universität wien

DISSERTATION

Titel der Dissertation

„A chemical genetics approach

to Polo-like kinase inhibition in *Trypanosoma brucei*“

Verfasserin

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angestrebter akademischer Grad

Doctor of Philosophy (PhD)

Wien, 2013

Studienkennzahl lt. Studienblatt:	A 094 490
Dissertationsgebiet lt. Studienblatt:	Molekulare Biologie
Betreuer:	Univ.-Prof. Dr. Graham Warren, FRS

I dedicate this thesis to my father, Carlos Lozano Rubio.

Dedico esta tesis a mi padre, Carlos Lozano Rubio.

"Life is what happens to you
while you are busy making other plans."

Beautiful Boy, John Lennon

"I may not have gone where I intended to go,
but I think I have ended up where I needed to be."

The long dark tea-time of the soul, Douglas Adams

Zusammenfassung

Polo-ähnliche Kinasen sind wichtige regulatorische Enzyme des eukaryotischen Zellzyklus. Der Erreger der Afrikanischen Trypanosomiasis, *Trypanosomabrucei*, besitzt nur eine einzige Polo-ähnliche-Kinase, genannt TbPLK. TbPLK ist notwendig für den Aufbau einer Reihe von Zytoskelettstrukturen, die mit dem Flagellum assoziiert sind. Das Ziel dieser Arbeit war die Bestimmung der molekularen Mechanismen durch die TbPLK die Biogenese dieser Strukturen reguliert. Bisherige Studien basierten auf der Nutzung von RNA-Interferenz oder Inhibitoren mit unbestimmter Spezifität. Im Gegensatz dazu haben wir eine analog-sensitive Strategie verwendet, die die selektive und akute Inaktivierung von Kinasen ermöglichte. Dazu wurde eine funktionell stille Mutation in das katalytische Zentrum der Kinase eingebaut. Ein abgeänderter Kinase-Inhibitor kann dann das katalytisches Zentrum der modifizierten Kinase besetzen und deren Aktivität hemmen. Alle endogenen Kinasen der Zelle werden von diesem modifizierten Inhibitor nicht beeinträchtigt. Die generierte analog-sensitive TbPLK wird *in vitro* und *in vivo* jedoch von diesem modifizierten Kinase-Inhibitor gehemmt. Normalerweise folgt TbPLK während des Zellzyklus einem charakteristischen Migrationsmuster. Nach der Inhibition verbleibt die analog-sensitive TbPLK aber im posterioren Ende der Zelle. In synchronisierten Zellkulturen konnten wir zeigen, dass TbPLK für die Biogenese des Bilobes, einem Organell, das mit dem Flagellum assoziiert ist, und für den Aufbau und die Verlängerung der Flagellum-Attachment-Zone (FAZ), welche das Flagellum mit der Zelloberfläche verbindet, direkt erforderlich ist. Außerdem konnten wir demonstrieren, dass die TbPLK Aktivität für die Rotation der duplizierten Basalkörper benötigt wird. Aufgrund all dieser beschriebenen Defekte kann die Zytokinese nicht beendet werden. Durch die Nutzung mehrerer quantitativer Proteomics-Methoden von jetzt an besteht die Möglichkeit,neue TbPLK-Substrate identifizieren zu können.

Abstract

Polo-like kinases are known to regulate different cell cycle stages in eukaryotes. In the protist parasite *Trypanosoma brucei*, the organism causing African trypanosomiasis, the single PLK homolog TbPLK is necessary for the assembly of a series of cytoskeletal structures associated with the flagellum. Since the molecular mechanisms that regulate the biogenesis of these structures are partially understood, this work set out to further investigate the different TbPLK functions. Previous studies have relied on RNA interference or inhibitors of undefined specificity, but here we used an analog sensitive approach to selectively and acutely inhibit TbPLK. This strategy consists of introducing a functionally silent mutation in the enzyme's catalytic pocket that makes it sensitive to an enlarged inhibitor, while all other non-engineered kinases remain unaffected. Although TbPLK is a highly motile kinase, inactive TbPLK cannot migrate effectively and remains trapped in the posterior of the cell throughout the cell cycle. Using synchronized cells, we showed the direct requirement of TbPLK activity for the replication of the bilobe, an organelle of still unclear function, and for the assembly and subsequent extension of the flagellum attachment zone, which maintains the flagellum adhered to the cell surface. TbPLK is also necessary for the rotation of the duplicated basal bodies, an event which positions the new flagellum so that it can extend without colliding with the old flagellum. As a consequence of all these unsuccessful events, cells develop defects in flagellar attachment and cannot complete cytokinesis. The next stepinvolves the application of quantitative proteomics methods aimed at identifying novel TbPLK substrates.

Acknowledgements

Very special thanks to:

Univ.-Prof. Dr. Graham Warren, FRS, for accepting me as a PhD student in his laboratory, promoting my research and my education, for his trust and understanding and for his valuable career advice. *I feel honoured to be your last PhD student ever.*

Ass. Prof. Dr. ChristopherLuis de Graffenried, for his constant support and help in getting this project started and developed over the last four years. Without his advice, troubleshooting and moving forward would have been much harder.

Many thanks to:

All the former and current members of the Warren Lab in Vienna**:** Our technicians Nina Pruckner and Katharina Havlicek, Kyojiro Ikeda, Dr. Heather Esson, Dr. Sevil Davidson, Dr. Lars Demmel, Dr. Brooke Morriswood, Dr. Marco Sealey and Dr. Katy Schmidt, for their hospitality and cooperation. *Kyo, thank you for all the shared muffins and bananas and for introducing me to X-Japan*. *Sevil, thank you for our trip to San Francisco, our visits to art galleries, the movie nights at your place and for binding my thesis. Marco, thanks for our long talks about Spain and life in general.*Thanks as well to the students Freia von Raußendorf, Elena Georgieva and Daniel Schönthaler, who spent some months with us and contributed to a great working atmosphere.

Ana Krolo, from Alwin Köhler's Lab, whose amazing help during the weekends made my experiments and my life much easier. *Ana, I am extremely grateful. I admire you as a scientist and as a person.*

Thomas Sauer, Head of the Biooptics-FACS Facility at the Max F. Perutz Laboratories, for his enormous contribution to get the elutriation protocol working and for all his stories about Vienna and Austria. *Vielen Dank!*

Dr. Elisabeth Froschauer-Neuhauser, manager of my PhD Program ¨Molecular Mechanisms of Cell Signaling¨, for her constant assistance in all kind of administrative and educational matters until the very end of this adventure.

All my colleagues and principal investigators in my PhD Program, for our scientific discussions and all the great times spent at retreats or symposia.

Dr. Andreas Brachner, Dr. Juliane Braun, Dr. Mirta Boban, Dr. Selma Osmanagic-Myers and Livija Zlopaša, from Roland Foisner's Lab, with whom I shared Lab space, equipment and many nice anecdotes.

My PhD Committee, Prof. Dr. Manuela Baccarini and Prof. Dr. Gerhard Wiche, for their valuable input in our yearly meetings.

My Thesis reviewers, Prof. Dr. Friedrich Propst and Prof. Dr. Jeremy Mottram.

My very best friends, Gala Guillán Carballeira and Marina Gómez Tutor, who encouraged and supported me through each single day of my PhD life. I feel the years we spent together at university united us for a lifetime. I enjoy so much our meetings, trips and celebrations and despite the distance, I could not feel you any closer.

Rubén Martínez Hernández, an amazing cyclist and friend. With him I shared my earliest passion about natural sciences and still today, we can discuss for hours about the similarities between research and sport.

My other very good long-term friends Camino Martínez Mancebo, Blanka Dutkowska, Blanca Miranda Bautista andDr. Albert Benseny Cases, who congratulated me on the good times and kept me going through the bad ones.

My former classmates, Dr. Daniel Ortiz Trujillo, Dr. Pablo Martín Gago, Miguel Garavís Cabello and Óscar Hernández Alba, members of our *PhD Chat*, a forum forchemistry and biology questions and a discussion platform about the directions our PhDs are heading to. They kept me motivated, cheered me up on those grey days and helped me to figure out what I want to do with my career.

Prof. Dr. Julio Casado Linarejos and Prof. Dr. Chema Hernández Hernández, from the University of Salamanca, and Dr. Manoj Mathews, Dr. Osamu Takayama and Dr. Susana Santos, from ICFO Barcelona. They are all part of the reason why I started this PhD.

My ¨Viennese¨ friends Anita Hunkar, Pernille Rude and Natalia Villanueva García, for all the nice evenings after work.

The whole Traunmüller family, Luise, Rudolf, Rudi, Christiane, Andrea, Maria and Matthias for making me feel in a foreign country like at home, and the Swingolf Linz community for their hospitality and kindness. *Vielen Dank, ihr seid wirklich nette Leute!*

The López-Varga family, Juan Carlos, Mercedes, Carlos, Javier y Miriam. I feel you are an extention of my own family.

Wolfgang Traunmüller, my partner, who was extremely patient with me and always optimistic, full of energy and ideas.

My parents, Carlos Lozano Rubio and Herminia Núñez Fernández. With them at my side, everything makes sense.

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Preface

In eukaryotes, cell division takes place in a series of regulated events known as the cell cycle. Chromosomes and organelles from a mother cell need to be duplicated and segregated into two daughter cells in a coordinated fashion. A complex network of regulatory proteins is involved in orchestrating this process. The most common mechanism used to modulate protein function in signaling pathways is phosphorylation by protein kinases. Among these, the Polo-like kinase family plays important roles in mitotic control and cytokinesis.

In the first chapter of this thesis, I will introduce the structure and regulation of the eukaryotic cell cycle and present a review of the literature regarding the Polo family. I will then describe the protist parasite *Trypanosoma brucei* as a model organism and its Polo-like kinase homologue (TbPLK). In contrast to other Polo-like kinases, TbPLK is excluded from the nucleus and does not seem to be involved in mitosis. Instead, it localizes to several cytoskeletal structures during their duplication. By the end of the chapter, I will introduce an analogue-sensitive approach that has been developed in order to achieve selective inhibition of protein kinases.

The results obtained in my experimental work are summarized in chapter 2 and chapter 3. Chapter 2 documents my studies aimed at understanding how acute and selective TbPLK inhibition in *Trypanosoma brucei* compromises the assembly of several cytoskeletal elements. Chapter 3 provides an overview of different mass spectrometry-based methods we have recently started to work on for the identification of novel TbPLK substrates.

My results are discussed in chapter 4. Finally, chapter 5 includes a description of the materials and methods that were necessary for this work.

Chapter 1. Introduction

1.1. The cell cycle

The cell cycle structure

Cellular reproduction is an essential process in all organisms. The duplication and subsequent segregation of cellular components into two daughter cells must be tightly regulated in order to ensure the fidelity of this process. To serve this purpose, cells have developed a complex machinery of regulatory proteins. This machinery controls the timing of the different cell cycle phases and includes checkpoints that monitor the correct completion of specific events. Dysregulation of the cell cycle is commonly associated with disease progression and linked to several types of cancers(Collins et al., 1997) (Williams and Stoeber, 2012).

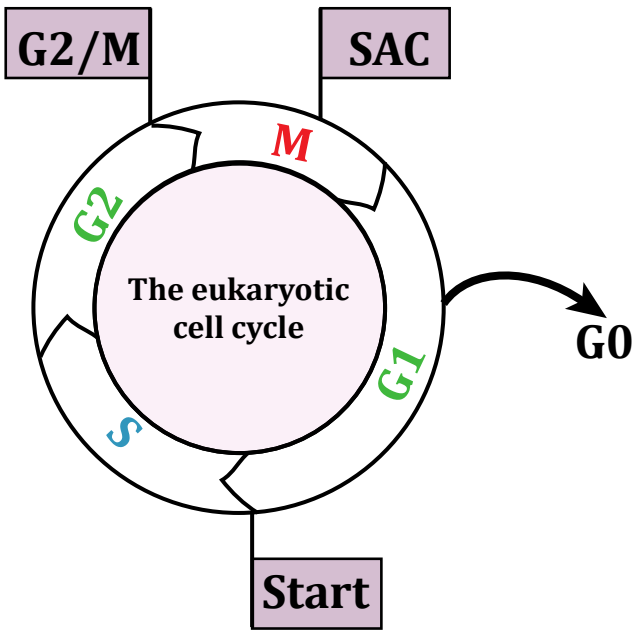

Figure 1 - Schematic representation of the main cell cycle phases and the corresponding gaps. Three main checkpoints control the right order between transitions: Start, G2/M and SAC.

The eukaryotic cell cycle can be generally divided into two main phases: S and M, and two gap interconnecting phases, G_1 and G_2 (Figure 1). The S phase is known as the synthetic phase, as it is during this period where the cellular components, including the DNA, are duplicated. The M phase corresponds to the second part of the cell cycle, when those duplicated components are divided into two daughter cells. It includes both mitosis (nuclear division) and cytokinesis (cytoplasm division). The two gap phases, G_1 and G_2, can be considered as preparatory phases for entering S phase and M phase, respectively. During G_1, cells typically grow in size in response to different stimulating signals, such as growth factors. G_2 also includes cell growth, specifically of those cytoplasmic components required for moving the duplicated chromosomes apart. When a cell is not prepared to divide or has reached a specific non-dividing state during development, it can become arrested in a phase known as $G_{0,}$ or quiescent, state. In this state, cell division ceases transiently or permanently. Whether G_0 is really a distinct phase or just a delay in the cell cycle is still under debate (Mendelsohn, 1962)(Pardee, 1974)(Coller, 2007).

In order to preserve fidelity during cell division, it is essential to control the timing of transitions between the different phases. The cell cycle checkpoints ensure that certain requirements have been met before the cell proceeds to the next phase of the cell cycle. The principles that govern these checkpoints and the consequences of bypassing them have been studied and discussed for a long time (Hartwell and Weinert, 1989)(Murray and Kirschner, 1989). Although multiple surveillance mechanisms have been described so far, we can still highlight three major checkpoints. First, the G1 to S checkpoint, also known as Start, is capable of detecting DNA damage or insufficient cell growth and triggers an arrest or even exit from the cell cycle. Second, The G2 to M checkpoint prevents cells from entering mitosis in case of incomplete DNA replication. The third main transition is the spindle assembly checkpoint (SAC). The mitotic spindle is an array of microtubules whose function is to segregate chromosomes to the daughter cells. If the spindle is not properly assembled, the separation of the duplicated chromosomes can be blocked at this point.

The fundamentals of the structure of the cell cycle and its main checkpoints have been described so far. Many of these are conserved amongst eukaryotes. In general, the cell cycle machinery has been highly conserved throughout evolution, yet there are some unique adaptations in specific eukaryotes.

Experimental organisms in cell cycle research

Budding and fission yeasts have proven to be very productive model organisms in cell cycle analysis because of their simplicity and ease of genetic manipulation. Paul Nurse and others pioneered the work on both budding yeast *S. cerevisiae* and fission yeast *S. pombe*(Forsburg and Nurse, 1991a)(Forsburg and Nurse, 1991b)(Nasmyth, 1996a)(Nasmyth, 1996b)(Mendenhall and Hodge, 1998). Cyclins, an important family of proteins among the cell cycle regulators and cyclin-dependent kinases (CDKs) have been extensively studied in yeast. Both *S. cerevisiae* and *S. pombe* consist of a single cell and can proliferate as haploids, which contain just one complete set of chromosomes instead of two copies. This feature facilitates the introduction of gene mutations and deletions. Conditional mutants that are sensitive to temperature or chemical compounds are also easy to generate. In *S. pombe*, the cell cycle structure and checkpoints are similar to the general eukaryotic ones described before. In *S. cerevisiae*, however, the G_1 phase can overlap with M phase and the existence of a proper G_2 is not very well defined (Kitamura et al., 2007). Mitotic entry is also not so strictly controlled as in other model organisms.

Among multicellular organisms, the frog *Xenopus laevis* has been used as a model for the understanding of embryonic division. Upon fertilization, *Xenopus* eggs start a series of very quick cell divisions that lack the conventional cell cycle gaps; the cells rapidly cycle between S and M phases with intervening cytokinetic events. This leads to an egg being divided into many small cells, called a blastula. The blastula will eventually become a tadpole after several morphogenetic transformations. Interestingly, *Xenopus* was the first organism in which a cell cycle clock or oscillator was identified (Hara et al., 1980)(Newport and Kirschner, 1984). This is a biochemical system based on different feedback loops and involving

cyclins and ubiquitin ligases, among others. The clock is independent of the cell cycle checkpoint system and is able to keep the cell division going even in the absence of certain cell cycle events. The original experiment involved the artificial division of a *Xenopus* egg into two halves, with only one of the two containing the nucleus. The non-nucleated part showed periodic waves of contraction with a similar timing to the divisions happening in the nucleated half (Hara et al., 1980). This shows that this autonomous oscillator in the cytoplasm operates without the major cell-cycle events taking place in the nucleus.

The *Drosophila melanogaster* fruit fly is another well-understood system in cell cycle genetics. Most efforts in the field have been oriented towards the links between development and cell division (Edgar and Lehner, 1996)(Edgar and Datar, 1996). In *Drosophila* development, the structure of the cell cycle varies depending on the stage of embryogenesis. Fertilization is followed by a series of quick cell divisions in the form of S-M cycles, without any gap phases. This is similar to the process described before for *Xenopus*. However, in *Drosophila*, the cytoplasm is not cleaved after each nuclear division, and therefore the duplicated nuclei share a single cytoplasm. It is then later in development when these nuclei eventually become compartmentalized. In such a way, one layer of cells termed the blastoderm is formed. Gastrulation is the process that follows, which consists of the generation of a more complex embryo containing several layers of cells. In later gastrulation, the cell cycle becomes longer and the gap phases G1 and G2 are introduced. The embryo eventually becomes a larva and metamorphoses into an adult fly. Apart from these special features, it is also remarkable that the first Polo homologue, from the Polo family of mitotic kinases, was identified in *Drosophila*(Sunkel and Glover, 1988).

Finally, mammalian cell systems have also been used to study the cell cycle. One of the first challenges was to remove primary cells from tissues or tumors and grow them in culture (Todaro et al., 1963)(Todaro and Green, 1963). The differences in the physiological conditions between the tissue and the culture can lead to senescence. It is however possible to immortalize a cell line, which consists of introducing certain mutations that arrest the cell line at a given

checkpoint. Another difficult challenge was the isolation of mutants from mammalian cell cultures. Chinese hamster cell lines were commonly used in the initial selection (Rosenstraus and Chasin, 1975). The identification and characterization of cell-cycle mutants was even harder mainly because many of them turned out to be lethal. Strategies to generate conditional mutants led to the development of temperature-sensitive mutants (Basilico, 1978). In addition, it was not trivial to choose the most interesting ones from the hundreds of cell-cycle genes. Previous findings in yeast shed light into the discovery of many mammalian cell-cycle genes. *Xenopus* was also used as a system to study the expression and regulation of mammalian mitosis-promoting factors, as it was possible to inject mammalian mitotic extracts into the frog's oocyte and follow its maturation (Nelkin et al., 1980).

Yeast, *Drosophila*, *Xenopus* and mammalian cells have proven to be, as described before, the most commonly used model organisms for cell cycle analysis. The general structure of the cell cycle might vary depending on the organism and the stage in development. The same is true for the different cell cycle regulators. Some species might have more homologues of a given protein than others. Or the same homologue might have specialized in slightly different functions.

However, these conventional model organisms are closely related in the evolutionary timeline. For this reason, it cannot be taken for granted that the molecular mechanisms and regulators identified in these experimental models would also apply for the rest of the eukaryotic kingdom. Whether these processes are universal or not is a subject of current investigation. Comparative studies with early-branched eukaryotes can therefore provide insight into how strongly conserved the cell division machinery is.

The protist parasite *Trypanosoma brucei (T. brucei)* is one of the organisms whose evolutionary distance from other commonly used model systems has become valuable for studying the cell cycle. According to the current eukaryotic phylogenetic tree, *T. brucei* belongs to Excavata. This group is distant in the

evolutionary time scale from Opisthokonta, the group including conventional eukaryotes (Walker et al., 2011). *T. brucei* is unicellular and belongs to the class Kinetoplastida, which includes several flagellated eukaryotes. *T. brucei* is genetically tractable and several molecular tools, such as epitope-tagging (Schimanski et al., 2005), homologous recombination (Kelly et al., 2007), RNA interference (Ngô et al., 1998) and live-cell imaging (He et al., 2004)are currently available. *T. brucei'*s genome has been sequenced. Its rapid doubling time, approximately 9 hours, facilitates the generation of transfectants and large-scale cultures. The cell cycle in *T. brucei* follows the G_1-S-G_2-M pattern described before. However, the cell possesses two DNA-containing organelles: the nucleus and the kinetoplast, the latter containing the mitochondrial DNA. The timing of nuclear replication is different in each of these organelles. The kinetoplast replicates and segregates first, while the nucleus is still in interphase (Woodward and Gull, 1990). The cell cycle control machinery in *T. brucei* seems to be divergent in several aspects. Some regulators are not present at all. And although several orthologues are conserved, some of them appear to have developed different functions. Interestingly, the spindle assembly checkpoint seems to be missing, as spindle mutants are still able to complete cytokinesis (Ploubidou et al., 1999)(Akiyoshi and Gull, 2013). If DNA replication is inhibited, cells are unable to undergo mitosis but do proceed through cytokinesis, producing cytoplasts that lack nuclei. These findings could suggest a cell cycle control mechanism with a different type of checkpoints, which probably monitors the duplication of the cell's elaborate cystokeleton instead of DNA replication. A more detailed description of *T. brucei* is provided in section 1.3.

In this introduction, the focus will be on M-phase, which includes mitosis and cytokinesis. Although a general description for conventional model organisms will be provided, some specific features for *T. brucei* will be highlighted. The progression through M-phase is mainly regulated by a key post-translational modification: reversible protein phosphorylation. In the next pages, the different phases in mitosis and the main families of mitotic kinases will be presented.

Mitosis and its regulation through mitotic kinases

The primary aim of mitosis is to divide the duplicated chromosomes, also known as sister chromatids, into the two daughter cells. After mitosis, cytokinesis produces the two daughter cells. Both mitosis and cytokinesis are differently organized depending on the organism. But generally, for mitosis every cell requires the action of a mitotic spindle, an array of microtubules capable of moving the chromosomes apart. Cytokinesis is also dependent on the action of the spindle. In addition, the cell cortex and an actomyosin ring (composed of actin and myosin) are often involved in cytokinesis.

Protein phosphorylationand protein degradation are the two most prominent post-translational modifications used to control mitosis. They are tightly related, in the sense that protein kinases might phosphorylate certain substrates and target them for degradation, and at the same time protein kinases can be degraded through proteolysis at some point during the cell cycle. As an example, the mitotic kinase Aurora B is able to phosphorylate p53, a tumor suppressor protein that negatively regulates the cell cycle. This phosphorylation targets p53 for degradation in an ubiquitin-dependent manner (Gully et al., 2012). The expression of Aurora B throughout the cell cycle can also be controlled by degradation via the ubiquitin pathway (Nguyen et al., 2005).

For the purpose of this thesis, the focus will be placed on the different families of mitotic protein kinases. The German cell biologist Walther Flemming was one of the pioneers in coining the term mitosis and describing the shape of sister chromatids (1878). During the first half of the 20th century, live imaging allowed a much better understanding of the molecular mechanisms governing mitosis. Today, however, some aspects concerning spindle dynamics and regulatory mechanisms are still not so well-understood.

A general model for mitosis in animal cells divides the cycle into several events with distinctive names (Figure 2). During prophase, chromatin condenses into chromosomes. The spindle position is defined by the prior migration of the

duplicated centrosomes (microtubule organizing centers) to the cell poles. During prometaphase, newly nucleated microtubules start to interact with the sister chromatids by means of a structure called the kinetochore. During metaphase, the sister chromatids align in the middle of the cell and are kept in this equatorial position by opposing yet balanced forces. At this point, the spindle assembly checkpoint (SAC) checks at the kinetochore that each chromosome is correctly attached to its corresponding spindle. After entry into anaphase, loss of cohesion leads to the separation of each sister chromatid, which gets pulled toward opposite poles of the cell. During telophase, the nuclear envelope is reassembled around the separated chromosomes, generating two distinct nuclei. Cytokinesis completes the process by physically cleaving both daughter cells.

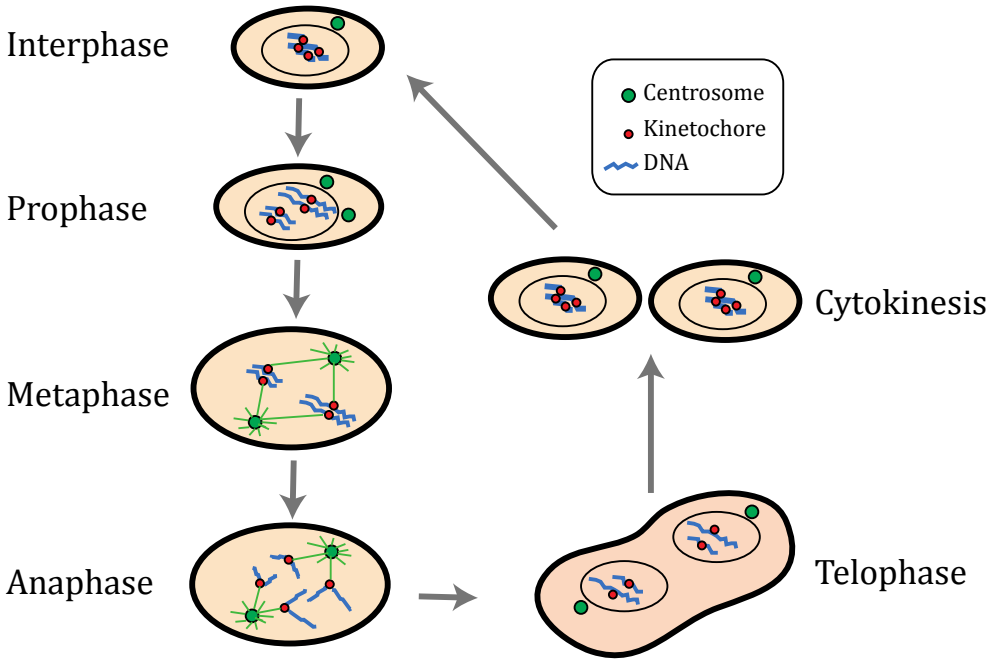

Figure 2 - Schema containing the main phases in mitosis. DNA is displayed in blue, the kinetochore in red and the centrosome (microtubule organizing center) in green. The interaction between DNA and microtubules is highly dynamic.

In *T. brucei*, the nuclear envelope remains intact during mitosis (Vickerman and Preston, 1970)and the mitotic spindle is assembled inside the nucleus. Fewer spindle-specific components have been identified and not a single kinetochore protein has been described so far (Berriman et al., 2005)(Akiyoshi and Gull,

2013). Cytokinesis is not based on an actomyosin ring, but orchestrated by the flagellum and the action of dynamic microtubules (Kohl et al., 2003).

Mitosis generally involves profound structural changes in the cytoskeleton, chromatin and the nuclear envelope, many of which are mediated by the action ofmicrotubules. Cell shape and polarization are some of the parameters under control during this process in many eukaryotes. Several molecular motors participate in the regulation of such dynamic rearrangements in the cell. Kinesins, for instance, are involved in microtubule elongation, assembly and stabilization. Kinesin-mediated processes are often controlled by protein kinases and its counteracting protein phosphatases. Phosphorylation can rapidly and reversibly regulate these events and their associated signaling pathways. For instance, Mklp2 is a molecular motor belonging to the kinesin-6 family and is known to participate in spindle assembly and dynamics (Cesario et al., 2006)(Vale et al., 2009). This kinesin is present in the nucleus during interphase. Upon nuclear envelope disassembly, the mitotic kinase CDK1 forms a complex with cyclin B, CDK1-Cyclin B, and then phosphorylates Mklp2. This is actually an inhibitory phosphorylation that prevents Mklp2 localization to the midzone and therefore the formation of premature spindles. Prior to anaphase, CDK1-cyclin B complexes become degraded. This leads to the dephosphorylation of Mklp2, which can then localize to the spindle and eventually participate in cytokinesis (Hümmer and Mayer, 2009). The main mitotic families include: CDKs, the Aurora, the NIMA and the PLK family.

The Cyclin-dependent kinases (CDKs)

Cyclin-dependent kinases (CDKs) form a complex with a regulatory cyclin subunit in order to become enzymatically active. In fact, CDK activation leads to several structural changes in the active kinase site (De Bondt et al., 1993). CDKs are considered the master regulators of cell cycle progression. Oscillations in the levels of cyclins and cyclin-CDK complexes can be correlated to cell cycle states. Cyclins can be divided in G1, G1/S, S or M cyclins depending on the cell cycle phase at which they are expressed. A given CDK can modulate its function by

binding different types of cyclins throughout the cell cycle. As cyclins are the limiting reagent in the formation of such complexes, the regulation of cyclin expression and degradation has a fundamental significance in cell cycle progression (Morgan, 1997).

Full activation of CDKs also requires the phosphorylation of a residue close to the active site by the CDK-activating kinases, CAKs (Fisher and Morgan, 1994). The Wee1 kinase and the Cdc25 phosphatase act together as a circuit to control CDK activity. Wee1 acts as a negative regulator (Russell and Nurse, 1987), whereas Cdc25 can reactivate CDKs (Strausfeld et al., 1991). Specifically, Wee1 is able to inactivate the cyclin-CDK complex by phosphorylating a conserved tyrosine residue (Tyr15) present in most CDKs. Tyr15 is located at the ATP-binding site and its phosphorylation is likely to compromise the binding to ATP phosphates. CDKs can be reactivated by means of Tyr15 dephosphorylation by Cdc25.

Yeast has only one CDK homologue, Cdk1p. *Xenopus* and *Drosophila* have at least two homologues, CDK1 and CDK2. In humans up to eleven classical homologues have been identified, although some of them are poorly described. The mammalian CDK1 homologue remains the most studied CDK.

CDK1 phosphorylates a variety of substrates, such as lamins, microtubule-binding proteins and motor proteins, or even Golgi components (Lowe et al., 1998). Lamins are structural components of the nuclear lamina. This structure becomes disassembled and reassembled during mitosis by means of reversible depolymerization. This process seems to be controlled partly by CDK1-mediated phosphorylation (Gerace and Blobel, 1980)(Heald and McKeon, 1990)(Heald et al., 1993). CDK1 regulates several aspects of microtubule dynamics in order to prevent aberrant chromosome segregation (Chen et al., 2011). In addition, the phosphorylation of several Golgi proteins has proved to be important for mitotic Golgi rearrangement. For instance, CDK1 is able to phosphorylate the peripheral Golgi component Nir2 and this promotes its release from the Golgi during mitosis. Phosphorylated-Nir2 is able to later interact with PLK1 during cytokinesis at the site of the cleavage furrow(Litvak et al., 2004).

Mitotic entry is regulated by a gradual activation of cyclin-CDK1 complexes (Gavet and Pines, 2010). CDK1 is also involved in promoting anaphase onset (Kramer et al., 2000)and CDK1 degradation triggers mitotic exit and subsequent cytokinesis (Noton and Diffley, 2000).For a long time, CDK2 was believed to be essential for mitotic entry (van den Heuvel and Harlow, 1993). But it was later shown that it is actually dispensable and CDK1 can take over CDK2's functions (Ortega et al., 2003).

Generally speaking, we can consider that CDKs are the driving force in cell cycle progression, whereas Aurora and Polo kinases have specialized in fidelity control. CDKs, Auroras and PLKs do not act in an isolated manner. There is, in fact, substantial evidence of different levels of crosstalk among their signaling cascades. As an example, CDK1 can act as a priming kinase for PLK1 (Elia et al., 2003)(Zhang et al., 2009). This is the case for Nedd1, a known PLK1 substrate. CDK1 phosphorylates Nedd1 so that Nedd1 can bind PLK1 and release it from its self-inhibitory state. Active PLK1 can afterwards phosphorylate Nedd1 at additional phosphorylation sites.

The Aurora kinases

Aurora was initially identified in *Drosophila* and yeast (Giet and Prigent, 1999)(Bischoff and Plowman, 1999). As mutations in Aurora led to monopolar spindles, the name "Aurora" was taken as a reference to the North Pole. Unfortunately, the nomenclature adopted to describe Auroras has now turned out to be quite confusing. For sake of simplicity, we can distinguish three main classes of mammalian Auroras: Aurora A, Aurora B and Aurora C. Aurora C is rather uncharacterized, it seems to be highly expressed in the testis and in some tumor lines (Kimura et al., 1999). In yeast, there is only a single Aurora homologue, which receives the name Ipl1p. *Xenopus* and *Drosophila* both have two homologues with several different names, but they correspond essentially to Aurora A and B.

Aurora A and B present different subcellular localizations. Aurora A is normally associated with centrosomes and the mitotic spindle throughout mitosis, whereas Aurora B seems to be rather more restricted to the equatorial zone during metaphase and anaphase, and later at the site of cytokinesis initiation. These localization patters correlate with their attributed functions. Thus, Aurora A seems to play a role in centrosome function and spindle assembly (Glover et al, 1995), whereas Aurora B has been related to microtubule-kinetochore attachment, spindle checkpoint and cytokinesis initiation (Murata-Hori et al., 2002)(Murata-Hori and Wang, 2002)(Kallio et al., 2002).

There are not many substrates identified and characterized for Auroras. Histone H3 and the motor protein Eg5 have been suggested as putative substrates. Phosphorylation and mitotic degradation by proteolysis are the most common ways to regulate Aurora activity.

Another significant example of interaction between the different families of mitotic kinases is the fact that Aurora A is able to activate PLK1. The activation of PLK1 prior to mitotic entry requires that Aurora A phosphorylates PLK1 in Thr-210 (Macurek and Medema, 2008). This activation process is enhanced by the presence of Bora, a cofactor for Aurora A. As it is also the case for PLKs, Auroras are overexpressed in several types of tumors. Several Aurora inhibitors have already been developed and are being tested at several clinical stages.

The NIMA-related kinases (NEKs)

NIMA (Never-in-mitosis A) kinase was originally identified in the fungus *Aspergillus nidulans*(Oakley and Morris, 1983). All the NIMA-related kinases are known today as the NEK family of kinases (NEKs). NEKs are probably the least well-understood family of mitotic kinases. However, in recent years many homologues have been identified in different eukaryotes, such as Drosophila, mice and humans. In particular, humans express eleven NEK proteins.

When NIMA was originally identified in *Aspergillus*, it was reported to be necessary for mitotic entry (Osmani et al., 1991)and its degradation was required for mitotic exit (Pu and Osmani, 1995). However, human NEKs do not seem to be necessary for mitotic entry. The latest findings suggest that NEKs might have specialized in regulating microtubule-dependent processes during the cell cycle (Fry et al., 2012). NEK2, which is the closest NIMA-homologue in mammals, seems to be involved in centrosome splitting and chromosome segregation (Fry et al., 1995). Among the best-studied human homologues, NEK6, NEK7 and NEK9 act together in a concerted way and seem to have a role in spindle formation (Yissachar et al., 2006)(Fry et al., 2012). NEK1, NEK4 and NEK8 have been implicated in cilia assembly and stability (Otto et al., 2008)(Thiel et al., 2011)(Coene et al., 2011). A general hypothesis is that those organisms that lack cilia, such as *Aspergillus*, do not conserve NEKs with cilia-associated functions, whereas ciliated organisms might contain more NEK homologues with a cilia-related function (Fry et al., 2012). In coming years we will probably gain a better understanding of the whole NEK family and their interactions with other mitotic kinases. A more detailed description of the contribution of NEKs to genomic stability will also increase the possibility of exploiting them as anti-cancer targets.

In *T. brucei*, the basic mitotic kinases seem to be conserved. The CDK/Cyclin machinery is present (Hammarton et al., 2003)and participates in the G_1/S and the G_2/M transitions (Tu and Wang, 2004)(Tu and Wang, 2005)(Li, 2012). *T. brucei* contains eleven CDKs (Parsons et al., 2005)and ten cyclins. There is an Aurora B-like kinase (TbAUK1) involved in mitosis and cytokinesis(Tu et al., 2006). TbAUK1 forms part of a complex known as chromosomal passenger complex (CPC) together with the CPC proteins TbCPC1 and TbCPC2 (Li et al., 2008a)(Li et al., 2008b). The localization of this complex varies according to cell cycle progression. It migrates from the chromosomes to the spindle during mitosis and localizes to the cleavage furrow during mitosis. There is as well a NIMA homologue, known as TbNRKC, which shares approximately 50% identity with both the humans NEK1 and NEK2 homologues. TbNRKC seems to be necessary for basal body segregation (Pradel et al., 2006).

The Polo-kinase family (PLKs)

Our final focus rests on the Polo-like kinase family (PLKs). All the experimental work in this thesis is related to the single PLK homologue in the protist *T. brucei*.

The first PLK was identified in *Drosophila* and was named Polo (Sunkel and Glover, 1988). PLKs turned out to be highly conserved throughout evolution and several other homologues were identified afterwards. In mammals, there are five PLK homologues (PLK1-5), with PLK5 having been described only recently (Andrysik et al., 2010)(de Cárcer et al., 2011). Yeast contains only one homologue corresponding to PLK1, named Plo1p in *S. pombe* and Cdc5p in *S. cerevisae*. In *Drosophila*, both PLK1 (Polo) and PLK4, also known as SAK, are present. In Xenopus, five PLK homologues have also been characterized (de Cárcer et al., 2011).

PLKs share common structural features that are unique to the protein family. They have a highly conserved kinase domain in the N-terminal region followed by a carboxy-terminal Polo-box regulatory domain (Elia et al., 2003)(Yu et al., 2012). This regulatory region, which actually consists of one or two polo-boxes, serves as a docking site for several phosphorylated substrates and targets PLKs to specific subcellular compartments (Barr et al., 2004).

Among mammalian PLKs, PLK2 seems to be present at the centrosomes and involved in centriole duplication (Warnke et al., 2004)(Chang et al., 2010). PLK3 is necessary for mitotic entry and promotes DNA replication(Zimmerman and Erikson, 2007). PLK4 (also called SAK) is a key regulator of centriole replication (Bettencourt-Dias et al., 2005)(Habedanck et al., 2005). The specific functions of PLK5 are not so well understood yet.

PLK1 and its orthologues Plo1p, Cdc5p and polo seem to have conserved mostly the same functions. In *T. brucei*, there is a single homologue, TbPLK, which strikingly is not involved in mitosis (Kumar and Wang, 2006)but has specialized in several functions associated with cytoskeletal inheritance (Hammarton et al., 2007a)(de Graffenried et al., 2008)(Ikeda and de Graffenried, 2012). A more

detailed description of the general PLK1 structure, regulation, and functions in these model organisms will be provided in the next sections of this chapter. The focus will eventually be TbPLK and the molecular mechanisms governing its unusual functions.

1.2. Polo-like kinase 1 (PLK1)

PLK1 is an important regulator of mitosis and its functions are conserved in a variety of organisms. PLK1, like all PLKs, presents a characteristic structure and its activity is tightly regulated. How exactly this regulation takes place depends on the organism, but generally, it is controlled by means of PLK transcript levels, PLK phosphorylation by upstream kinases and PLK proteolytic degradation.

PLK1 has a conserved structure

PLK1 is a Ser/Thr kinase composed of two distinct domains. The kinase domain in the N-terminus is the catalytic domain, whereas the polo-box domain (PBD) in the C-terminus acts as a regulatory domain (Lowery et al., 2005). The kinase domain is very conserved throughout all the PLK homologues and is similar to the Aurora kinase domain. The PBD comprises two polo-boxes (PBs). A linker region and a small polo-cap region (Pc) are responsible for connecting these two domains (Barr et al., 2004). In the kinase domain, there is a conserved key Thr210 residue located at the activation T-loop. Thr210 phosphorylation by Aurora A is essential for PLK1 activation (Macůrek et al., 2008).

Several substrates for PLK have been proposed so far, such as the phosphatase Cdc25 (Nakajima et al., 2003) the kinase Wee1 (Sakchaisri et al., 2004)(Watanabe et al., 2004)the kinetochore protein Bub1(Qi et al., 2006)and the Golgi protein GRASP65 (Lin et al., 2000).

A pocket in the PBD comprises the residues Trp-414, His-538 and Lys-540, which constitute a phosphopeptide-binding motif. In this way, PLK is able to bind proteins that have previously been phosphorylated (Elia et al., 2003). The

effectors of these priming phosphorylations are usually CDKs or other mitotic kinases that precede PLK1 and target it to specific substrates.

Two models have been proposed in order to describe how the PBD might exert its regulatory control over the kinase domain (Lowery et al., 2005). In the first model, also called the *processive* model, when the PBD binds a certain protein, this one becomes later phosphorylated on a different residue by the kinase domain of PLK1. According to this model, PLK substrates should contain both kinase and PBD- binding domains. An alternative *distributive* model suggests that the binding of the PBD domain to a specific protein targets the kinase to a given cellular localization with specific PLK substrates. In this model, the ligands for the PBD and the substrates for PLK are therefore different. These two models do not necessarily exclude each other and might vary depending on the substrates.
Interestingly, an inhibitory interaction between the kinase domain and the PBD has also been described. The C-terminal domain is able to bind either the full-length PLK or just its catalytic domain. The binding is destroyed if the Thr210 residue is mutated. The exact mechanisms for this interaction are not so well understood, but it certainly reduces the levels of kinase activity and the binding to phosphopeptides, as well as the phosphospecificity (Jang et al., 2002).

PLK1 regulation by transcription, phosphorylation and protein degradation

PLK1 is regulated at the transcriptional level in a variety of organisms. PLK levels are higher at the time of mitotic entry (Uchiumi et al., 1997). During G1, PLK1 expression is repressed and p53 has been proposed as a negative transcriptional regulator (Martin and Strebhardt, 2006).

As mentioned before, phosphorylation at the Thr210 residue within the T-loop region by Aurora A is a conserved mechanism for PLK activation. Bora, an activator of Aurora A, is required for this phosphorylation event. At the same time, PLK regulates Aurora A by targeting Bora for degradation (Seki et al., 2008). Reversibly, the protein phosphatase 1 (PP1) is capable of inactivating PLK by dephosphorylating Thr210 (Yamashiro et al., 2008). But Thr210 is not the only

residue involved in PLK regulation. Phosphorylation might also take place at sites outside the T-loop, although they do not seem to be so well conserved. As an example, phosphorylation at Ser49 by the PAK1 kinase enhances PLK1 activity at the metaphase to anaphase transition (Maroto et al., 2008).

The third main mechanism for regulation is protein degradation. PLK1 can be ubiquitinated and then targeted for degradation by the proteosome (Ferris et al., 1998). In fact, PLK1 is a target of the Anaphase Promoting Complex (APC), a ubiquitin ligase complex that is able to recognize a destruction box in the PLK sequence (Lindon and Pines, 2004). PLK1 usually becomes degraded during G1 and at the end of mitosis.

PLK1 dysregulation is commonly associated with disease in humans. PLK1 overexpression is frequent in several types of tumors, such as melanomas or gliomas, ovarian, prostatic and breast cancers. Very high PLK levels are usually associated with the worst prognosis. Both the PLK1 kinase domain and the PBD are interesting targets for drug development. The dihydropteridinone BI 2536 is one the strongest and most selective PLK1 inhibitors available (Steegmaier et al., 2007)and it is being clinically tested at the moment.

PLK1 localization and functions

PLK1 localization normally depends on its interaction with microtubules and microtubule-associated proteins (Figure 3). In mammals, PLK1 initially localizes to the centrosomes during G2 and later moves to the centromeres in prophase and to the kinetochores in metaphase. During anaphase and telophase, PLK1 migrates to the mitotic spindle. It also localizes to the midbody during cytokinesis(Barr et al., 2004)(Archambault and Glover, 2009). In Drosophila and yeast, there are some slight variations in these localization patterns, but in all cases PLK1 localizes to the spindle poles and to the site of cytokinesis/cell fission initiation. The centromere localization does not seem to be conserved in Drosophila or yeast. The same is true for the kinetochores in yeast. The fact that these mammalian localizations are not conserved in lower eukaryotes might imply

that the PLK roles at these sites are not absolutely essential. For instance, PLK1 levels are normally high on unattached kinetochores, which would suggest that PLK might be involved in the Spindle Assembly Checkpoint (SAC). In fact, PLK1 phosphorylates Bub1, a conserved SAC protein (Ahonen et al., 2005)(Marchetti and Venkatachalam, 2010). However, PLK1 does not seem to be completely essential for SAC activation, as SAC-driven mitotic arrest proceeds normally upon PLK1 RNAi or inhibition (Elowe et al., 2007)(Petronczki et al., 2008).

PLK1 is required for entry into mitosis (Sumara et al., 2004)(van de Weerdt and Medema, 2006). Although the CDK1-cyclin B complex is the strongest promoter of mitotic entry, PLK1 has been described as part of the positive feedback that leads to CDK1 activation. Specifically, PLK1 can phosphorylate the phosphatase Cdc25, which in turn is able to activate CDK1. This function is highly conserved from yeast to mammals.

PLK1 is also involved in promoting centrosome maturation both in humans and Drosophila. The centrosomes are usually composed of a pair of centrioles and some pericentriolar material (PCM), and act as a nucleating center for microtubules. Specifically, PLK1 seems to be required for the recruitment of the γ-tubulin ring complex, which is necessary for the nucleation of microtubules at the centrosomes (do Carmo Avides et al., 2001).

One of the most important moments during mitosis is the segregation of the sister chromatids to the two daughter cells. PLK1 also participates in controlling this crucial transition. The sister chromatids are bound together by means of cohesin-complexes. PLK1 is involved in promoting the cleavage of SSC1, one sister-chromatid cohesion protein (Losada et al., 2002)(Sumara et al., 2002). Mitotic exit is triggered by the degradation of the CDK1-Cyclin B complex and this event requires the action of the APC. PLK1 is able to activate the APC complex in mammals (Golan et al., 2002)(Kraft et al., 2003).

Finally, PLK1 is required for cytokinesis and this is probably its most conserved role, from yeast to humans. In mammals, PLK1 has been described to interact

with RhoA, a GTPase involved in the assembly of the actomyosin ring during cytokinesis (Burkard et al., 2007).

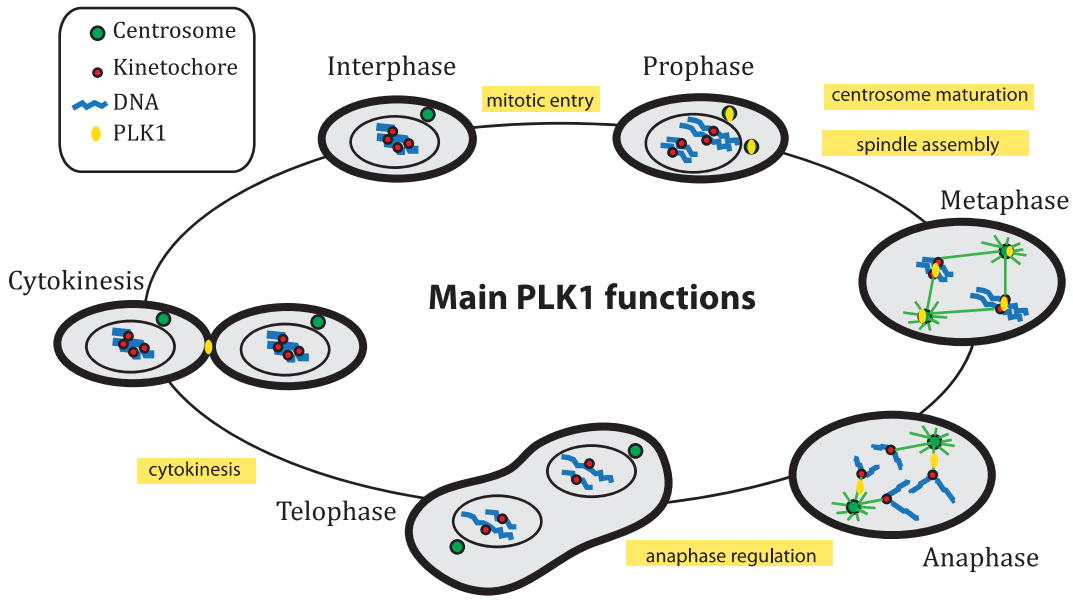

Figure 3 - Schematic representation of the main PLK1 functions at different cell cycle stages. The centrosome is depicted in green, the kinetochore in red, DNA in blue and PLK in yellow. The functions represented here mainly correspond to mammalian cells.

As described so far, PLK1 is an important regulator of mitosis and is involved in several mitotic events. Some of its functions are highly conserved, although the exact mechanisms might differ between species. The main functions associated to PLK1 at the moment can be summarize as follows: mitotic entry, centrosome assembly, sister chromatid separation, exit from mitosis and cell division.

In *T. brucei*, TbPLK does not localize to the nucleus at any time, which implies that most of the previously described functions are not conserved. In addition, its contribution to cytokinesis seems to be indirect. TbPLK has a dynamic and very characteristic localization pattern during the cell cycle. It migrates from the posterior to the anterior end of the cell by localizing to several cytoskeletal structures. The next section will present *T. brucei* as a model organism in cell biology and will offer a careful description of TbPLK, its localization and its attributed functions.

1.3. Polo-like kinase in *Trypanosoma brucei* (TbPLK)

Trypanosoma brucei as a pathogen: sleeping sickness

Trypanosoma brucei (*T. brucei*) is a unicellular protist parasite from the kinetoplastids class. Kinetoplastids posses a distinctive organelle with a network of circular DNA inside a single mitochondrion (Maslov et al., 2002).*T. brucei* is known for its clinical and veterinary relevance: it is the causative agent of African trypanosomiasis. Colloquially, trypanosomiasis is referred to as *sleeping sickness* in humans and *nagana*in cattle (Cox, 2004). The parasite is transmitted between hosts through the bite of the hematophagous tsete fly (*Glossina* genus), meaning that its life cycle alternates between the mammalian bloodstream and the fly's midgut (Marcello and Barry, 2007). Upon infection, the disease goes through different clinical stages and can become fatal if the patient does not receive treatment. In the first phases, trypanosomes multiply in the bloodstream and the lymph, and the symptoms are those similar to an infection, including fever, lymphadenopathy and hepatosplenomegaly (Bouteille et al., 2003)(Stich et al., 2002). Later in disease progression, they are able to cross the blood-brain barrier and induce severe neurological damage that leads to a permanent state of behavioral changes, confusion and somnolence, which can eventually end up in coma. Over the last century, major epidemics occurred in several African countries but thanks to control measures the number of reported cases was highly reduced by the 1960s. However, and mostly due to relaxation in these control policies, the parasite is rebounding in several African countries. According to the World Health Organization (www.who.int, stand June 2013), the number of people at risk at the moment is around 70 million and the number of estimated cases is approximately 30,000/year. Most of them have been reported in the Democratic Republic of Congo, Sudan and Chad. The disease still has a big impact on human health, agriculture and farming in the affected regions. The treatment for the patients is different depending on the stage of infection, but it generally involves the administration of highly toxic drugs (for instance, melarsoprol, an arsenic compound) with severe side effects. To date, there has not been a successful vaccine. Trypanosomiasis is often associated with the so-called *neglected tropical*

diseases, those diseases that only affect poor rural areas and therefore attract less attention from the international community.

T. brucei as a model organism

Three subspecies of *T. brucei* have been described: *T.brucei brucei*, *T. brucei gambiense*, and *T. brucei rhodesiense*. Among those, *T. b. brucei* can infect cattle but not humans (Hoare, 1966)(Cox, 2004). Human serum includes a subfraction of high-density lipoprotein (HDL) containing the apolipoprotein L-1 (APOL1), which has trypanocidal activity towards *T. b. brucei* but not towards the other two subspecies, which apparently have become resistant (Hawking, 1973)(Raper et al., 1996)(Vanhamme et al., 2003)(Pérez-Morga et al., 2005). A recent paper has shown that this resistance might be mediated via a specific glycoprotein (TgsGP) in *T. brucei gambiense*. This glycoprotein seems to stifften the trypanosome lysosomal membrane and prevent APOL1 incorporation into the membrane, which in non-resistance species leads to disturbance of the lysososmal physiology and eventually cell bursting and death (Uzureau et al., 2013).

T. b. brucei is probably the most commonly used subspecies in biology research due to the fact that it is unable to infect humans. The experimental work in this thesis has been done exclusively in *T. b. brucei*. For simplicity purposes, it will be referred to hereinafter only as *T. brucei*. The life cycle in *T. brucei* shuttles between the tsetse fly and the mammalian host. This life cycle includes several cell states, which differ in morphology and metabolic properties. The two most characteristic states are the procyclic form (PCF), resident in the fly, and the bloodstream form (BSF), which can be found in mammals. The PCF form has been used for the totality of the work included in this thesis.As previously mentioned, *T. brucei* has gained interest as a model organism in biology mostly because of its medical importance and its evolutionary distance from conventional model organisms. In addition, a sequenced genome (Berriman et al., 2005)and an inheritable and inducible RNAi system facilitate its genetic manipulation (Lee and Van der Ploeg, 1990)(Wirtz et al., 1999)(Wang et al., 2000).

The cytoskeleton of *T. brucei*

The trypanosome cell differs greatly from a typical mammalian cell in shape, morphology and organelle topology. In order to understand how cell division takes place in this parasite, a careful understanding of its highly ordered cytoskeleton is essential. The cell is highly polarized and presents a leaf-like shape with the pointed part situated at the anterior pole (Figure 4). Strikingly, the cell does not contain cytoplasmic microtubules, but a microtubule corset under the plasma membrane that maintains the cellular architecture. The corset is tightly associated with the cytoplasmic side of the plasma membrane. These microtubules are nucleated at the anterior part of the cell and stretch towards the posterior end (Hemphill et al., 1991a)(Hemphill et al., 1991b)(Robinson et al., 1995). At this end, an invagination of the plasma membrane known as the flagellar pocket (FP) can be found. Adjacent to the FP, the mature basal body nucleates the flagellum, which emerges from the FP and extends towards the anterior pole of the cell, remaining all the time adhered to the cell body (Henley et al., 1978)(Field and Carrington, 2009)(Lacomble et al., 2009). The Flagellar Attachment Zone (FAZ) is responsible for keeping the flagellum attached, meaning that the flagellum beating leads to the movement of the entire cell. The FAZ is comprised of a proteinaceous filament (EDF) and a set of four microtubules or microtubule quartet (MTQ) (Vickerman, 1969). A probasal body can be found orthogonal to the mature one, and the MTQ actually nucleates between both basal bodies, then goes around the FP and runs parallel to the plasma membrane. Interestingly, the polarity of the MTQ is opposite to the one corresponding to the corset(Sherwin and Gull, 1989a). The new flagellum exits the flagellar pocket at the site of the flagellar pocket collar (FPC), which maintains the FP structure (Gull, 2003)(Bonhivers et al., 2008). Surprisingly, and despite the fact that *T. brucei* is characterized by a very active membrane trafficking, all the exocytic and endocytic events in the cell are restricted to the FP (Engstler et al., 2004). The cell typically presents a single Golgi complex, which is situated close to the anterior part of the FP. An additional cytoskeletal structure termed the bilobe is localized nearby. Several bilobe components have been identified so far, including the centrins TbCentrin2 and TbCentrin4, a type of small-calcium binding proteins, and the MORN-like repeat protein TbMORN1 (Morriswood et al., 2009). A recently developed proximity-

based biotynilation assay (Roux et al., 2012)also identified seven new bilobe components when applied for the first time to *T. brucei*(Morriswood et al., 2013). The bilobe has been proposed to be involved in Golgi duplication, although its specific functions are still unclear (He et al., 2005)(Morriswood et al., 2009)(Zhou et al., 2010)(Esson et al., 2012). *T. brucei* presents a two-unit genome, as the DNA is organized in two organelles: the nucleus and the kinetoplast. The kinetoplast contains a network of mitochondrial DNA (kDNA). The basal body is connected to the kinetoplast by means of a series of filaments that constitute the tripartite attachment complex (TAC) (Ogbadoyi et al., 2003).

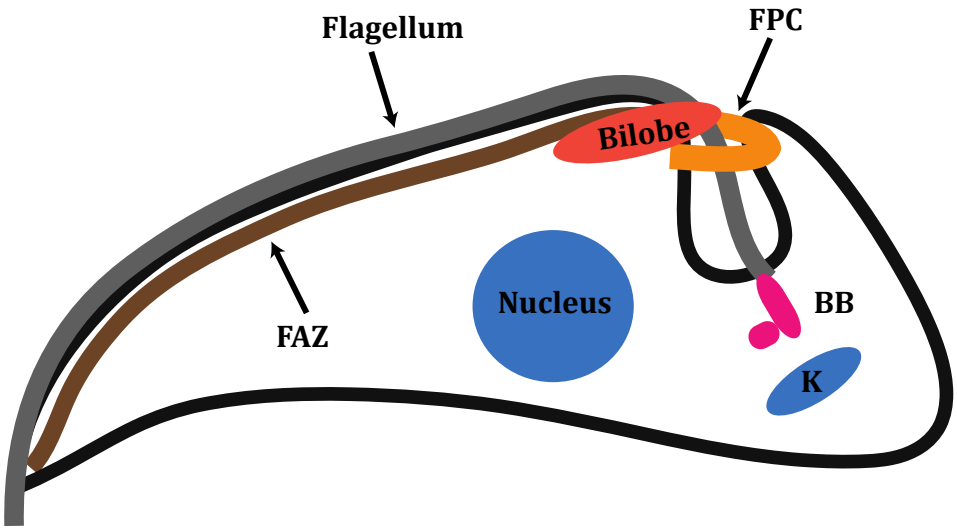

Figure 4- Diagram of a *T. brucei*'s cell including the main organelles. BB stands for Basal Body, FPC for Flagellar Pocket Collar, K for Kinetoplast and FAZ for Flagellar Attachment Zone. The flagellum nucleates at the posterior end of the cell and extends towards the anterior pole.

Several unique organelles make this cell distinctive in comparison with conventional mammalian cells. In addition, some processes during mitosis seem to work differently. For instance, the nuclear envelope remains intact during mitosis. But in common with other eukaryotes, *T. brucei* needs to coordinate a series of events during its cell cycle. A clear fact is that cell motility, essential for viability and important in the infection process, also plays a role in cell division (Ralston et al., 2006)(Broadhead et al., 2006). As described before, most of the organelles in *T. brucei* are related to the flagellum, which can be considered a key player in the cell division process.

The cell cycle in *T. brucei*

Most organelles in the cell are present in a single copy, which makes *T. brucei* a simple model system to study organelle inheritance. The cell cycle lasts approximately 9h. The duplication of the flagellum and its associated structures occurs in a precise order and is highly regulated (Sherwin and Gull, 1989b). However, this regulation seems to be achieved through some novel cell cycle control checkpoints different from mammalian cells. The fact that *T. brucei* is able to start cytokinesis without having completed mitosis, strongly suggests that some of the conventional eukaryotic checkpoints are missing (Robinson et al., 1995)(Ploubidou et al., 1999). The current hypothesis implies that the cell monitors the status of the cytoskeleton division, instead of DNA replication. For instance, it has been proposed that cytokinesis entry might be mediated by controlling the status of kinetoplast division (Ploubidou et al., 1999). The following analysis of *T. brucei*'s cell cycle will be based on the duplication of the main cytoskeletal structures. *T. brucei's* cell division begins with the assembly of a new MtQ prior to the elongation and maturation of the probasal body (McKean, 2003)(Lacomble et al., 2010). The nucleation of the new flagellum is next, an event that seems to be dependent on the recruitment of γ-tubulin (McKean et al., 2003). The basal bodies then duplicate, and the FP and FPC duplication follows. This allows each flagellum to be contained in an individual FP (Gadelha et al., 2009)(Ikeda and de Graffenried, 2012). The bilobe organelle duplicates shortly after that, and when the new flagellum grows out of its corresponding FP, the EDF (FAZ component) starts to assemble. Flagellum and FAZ growth are then coupled to ensure flagellar adhesion. Both the EDF and the MtQ grow parallel to the plasma membrane towards the anterior pole of the cell(Lacomble et al., 2009)(Ikeda and de Graffenried, 2012). During this elongation process, the basal bodies segregate completely, the kinetoplast duplicates and nuclear replication occurs. An additional element known as the flagellar connector (FC) is able to link the old and the new flagellum, facilitating control over the new flagellum's position (Moreira-Leite et al, 2001). The last step corresponds to cytokinesis, which is not based on an actomyosin ring like the classical model in animal cells. The cleavage site for furrow initiation seems to be determined by the position of the new flagellum or the new FAZ, as flagellar mutants with very short new flagella present

abnormal selection of the cleavage site (Kohl et al., 2003). There is currently no evidence of the participation of actin, myosin or septin, common regulators of cytokinesis in other organisms, in furrow ingression (García-Salcedo et al., 2004)(Hammarton et al., 2007b).

The biology governing this parasite's cell cycle is quite intriguing. Many regulators and organelles have not yet been well characterized. For instance, there are rather few FAZ components identified, FLA1 and FAZ1 are the most characterized ones (Nozaki et al., 1996)(Vaughan et al., 2008). Little is known about the functional relevance of the bilobe structure. The uncoupling of mitotic and cytokinetic regulation is also not well understood. And the existence of unique checkpoints, very different to the conventional mechanisms, could be exploited for evolutionary studies as well as drug discovery.

Despite this divergence, several mitotic kinases have been conserved, some of them adapting their function to specific needs in *T. brucei*. There is evidence that CDKs are involved in progression through the cell cycle. For instance, CYC2 (a G1 cyclin) depletion results in G1 arrest(Li and Wang, 2003)and a compromised morphology originated by abnormal microtubule extension at the posterior pole of the cell (Hammarton et al., 2004). The mitotic CDK1 homologue, CRK3, and its corresponding mitotic cyclin CYC6 have been involved in the G2/M transition as their knockdown resulted in mitotic arrest (Tu and Wang, 2004). TbAUK1 depletion leads to impaired mitotic spindle formation and cytokinesis failure (Tu et al., 2006). NRKC, the NIMA-homologue in *T. brucei*, has also been implicated in basal body separation (Pradel et al., 2006). And finally, TbPLK, is of special interest because of its unusual localization and its specialization in flagellar inheritance.

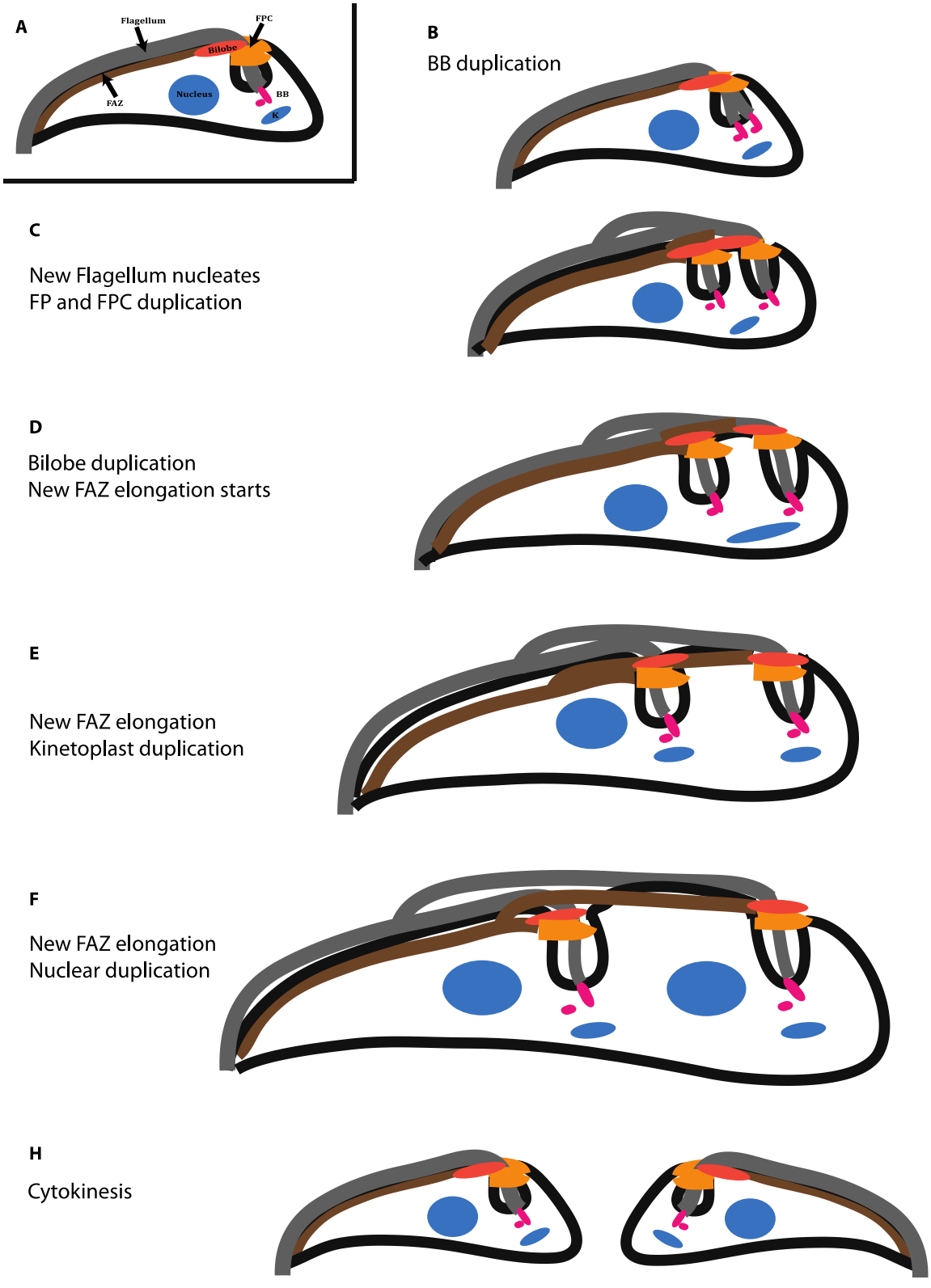

Figure 5 - The main events in *T.brucei*'s cell cycle. The BB duplication is one of the earliest events, followed by the duplication on the organelles present in the pocket area. The new FAZ assembles and extents towards the posterior end of the cell. Cytokinesis gives rise to two daughter cells.

TbPLK

TbPLK shares approximately 50% identity with mammalian PLKs in its catalytic domain (Graham et al., 1998). The fact that TbPLK depletion produces cells that are unable to undergo cytokinesis suggests a role in cell division. However, whether this is a direct effect, or rather a consequence of the abnormal assembly of other organelles has been debated.TbPLK's localization pattern during the cell cycle starts at the MtQ and continues at the basal bodies. TbPLK depletion does not however play a role in basal body duplication or maturation, only on their segregation (Ikeda and de Graffenried, 2012). This is in agreement with PLK1 function in mammals, whose depletion does not affect centrosome duplication but separation(Lane and Nigg, 1997)(Tsou et al., 2009). Basal body segregation in *T. brucei* is thought to be mediated by the new flagellum (Davidge et al., 2006)but how exactly TbPLK might participate in this process in unknown. TbPLK later localizes to the bilobe and to the FPC and is involved in the duplication of both structures: its depletion causes defects in new bilobe assembly (de Graffenried et al., 2008) and compromises the formation of a new FPC (Ikeda and de Graffenried, 2012).TbPLK is next observed on the tip of the growing new FAZ. Interestingly, the kinase can be observed simultaneously at the posterior part of the new bilobe. This suggests that the bilobe and the EDF part of the FAZ could be elements of a continuous cytoskeletal structure (Esson et al., 2012) (Ikeda and de Graffenried, 2012). TbPLK then migrates along the FAZ as long as this grows and disappears shortly before the cleavage furrow starts (Ikeda and de Graffenried, 2012). TbPLK depletion compromises the assembly of the new FAZ, which in turn leads to flagellar detachment (de Graffenried et al., 2008) (Ikeda and de Graffenried 2012). As previously mentioned, the position of the new FAZ could influence the site of cleavage furrow initiation prior to cytokinesis (Vaughan, 2010). The kinase might also be necessary for the proper assembly of the new MtQ (Ikeda and de Graffenried, 2012). As the MtQ is situated in the path that TbPLK follows and PLK1 is often associated with microtubules (Peters et al., 2006)one could hypothesize that the kinase benefits from the microtubule quartet for its migration towards the anterior end (Ikeda and de Graffenried, 2012). Finally, TbPLK has also been related to the biogenesis of the Golgi apparatus, as kinase depletion led to a heightened Golgi number (de Graffenried et al., 2008). However,

how exactly these additional Golgi originate and how TbPLK could mediate this process is still unknown.

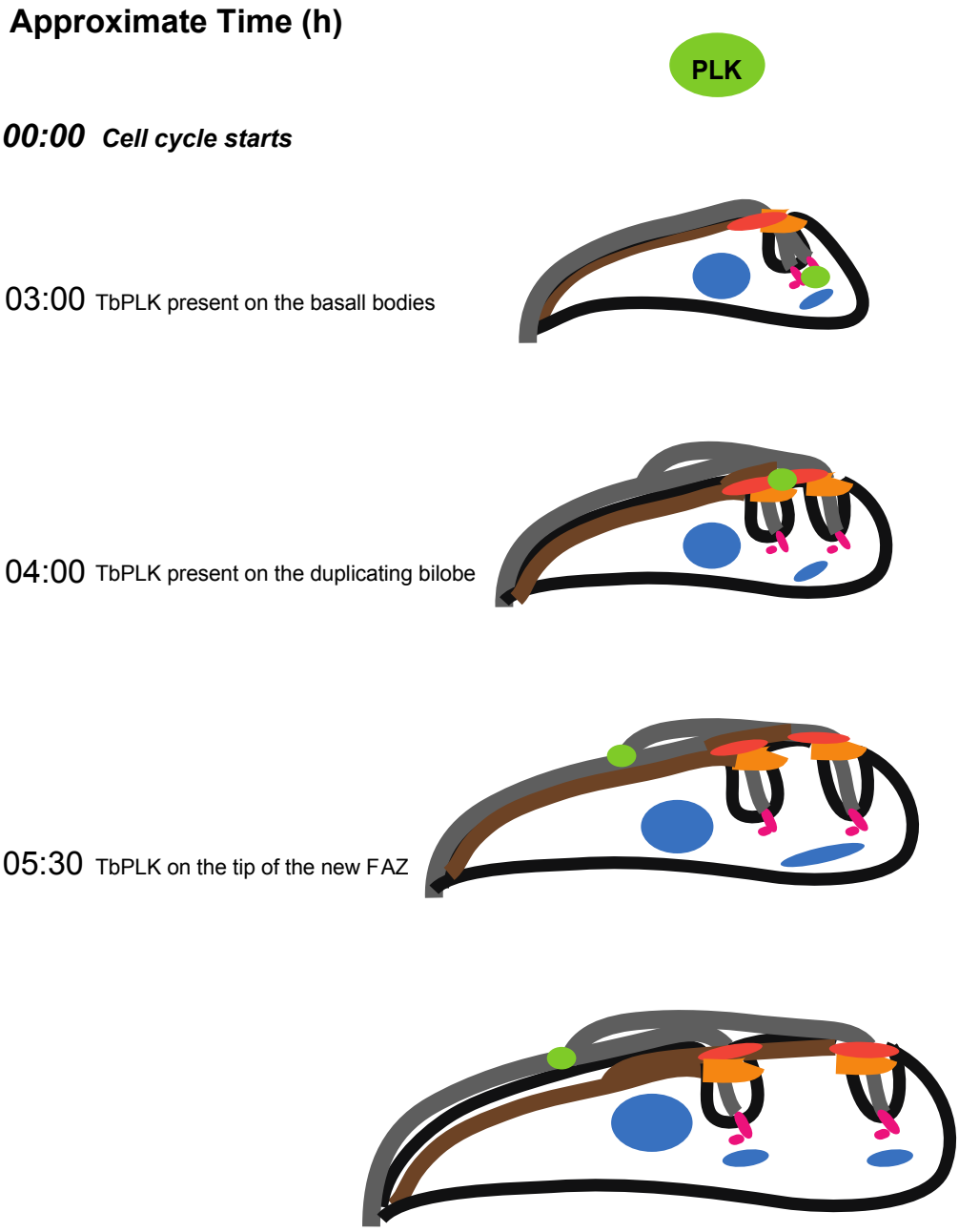

Figure 6 - TbPLK dynamic positioning during cell division. The kinase localizes to dividing structures during their duplication. Timing based on Ikeda and de Graffenried, 2012.

Ikeda and de Graffenried (Ikeda and de Graffenried, 2012)have provided a careful temporal analysis of PLK positioning during the cell cycle. In addition, there is substantial evidence of a requirement for TbPLK in the duplication of several cytoskeletal structures. However, the exact contribution of PLK to bilobe and FAZ duplication has not been well described. TbPLK depletion defects in FAZ assembly might just be a consequence of abnormal bilobe replication. And whether the kinase is required for the whole FAZ elongation or just for its initiation also needs to be clarified. In addition, Centrin2 is the only known TbPLK substrate (de Graffenried et al., 2013). The identification of new TbPLK substrates and interactors might facilitate the understanding of some TbPLK-mediated processes. Some of these studies have lagged behind because of several experimental concerns. TbPLK depletion by means of RNAi is a powerful technique, but poses important drawbacks and limitations. As an alternative, small-molecule inhibition provides some advantages that will be discussed in the next section.

1.4. Analogue-sensitive kinases for small-molecule inhibition

RNA interference and the identification of small-molecule inhibitors

RNA interference (RNAi) is probably one of the most important technologies available in biology today. Being a natural cellular process, RNAi has been turned into a powerful tool for loss-of-function studies in different systems. The phenomenon was originally observed in the worm *C. elegans*, where double-stranded RNA (dsRNA) led to sequence-specific gene silencing. They named this phenomenon “RNA interference” (Timmons and Fire, 1998). Later, the efficiency of a dsRNA mixture versus sense and antisense RNAs was tested and the finding was that the mixture was a stronger silencer than the individual RNAs. In the original studies in *C. elegans*, dsRNA or recombinant dsRNA was injected into the worm's gonad or provided through the diet (Timmons and Fire, 1998) (Hannon, 2002). RNAi has now been described in a variety of organisms, including trypanosomes (Ngô et al., 1998). But using RNAi in other organisms was hard at the beginning because the delivery of long dsRNA (>30 nt) into mammalian cells, for instance, turned out to be unsuccessful. The introduction of long dsRNAs into

different types of mammalian cultures led to non-specific gene silencing (Caplen et al., 2002)(Knight and Bass, 2001). This was explained by the fact that long dsRNA could actually interfere with enzymes forming part of the IFN defense pathway (Leaman et al., 1998). Additional studies in other organisms showed that RNAi was actually initiated by short dsRNA oligonucleotides (21 nt), instead of long dsRNAs The transfection of such short RNAs into mammalian cells was able to bypass the previously observed non-specific effects (Elbashir et al., 2001). These siRNAs are processed or "diced" from long dsRNAs by an enzyme known as Dicer, a type of RNase III nuclease (Bernstein et al., 2001). Current RNAi technology makes use of the cell's natural system to effectively turn off a gene of interest. At the moment, there are several strategies to induce RNAi, such as synthetic molecules or vectors. In general, siRNAs can be designed to match any gene. The siRNAs are able to degrade their corresponding target mRNAs in the cell. Specifically, the antisense strand of a siRNA forms part of a complex constituted by several proteins, the RNA-induced silencing complex, known as RISC, which is able to identify the corresponding mRNA and cleave it specifically. This cleaved mRNA is then targeted for degradation. This results in the depletion of the corresponding protein.

RNAi has been used to study the function of several mitotic kinases, including trypanosomal TbAUK1 (Tu et al., 2006)and TbPLK (de Graffenried et al., 2008) (Ikeda and de Graffenried, 2012). As mitotic kinases have become one of the most interesting classes of drug targets, there is a growing effort in the development of specific kinase inhibitors. Some of these small drugs are currently used in cancer therapies or clinical trials. A protein kinase catalyzes the transfer of a phosphate group from ATP to a Ser/Thr/Tyr residue on its corresponding substrate. Many kinase inhibitors developed so far are ATP-competitive and target the kinase's ATP-binding site (Johnson et al., 1998)(Denessiouk et al., 1998). The so-called Type 1 inhibitors usually recognize the active kinase confirmation, whereas Type 2 inhibitors bind to the inactive kinase version (Zhang et al., 2009). Covalent inhibitors rely on a covalent and irreversible binding to the kinase's active site (Cohen et al., 2005). A fourth type of inhibition is the allosteric one; in this case the inhibitor binds the kinase outside the active site. Despite the big number

of inhibitors discovered so far, the search for more potent and selective kinase inhibitors attracts great interest in chemistry and biology. High-throughput screening of compound libraries, cell-based assays and analogue-based synthesis have been used in the field of drug discovery (von Ahsen and Bömer, 2005). These processes are often laborious and the main challenges when developing an inhibitor are probably the specificity and achieving the ideal physical and pharmacological properties.

RNA interference and small-molecule inhibition have been often used together to study the function of a given protein. The phenotypes observed by using both methods are complementary in some cases, while incongruent in others. For instance, the small drug hesperadin is able to inactivate Aurora B (Hauf et al., 2003), but the spindle checkpoint is still active upon nocodazole-induced microtubule depolymerization. However, Aurora B depletion impedes the activation of the spindle checkpoint in the same physiological conditions(Keen and Taylor, 2004). This result could be explained by the fact that Aurora B belongs to the chromosomal passenger complex (CPC). Aurora B RNAi removes the kinase from the CPC and likely also destabilizes the whole complex. In contrast, a CPC containing inactive Aurora B can probably still achieve some of its functions (Ditchfield et al., 2003). All together, these findings highlight the importance of understanding the differences in both methods when analyzing a phenotype.

Proof that an inhibitor is selective for a certain kinasethe use of a small-molecule can provide certain advantages over RNAi. Drug administration is quick and direct and can be reversible if the inhibitor is not covalent. As many small molecules can rapidly go through the cell membrane, they can diffuse quickly once inside the cell. Drugs can be dosed at multiple concentrations, meaning that the effects of partial or total inhibition can be evaluated. In contrast, RNAi relies on protein turnover, which takes much longer and can give rise to the upregulation or downregulation of related kinases in a certain pathway. In addition, as illustrated before, protein depletion can compromise the stability of related protein complexes.

An analogue-sensitive approach for selective kinase inhibition

Unfortunately, obtaining a small molecule can require a very big screening effort and ensuring that this molecule is specific is a big concern. Given that the binding site for ATP is highly conserved in many kinases, small molecules might slot into the active site of more than one protein (Cohen, 1999). In addition, non-specific effects from the interaction of chemical groups with biomolecules can also occur. The current drug design approaches make use of subtle binding and conformational differences in the active site of the kinase in order to design more specific inhibitors. Typically, the specificity can be evaluated by means of kinase and binding assays. For instance, binding assays showed that VX-680, an Aurora inhibitor, also binds to a BCR-ABL1 mutant that was previously resistant to the available inhibitors at that time (Harrington et al., 2004) (Carter et al., 2005). BCR-ABL1 is an oncogene implicated in leukemia. However, inhibitor screenings against a big number of kinases are often laborious and require high costs.

A general chemical-genetics method has been devised to generate analogue-sensitive mutant kinases (Bishop et al., 1998). This approach takes advantage of the high conservation of the kinase ATP binding site. The kinase active site becomes engineered by introducing a silent mutation at the gatekeeper residue. This is normally a large and hydrophobic residue contacting the N6 group of ATP. By mutating the gatekeeper to glycine or alanine, a novel and unique 'pocket' is created at the kinase active site. The derivatization of a known ATP-kinase inhibitor with a bulkier substituent allows the drug to dock into the pocket, but not into any other wild-type kinase. In this way, a complementary inhibitor-kinase duo is designed. As the mutant kinase and the wild-type kinase have the same function in the cell, the inhibition should affect the associated pathways to the same extent a selective inhibitor would do. When generating an analogue-sensitive cell line, a proper strategy has to be adopted to ensure that the mutant kinase is the only version present in the cell. This is a rapid methodology to study the roles of individual kinases without a big concern about unspecific effects. One of the first analogue-sensitive kinases was v-Src, from the Src-family of protein tyrosine kinases (Bishop et al., 1998) (Bishop and Shokat, 1999). The aminoacid

Ile338 in the catalytic pocket was mutated to Gly. The core structure of a previously reported Src-inhibitor was pyrazolo[3,4-d]pyrimidine (Hanke et al., 1996). From this original inhibitor, different analogues were synthesized and screened against the wild-type and mutant Src kinase. Finally, the most potent analogue-inhibitor was selected. Since then, the analogue-sensitive strategy has been applied to many different kinases with successful results. However, for a subset of kinases, introducing the gatekeeper mutation results in a big loss in catalytic activity and function, which suggests that the mutation is not well tolerated. To rescue the activity in such kinases, several second-site suppressor mutations in the kinase domain were identified by using a bacterial genetic screen in an aminoglycoside kinase. These results illuminated the beta sheet region in the N-terminal kinase subdomain. From this starting point, Zhang and colleagues did a sequence alignment according to structure for several protein kinases in this specific region. This approach led to the identification of the corresponding compensatory mutations in different types of kinases (Zhang et al., 2005).

Some examples of successfully engineered kinases are Aurora in yeast (Koch et al, 2012), CDPK1 in Toxoplasma gondii (Lourido et al., 2010) (Lourido et al., 2013), Protein kinase C in zebrafish (Cibrián Uhalte et al., 2012) or CDK2 in human cells (Horiuchi et al., 2012). In addition, the analogue-sensitive strategy has also been achieved in Cdc5p, the yeast PLK homologue (Snead et al., 2007) and in human PLK1. In the latter, the results brought novel findings about the role of PLK1 in RhoA-mediated cytokinesis (Burkard et al., 2007). Interestingly, recent work has shown that analogue-sensitive PLK1 is resistant to conventional PLK1 inhibitors such as BI-2536. The reason for this seems to be the second-site suppresor mutation, which sterically impedes the binding of BI-2536 to the catalytic pocket (Burkard et al., 2012).

Trypanosome drug discovery often tries to exploit the unusual biochemical pathways present in trypanosomes. A chemoproteomics-based strategy has been recently developed to interrogate the parasite's kinome (Urbaniak et al., 2012a) (Urbaniak et al., 2012b). Nevertheless, a specific inhibitor against TbPLK is not available at the moment. Li and colleagues have reported that the mammalian

PLK1 inhibitor GW843682X is able to inhibit TbPLK in vitro and leads to aberrant cytokinesis in vivo (Li et al., 2010) although its specificity has not been so extensively characterized. However, in our hands, neither GW843682X nor the other well-known PLK1 inhibitor BI2536 had a significant impact on *T. brucei*'s viability when used at the concentration of 10 µM (unpublished data).

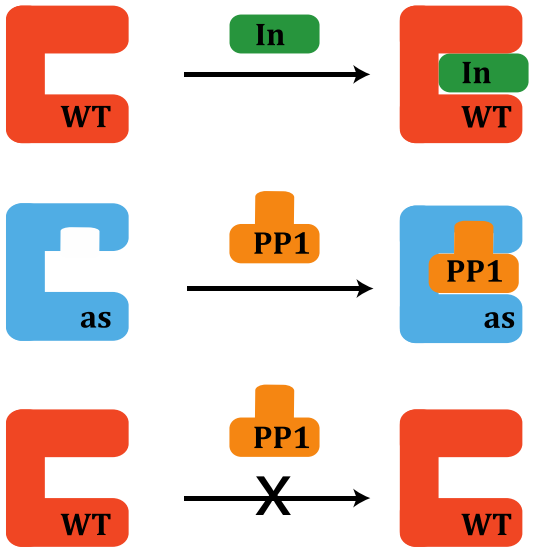

Figure 7 - Fundamental principles of the analogue-sensitive strategy for protein kinases. WT represents wild-type, as stands for analogue-sensitive, In is a general inhibitor and PP1 a modified inhibitor.

1.5. Aims of this thesis

The main aim of this work is to achieve a better understanding of the different TbPLK functions during *T. brucei*'s cell division, specifically, at the basal bodies, the bilobe and the FAZ. For this purpose, we set out to generate for the first time in trypanosomes a cell line expressing only an analogue-sensitive version of TbPLK (TbPLK^{as}), in which we could inhibit the enzyme acutely and specifically with a corresponding engineered drug. Our second aim pursues the achievement of a highly synchronous *T.brucei*'s cell culture in which the different TbPLK functions can be investigated with a temporal control. Finally, we expect to combine these strategies with the proteomics methods SILAC and iTRAQ with the objective of identifying novel PLK substrates and with BioID for the search of potential interactors.

Chapter 2. Published results

2.1. Prologue

Our results have been compiled in the following publication:

Lozano-Núñez, A., Ikeda, K.N., Sauer, T., and de Graffenried, C.L. (2013). An analogue-sensitive approach identifies basal body rotation and flagellum attachment zone elongation as key functions of PLK in Trypanosoma brucei. Mol. Biol. Cell *24*, 1321–1333.

This work reports the first application of the protein kinase analogue-sensitive strategy in kinetoplastids. Acute and specific inhibition was combined with a synchronous cell culture growth. Synchronization was achieved by means of centrifugal elutriation, following a recently published protocol specific for trypanosomes (Archer et al., 2011). Centrifugal elutriation allows the isolation of cells by means of their sedimentation rate, which depends on their size and density. A population of cells is exposed to the balance of two opposing forces: the centrifugal force and the counterflow force from the buffer. The smaller the cells, the earliest they can be washed out from the elutriation chamber. A gradual increase in the buffer flow rate allows the isolation of larger cells. Elutriation has been used for a long time and it is a well-established procedure, for instance, in yeast biology. Archer and colleagues (Archer et al., 2011) improved the separation protocol when applied to trypanosomes. In order to increase the overall yield, they developed a double-cut elutriation procedure. In the first step, and by using a fixed flow rate, they isolated a fraction of big cells and set them in culture for a short time, with the purpose of letting them divide. These cells were afterwards elutriated again by using a lower flow rate, which yielded a population of highly pure small cells. This culture was in the G1 phase and proceeded very synchronously through the cell cycle over time.

Our key findings are the following:

TbPLK is necessary for the rotation of the basal bodies after their duplication, and this is required for proper positioning of the new flagellum.

TbPLK is not only required for the initiation of a new FAZ, but also for its elongation to achieve a proper length.

Our results illuminate our current understanding into how *T. brucei* duplicates and partitions its cytoskeleton and attribute additional functions to TbPLK.

Contributions:

C.L.D and A.L.N conceived and designed the experiments. K.N.I and T.S provided valuable help with the planning and execution of the elutriation experiment. A.L.N performed the experiments with the help of C.LD, who contributed in cell line generation and Western Blotting, and K.N.I, who was responsible for electron microscopy. The data was analyzed by A.LN. C.LD and A.L.N interpreted the data and wrote the manuscript. A.L.N designed and made all the figures, except for Figure 6, which was made by K.N.I.

2.2. Publication

MBoC | ARTICLE

An analogue-sensitive approach identifies basal body rotation and flagellum attachment zone elongation as key functions of PLK in *Trypanosoma brucei*

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ABSTRACT Polo-like kinases are important regulators of cell division, playing diverse roles in mitosis and cytoskeletal inheritance. In the parasite *Trypanosoma brucei*, the single PLK homologue TbPLK is necessary for the assembly of a series of essential organelles that position and adhere the flagellum to the cell surface. Previous work relied on RNA interference or inhibitors of undefined specificity to inhibit TbPLK, both of which have significant experimental limitations. Here we use an analogue-sensitive approach to selectively and acutely inhibit TbPLK. *T. brucei* cells expressing only analogue-sensitive TbPLK (TbPLK^{as}) grow normally, but upon treatment with inhibitor develop defects in flagellar attachment and cytokinesis. TbPLK cannot migrate effectively when inhibited and remains trapped in the posterior of the cell throughout the cell cycle. Using synchronized cells, we show that active TbPLK is a direct requirement for the assembly and extension of the flagellum attachment zone, which adheres the flagellum to the cell surface, and for the rotation of the duplicated basal bodies, which positions the new flagellum so that it can extend without impinging on the old flagellum. This approach should be applicable to the many kinases found in the *T. brucei* genome that lack an ascribed function.

Monitoring Editor
Monica Bettencourt-Dias
Instituto Gulbenkian de Ciência

Received: Dec 3, 2012
Revised: Feb 14, 2013
Accepted: Feb 19, 2013

INTRODUCTION

Trypanosoma brucei causes severe illnesses in humans and animals that create substantial health and economic problems in sub-Saharan Africa. The few viable treatments for trypanosomiasis are extremely toxic, and parasite resistance to available drugs is a worsening problem (Bouteille *et al.*, 2003). *T. brucei* is an obligate extracellular parasite that confines all of its exocytosis and endocytosis to a single compartment in the posterior of the cell (Gull, 2003; Field and Carrington, 2009). This compartment, known as the flagellar pocket, also contains the trypanosome's single flagellum, which is nucleated by the basal body docked at the base of the pocket (Lacomble *et al.*, 2009). The flagellum traverses the pocket and then passes through the top of the compartment, which is cinched tightly against the flagellum. The top of the pocket is marked by two cytoskeletal structures—the flagellar pocket collar (FPC) and the bilobe—which encircle the flagellum and may be responsible for ensuring the tight connection between the plasma membrane and flagellum, which delineates the pocket as a distinct membrane compartment (Bonhivers *et al.*, 2008; Gadelha *et al.*, 2009; Esson *et al.*, 2012).

Once outside the cell, the flagellum is adhered to the cell surface and extends toward the anterior of the cell. The attachment of the flagellum is mediated by a structure found within the cell body known as the flagellum attachment zone (FAZ). The FAZ comprises an electron-dense filament and a unique set of four microtubules termed the quartet (MtQ). The filament links together a series of junctions that contact the flagellar membrane, which keep the

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E12-12-0846) on February 27, 2013.

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Abbreviations used: BB, basal body; CDK1, cyclin-dependent kinase 1; CDK2, cyclin-dependent kinase 2; DMSO, dimethyl sulfoxide; EM, electron microscopy; FAZ, flagellum attachment zone; FPC, flagellar pocket collar; HU, hydroxyurea; MtQ, microtubule quartet; PLK, Polo-like kinase; RNAi, RNA interference; snFAZ, short new flagellum attachment zone; TbPLK, *Trypanosoma brucei* Polo-like kinase; TbPLK^{as}, analogue-sensitive *Trypanosoma brucei* Polo-like kinase.

Supplemental Material can be found at:
http://www.molbiolcell.org/content/suppl/2013/02/25/mbc.E12-12-0846.DC1.html

flagellum firmly attached to the cell body (Vickerman, 1969; Gull, 1999). The MtQ nucleates next to the basal body, wraps around the flagellar pocket, and then extends beside the FAZ filament along the whole length of the cell body (Lacomble *et al.*, 2009). Depletion of bilobe, FPC, or FAZ components causes defects in flagellar attachment and in cytokinesis, usually leading to growth arrest (LaCount *et al.*, 2002; Selvapandiyan *et al.*, 2007; Bonhivers *et al.*, 2008; Shi *et al.*, 2008; Vaughan *et al.*, 2008).

The genome sequence of *T. brucei* shows that the parasite has undergone substantial selection by its environment. The trypanosome cytoskeleton has reduced the role of the acto-myosin network to the point that actin appears to be dispensable in one life stage of the parasite, whereas the role of tubulin has been enhanced (García-Salcedo *et al.*, 2004; Berriman *et al.*, 2005). *T. brucei* possesses a large complement of protein kinases, comprising almost 2% of its genome (Parsons *et al.*, 2005). The kinome includes members of most eukaryotic kinase families, along with many unique kinases that may be important for the unique features of the trypanosome life cycle and for pathogenicity.

Among the *T. brucei* kinases that have been studied is the single Polo-like kinase homologue TbPLK (Kumar and Wang, 2006; Hammarton *et al.*, 2007; de Graffenried *et al.*, 2008). In mammalian cells the four PLK homologues play important roles in mitosis, centriole biogenesis, and the DNA damage response (Archambault and Glover, 2009). The function of TbPLK has been specialized toward the inheritance of the flagellum, which is duplicated during cell division (Ikeda and de Graffenried, 2012). TbPLK is expressed early in the cell cycle, initially localizing to the new MtQ as it nucleates. The kinase is then found on the basal body as it duplicates and forms a new flagellum, which then rotates around the old flagellum to assume a position in the posterior of the cell (Lacomble *et al.*, 2010). This rotation event also coincides with the formation of a new flagellar pocket (Gadelha *et al.*, 2009). TbPLK then migrates to the bilobe as the structure duplicates. After bilobe duplication, TbPLK localizes to the tip of the new FAZ as it extends toward the anterior of the cell, disappearing just before cytokinesis (Ikeda and de Graffenried, 2012). Depletion of TbPLK causes defects in bilobe, FPC, and FAZ duplication (de Graffenried *et al.*, 2008; Ikeda and de Graffenried, 2012). Basal body duplication does occur, but the replicated structures cannot separate.

Small-molecule inhibitors have played an essential role in elucidating the function of important mitotic kinases such as CDK1 and PLK1. These molecules, which are usually modeled after the universal phosphate donor ATP (Peters *et al.*, 2006; Vassilev *et al.*, 2006; Lénárt *et al.*, 2007), rapidly block the activity of kinases within cells and allow the consequences of this acute inhibition to be assessed. Unfortunately, the utility of many inhibitors is limited by their lack of specificity. Early kinase inhibitors used ATP as a starting point for inhibitor design because its binding site is highly conserved, but this made it difficult to develop drugs that targeted a single kinase (Cohen, 1999). The large number of kinase crystal structures has allowed subtle differences in the ATP-binding site and conformational effects to be exploited to generate specific inhibitors, but this process is synthetically laborious and requires extensive screening to ensure that the compounds are truly specific (Tong *et al.*, 1997; Gray *et al.*, 1998; Schindler *et al.*, 2000). The advent of RNA interference (RNAi) provided a new method for inhibition by depleting the kinase of interest, which frequently can be done without targeting related proteins. However, inhibition by RNAi requires protein turnover, which takes much longer than small-molecule inhibition and may be more prone to the up-regulation of other kinases that compensate for the loss of activity (Weiss *et al.*, 2007). Kinase depletion may also lead to additional phenotypes if the protein is part of a complex or has other functions.

Considering its importance in cell division, a specific inhibitor of TbPLK would be extremely useful to study the acute effects of kinase inhibition. An inhibitor of mammalian PLK1 has been shown to inhibit TbPLK in vitro and causes cytokinesis defects in vivo, but the specificity of this drug is not well established (Li *et al.*, 2010). Parasite-specific inhibitors are under development (Urbaniak *et al.*, 2012), but it is unlikely that inhibitors against the many different kinases present in *T. brucei* will be available in the near future. The ability to clearly identify the function of individual kinases would also facilitate the discovery of potential drug targets.

A general method for kinase inhibition has been established that takes advantage of the conservation within the ATP-binding site (Bishop *et al.*, 1998, 2000; Garske *et al.*, 2011). A conserved large hydrophobic residue (known as the gatekeeper) that contacts the N6 group of ATP is mutated to alanine or glycine, generating a novel pocket within the active site. A potent but nonspecific ATP-competitive kinase inhibitor is then modified with a bulky substituent that docks into this additional pocket, which renders the modified compound incapable of inhibiting kinases that lack the accommodating mutation. When introduced into cells the mutated, termed analogue-sensitive, kinase should be the only kinase whose activity is blocked by addition of the modified inhibitor. The specificity of the modified inhibitor can be further tested by adding it to wild-type cells, which should not show any response. By generating orthogonal kinase-inhibitor pairs, it is now possible to use modified inhibitors to acutely inhibit many different protein kinases with limited concerns about off-target effects.

In this work we generated procyclic *T. brucei* cells that exclusively express the analogue-sensitive variant of TbPLK (TbPLK^{as}). Using acute inhibition and cell cycle synchronization achieved by elutriation, we were able to dissect key roles played by TbPLK in new FAZ formation and basal body rotation during cell division. Our results show the utility of the analogue-sensitive method in *T. brucei*, which will allow the acute and specific inhibition of many of the kinases present in the parasite without the burden of identifying new inhibitors.

RESULTS

The PLK family belongs to a small subset of kinases that lose the bulk of their activity upon mutation of the gatekeeper residue within the ATP-binding site that is necessary for the analogue-sensitive strategy. A second site suppressor screen identified a compensatory mutation that restores sufficient activity to PLK to support growth (Zhang *et al.*, 2005). We were able to identify both the gatekeeper residue (L118) and the second site suppressor (C57) in TbPLK due to the high degree of conservation with other PLK family members within the kinase domain (Burkard *et al.*, 2007). We generated a TbPLK^{as} mutant by mutating L118 to glycine and C57 to valine. Recombinant baculoviruses containing the hexahistidine-tagged wild-type and TbPLK^{as} sequences were generated and used to infect insect cells to produce the proteins. Affinity-purified kinases were tested for their sensitivity to the bulky purine analogue 3MB-PP1, which was previously used to inhibit PLK1^{as} (Burkard *et al.*, 2007). TbCentrin2, a small calcium-binding protein phosphorylated by TbPLK in vitro, was expressed in *Escherichia coli* and used as a substrate (de Graffenried *et al.*, 2008; Yu *et al.*, 2012). Wild-type TbPLK was not inhibited by 3MB-PP1 within the concentration range tested (50 nM to 10 μM), whereas TbPLK^{as} showed an IC_{50} value of ~250 nM (Supplemental Figure S1). TbPLK^{as} was less active than the wild-type kinase, requiring a longer exposure. This result shows that

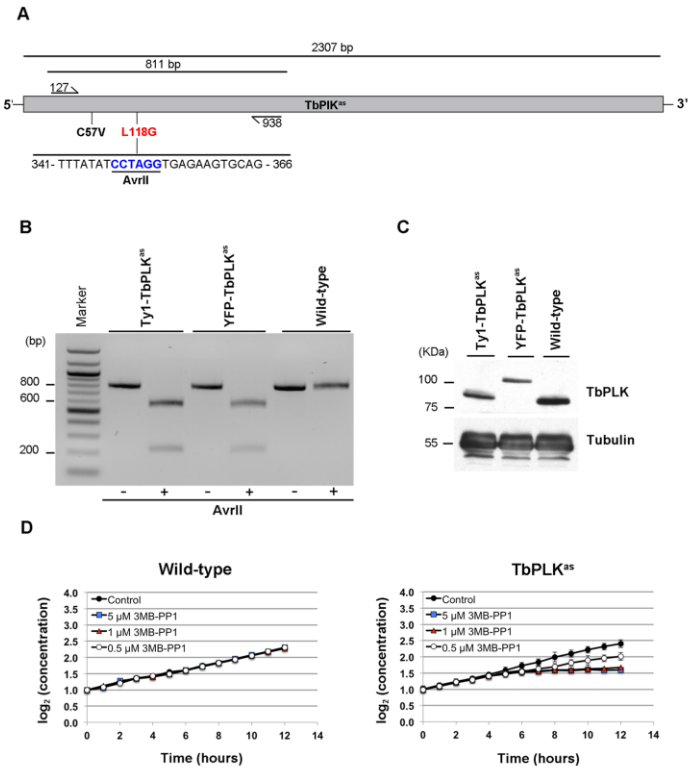

FIGURE 1: Incorporation of analogue-sensitive mutations into TbPLK in procyclic cells. (A) A schematic of the TbPLK loci and the strategy used to introduce analogue-sensitive mutations. Primers flanking the positions of the C57V (black) and L11G (red) mutations were used to amplify an 811–base pair fragment of the TbPLK gene. If the L11G mutant is present, a unique *Avr*II restriction site is present in the fragment. (B) PCR using the primers described in A from genomic DNA isolated from cells expressing only Ty1-TbPLK^{as}, YFP-TbPLK^{as}, and wild-type cells. The fragment was digested with *Avr*II to test for incorporation of the analogue-sensitive mutations. The fragment and the product of the *Avr*II digest for each isolated genomic DNA were run on an agarose gel. Only the fragments isolated from the TbPLK^{as} cell lines were sensitive to *Avr*II, showing that the mutations were present. (C) Cell lysates from Ty1-TbPLK^{as}, YFP-TbPLK^{as}, and wild-type cells were fractionated by SDS–PAGE, transferred to nitrocellulose, and incubated with an antibody against TbPLK and tubulin as a loading control. (D) Wild-type and TbPLK^{as} cells were treated with different concentrations of 3MB-PP1 or a vehicle control, and their growth was monitored with a cell counter for 12 h. Error bars, SD of three biological replicates.

TbPLK tolerates the gatekeeper and second-site suppressor mutations and is sensitive to 3MB-PP1. Of importance, the wild-type kinase was not inhibited by the drug.

Once the in vitro activity and 3MB-PP1 sensitivity of TbPLK^{as} were established, we made a procyclic *T. brucei* cell line that exclusively expressed the mutant kinase. Modifying the endogenous TbPLK loci using homologous recombination was necessary because the kinase is mitotically regulated, and constitutive overexpression can cause premature cytokinesis (Kumar and Wang, 2006). We generated a cell line in which one TbPLK allele was replaced with a puromycin resistance gene and the second one with a construct containing both analogue-sensitive mutations and a blasticidin resistance cassette. To allow us to identify clones in which both mutations were incorporated into the TbPLK loci, the nucleotides that introduced the L118G mutation also included a unique *Avr*II restriction site (Figure 1A). Two versions of the targeting construct were used, with either an N-terminal yellow fluorescent protein (YFP) or a Ty1 tag, so that the TbPLK^{as} allele could be identified directly. Amplified genomic DNA from doubly resistant clones was screened by PCR and digestion with *Avr*II to identify clones in which both mutations had been incorporated (Figure 1B). Lysates from the Ty1-TbPLK^{as}, YFP-TbPLK^{as}, and wild-type cells were blotted and probed with a monoclonal antibody against TbPLK. The Ty1 and YFP-TbPLK^{as} cell lines both lack untagged TbPLK and express slightly lower levels of kinase than the wild-type cell line (Figure 1C).

The effect of 3MB-PP1 on growth of wild-type and TbPLK^{as} cells was tested to ensure that the introduced mutations had made the kinase sensitive to the drug in vivo. Wild-type and TbPLK^{as} cells were seeded in culture, and different concentrations of 3MB-PP1, ranging from 5 μM to 500 nM, or a vehicle control (dimethyl sulfoxide [DMSO]) were added. The growth of the treated cultures was monitored every hour for 12 h using a cell counter. A full round of cell division in procyclic *T. brucei* takes ~8.5 h (Sherwin and Gull, 1989). In the absence of drug, the growth of the wild-type and TbPLK^{as} cell lines was almost identical, showing that the mutations in the TbPLK^{as} allele were well tolerated (Figure 1D). The growth of wild-type cells was not affected by the drug at any concentration tested. The intermediate cell line lacking one TbPLK allele that was used to construct the TbPLK^{as} cell line was also insensitive to the drug at all concentrations (Supplemental Figure S2). The growth of the TbPLK^{as} cells was strongly inhibited at 1 and 5 μM, with a clear growth defect appearing 6 h after the addition of drug. At this point the cells ceased to divide for the duration of the experiment. This result shows that TbPLK^{as} cells treated with at least 1 μM 3MB-PP1 do not undergo cytokinesis within the first cell cycle. TbPLK^{as} cells treated with 500 nM drug grew at ~50% the rate of control cells.

The cell cycle phenotypes produced by TbPLK^{as} inhibition were identified to determine whether they were similar to previously published results using other methods for inhibiting or depleting TbPLK. Early in the cell cycle trypanosomes contain one nucleus and one kinetoplast (1N1K). The kinetoplast duplicates before the nucleus (1N2K); subsequent nuclear division results in a 2N2K cell, which then undergoes cytokinesis. An exponentially growing culture contains ~80% 1N1K cells; the remaining 20% of the culture is near evenly split between 1N2K and 2N2K cells. TbPLK activity has been depleted from cells using RNAi and inhibited using a drug that

inhibits human PLK1 (Kumar and Wang, 2006; Hammarton *et al.*, 2007; de Graffenried *et al.*, 2008; Li *et al.*, 2010). Cells lacking TbPLK activity have fewer 1N1K cells, an increased number of 2N2K cells, and aberrant 2N1K cells where the nucleus has duplicated prior to the kinetoplast.

These 2N1K cells are a hallmark of TbPLK inhibition and arise due to a lack of segregation of the duplicated basal bodies (Ikeda and de Graffenried, 2012). To compare these results with TbPLK^{as} inhibition, we treated TbPLK^{as} cells with 3MB-PP1 or vehicle control for 9 h and then fixed and stained them with 4′,6-diamidino-2-phenylindole (DAPI) to assess their DNA state. Vehicle control–treated TbPLK^{as} cells had a DNA distribution identical to that of wild-type cells (Figure 2A). Cells treated with 3MB-PP1 showed a significant change in DNA state, with the 1N1K population dropping to only 30% of total. The number of 1N2K cells decreased slightly, whereas the number of 2N2K cells doubled. 2N1K cells also appeared, comprising >20% of the population. These results show that TbPLK^{as} inhibition has a very similar effect on the cell cycle as depletion of the kinase by RNAi.

The cell cycle defects observed upon TbPLK depletion are caused by difficulties in assembling and separating cytoskeletal elements that are necessary for kinetoplast segregation and cytokinesis (Ikeda and de Graffenried, 2012). In TbPLK-depleted cells the basal bodies are able to undergo the normal maturation and duplication cycle, producing a new flagellum, but the replicated structures are not able to separate. The bilobe structure and the FAZ, which are involved in positioning and adhering the flagellum to the cell surface, do not duplicate, generating a new flagellum that is detached from the cell body. In TbPLK^{as} cells treated with 3MB-PP1 for 9 h, cells at all cell cycle stages showed an increase in detached new flagella compared with vehicle control-treated samples (Figure 2B). In 2N cells, more than half of the population had detached new flagella. The small number of detached flagella present in the vehicle control samples is most likely due to a fixation artifact, as detached flagella were never observed in live cells in this sample.

To test whether bilobe duplication was perturbed, TbPLK^{as} cells were treated with 3MB-PP1 or vehicle control for 9 h, fixed, and then stained for the bilobe and basal body marker TbCentrin4 (Figure 2C; Selvapandiyan *et al.*, 2007; Shi *et al.*, 2008). In wild-type cells the bilobe duplicates at the 1N1K state (Figure 2C, b, arrowhead). The first evidence of duplication is the elongation of the bilobe to 2.2 μm or longer. This elongated structure then is separated into two distinct bilobes, which are moved further apart as the cell goes through the 1N2K and 2N2K states (Figure 2C, c, arrowhead). In vehicle control-treated cells, ~18% of 1N1K cells contained either elongated or duplicated bilobes, whereas all 2N2K cells contained two bilobes (Figure 2E). In 3MB-PP1-treated cells, there was no evidence of elongated (>2.2 μm) or duplicated bilobes in 1N1K cells (Figure 2E). Greater than 50% of 2N2K cells had only one bilobe (Figure 2, E and C, d and e), whereas all the aberrant 2N1K cells had a single bilobe (Figure 2, E and C, f). As noted previously, the 3MB-PP1–treated cells frequently had detached flagella (Figure 2C, d–f, arrows).

Considering the extent of new flagellum detachment and the defects in bilobe assembly, it was likely that FAZ assembly was also impeded by the absence of TbPLK activity. To evaluate the new FAZ, we treated TbPLK^{as} cells with 3MB-PP1 or vehicle control for 9 h and then fixed and stained them with an antibody against FAZ1, a FAZ component (Figure 2D; Kohl *et al.*, 1999; Vaughan *et al.*, 2008). In vehicle control–treated cells new FAZ assembly begins in 1N1K cells and continues through the 1N2K and 2N2K stages, reaching completion just before cytokinesis (Figure 2D, b and c, open arrowheads). In vehicle control–treated cells, FAZ duplication proceeds as in wild-type cells, with ~10% of 1N1K cells containing two FAZ, whereas all 1N2K and 2N2K cells had two FAZ (Figure 2F). In 3MB-PP1–treated cells the number of 1N1K cells with two FAZ decreased (Figure 2, D, d, and F). In the 2N2K population, 23% had only a single FAZ, whereas 10% contained a short new FAZ (snFAZ), <5 μm, which is the shortest-length new FAZ that was observed in wild-type 2N2K cells (Figure 2, D, e, and F). We hypothesize either that cells with snFAZ prematurely aborted the assembly of the new FAZ or that the assembly process was somehow delayed. FAZ defects were very evident in 2N1K cells, with 90% of these cells containing only a single FAZ (Figure 2, D, f, and F). Cells that lacked a new FAZ or had an snFAZ almost always had detached new flagella (Figure 2D, d–f, arrows).

To further confirm that the defects observed in drug-treated TbPLK^{as} cells were specific, we treated wild-type cells with 3MB-PP1 or a vehicle control for 9 h and evaluated their DNA state and ability to assemble a new bilobe and FAZ. Wild-type cells treated with drug or the vehicle control had nearly identical distributions of 1N1K, 1N2K, and 2N2K states and lacked any cells with aberrant DNA content, such as 2N1K (Supplemental Figure S3A). Wild-type cells were able to duplicate their bilobe and FAZ in the presence of 3MB-PP1, showing that the drug only has an effect on cells carrying the TbPLK^{as} allele (Supplemental Figure S3, B and C).

Inhibition of TbPLK^{as} is phenotypically similar to depletion of TbPLK by RNAi. Both methods produce cells that contain aberrant DNA states, detached flagella, and defects in bilobe and FAZ duplication. The onset of the phenotype appears to be more rapid and severe when the TbPLK^{as} system is used, which is expected because the small molecule can directly inhibit the kinase, whereas RNAi relies on protein turnover for its effects. One important difference is that small-molecule inhibition leaves its target intact, allowing changes in the localization pattern of the enzyme to be established. Because TbPLK is highly motile and PLK homologues rely on their activity to generate some of their own binding sites, identifying what happens to the kinase upon inhibition may provide some insight into how TbPLK localizes to different positions within the cell (Neef *et al.*, 2003).

TbPLK^{as} was localized in 3MB-PP1– and vehicle-control–treated cells to determine whether inhibition of the kinase affected its localization. TbPLK^{as} cells were treated for 9 h with 3MB-PP1 or vehicle control, then fixed and labeled with antibodies against TbPLK and either TbCentrin4 or FAZ1 antibody to mark the bilobe or FAZ, respectively. In control cells, TbPLK was first present on a structure next to the kinetoplast, which corresponds to the new MtQ (Figure 3, A, a, and B, a; arrowheads; Ikeda and de Graffenried, 2012). The kinase then moved to the basal body and bilobe (Figure 3, A, b, and B, b; arrowheads), followed by accumulation on the tip of the new FAZ (Figure 3, A, c and d, and B, c and d; arrowheads). The location of the kinase in 3MB-PP1–treated and vehicle control cells was quantitated and placed in two categories: 1) TbPLK localization to the MtQ, basal body, and bilobe (pocket region), and 2) TbPLK presence on the new FAZ. Sixty percent of cells expressed TbPLK in both conditions, arguing that inhibition does not lead to changes in kinase expression (data not shown). In vehicle control–treated cells, 80% of 1N1K cells expressing TbPLK had labeling in the pocket region, whereas in 20% the kinase had already migrated onto the growing tip of the new FAZ (Figure 3C). In 2N2K cells, all cells expressing TbPLK^{as} had labeling exclusively on the FAZ. In the 3MB-PP1–treated samples 1N1K cells showed slightly elevated levels of pocket region localization. There was a striking change in localization in 2N2K cells. The kinase, which localized exclusively to the FAZ in untreated cells, was now found within the pocket region >80% of the time (Figure 3, A, e–h, and B, e–h, arrowheads, and C). 2N2K cells with pocket region TbPLK^{as} frequently had defects in bilobe

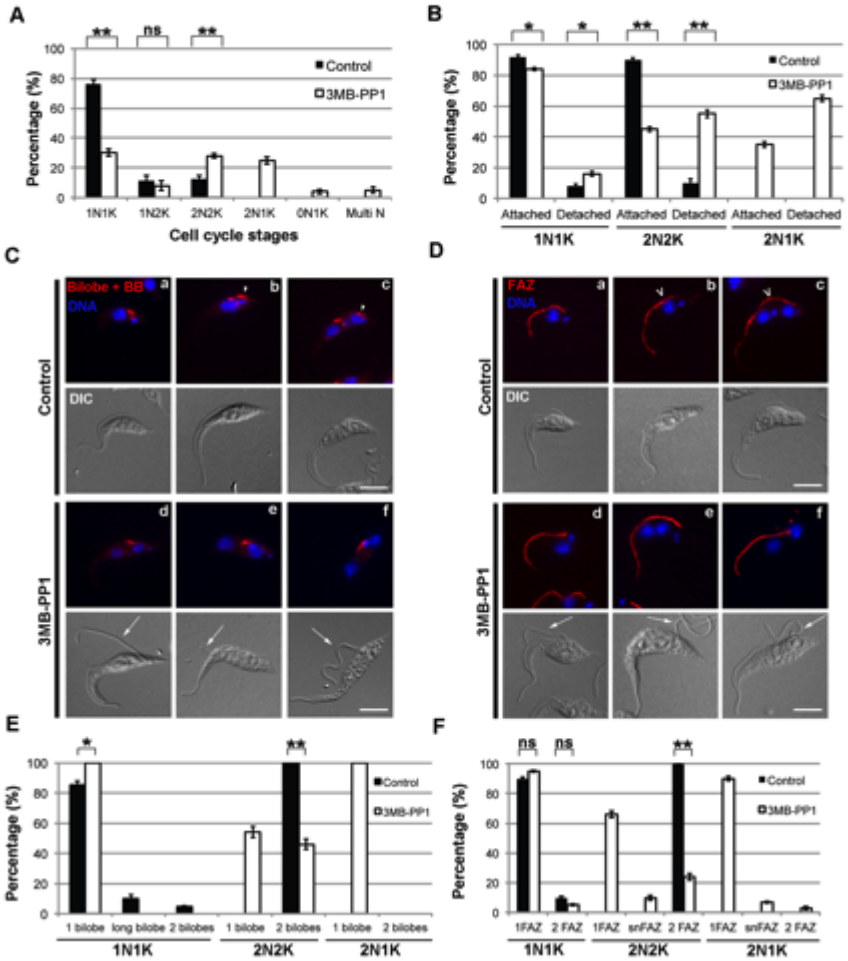

FIGURE 2: Cells expressing TbPLK^{as} and treated with 3MB-PP1 have DNA, bilobe, and FAZ defects. (A) TbPLK^{as} cells were treated with 3MB-PP1 or vehicle control for 9 h and then fixed and stained with DAPI to label DNA. The DNA content of the cells was determined by fluorescence microscopy. Cells treated with 3MB-PP1 showed an increase in 2N2K cells and the appearance of aberrant 2N1K cells. (B) TbPLK^{as} cells treated as in A were fixed and evaluated for flagellar attachment by differential interference contrast microscopy. Cells treated with 3MB-PP1 showed high levels of flagellar detachment. (C) TbPLK^{as} cells were treated as in A and then fixed and labeled with anti-TbCentrin4 antibody (Bilobe + BB; red) and DAPI (DNA; blue) to label DNA. The bilobe is present between the kinetoplast and nucleus (a). The new bilobe forms toward the posterior of the cell (arrowhead) in a subset of 1N1K cells (b) and moves away from the old bilobe as the cell cycle progresses (c). Cells treated with 3MB-PP1 had difficulty duplicating the bilobe (d–f) and have detached new flagella (arrows). (D) TbPLK^{as} cells were treated as in A and then fixed and labeled with anti-FAZ1 antibody (FAZ; red) and DAPI (DNA; blue) to label DNA. The FAZ underlies the flagellum and runs from the posterior to the anterior end of the cell (a). The new FAZ (empty arrowhead) forms toward the posterior of the cell (b), then extends until just before cytokinesis (c). Cells treated with 3MB-PP1 were not able to assemble a new FAZ and had detached flagella (d–f; arrows). (E) Quantitation of the cells in C. (F) Quantitation of the cells in D. Scale bars, 5 μm. Error bars, SD of three biological replicates with 300 cells counted per condition. *$p < 0.05$, **$p < 0.01$, ns, not significant.

and FAZ duplication, along with detached new flagella (Figure 3, A, e–f, and B, e–f; arrows). In 2N1K cells TbPLK^{as} was found exclusively in the pocket region (Figure 3, A, g–h, and B, g–h; arrows).

Because TbPLK^{as} plays multiple roles at different time points and locations during cell division, a cell cycle synchronization approach would allow a much more precise assignment of kinase

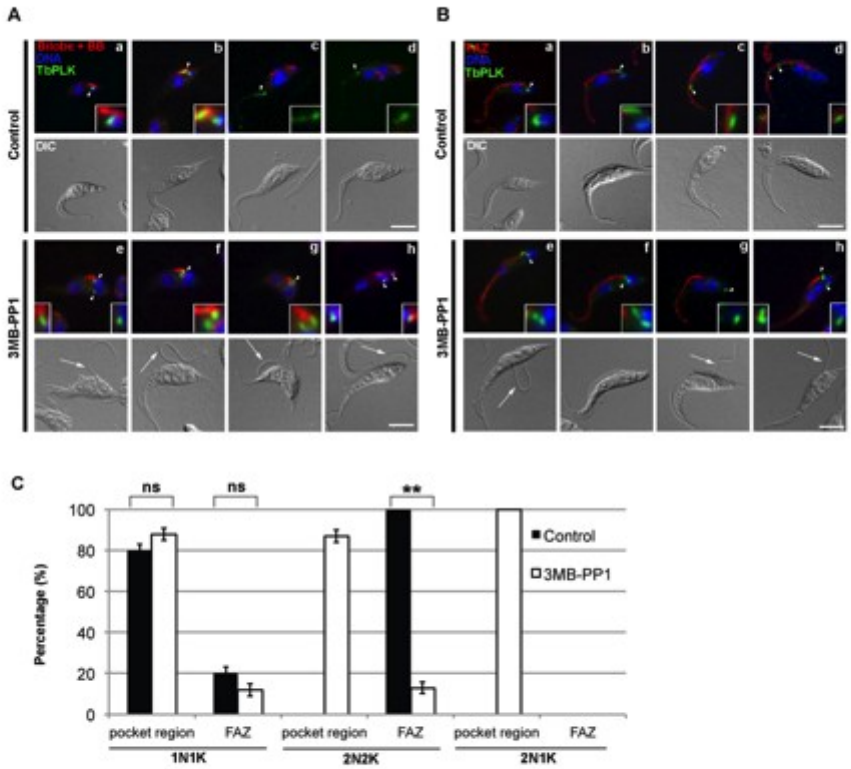

FIGURE 3: Inhibition of TbPLK causes the kinase to remain in the pocket region. TbPLK^{as} cells were treated with 3MB-PP1 or vehicle control for 9 h, then fixed and stained with anti-TbPLK antibodies and organelle markers to identify the location of TbPLK. Insets in A and B are threefold magnifications of the area around the TbPLK signal. (A) Cells were stained with anti-TbCentrin4 (Bilobe + BB; red), anti-TbPLK (TbPLK; green), and DAPI (DNA; blue). In control cells TbPLK is initially present in the pocket region, which consists of the basal body, MtQ, and bilobe (a, b; arrowheads), then migrates out toward the anterior of the cell (c, d; arrowheads) once kinetoplast and nuclear duplication occur. In 3MB-PP1–treated cells, the kinase remains in the pocket region even after nuclear duplication had occurred (e–h; arrowheads). (B) Cells were stained with anti-FAZ1 (FAZ; red), anti-TbPLK (TbPLK; green), and DAPI (DNA; blue). In the control cells the TbPLK signal shows the same migration pattern as in A (a–d; arrowheads). In 3MB-PP1–treated cells, the kinase does not migrate toward the posterior of the cell (e–h; arrowheads) and remains near the flagellar pocket. (C) Quantitation of the data in A and B. Scale bars, 5 μm. Error bars, SD of three biological replicates with 300 cells counted per condition. **$p < 0.01$, ns, not significant.

function. The ability to couple the rapid inhibition afforded by the TbPLK^{as} system with cell synchronization would allow us to dissect the roles of the kinase at specific points of the cell cycle. Synchronization techniques for trypanosomes have been established that rely on either treatment with hydroxyurea (HU) or cell starvation (Gale and Parsons, 1993; Chowdhury *et al.*, 2008). Treatment with HU produces a synchronous population of cells on the 1N1K/1N2K boundary, which proceeds through the cell cycle upon washout. However, the point of synchronization is after many of the critical events that require TbPLK, such as bilobe duplication and the initiation of the new FAZ. Cell starvation can be used to produce synchronized cells at an earlier point in the cell cycle corresponding to nuclear G_1, but the cells take 10 h to progress into S phase, suggesting that the cells may be in a quiescent G_0-like state due to starvation (Li *et al.*, 2010). This makes it uncertain whether any phenotypes identified using this method are due to conventional cell cycle progression.

A promising double-cut elutriation strategy has been described that synchronizes cells at a very early point in the cell cycle, allowing all subsequent events to be monitored (Archer *et al.*, 2011). Elutriation discriminates between cells based on their size and density, which correlates well with their cell cycle stage (Banfalvi, 2008). In the double-cut protocol, the largest cells isolated during elutriation are put back into culture for 1.5 h (Figure 4A). Because the largest cells are most likely ones that are about to undergo cytokinesis, this incubation time produces a large number of cells that have just finished dividing. This population of recently divided cells is then isolated by a second

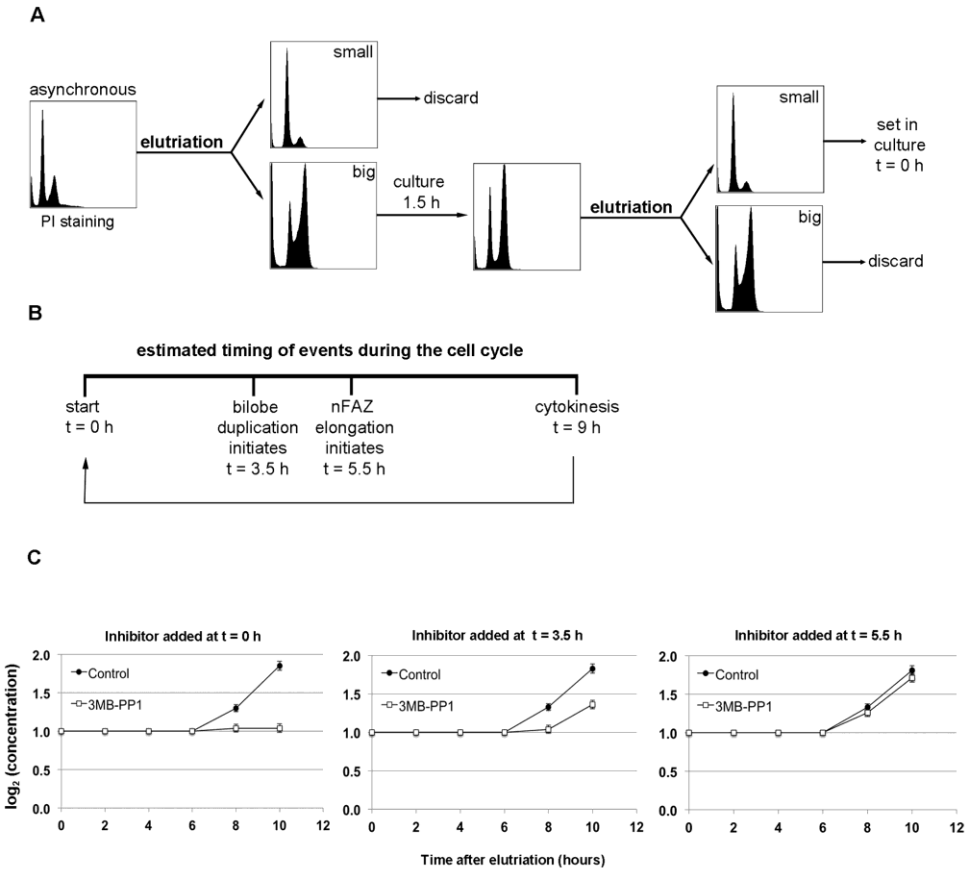

FIGURE 4: Double-cut elutriation of TbPLK^{as} cells produces synchronous cultures. (A) The protocol for double-cut elutriation. An asynchronous culture is elutriated, selecting for the largest cells, which are set in culture for 1.5 h so that they can undergo division. The population is elutriated again, and the smallest cells, which have just finished dividing, are selected and used for further experiments. (B) A simplified view of the cell cycle in *T. brucei*, highlighting bilobe duplication (t = 3.5 h) and new FAZ elongation (t = 5.5 h). The cell cycle completes with cytokinesis at 9 h. (C) TbPLK^{as} cells were synchronized by double-cut elutriation, and then 3MB-PP1 or vehicle control was added to the cells at different points in the cell cycle. Addition of drug just after elutriation (t = 0) totally blocked cell division, whereas addition at t = 3.5 caused a 50% block. Addition at t = 5.5 had essentially no effect on division. Error bars, SD of three biological replicates.

round of elutriation, this time selecting for the smallest cells in the culture.

We used the double-cut elutriation protocol to synchronize TbPLK^{as} cells. Elutriated cells were fixed and stained at different time points with propidium iodide to monitor DNA synthesis (Supplemental Figure S4). Cells fixed just after the elutriation procedure (t = 0 h) showed a substantial increase in G_1 cells, very few cells in S phase, and a suppressed G_2 population compared with an asynchronous culture. At t = 3 h, the population shifted into S phase, followed by most of the cells progressing to G_2 at t = 5 h. The cells began to return to G_1 at t = 7 h, indicating that cytokinesis was beginning. The cells then started another round of division, shifting into S phase at t = 10 h. Labeling elutriated cells with DAPI and TbPLK antibodies to look at individual cells by fluorescence microscopy showed that the culture was 99% 1N1K cells at t = 0 h, with only 10% of the population expressing TbPLK, compared with 60% of the 1N1K population in an asynchronous culture (data not shown). By following the TbPLK expression profile of the synchronized cells over time, we showed that all the cells in our synchronized cultures were within a 2.5-h portion of the cell cycle (data not shown).

Once the efficacy of the double-cut elutriation strategy in the TbPLK^{as} background had been established, we synchronized cells and treated them with 3MB-PP1 or vehicle control at specific points of the cell cycle. Cells were treated at four points in the cell cycle:

3MB-PP1 added at *t* (h)	Cells harvested at *t* = 7.5 h		
	Abnormal DNA states?	Bilobe duplication?	FAZ duplication?
0	Yes	Impaired	Impaired
3.5	No	Normal	Partial
5.5	No	Normal	Normal

Cells synchronized by elutriation were treated with 3MB-PP1 after 0, 3.5, or 5 h in culture. The cells were then fixed and stained with DAPI to evaluate their DNA state, along with antibodies against the FAZ or bilobe to assess the status of these structures.

TABLE 1: Summary of the DNA, bilobe, and FAZ states of synchronized cells upon treatment with 3MB-PP1 at different cell cycle stages.

immediately after completing the elutriation (*t* = 0 h), just before TbPLK expression (*t* = 1.5 h), at the point when TbPLK is on the bilobe and the structure begins to duplicate (*t* = 3.5 h), and when TbPLK is present on the new FAZ (*t* = 5.5 h; Figure 4B). These points were chosen based on previous work that established the timing of these cell cycle events and the position of TbPLK (Ikeda and de Graffenried, 2012). Elutriated TbPLK^{as} cells treated with vehicle control began to divide after 7 h in culture and completed a full round of division within 10 h (Figure 4C, control). Treatment with drug at *t* = 0 or 1.5 h caused total growth arrest (only *t* = 0 h is shown), whereas treatment at *t* = 3.5 h caused a 50% decline in growth. Cells treated at *t* = 5.5 h showed little or no growth defects compared with vehicle control within the time frame of the experiment.

The elutriated TbPLK^{as} cells were treated with 3MB-PP1 or vehicle control at *t* = 0, 1.5, 3.5, or 5.5 h and then harvested at *t* = 7.5 h to ensure that most of the cells had not undergone cytokinesis. The cells were then fixed and stained with DAPI and antibodies against bilobe and FAZ markers. Treatment with drug at *t* = 0 or 1.5 h led to decreased numbers of 1N2K cells and the appearance of 2N1K cells, but adding the drug at later points had little effect on DNA state (Supplemental Figure S5A). We chose to concentrate on 2N cells for bilobe and FAZ analysis to be certain that we were analyzing cells that had not divided since the drug had been added. The duplication of the bilobe was impaired by the addition of 3MB-PP1 at *t* = 0 or 1.5 h, leading to 2N cells with a single bilobe (Supplemental Figure S5B). Treatment at later time points had no effect on bilobe duplication. FAZ duplication was completely blocked by 3MB-PP1 treatment at *t* = 0 or 1.5 h, whereas treatment at *t* = 5.5 h had no effect (Supplemental Figure S5C). Treatment at 3.5 h produced an intermediate phenotype, where 40% of 2N cells had either a single FAZ or an snFAZ. A summary of the effects of TbPLK^{as} inhibition at different time points on DNA content, bilobe, and FAZ is presented in Table 1. We chose to study the *t* = 3.5 h time point to establish the precise bilobe and FAZ phenotype in more detail.

Considering the close spatial association of the bilobe and FAZ and how the structures duplicate within a narrow time window, it has been difficult to say with certainty whether one requires the other for assembly (Esson *et al.*, 2012; Ikeda and de Graffenried, 2012). If bilobe duplication is necessary for new FAZ assembly, then the absence of new FAZ in cells lacking TbPLK activity could be due to defects in bilobe duplication. To address this issue, we treated TbPLK^{as} cells with 3MB-PP1 or a vehicle control 3.5 h after synchronization, then harvested them at 7.5 h and fixed and stained them with TbCentrin4 or FAZ1 antibodies (Figure 5A). Cells treated with 3MB-PP1 were able to assemble new bilobes but frequently had detached flagella, suggesting a FAZ defect (Figure 5B, d–f; arrowheads depict new bilobes, arrows show detached flagella; Figure 5D). Forty percent of 2N cells had either no new FAZ or an snFAZ (Figure 5C, d–f, empty arrowheads, and E). This result shows that TbPLK activity is directly necessary for FAZ elongation. We measured the length of the new FAZ in 2N cells in vehicle control– and drug-treated samples to further establish the FAZ phenotype. In vehicle control–treated samples, new FAZ length ranged from 5 to >11 µm, with a median length of 10.1 µm (Figure 5F). There were no FAZ <5 µm. In 3MB-PP1–treated samples, cells with aberrant snFAZ of <5 µm were observed, but the median length of the new FAZ also dropped to 7.1 µm. TbPLK activity is not only necessary for the initiation of a new FAZ, but it also is required for the structure to reach its full length.

Having shown that cell synchronization along with TbPLK^{as} inhibition allows us to study cell cycle events with high temporal resolution, we decided to revisit the function of TbPLK in the duplication and inheritance of the basal body. Recent work shows that the newly assembled basal body, which nucleates the new flagellum, rotates around the old flagellum in a counterclockwise motion before the new flagellum emerges from the flagellar pocket (Lacomble *et al.*, 2010). This rotation may facilitate the production of a new flagellar pocket and situates the new flagellum in the posterior of the cell, where it can extend properly. This rotation occurs early in the cell cycle when TbPLK is still in the pocket region; if this process does not occur, it could lead to basal body segregation defects due to steric clashes between the assembled organelles.

To test for defects in basal body rotation, we synchronized TbPLK^{as} cells, treated them with 3MB-PP1 or vehicle control at *t* = 0 h, and then harvested them at *t* = 4 h (Figure 6A). This endpoint was chosen because almost all cells should have undergone rotation by this time. The cells were placed on grids, and whole-mount cytoskeletons were prepared by extraction with detergent, followed by labeling with aurothioglucose for negative-stain electron microscopy (Höög *et al.*, 2010). At early points in the cell cycle, the probasal body is found on the anterior side of the mature basal body. When the probasal body matures, it docks with the flagellar pocket membrane and nucleates a new flagellum, which contacts the old flagellum (Figure 6B). Before the new flagellum emerges from the flagellar pocket, the new basal body rotates in a counterclockwise direction around the old flagellum, assuming a position in the posterior of the cell (Figure 6B). In vehicle control–treated cells the new basal body was positioned on the posterior side of the old basal body in 90% of cells, indicating that rotation had occurred (Figure 6, C, a–b, and F). If basal body rotation failed to occur, the new basal body would remain on the anterior side of the old basal body (Figure 6D). In 3MB-PP1–treated cells the new flagellum was frequently detached from the cell body, indicating a defect in new FAZ formation (Figure 6E, a–b). The new basal body had not rotated in 70% of the cells (Figure 6, E, a–b, and F). The flagella appeared to cross one another at the point where they emerged from the flagellar pocket. Additional images of 3MB-PP1–treated cells where basal body rotation has been inhibited are shown in Supplemental Figure S6.

DISCUSSION

TbPLK tolerates the analogue-sensitive mutations and responds to the modified inhibitor 3MB-PP1 in vitro and in vivo. TbPLK^{as} cells responded to 3MB-PP1 treatment while treatment of wild-type cells with drug had no effect on growth. This clearly shows that the phenotypes observed in the TbPLK^{as} cell line upon drug treatment are due to inhibition of TbPLK^{as}. The recombinant analogue-sensitive kinase was not as active as wild-type TbPLK. However, the activity of

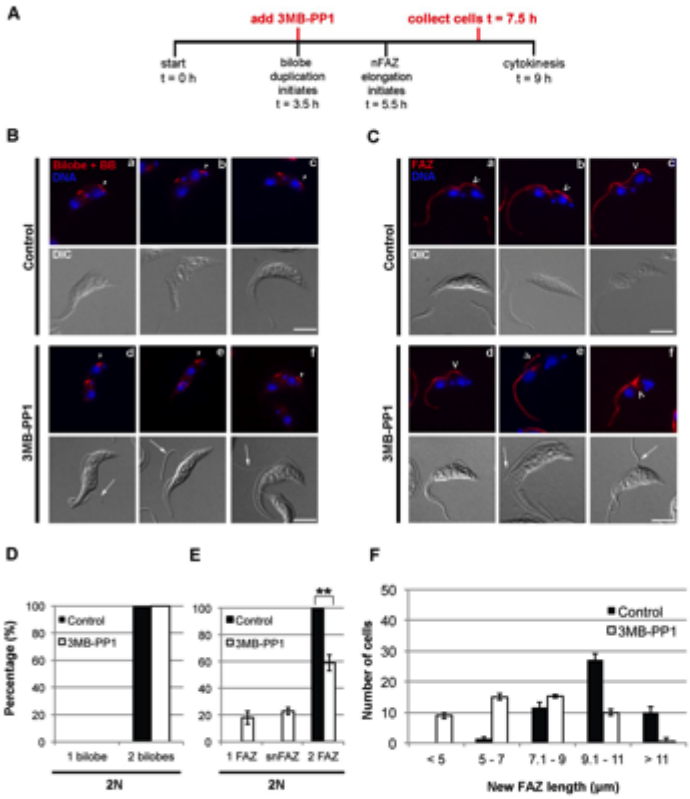

FIGURE 5: Treatment of synchronized TbPLK^{as} cells with 3MB-PP1 at $t = 3.5$ h produces cells that duplicate the bilobe but have new FAZ defects. (A) TbPLK^{as} cells were synchronized and treated with 3MB-PP1 or vehicle control at $t = 3.5$, followed by fixation at $t = 7.5$. (B) Cells treated as in A were labeled with anti-TbCentrin4 antibody (Bilobe+BB; red) and DAPI (DNA; blue). Cells that had reached the 2N state after treatment with 3MB-PP1 at $t = 3.5$ were able to duplicate their bilobe (arrowheads, d–f) but frequently had detached flagella (arrows). (C) Cells treated as in A were labeled with anti-FAZ1 antibody (FAZ; red) and DAPI (DNA; blue). Cells that had reached the 2N state after treatment with 3MB-PP1 at $t = 3.5$ frequently had FAZ that were too short (d–f; empty arrowheads) and had detached flagella (arrows). (D) Quantification of the bilobe state of the 2N cells in B. (E) Quantification of the FAZ state of the 2N cells in C. (F) Quantification of new FAZ length in 2N cells in C. Cells treated with 3MB-PP1 at $t = 3.5$ have shorter new FAZ than cells treated with vehicle control. Scale bars, 5 µm. Error bars, SD of three biological replicates. For D and E, 80 2N cells were counted per condition. $^{**}p < 0.01$. For F, 50 cells were counted per condition. A Student's t-test was performed to compare the means of the two distributions, giving $p < 0.05$.

the sensitized kinase was sufficient to support near-wild-type growth in procyclic cells, even at slightly lower expression levels. Previous work showed that a human PLK1 inhibitor blocks the activity of TbPLK in vitro and causes defects in cytokinesis in vivo, but the specificity of the inhibitor in trypanosomes was not established (Li *et al.*, 2010). The drug inhibits other human kinases such as Aurora and CDK2 in vitro with IC_{50} values that fall within the concentration range used for in vivo experiments with trypanosomes (5 µM), raising the possibility that the trypanosome homologues of these kinases were also affected and contributed to the observed phenotypes.

In asynchronous cells, TbPLK^{as} inhibition and TbPLK depletion by RNAi lead to similar phenotypes. The fact that two distinct methods give very similar phenotypes argues strongly that TbPLK plays an important role in FAZ and bilobe duplication. The similarity between the RNAi and small-molecule inhibition phenotypes suggests that the essential functions of TbPLK are mediated by its enzymatic activity. TbPLK is the sole PLK family member in trypanosomes, making up-regulation of a compensatory enzyme unlikely. TbPLK, like other polo homologues, appears to be degraded at the end of each cell cycle, which improves the effectiveness of RNAi (Lindon and Pines, 2004). Previously we were able to observe near-quantitative depletion of TbPLK from cultures using RNAi within 8 h of induction (de Graffenried *et al.*, 2008; Ikeda and de Graffenried, 2012). Even with this rapid effect, the TbPLK-depleted cultures did not show any growth defects at this point, whereas TbPLK^{as} inhibition yields growth defects within 6 h of treatment.

Inhibition of TbPLK causes the kinase to remain in the flagellar pocket region of the cell throughout the cell cycle. The inability of the kinase to migrate may be due to the absence of the new FAZ, which could recruit the kinase and function to transport it toward the anterior of the cell. At early points in the cell cycle TbPLK localizes to the MtQ (Ikeda and de Graffenried, 2012). If these microtubules are not initiated or if their extension is cut short, the kinase may be trapped within the flagellar pocket region. PLKs are frequently recruited by upstream kinases such as Cdk1 that generate phosphosites recognized by the polo-box domain of PLK (Preisinger *et al.*, 2005; Crasta *et al.*, 2006). The upstream kinases that control TbPLK localization may continue to generate binding sites for TbPLK within the pocket region even though its activity is blocked. Migration of TbPLK may require the kinase to generate its own binding sites on the FAZ, which would explain its inability to localize to this structure upon inhibition. PLK1 creates a subset of its own binding sites during anaphase (Neef *et al.*, 2007). Previous work showed that kinase-dead versions of TbPLK, the use of which is analogous to the inhibition strategy used here, remain associated with the FAZ (Sun and Wang, 2011; Yu *et al.*, 2012). However, the kinase-dead TbPLK was overexpressed using a very strong promoter, which can lead to mislocalization of the protein. It is also possible that the kinase-dead mutants produce a different phenotype from small-molecule inhibition, perhaps allowing the new FAZ to form to some extent.

Combining analogue-sensitive inhibition with cell synchronization established the precise role of TbPLK at different points of the cell cycle. Previous work with TbPLK RNAi on asynchronous cultures

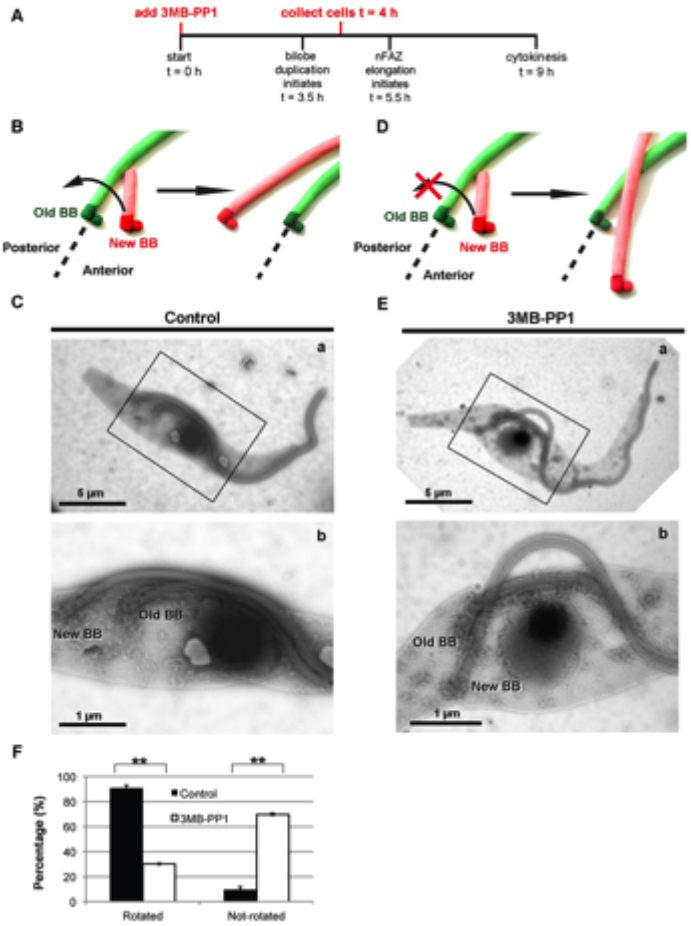

FIGURE 6: Treatment of synchronized TbPLK^{as} cells with 3MB-PP1 at t = 0 h blocks rotation of the duplicated basal body. (A) TbPLK^{as} cells were synchronized and treated with 3MB-PP1 or vehicle control at t = 0 and then collected at t = 4 and processed for negative-stain electron microscopy. (B) A schematic of the progression of basal body rotation. The old basal body (Old BB) and flagellum are shown in green, and the new basal body (New BB) and flagellum are shown in red. The new basal body rotates around the old basal body before the new flagellum emerges from the flagellar pocket. (C) A 1N cell from a vehicle control–treated (Control) sample. The new flagellum is present in the posterior of the cell, having rotated from the anterior side of the old flagellum during its maturation process. (D) A schematic of the aberrant case in which basal body rotation does not occur. (E) In the 3MB-PP1–treated cell, the new flagellum is detached from the cell body and has not rotated around the old flagellum (a, b). (F) Quantification of the data in B. Scale bar size is noted in the individual images. Error bars, SD of three biological replicates with between 55 and 65 cells counted per condition. **$p < 0.01$.

was hampered by the fact that TbPLK plays many different roles in cell division, and loss of activity at different points in the cell cycle most likely has different outcomes. Cells that are affected by TbPLK depletion early in the experiment may change their appearance over time due to the progression of cell cycle events that are not governed by TbPLK, such as karyokinesis. Using synchronous cells, we were able to show that inhibition at early points of the cell cycle blocks cell division and causes severe defects in bilobe and FAZ duplication. Treatment at late time points had little or no effect on growth, which suggests that early phosphorylation events that trigger cytoskeletal duplication are essential events in the *T. brucei* cell cycle.

Using synchronous cells, we showed that TbPLK^{as} inhibition led directly to FAZ defects. Previous work suggested that FAZ assembly requires TbPLK, but this result relied on RNAi in asynchronous cells (Ikeda and de Graffenried, 2012). This limitation meant that although TbPLK depletion led to cells with FAZ defects, these cells almost always had defects in bilobe duplication or basal body segregation. Because depletion of bilobe and basal body components blocks formation of the new FAZ, it seemed possible that the FAZ defects in TbPLK RNAi cells could be secondary effects (Selvapandiyan *et al.*, 2007; Shi *et al.*, 2008). By using synchronous cells and acute inhibition, we can inhibit TbPLK activity at the point when FAZ duplication occurs but after bilobe duplication. This allows us to clearly show that FAZ duplication and extension rely on TbPLK activity and are not just blocked due to bilobe defects. Inhibition of TbPLK also shortened the overall length of the new FAZ. Considering that treatment of cells with drug at *t* = 3.5 h had a partial effect on growth, it is likely that some of the shorter new FAZ were still functional. This result argues that TbPLK is involved in FAZ extension late in the cell cycle but that once the new FAZ elongates beyond a critical length, which is at least 5 µm, cytokinesis can proceed.

Inhibition of TbPLK^{as} early in the cell cycle leads to defects in basal body rotation. The rotation of the new basal body resolves a series of steric clashes between the new flagellum and MtQ that would arise if it remained on the anterior side of the old basal body and may allow the new flagellar pocket to form by pulling membrane along the old MtQ, which delineates the new pocket (Gadelha *et al.*, 2009; Lacomble *et al.*, 2010). TbPLK is present on the basal bodies early in the cell cycle, just before bilobe duplication, which is consistent with the timing of the rotation (Ikeda and de Graffenried, 2012). The forces responsible for basal body rotation, which includes movement of the new MtQ and probasal body, are not known. PLK1 plays an important role in centriole separation in mammalian cells by acting on the kinesin Eg5 (Le Guellec *et al.*, 1991; Hagan and Yanagida, 1992; Smith *et al.*, 2011). It is possible that the rotation is mediated by a related kinesin. It is important to note that the basal bodies in trypanosomes contact the flagellar pocket membrane and are linked

to the kinetoplast, which puts additional constraints on their movement (Robinson and Gull, 1991; Lacomble *et al.*, 2009). Basal body rotation usually occurs before emergence of the new flagellum and may be essential for the formation of a new flagellar pocket (Lacomble *et al.*, 2010). In cells that have not managed to undergo rotation, the new flagellum may emerge from the same flagellar pocket collar, as is the case with TbPLK RNAi cells (Ikeda and de Graffenried, 2012). It appears likely that once the two flagella are threaded through the same FPC, the cell will not be able to generate a new pocket and the two flagella may be confined within the same membrane compartment, making their separation unlikely.

To our knowledge, our modification of TbPLK is the first time that the analogue-sensitive method has been used in the kinetoplastids. Kinases comprise approximately 2% of the sequenced kinetoplastid genomes, which argues that protein phosphorylation plays important and diverse roles in these organisms (Parsons *et al.*, 2005). In novel kinases in which the gatekeeper residue can be easily identified and its mutation tolerated, the analogue-sensitive approach should provide a viable alternative to depletion by RNAi. Many of the kinetoplastid kinases are members of kinase families that are amenable to the analogue-sensitive strategy, such as the CDKs and MAPKs (Naula *et al.*, 2005; Parsons *et al.*, 2005; Morand *et al.*, 2012). It is likely that many of these kinases could be studied using this method, allowing acute inhibition without concerns for off-target effects.

MATERIALS AND METHODS

Cell culture

All experiments were performed in the procyclic *T. brucei brucei* 427 strain. Cells were cultured at 27°C in SDM-79 medium supplemented with 7.5 µg/ml hemin and 20% heat-inactivated fetal calf serum (Sigma-Aldrich, St. Louis, MO). Cell growth was monitored using a particle counter (Z2 Coulter Counter; Beckmann Coulter, Brea, CA).

Sf9 and High Five insect cells were cultured according to the manufacturer's instructions (Growth and Maintenance of Insect Cell Lines; Invitrogen, Carlsbad CA).

TbPLK inhibitor

3MB-PP1 was originally obtained from Kevan Shokat (University of California, San Francisco, San Francisco, CA) and later purchased from Calbiochem (La Jolla, CA).

Antibodies

The anti-FAZ1 (L3B2) was provided by Keith Gull (University of Oxford, Oxford, United Kingdom). The mouse anti-TbCentrin4 monoclonal and rabbit anti-TbPLK polyclonal antibodies were reported previously (de Graffenried *et al.*, 2008; Ikeda and de Graffenried, 2012). The rat monoclonal anti-TbPLK will be described in a subsequent publication (de Graffenried and Warren, unpublished data).

TbPLK^{as} cell line construction

A knockout construct comprising the puromycin resistance gene flanked by 500 base pairs of TbPLK 5′ and 3′ untranslated regions (UTRs) was transfected into 427 cells, which were cloned and put under puromycin selection. Puromycin-resistant clones were screened by PCR to assure proper targeting of the puromycin cassette. One positive clone was chosen and transfected with an endogenous tagging construct that appends either Ty1 or YFP to the N-terminus of proteins under blasticidin resistance (Morriswood *et al.*, 2009). This construct was directed against the TbPLK genomic loci using 500 base pairs of the TbPLK 5′ UTR and first 456 base pairs of the open reading frame. The portion of the open reading frame used for targeting contained the two point mutations (L118G and C57V) necessary to produce TbPLK^{as}. The sequence around the L118G mutation included silent mutations that introduced an *Avr*II restriction site (nt 341-TTTATAT**CCTAGG**TGAGAAGTGCAG-nt 366). After transfection, cells were cloned and selected with puromycin and blasticidin. Genomic DNA was isolated from doubly resistant clones, and PCR was performed to amplify an ~800–base pair fragment of TbPLK using the primers 5′-TTCCGTTGCGGAAGGATGCTCG-3′ and 5′-TGTAGAAGAGTGTGGTAGGTGCTGTCGTCG-3′. This fragment contains both mutation sites. The PCR product was subjected to digestion with *Avr*II to determine whether both mutations were present. The fragment was then submitted for DNA sequencing.

Western blotting

Cells were washed once in phosphate-buffered saline (PBS) and then lysed directly in SDS–PAGE loading buffer. Then 3×10^6 cell equivalents were loaded per sample and fractionated by SDS–PAGE. Proteins were then transferred to nitrocellulose and probed with rat anti-TbPLK, followed by horseradish peroxidase–labeled secondary antibodies. Enhanced chemiluminescence and film were used for detection.

In vitro TbPLK^{as} expression

The gatekeeper mutation (L118G) and the compensatory mutation (C57V) were introduced into N-terminal hexahistidine-tagged TbPLK (de Graffenried and Warren, unpublished data) using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA) according to the manufacturer's instructions. This construct was cloned into pFastBac HT, and recombinant baculovirus was generated using the Bac-to-Bac Expression System (Invitrogen). Sf9 cells were transfected with the baculovirus DNA to produce virus, which was then used to infect additional Sf9 cells to produce high-titer virus. The obtained high-titer virus was then used to infect High Five cells, which were then grown for 60 h before lysis in lysis buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 1% Triton X-100, 1% glycerol, 300 mM NaCl, and 15 mM imidazole). The recombinant TbPLK was purified using TALON metal affinity resin (Clontech, Mountain View, CA) according to the manufacturer's conditions.

Kinase assays

The kinase assay protocol was adapted from a previously published protocol (Hammarton *et al.*, 2007). Purified TbPLK or TbPLK^{as} were mixed with 10 µg of recombinant TbCentrin2 and different concentrations of 3MB-PP1 or vehicle control (DMSO) in kinase assay buffer (50 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.2, 20 mM $MgCl_2$, 10 mM ethylene glycol tetraacetic acid, and 2 mM dithiothreitol). ATP, 20 µM, containing 0.5 µCi of [γ-^{32}P]ATP was then added to the reactions, which were then incubated for 30 min at 30°C. SDS–PAGE loading buffer was then added, followed by boiling. Thirty percent of each reaction was fractionated using SDS–PAGE, then stained with GelCode (ThermoScientific, Waltham, MA) to visualize proteins. The gel was dried under vacuum and exposed to a phosphorimaging plate, followed by visualization with a Typhoon scanner (GE Healthcare, Piscataway, NJ). The resultant image file was quantified in ImageJ (National Institutes of Health, Bethesda, MD; 1.47h) using the "plot lanes" tool.

Statistics

All quantifications were generated from three biological replicates. For comparisons of groups where the outcome variable is

categorical the chi-squared test was used. For comparisons with data that fit a normal distribution, the Student's *t* test (two tailed, equal variance, unpaired) was used. The *p* value and number of cells counted are given in the figure legends.

Immunofluorescence microscopy

Cells were taken from culture and washed once with PBS and then adhered onto coverslips before fixation in −20°C methanol for 15 min. Samples were rehydrated with PBS and blocked overnight at 4°C in 3% bovine serum albumin in PBS.

Primary antibody incubations (anti-FAZ1, diluted 1:200; anti-TbCentrin4, 1:400; rabbit anti-TbPLK, 1:200) were done in blocking buffer for 1 h at room temperature. Coverslips were then washed with PBS, blocked for 20 min, and incubated with the appropriate Alexa dye–conjugated secondary antibodies (Invitrogen) for 1 h at room temperature. Cells were then washed three times in PBS and mounted using DAPI Fluoromount G (Southern Biotech, Birmingham, AL).

Images were acquired using an inverted microscope (Axio Observer Z1; Carl Zeiss MicroImaging, Jena, Germany) equipped with a PCO 1600 camera. Image processing was done using ImageJ and Photoshop (Adobe, San Jose, CA). For each condition and time point, 300 cells were analyzed in asynchronous cultures and 100 in synchronous ones.

Bilobe and FAZ length measurements

Bilobe and FAZ length measurements were done using the Freehand Line and Measure tools in ImageJ.

Electron microscopy

Negative-stain electron microscopy was performed as described previously (Höög *et al.*, 2010; Ikeda and de Graffenried, 2012).

Double-cut elutriation

Cells were cultured in MEM (Life Technologies, Carlsbad, CA) supplemented with MEM nonessential amino acids (Life Technologies) and with 20% heat-inactivated fetal calf serum. Approximately 4×10^9 cells were collected for double-cut elutriation, which was performed as previously described (Archer *et al.*, 2011), except for the interval between the first and second cuts, which was extended to 90 min. Synchronous cells were set back in culture in supplemented MEM, and growth was monitored for the next 10 h.

Flow cytometry analysis

Approximately 2×10^6 cells were harvested by centrifugation, washed once with PBS, and fixed by dropwise addition of ice-cold 70% ethanol/30% PBS while vortexing. Cells were kept at 4°C overnight, then collected by centrifugation and resuspended in 500 µl of PBS containing RNase A (10 µg/ml) and propidium iodide (10 µg/ml) and incubated at 37°C for 30 min. Cells were analyzed using a FACSCalibur cytometer (BD Biosciences, San Diego, CA).

ACKNOWLEDGMENTS

We thank Graham Warren for support and critical comments and the members of the Warren laboratory for comments on the manuscript. We also thank Stuart Archer (Zentrum für Molekulare Biologie, Heidelberg, Germany) and Cristina Purificato (Istituto Superiore di Sanità, Rome, Italy) for valuable advice on elutriation, Marisi Castañón for help with baculovirus expression, and Andras Aszodi for help with statistics. This work was supported by the Austrian Science Fund (FWF) by Grant P21550-B12 to Graham Warren and C.L.dG. and via the doctoral program Molecular Mechanisms of Cell Signaling (W1220-B09).

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Chapter 3. Unpublished results

3.1. Prologue

The following section includes my contribution to several ongoing projects. This work was initiated at the University of Vienna and will be continued at Brown University, Providence, USA, where Ass. Prof. de Graffenried has recently established a new research group.

The previously published work (Lozano-Núñez et al., 2013) complemented and expanded our current understanding of TbPLK-mediated cytoskeletal replication in *T. brucei*. At the moment, the calcium binding protein Centrin2 has been identified as TbPLK substrate in vitro and in vivo(de Graffenried et al., 2013). Centrin2 localizes to the bilobe during its replication, and it is also present at the basal bodies and the FAZ. The residue S54 in the N-terminal domain is of special importance because it needs to be phosphorylated during bilobe duplication. Dephosphorylation of S54 seems to be required for triggering the end of bilobe duplication and proceeding through the cell cycle.

As additional TbPLK substrates have not been identified yet, we adopted several screening approaches independent from each other but based on proteomics. The advent of proteomics allows nowadays the interrogation of different kind of systems with high throughput analysis. I actively contributed in the sample preparation.

Our hope with these screenings is to obtain an initial list of candidate proteins, which will later be analyzed by means of extensive functional studies.

3.2. SILAC and iTRAQ

SILAC - Stable Isotopic Labeling of Aminoacids in Cell Culture

SILAC is a strategy based on the labeling of proteins for Mass Spectrometry (MS) proteomics methods(Ong et al., 2002). It is normally used to compare two cell populations exposed to different conditions or where a protein becomes depleted or inhibited, for instance. The label just consists of a different version of a specific amino acid. It gets incorporated into one of two cell populations by growing these cells in a media supplemented with this analogue. The natural form is usually called "light" and the analogue "heavy" because it contains a different stable isotope of a given atom. For instance: 12C vs. 13C or 14N vs. 15N. After several rounds of cell division, the population containing the heavy amino acid is able to replace all the light versions (up to 100%) by means of protein synthesis. The two cell populations are almost chemically identical but can be easily distinguished by MS because of their different m/z ratio. One of the main advantages of SILAC is that the two samples can be mixed and processed together, which ensures that the operating conditions are the same.

SILAC has been already successfully implemented in Trypanosomes (Urbaniak et al., 2012b)(Gunasekera et al., 2012). In our case, we want to compare the overall changes in the phosphoproteome of two asynchronousTbPLK^{as} cell populations, one used as a control sample and the other onetreated with 3MB-PP1, TbPLK inhibitor, for a short period (2h).This experiment requires a big number of starting cells and a laborious data analysis and quantification. We have performed several independent experiments and the data is currently under analysis at the MS facility.

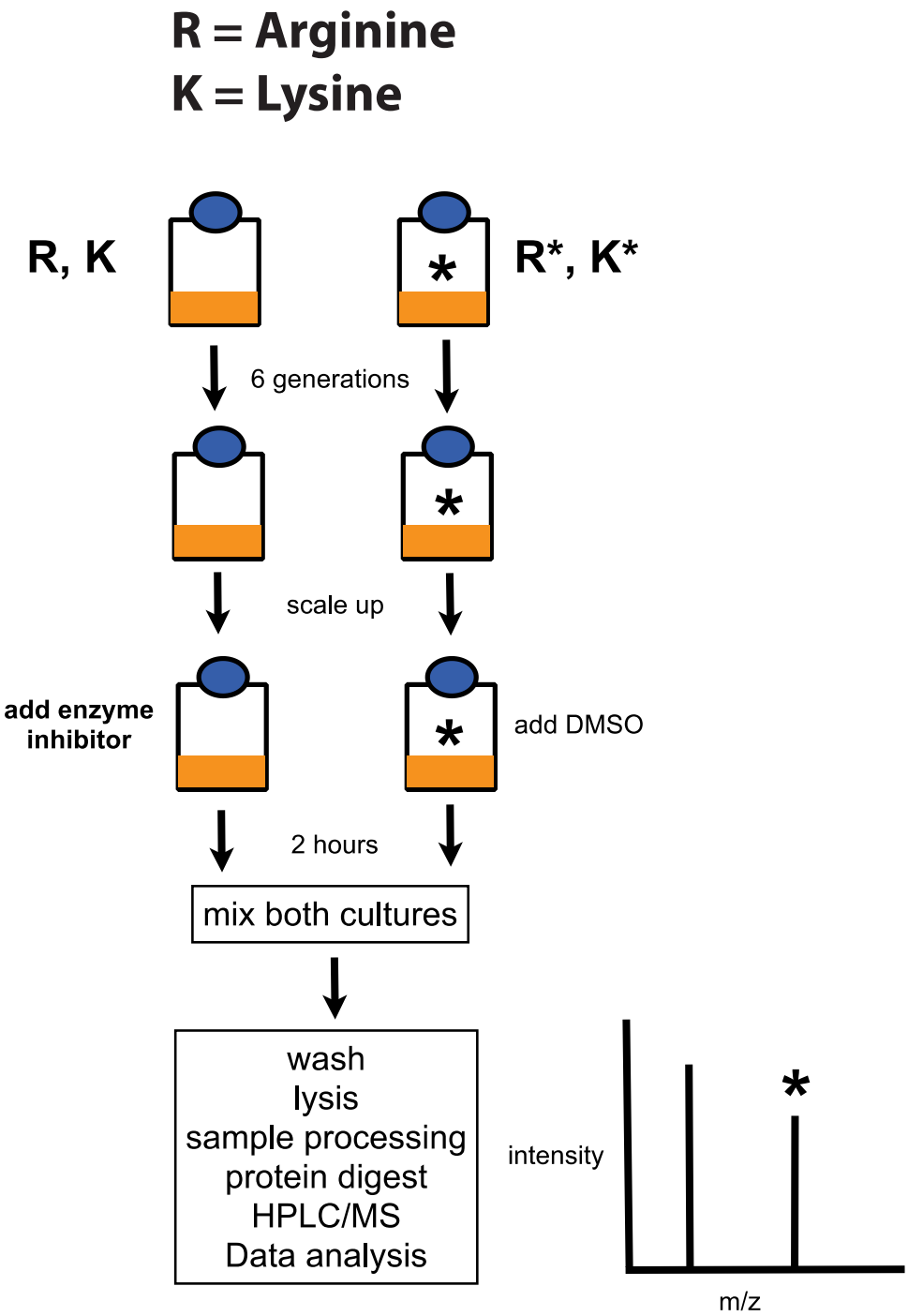

Figure 8 - Workflow for our SILAC experimental setup.

iTRAQ - Isobaric Tags for Relative and Absolute Quantification

As it was the case for SILAC, iTRAQ also allows the comparison of two cell conditions: control and drug treated cells, for instance. iTRAQ is as well based on the different labeling of two cell populations, but this time the label is done in a completely different way by using the corresponding iTRAQ reagents (Ross et al., 2004). There are different types, but they all consist of a group named reporter and a balance group that is able to react with peptides via their primary amines. The mass of the reporters and balancers is different for each reagent, but importantly, they are designed in such a way that the final mass of the peptide is always the same, independently of the reporter and balance combination used. One of the positive features of iTRAQ is that you do not need to work with cell cultures. It is a method one could apply directly after cell lysis. In addition, the procedure allows multisampling, as one can use several iTRAQ reagents in a single MS experiment.

The whole peptide mixture is analyzed by LC-MS/MS. The peptide identity is obtained from the fragmentation pattern of the different peptides. The quantity is analyzed by looking at the abundance of the different reporters.

In our setting, we have used again the TbPLK^{as}cell line, but this time a synchronous cell culture after elutriation. The starting material is lower than for SILAC, but it requires the collection and pooling together of 1N1K cells from three elutriations. One of the two conditions is treated with 3MB-PP1 for 2 hours. For the initial experiments, we chose a time interval around bilobe duplication, but the timeframe can be selected according to the specific needs. We have performed several independent experiments and the data is also under analysis at the MS facility.

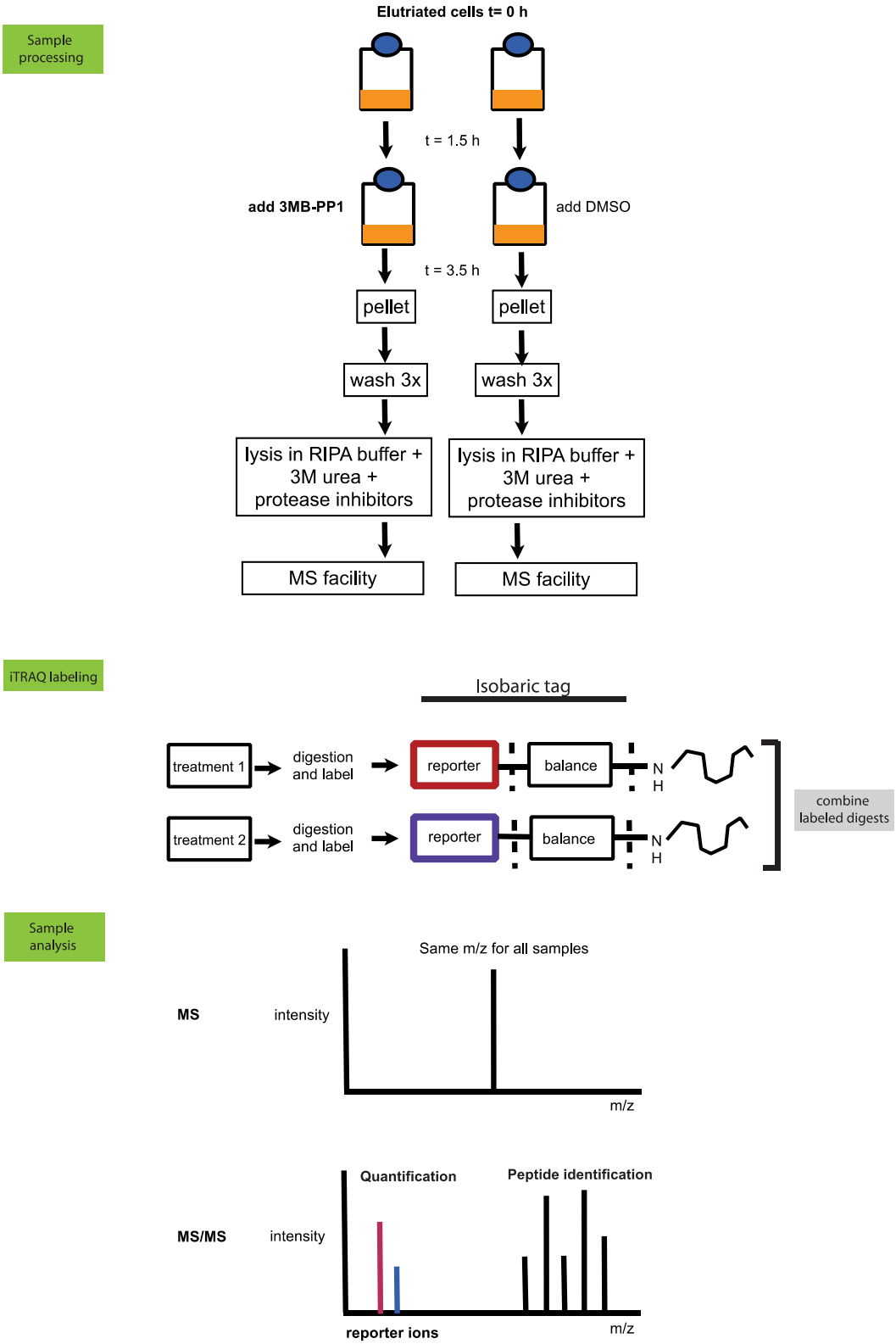

Figure 9 - Experimental diagram explaining the basic steps in iTRAQ.

Chapter 4. Discussion

In this thesis, we have investigated PLK in *T. brucei* by modifying TbPLK to become sensitive to 3MB-PP1, a modified kinase inhibitor. Our approach has the fundamental advantage that we can verify the specificity of this inhibitor to TbPLK by using the wild-type version as a negative control. Our work highlights the importance of small molecule inhibitors for exploring protein kinase signaling pathways. We demonstrate here that the analogue-sensitive approach can also be implemented in Kinetoplastids. We believe that this approach could also be applied to other kinases in *T. brucei*'s genome of yet unknown function. The method could potentially also be extended to other kinetoplastid parasites. However, we are aware that for our study we had the benefit of a high level of conservation in the PLK sequence. For other not so well-conserved kinases, the approach would also probably involve additional screenings for the identification of second-site suppressor mutations.

Our results confirmed previous RNAi studies and we gained insight into TbPLK function in basal body, bilobe, flagellar attachment and cytokinesis functions. We used a synchronized cell population that allowed us to inhibit TbPLK in narrow windows of *T.brucei*'s cell cycle. We found that the enzyme is necessary for the duplication of both the bilobe and the FAZ structures, and also for the rotation of the basal bodies. This rotation in *T. brucei* is necessary for the new flagellum to be able to grow without colliding with the old one. We hypothesize whether this rotation might also be necessary in other types of highly polarized cells, such as worm embryos, also for reasons related to space constraints.

TbPLK inhibition at different cell cycle points can completely block the new FAZ assembly or shorten its total length, depending on the time at which the inhibitor is added. How exactly FAZ lengths contributes to the overall cell length is not so well studied, but if a minimum cell length is not achieved, the new cell is not viable. Our studies did not show any direct effect of TbPLK on cytokinesis, suggesting that

failure to divide might probably be an effect of a defective cytoskeleton and rather not a specific failure in abscission.

Work done by us and others has shown that TbPLK is a highly motile kinase which localizes to different cytoskeletal structures during their duplication. Our contribution here added the observation that inactive TbPLK remains in the pocket region and is not able to migrate from the posterior to the anterior end of the cell along the FAZ.

In the next years, one of the priorities in the field will probably be the identification of TbPLK substrates and interactors. If we manage to find, for instance, kinesins or motor proteins that interact with TbPLK or become phosphorylated, we will be able to explain the molecular mechanisms that make this such a motile kinase. Along these lines, we have initiated quantitative proteomics screenings based on SILAC and iTRAQ methods that will hopefully provide us with a comprehensive list of candidate proteins. We have as well started to work with the BioID technology, a proximity-dependent biotin identification method that has been recently implemented in *T. brucei* by members of our Laboratory. By using TbPLK as a probe, we will probably be able to identify neighbors and interacting partners, which might as well be substrates. BioID will also be used in the next years for other proteins in *T.brucei* that localize to structures with rather few identified components.

This thesis has implemented the analogue-sensitive strategy in *T.brucei* and the technology has a high potential to be used in the next years in a variety of settings. Some applications could include: further interrogation of TbPLK's functions, exploration of other protein kinase pathways, uncharacterized organelles or cytoskeletal rearrangements during cell differentiation.

Chapter 5. Materials and Methods

For methods corresponding to the results in Chapter 2, please refer to the publication:

Lozano-Núñez, A., Ikeda, K.N., Sauer, T., and de Graffenried, C.L. (2013). An analogue-sensitive approach identifies basal body rotation and flagellum attachment zone elongation as key functions of PLK in Trypanosoma brucei. Mol. Biol. Cell *24*, 1321–1333.

Additional methods corresponding to Chapter 3:

Preparation of SILAC medium

Light and heavy SILAC mediawereprepared with SDM-79 depleted of Arg andLys, 20% dialyzed serum and other media supplements. Arg, Lys, *Arg and *Lys were added later to each medium.

Preparation of Lysis Buffer for SILAC and iTRAQ

The Lysis Buffer is a RIPA buffer containing 0.3 M urea and phosphatase inhibitors.

Sample preparation for SILAC

The total number of cells required was 1.24 x 10^9 at a concentration of 5x10^6 cells/mL. The amount of protein expected for this cell density was 8mg. Some days prior to the experiment, two cell cultures from the same line were established in light and heavy SILAC medium respectively, and passaged at low density to promote the adjustment to the new medium conditions. After at least six

generations, the cultures were scaled up with the aim of achieving the desired concentration for sample collection.

Once this cell number was achieved, the light culture was treated with 3MB-PP1 (5 µM) for 2 hours. The heavy culture was treated with a vehicle control. After incubation, both cultures were mixed together, washed twice with SILAC medium and finally resuspended in 660 µL Lysis Buffer. The lysate was vortexed vigorously and kept on ice for 30 minutes. The sample was handed in to the MS facility. A small aliquote was kept to perform a protein concentration assay.

Sample preparation for iTRAQ

The cell culture was synchronized in early 1N1K via centrifugal elutriation. 3MB-PP1 (5 µM) was added to the culture at t=1.5 h and incubated for 2 hours. Cells were then pelleted and snap-frozen in liquid nitrogen. Different pools of cells from several elutriations were frozen down and then pooled together. The total number of cells required per condition was 18 x 10^7, which corresponds to 1 mg of protein. After combination of the different fractions, proceed with the lysis as indicated for SILAC.

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Annex

Ana Lozano - Curriculum Vitae

Ana Lozano

Chemist and molecular biologist

Career history

2009 - present	University assistant (predoc)	Max F. Perutz Laboratories GmbH University of Vienna, Austria

Education

2009 - present	PhD in Molecular Biology	University of Vienna, Austria
2003 - 2008	Licenciado (MSc) in Chemistry	University of Salamanca, Spain

Research

Dissertation

Member of the International PhD Program "Molecular Mechanisms of Cell Signaling"
PhD thesis on the role of Polo-like kinase in cell division in trypanosomes
Supervisor: Prof. Graham Warren, University of Vienna

Short research internships

2009	6 months	School of Chemistry, University of Edinburgh, UK
2008	2 months	Institute of Nuclear Chemistry and Technology, Warsaw, Poland
2008	2 months	Institute of Molecular Biology - CSIC, Barcelona, Spain
2007	3 months	Institute of Photonic Sciences (ICFO), Barcelona, Spain

Main scientific skills

Trypanosomes cell culture, mammalian and insect cells culture, Baculovirus expression system, bacterial culture, gene cloning, Western blotting, immunofluorescence and live cell imaging, cell cycle and cell death analysis, flow cytometry, quantitative proteomics

Publications

Lozano A, Ikeda KN, Sauer T, de Graffenried CL: *An analog-sensitive approach identifies basal body rotation and Flagellar Attachment Zone elongation as key functions of PLK in Trypanosoma brucei.* Mol Biol Cell, 2013

Software

- Microsoft Office, iWork, Adobe Illustrator and Photoshop, ImageJ, R for biologists, SPSS statistical package

Management and organization

- **European Business Competence Licence (EBC*L)** levels A and B: Business law, business objectives, accounting, costing and pricing, business plan, marketing and sales
- **Patent searching** training workshop, European Patent Office and University of Vienna
- **Project management** course, Pentalog Unternehmensberatung Vienna
- **Volunteer organizer** of several events at the Max F. Perutz Laboratories, such as PhD & PostDoc retreat 2010, Career Day 2011 or Lange Nacht der Forschung 2012

Presentation and communication

Presentation awards

- **Best poster award** at the 7th PhD Symposium Medical University of Vienna, Austria
- **Winner Science Slam** Vienna 2012, supported by Austrian Ministry of Research
- **2nd place at Falling Walls Lab** Vienna 2012, organized by Falling Walls and A.T. Kearney

Poster presentations at international meetings

2012	San Francisco, USA	American Society of Cell Biology Annual Meeting
2012	Roscoff, France	CNRS Conference on Cell division
2011	Heidelberg, Germany	EMBL Symposium "Imaging the processes of life"
2011	Woods Hole, USA	22nd Molecular Parasitology Meeting

Languages

Spanish	mother tongue
English	excellent (Certificate of Proficiency in English, C2)
German	advanced (Österreichisches Sprachdiplom Deutsch, C1)
French	intermediate (B1)
Serbo-Croatian:	intermediate (B1)

Volunteer translator for *Science in School*, a science teaching journal based at EMBL Heidelberg

Miscellanea

- Participant in Roche Continents 2012, a one-week seminar about creativity in science for 100 European students selected by Roche
- Representative of Spain in the 28th Study trip to Japan for the European Youth, organized by the Japanese Ministry of Foreign Affairs

References

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Scientific Director Max F. Perutz Laboratories (MFPL), University of Vienna, Austria

Dr. Susana Santos (susana.santos@icfo.es)
Program Manager, Institute of Photonic Sciences (ICFO), Barcelona, Spain