-PTP purification and is expected to be a false positive since this protein is predicted to be in the nucleus. Additionally, no classical histones have been found in trypanosomal mitochondrial, instead a family of four H1 histone-like proteins (KAP1, 2, 3 and 4) are associated with the kinetoplast DNA in the trypanosomatid *Crithidia fasciculata* (22).

PTP TAP has been successfully used to purify protein complexes in trypanosomes (Table 4.1). However, we were unable to isolate a protein complex after PTP TAP of all three mitochondrial DNA polymerases (POLIB, IC, ID). One explanation is that the interacting proteins are likely low in abundance and cannot be detected because they are too dilute in solution. The interaction of these polymerases with other proteins may be weak and lost during washing steps. Alternatively, kDNA replication may be governed by transient interactions that are constantly formed and dissociated during kDNA duplication. Interestingly, we have demonstrated that the localization of POLIC and POLID is dynamic and changes in respect to the *T. brucei* cell cycle (7 and chapter 3). It is likely that interactions of these polymerases with other proteins are coordinated with kDNA replication, and only 35 % of *T. brucei* cells are undergoing kDNA replication in an unsynchronized population (7). Furthermore, POLID and POLIC are detected to the sites of kDNA replication in only 25% of the cells. Thus, our PTP purification experiments may not identify time-dependent interactions during the kDNA replication process. Our data suggest that classical TAP is not suitable for detecting cell cycle-dependent and transient interactions.

Most PPI within cells are predicted to be transient, occurring for only a short time and disassociating frequently during specific events. Transient PPI interactions are
expected to control a large number of cellular processes (e.g., cell regulation and signal transduction) and many of these remain uncharacterized since they are technically difficult to detect experimentally. Several methods have been modified to detect unstable and transient PPI. Yeast two hybrid (Y2H) assay is able to detect some of these interactions. However, it can only detect binary interactions and it has additional limitations, such as detection of false positives. Efforts to decrease the frequency of Y2H false-positives include a recent study that demonstrated that by pooling interaction data readouts from repeated Y2H screens investigators achieve higher detection coverage and were able to detect even transient interactions (38). TAP is one of the most popular methods for detection PPI and allows the identification of protein complexes (26, 41). However, it is not the method of choice for isolation of transient and low affinity complexes (20, 40). In many cases PPI are not retained during cell lysis or washing steps, which are necessary to remove non-specific interactions. The use of chemical cross-linking reagents such as formaldehyde has allowed researchers to freeze transient interactions in vivo and isolate transient complexes by TAP (12, 35, 40). We used formaldehyde cross-linking in combination with PTP TAP to attempt isolation of transient/weak interactions. This method was not successful for isolating T. brucei mitochondrial DNA polymerase IB (Fig. 4.6), IC and ID complexes (data not shown). After using several concentrations of formaldehyde cross-linking we were unable to purify PTP-tagged proteins because the IgG affinity purification step was highly inefficient (Fig. 4.6A). We detected PTP-tagged proteins in the IgG eluate, which suggests that after formaldehyde cross-linking the protein A domain within the PTP tag is blocked or that the amino acids essential for affinity binding are being modified (35, 37).
Mitochondrial DNA replication in all systems may be regulated by transient PPI. One line of evidence for this hypothesis is that no mitochondrial replisome has ever been purified in vivo. Only a minimal replisome composed of Twinkle, Pol γ and SSB has been reconstituted in vitro (17). Our data further suggest that the association of mitochondrial DNA polymerases with other proteins is transient/weak. Additional PTP TAP experiments using other replication proteins as bait are necessary and will help determine whether mitochondrial kDNA replication is governed by transient/weak PPI while establishing novel methods to identify these interactions.

4.10 Bibliography


CHAPTER 5

5 EXPLORING THE MECHANISMS OF TBPOLIC AND TBPOLID
DYNAMIC LOCALIZATION FROM MULTIPLE ANGLES

5.1 Perspective and future directions

*Trypanosoma brucei* mitochondrion contains multiple DNA polymerases, two are pol β-like (pol β and pol β-PAK) and four are pol I-like DNA polymerases (IA, IB, IC and ID) (5,7). The Klingbeil lab aims to understand the molecular mechanism and precise role of *T. brucei* pol I-like DNA polymerases during kDNA transactions. These proteins have a C-terminal family A DNA polymerase domain that contains amino acids critical for polymerase activity, including two aspartic acid residues required for coordination of Mg$^{2+}$. In addition to the polymerase domain, TbPOLIB and TbPOLID have a 3’ exonuclease domain presumably involved in proofreading. Using RNA interference (RNAi) studies it was previously demonstrated that POLIB, IC and ID are essential for cell viability and kDNA replication (1,2,5). However, it is not clear if the essential role of these proteins resides exclusively in the polymerase and exonuclease domains. One possibility is that their essential activities in kDNA replication are not limited to nucleotide incorporation and proofreading. Although protein localization does not necessarily define the function of a protein it is tempting to speculate that cell cycle dependent localization of some of these proteins could control their role in kDNA replication. One potential line of evidence is provided in this thesis. Using multiple immunofluorescence microscopy tools we demonstrated that TpPOLIC and TpPOLID localize to the sites of kDNA replication only during kDNA S phase. We provided a comprehensive analysis of the spatial-temporal localization of these two essential
mitochondrial DNA polymerases and developed a model based on the localization dynamics of these proteins during the cell cycle (Fig. 5.1).

Using the five different kDNA replication stages described by Gull (3) and colleagues we defined TbPOLIC and TbPOLID localization during the cell cycle of *T. brucei* (Chapters 2 and 3). Our working model for TbPOLIC and TbPOLID dynamic localization suggest that during stage I (a single unit kDNA and one basal body (bb)/pro-basal body (probb pair) TbPOLID is distributed throughout the mitochondrial matrix and low levels of TbPOLIC are detected presumably to the KFZ region (Fig. 5.1). During stage II (dome network, 2bb/pro-bb pair and visible antipodal sites) the fraction of TbPOLID detected at the mitochondrial matrix redistributes as two discrete foci that localize to the antipodal sites along with replicating minicircles. At this stage, TbPOLIC is detected by IF and it colocalizes with TbPOLID antipodal sites foci. As the cells transition from stage II to stage III (bilobe shape kDNA network, 2bb/pro-bb pair and antipodal sites that are detected occasionally), TbPOLIC and TbPOLID undergo major changes in their localization. TbPOLIC levels decrease and it becomes undetectable by IF and TbPOLID goes back to the mitochondrial matrix were it remains until the next round of kDNA replication (Fig. 5.1). We propose that dynamic localization of kDNA replication proteins provides a novel mechanism for regulating kDNA replication. Our studies open up new avenues to explore and are the foundation for developing and testing new hypothesis.

Prior to the initiation of DNA replication TbPOLIC intensity is low and is detected as a diffuse signal that is presumably located at the KFZ. Additional markers for detection of the KFZ are necessary to be able to precisely determine if this signal is
present in this region. KFZ localization was reported for proteins such as TbPOLIB, UMSBP and TbPIF2. To confirm this data and be able to use any of these proteins as KFZ markers it would be necessary to perform additional localization analysis using basal body labeling and DAPI staining. This tool will allow us to confirm that these proteins localize between the kDNA disk and the basal bodies, which is the region of the KFZ.

The KFZ is also known to be transverse by a filament system called the tripartite attachment complex (TAC), which connects the basal body with the kDNA disk. The TAC consist of (1) exclusion zone filaments that are directly linked to the proximal end of the basal body and the outer mitochondrial membrane, (2) a detergent resistant mitochondrial membrane region and, (3) unilateral filaments that run from the inner mitochondrial membrane to one face of the kDNA disk or through the region that is best known as the KFZ (6). Only one stable component of the TAC known as p166 has been characterized (8). This protein localized between the kDNA disk and the basal bodies and was suggested to have an association with the unilateral filaments. In future studies, p166 could also serve as a marker for the KFZ region. This will require the generation of a cell line expressing an epitope tag in p166 since no antibody effective for IF that detects the endogenous protein is available. Additional information regarding the localization of proteins within the KFZ can be obtained using immunogold studies. Using transmission electron microscopy and cytochemical analysis the Gull’s lab identified subdomains of the KFZ that were transverse by two biologically different unilateral filaments termed the inner and outer unilateral filaments (4). The inner filaments were located at the base of the kDNA disk and did not extend to the inner mitochondrial membrane. This region was
sensitive to EDTA bleaching and uracyl acetate, which suggested that minicircles are not randomly distributed through the KFZ but rather they are contained within a specific region of the KFZ. It will be interesting to determine to which specific KFZ subdomain TbPOLIC localizes prior to kDNA replication. It is possible that recruitment of TbPOLIC to a KFZ subdomain prior to kDNA replication could determine if this protein participates in specific kDNA replication stages. Interestingly, independent RNAi of TbPOLIB and TbPOLID showed inhibited minicircle replication as previously determined, as well as, TbPOLIC localization to the antipodal sites. One explanation why TbPOLIC localization to the antipodal sites is impaired could be attributed to a reduction of TbPOLIB or TbPOLID protein levels, which will have a direct affect in kDNA replication. Future experiments in which we can monitor TbPOLIB and TbPOLID protein levels post RNAi-mediated silencing in a cell line expressing POLIC-PTP are necessary to confirm the decline of TbPOLIB and TbPOLID protein levels. Inhibition of kDNA replication is not only preventing TbPOLIC recruitment to the antipodal sites but its detection to the KFZ suggesting that early replication events at the KFZ are essential for TbPOLIC localization to the antipodal sites. Additional RNAi studies of kDNA replication proteins and subsequent localization of TbPOLIC will allow us to determine if localization of TbPOLIC to the antipodal sites is directly affected by inhibiting replication or by silencing only the essential mitochondrial DNA polymerases.

Recently, three arginine-methylated sites within the TbPOLIC sequence were detected in a proteomic study. It is important to determine if these sites are involved in kDNA replication or cell cycle dependent localization of TbPOLIC. It will be fundamental to perform mutation analysis to determine if TbPOLIC arginine-methylated
sites are essential for the protein in vivo function. Substitution of arginine by other positively charged residues such as lysine will inhibit the protein arginine methyltransferase recognition site and will cause TbPOLIC to remain unmethylated at all times. It is not clear if protein arginine methylation (PRMT) plays a role in kDNA replication. RNAi studies of the mitochondrial PRMT and evaluation of the effect on kDNA replication will help confirm the role of putative mitochondrial PRMT in kDNA replication. Alternatively, kDNA loss can be monitored after inhibiting protein arginine methylation by using compounds that block S-adenosylmethionine-dependent methylation. To gain information regarding the temporal and spatial methylation of TbPOLIC, a potential tool could be to develop an antibody that preferentially recognizes methylated TbPOLIC. Defining the biological function of TbPOLIC arginine-methylation will have a major impact for the field of mitochondrial DNA replication in all organisms. The function of post-translational modifications for mitochondrial proteins is not well characterized. Uncovering the mechanism and role of TbPOLIC arginine-methylation could represent the groundwork for understanding the role that post-translational modifications play in mitochondrial processes.

TbPOLIC and TbPOLID localization to the antipodal sites occurs strictly during kDNA replication. Colocalization analysis of these two proteins suggested that they precisely colocalized at early stages of kDNA replication. At later stages of kDNA replication these proteins exhibit only partial colocalization suggesting that at that time a fraction of TbPOLID initiates its shift to the mitochondrial matrix. The elements that control the dynamic localization of these proteins remain unknown. One way to identify the region of the protein that contains the antipodal site localization signal is by
generating truncated forms of the proteins and monitoring its localization. These studies could provide insights into the antipodal sites localization signal and will help determine if this is a common signal between proteins that localize to this region.

Currently, we are also investigating if post-translational modifications play a role in TbPOLID dynamic localization. It is not likely that arginine-methylation directly affects TbPOLID localization since no arginine methylated sites within the TbPOLID protein sequence have been detected in proteomic studies. However, PRMT could indirectly influence the localization of TbPOLID. For example, the interaction of TbPOLID with other proteins could be dependent on the methylation status of the interacting protein. We have also explored TbPOLID phosphorylation sites using tandem affinity purification and Mass Spectrometry. No phosphopeptides within the TbPOLID sequence were identified by Mass Spectrometry after 40% protein coverage (Michele Klingbeil and Alice Tran personal communication). We have not eliminated the possibility that phosphorylation sites are present in the TbPOLID sequence since Western Blots probed with phosphotyrosine specific antibody detected a band at about the same molecular weight of TbPOLID after POLID-PTP immunoprecipitation. However, additional studies are required to confirm if TbPOLID is phosphorylated.

Using the tools we reported in these studies we can now assess dynamic localization for other kDNA replication proteins. We anticipate that other kDNA replication proteins such as the multiple helicases will undergo dynamic localization to be able to participate in the different stages of the kDNA replication process. Understanding the spatial temporal localization of kDNA replication proteins will provide new insight into the complex mechanism of kDNA replication. Given that in other organism the
replication of the mitochondrial genome has no cell cycle control, *T. brucei* could be the best model system for studying the dynamic localization of mitochondrial proteins.
Figure 5.1 – Working Model of TbPOLIC and TbPOLID dynamic localization

Light blue area represents the regions when the kDNA is negative for BrdU incorporation and the grey area corresponds to the time when BrdU is detected at the antipodal sites. kDNA duplication stages (I-V) are indicated on top of the table. Schematic representation of cytological changes within stages are indicated above the table.

5.2 Bibliography


APPENDIX

RNAI-MEDIATED SILENCING OF TBPOLIB IMPACTS TBPOLIC FOCI

I. Introduction

Trypanosomes, like no other eukaryotic organism, contain six mitochondrial DNA polymerases (6, 7). Two of them are Pol β-like DNA polymerases (Pol β and Pol β-PAK) and the other four are pol I-like DNA polymerases (TbPOLIA, IB, IC and ID). Studies on Pol β and Pol β-PAK demonstrated that both of them have DNA polymerase and dRP lyase activity. It is suggested that these two enzymes participate in later stages of minicircle replication. The proposed model for kinetoplast (kDNA) replication indicates that Pol β fill most of the minicircle gaps between Okazaki fragments in the antipodal sites. As minicircles reattach to the network, the remaining gaps are presumably filled by Pol β-PAK. It is not known if these enzymes are essential for kDNA replication. However, using RNAi, three of the pol I-like DNA polymerases (TbPOLIB, IC and ID) were demonstrated to be essential for cell viability and kDNA replication. TbPOLIB silencing resulted in growth inhibition, kDNA loss and the accumulation of a novel population of free minicircles that is mainly comprised of covalently closed minicircle dimers (1). It was also demonstrated to contribute to both leading and lagging strand synthesis. Individual silencing experiments on TbPOLIC and TbPOLID resulted in growth inhibition and kDNA loss suggesting that pol I-like DNA polymerases have non-redundant roles in the kDNA replication process (2, 5).

In addition to their essential role in the replication process we now know that they are located in the two main regions where kDNA replication events occur: the
kinetoflagellar zone (KFZ) and the antipodal sites. Using peptide antibodies against TbPOLIB C-terminal region this protein was reported to localize to the KFZ. We have demonstrated in chapters 2 and 3 that the localization of TbPOLID and TbPOLIC is not static and varies throughout the cell cycle. We showed that TbPOLID redistributes from the mitochondrial matrix to the antipodal sites during kDNA replication. These studies were the first report of cell cycle dependent protein redistribution during kDNA replication. In contrast to TbPOLID, TbPOLIC becomes undetectable by IF when is not present at the site of replication. In 1N1K cells when TbPOLIC was not organized as discrete foci, it was detected as a faint signal. A fraction of this faint signal resembled to the previously characterized localization to the KFZ and also to the kDNA network. It was detected that TbPOLIC foci colocalizes with TbPOLID foci only during initial stages of kDNA replication. At later stages of replication, only a fraction of these proteins colocalize and TbPOLID initiates to disperse throughout the mitochondrial matrix. So far, it is not known if TbPOLIB colocalizes with any of the other polymerases or if the localization of this protein is also dynamic through the cell cycle.

We hypothesize that the role of these polymerases is highly coordinated during kDNA replication. We explored if silencing of individual DNA polymerases (TbPOLID and TbPOLIB) impaired TbPOLIC foci and protein stability. In chapter 3, we demonstrated that TbPOLID silencing affects TbPOLIC foci positive cells and protein levels. We asked if this effect was specific for TbPOLID silencing and proceeded to investigate the effect of TbPOLIB silencing on POLIC-PTP foci and protein levels. The data presented here supports that TbPOLIB silencing also affects TbPOLIC foci and has a greater impact on TbPOLIC protein stability.
II. Materials and methods

**Plasmid construction.** (i) **POLIC** knockout construct pKOPOLIC\(^{\text{BSR}}\). The puromycin resistance cassette from pKOPOLIC\(^{\text{Puro}}\) was replaced with blasticidin sequence to generate pKOPOLIC\(^{\text{BSR}}\). Briefly, the puromycin cassette was released from pKOPOLIC\(^{\text{Puro}}\) after Ascl and PacI digestion and the blasticidin sequence from the pKO\(^{\text{BSR}}\) vector was ligated into pKOPOLIC vector to generate the pKOPOLIC\(^{\text{BSR}}\).

(ii) **PTP tag constructs.** pPOLIC-PTP-PURO was generated as described (1).

**Generation of cell line**

**TbIC-PTP/SLIB.** The pSLIB vectors were generated as previously described (1). AflII-linearized pPOLIC-PTP-PURO was transfected into SLIB RNAi cell line. Cells expressing a POLC-PTP and the intramolecular stem-loop vector to target *TbPOLIB* RNAi were subsequently transfected with pKOPOLIC\(^{\text{BSR}}\) to knockout the *TbPOLIC* wild type allele. Cells were then selected with 15 μg/ml G418, 50 μg/ml hygromycin, 2.5 μg/ml phleomycin, 1 μg/ml puromycin and 10 μg/ml blasticidin resulting in cell lines expressing a single PTP-tagged POLIC allele (POLIC-PTP/SLIB/ICKO\(^{\text{BSR}}\)). Single knockout was confirmed as described above. Clonal cell line P1A8 was selected for this study. We named this cell line TbIC-PTP/SLIB.

**TbPOLIB RNAi.** TbIC-PTP/SLIB cells were induced for RNAi by adding 1 μg/ml tetracycline, and cell growth was monitored daily using a Z2 model Coulter Counter (Beckman Coulter).
**In situ TdT labeling and quantification.** Cells were fixed in 4% PFA, permeabilized in methanol and labeled *in situ* with TdT as previously described (4). TdT-positive and negative cells were quantified at indicated times.

**Immunofluorescence (IF).** TbIC-PTP/SLIB uninduced and RNAi induced cells (day 4, 6 and 8) were harvested by centrifugation for 5 minutes at 1,000 x g; resuspended in 1X phosphate-buffered saline (PBS), and adhered to poly-L-lysine (1:10) coated slides for 5 minutes. Cells were then fixed for 5 minutes using 4% paraformaldehyde (PFA) and washed three times (5 minutes each) in 1X PBS containing 0.1 M glycine (pH 7.4). Cells were permeabilized with 0.1% Triton X-100 for 5 minutes and washed in 1X PBS 3 times for 5 minutes. PTP-tagged proteins were detected by incubating with anti-protein A serum (Sigma, 1:3000) for 60 minutes followed by Alexa Fluor® 594 goat anti-rabbit (1:250) for 60 minutes. DNA was stained with DAPI (3 μg/ml). Slides were then washed 3 times in 1X PBS prior to mounting in Vectashield (Vector Laboratories).

**Western Blotting.** Cells were harvested at 3,500 x g for 10 minutes (4°C) and pellets were washed once in PBS supplemented with protease inhibitor cocktail Set III (1:100) (CalBioChem). Cells were lysed in 4X SDS sample buffer (BioRad) containing 5% beta-mercaptoethanol and incubated at 90°C for 5 minutes. Proteins were separated by SDS-PAGE on a 8% acrylamide gel and transferred to a PVDF membrane overnight at 4°C at 90 mA in transfer buffer containing 0.1% methanol. Membranes were incubated in 1% Roche blocking reagent (60 minutes) followed by incubation with antibodies (60 minutes) diluted in 0.5% blocking reagent (60 minutes). PTP (ProteinC-TEV-ProteinA) tagged proteins were detected with 1:2000 Peroxidase-Anti-Peroxidase soluble complex
(PAP) reagent (Sigma). For subsequent detections, membranes were stripped for 15 minutes at 37 °C with 0.1 M glycine (pH 2.5), washed in TBS with 0.1 % Tween-20, blocked and re-probed with one the following primary/secondary antibody combinations: *C. fasciculata* specific anti-Hsp70 (1:10,000) (12) /chicken anti-rabbit (1:10,000, Roche), *T. brucei* anti-Pol β (1:1000) (7)/goat anti-rat (1:5000) and anti-TAO (*T. brucei* alternative oxidase; 1:100) (3)/ goat anti-mouse (1:1000) and anti-tubulin (1:20,000, Sigma) /goat anti-mouse (1:1000). All secondary antibodies were HRP conjugated. Signal was detected with BM Chemiluminescence Western Blotting Substrate (POD) from Roche.

**RNA isolation and quantitative PCR.**

*TbIC-PTP/SLIB* uninduced and induced cells (0 and 2 days) were harvested at 4°C (3,500 rpm for 10 minutes) and pellets were washed with cytomix (8). Total RNA was extracted from 5x10⁷ cells using the TRIsol reagent (Ambion) according to the manufacturer's protocol. 10 μg of RNA were treated with 10 units (30 minutes at 37°C) of RNase-free DNase I (BioRad) to remove any DNA contamination. Subsequently, RNA was cleaned using the RNA clean and concentrator kit (Zymo Research). The High Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Ambion) and the Multi-Scribe Reverse Transcriptase were used to convert 500 ng of total RNA to cDNA. RT-PCT was performed in a 10-μl reaction contained 1 μl cDNA template, 5 μl FastStart universal SYBR Green master (Rox) kit (Roche Diagnostics Corp., Indianapolis, IN), 300 nm forward and reverse primers each, and nuclease-free water. Primers used for this analysis are listed in Table 1. All data was normalized to GAPDH. The normalized values
from induced samples were compared against uninduced controls for the relative expression levels of mRNA. Relative mRNA levels shown in Fig. 1A and B are represented as the mean of two experimental replicates and three separate RNAi induction experiments.

III. Results

1. **TbPOLIB RNAi-mediated silencing causes a reduction in POLIC-PTP foci positive cells**

   We demonstrated in chapter 3 that silencing TbPOLID altered POLIC-PTP foci. We investigated if POLIC-PTP foci were also affected after silencing another essential mitochondrial DNA polymerase, TbPOLIB. We generated a single expresser POLIC-PTP tagged cell line and transfected it with a POLIB stem-loop RNAi vector (TbIC-PTP/SLIB clone P1A8). We induced formation of TbPOLIB-specific intramolecular stem-loop dsRNA by adding tetracycline to the cells to knockdown the expression of TbPOLIB.

   Loss of TbPOLIB caused growth inhibition starting at day 4 (uninduced; 7.3 ± 0.2, N=6 and induced; 7.2 ± 0.2, N=6) and persisted throughout the course of an 8 day induction, in agreement with a previous report on TbPOLIB silencing (Fig. 1A) (1). At day two of the induction, the relative amount of TbPOLIB mRNA decreased by 63 %.

   We evaluated the relative mRNA levels for the two other essential mitochondrial DNA polys (TbPOLID and TbPOLIC) and several kDNA replication proteins (TopoII, UMSBP, PRI1 and PIF1). No significant reduction in the mRNA levels was detected for all of these genes by quantitative PCR analysis (Fig. 1B and C).
To assess the effect of TbPOLIB knockdown on kDNA networks, we monitored progressive loss of kDNA at day 4, 6 and 8 of induction (Fig. 1D). We quantified 200 individual cells (uninduced and induced) from three separate RNAi inductions per time point for normal, small and loss of kDNA (Fig. 1D). DAPI-stained networks that exhibited normal-sized networks represented 97% in an uninduced population. At day 4 of the induction, the percentage of cells with normal-sized networks decreased to 30% while the percentage of cells with small kDNA increased to 59%. Only 2% of the cells contained normal-sized kDNA following 8 days of TbPOLIB RNAi. At this time, there was a dramatic increase in cells with no detectable kDNA (68%) as the percentage of cells with small kDNA declined to 30%. Kinetics of kDNA loss in TbIC-PTP/SLIB cells is comparable to those previously reported (Fig 1D).

We evaluated the effect of TbPOLIB RNAi on the accumulation of gapped/replicating minicircles at the antipodal sites using TdT labeling. Additionally, POLIC-PTP foci positive cells were monitored during TbPOLIB RNAi. Uninduced and induced (day 4 and 8) cells were fixed and labeled with DAPI, anti-protein A and TdT. In an uninduced population, 27% of the cells are TdT-positive and have the same labeling patterns as described in Fig 3 (Fig. 2A). In these cells, POLIC-PTP foci colocalized with gapped/replicating minicircles at the antipodal sites (Fig. 2B, day 0). After 4 days of TbPOLIB RNAi, the number of TdT-positive cells decreased to 17% (Fig. 2A). Additionally, the population of cells with POLIC-PTP foci decreases to 11% after TbPOLID silencing (Fig 2C). On day four of the induction, POLIC-PTP foci were present only in TdT-positive cells (Fig. 2B, day 4), which represented 17% of the population (Fig. 2B and C). Nearly all cells were negative for TdT labeling and POLIC-
foci on day 8 of the induction (Fig. 2). These data suggest that accumulation and assembly of POLIC-PTP foci to the site of replication is dependent on POLIB expression and kDNA replication.
Figure 1 – Effect of *TbPOLIB* RNAi

(A) *TbIC-PTP/SLIB* clonal cell P1A8 was grown in the absence (open circles) or presence (filled squares) of tetracycline (1 μg/ml) to express the *TbPOLIB* stem-loop dsRNA. Cell density was plotted as function of cumulative doublings. Values represent the mean of six independent RNAi induction experiments. (B and C) qRT-PCR analysis of the relative amounts of *TbPOLIB, TbPOLIC, TbPOLID, TopoII, UMSBP* and *PIF1* mRNA levels following two days (D2) of *TbPOLID* RNAi. Uninduced (day 0) GAPDH was used as our normalizer. Normalized values from induced samples were compared against uninduced controls for the relative expression levels of mRNA. Values represent the mean from three separate experiments. Error bars represent the SEM. (D) Quantitation of kinetics of kDNA loss by microscopy. More than 200 cells per timepoint were scored for normal sized kDNA (open circles), small kDNA (filled squares) or no kDNA (open squares). Others (filled triangles) represent cells with abnormal karyotypes. Values represent the mean from three independent experiments. Error bars represent the SEM.
Figure 2 – Effect of *TbPOLIB* RNAi induction on POLIC-PTP localization

Quantification of TdT-positive cells after 4 and 8 days of *TbPOLIB* silencing (200 cells per time point). (B) Detection of POLIC-PTP (red) and gapped/replicating minicircles after TdT labeling (green) during *TbPOLIB* silencing. DAPI-stained DNA is shown in blue. Representative images are shown. Scale bar, 10 µm. (C) Quantification of TdT-positive (dark grey) and TdT-negative (lighter grey) in uninduced and POLID RNAi-induced cells (Day 4, 6 and 8). Values represent the mean of three separate experiments (200 cells per time point). Error bars represent the SEM.
2. **POLIB knockdown affects POLIC protein levels**

We investigated if POLIC-PTP protein levels were affected by perturbation of *TbPOLIB*. POLIC protein levels and other mitochondrial proteins (mtHsp70 and alternative oxidase (TAO)) were monitored in uninduced and induced TbIC-PTP/SLIB cells (day 4, 6 and 8) (Fig. 3A). Membranes corresponding to three separate experiments were probed with PAP, anti-TAO, anti-mtHsp70, and anti-tubulin (Fig. 3A). Protein levels from a representative induction are shown in fig 3A. The intensities of each band were quantified using Image J and were normalized with the corresponding β tubulin control (Fig. 3B). No significant changes in POLIC-PTP protein levels were detected at day 4 (8%) and 6 (2%) of *TbPOLID* silencing (Fig. 3B). At this time point, TAO and Hsp70 show a slight decrease of less than 10%. After 8 days of *TbPOLIB* RNAi, a dramatic decrease in POLIC-PTP proteins levels was detected (66%) (Fig. 3B). However, mitochondrial proteins TAO and Hsp70 were not dramatically affected. We did not detect proteolytic processing of POLIC-PTP at different time points of the induction (data not shown). Here we demonstrate that following *TbPOLIB* silencing POLIC-PTP protein levels were dramatically affected.
Figure 3 – POLIC-PTP protein levels following *TbPOLIB* silencing

(A) Western blot detection of POLIC-PTP, alternative oxidase (TAO) and Hsp70, during *TbPOLIB* RNAi. Cells were harvested 4, 6, and 8 days post-induction, and 5x106 cells were loaded into each lane. The membrane was probed with antibodies against each individual protein.

(B) Quantification of the relative protein levels during *TbPOLIB* RNAi. Values were normalized against tubulin. Values represent the mean of three independent induction experiments. At day 8, a statistical significant decreased in POLIC-PTP protein levels was detected (p<0.01). Error bars represent the SEM.
IV. Discussion

We conducted RNAi experiments of TbPOLIB to evaluate if perturbing TbPOLIB has an impact on TbPOLIC foci. The localization of POLIC-PTP was evaluated in a single expresser cell line that expressed the stem-loop vector for TbPOLIB RNAi silencing. Previously, we demonstrated that TbPOLIB silencing resulted in growth inhibition, kDNA loss, and a decline in nicked/gapped (replicated) minicircles species (2). A similar phenotype for after TbPOLIB silencing was detected in TbIC-PTP/SLIB cells. Consistent with previous reports we detected growth inhibition and an increase in small kDNA by day 4 of the induction. Importantly, mRNA levels for TbPOLIC, TbPOLID and few kDNA replication proteins were not affected (Fig. 1B and C) indicating that observed phenotype is specifically due to downregulation of TbPOLIB. Moreover, using TdT in situ labeling we demonstrated that TbPOLIB silencing caused a rapid decline (Day 4) of gapped/replicating minicircles at the antipodal sites, indicating that minicircle replication is impaired (Fig. 2A). POLIC-PTP foci were also affected during TbPOLIB silencing (Fig. 2B). At day 4 of the induction, only cells in which replication was not yet inhibited (TdT positive) had POLIC-PTP foci (Fig. 2B). POLIC-PTP was never detected in cells with small kDNA and no kDNA. Gradual accumulation of TbPOLIC foci to the antipodal sites seems to depend on the expression of the two essential DNA polymerases (TbPOLIB and TbPOLID). It is not clear how individual silencing of TbPOLIB and TbPOLID affects TbPOLIC protein stability.
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