Functional characterisation of cohesin subunit SMC3 and separase and their roles in the segregation of large and minichromosomes in *Trypanosoma brucei*

being a Thesis submitted for the Degree of Doctor of Philosophy

in the University of Hull

by

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Alexandria University, Egypt

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<tr>
<td>APC</td>
<td>Anaphase Promoting Complex</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine TriPhosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic Acid Protein</td>
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<tr>
<td>bp</td>
<td>base pair</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>CARs</td>
<td>Cohesin Attachment Regions</td>
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<td>CDK</td>
<td>Cyclin-Dependent Kinase</td>
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<td>Cdc-Related Kinase</td>
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<td>Cyclin</td>
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<td>DAPI</td>
<td>4',6-DiAmidino-2-PhenylIndole</td>
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<td>Endoplasmic Reticulum</td>
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<td>ESAGs</td>
<td>Expression Site Associated Genes</td>
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<td>FEAR</td>
<td>Cdc Fourteen Early Anaphase Release</td>
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<td>Gap2 phase</td>
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<td>HR</td>
<td>Homologous Recombination</td>
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<td>HRP</td>
<td>HorseRadish Peroxidase</td>
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<td>HYG</td>
<td>Hygromycin</td>
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<td>ICs</td>
<td>Intermediate Chromosomes</td>
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<tr>
<td>IPTG</td>
<td>IsoPropyl β-D-1-ThioGalactopyranoside</td>
</tr>
<tr>
<td>Kbp</td>
<td>Kilo base pair</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
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<tr>
<td>K</td>
<td>Kinetoplast</td>
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<tr>
<td>LB broth</td>
<td>Lauria-Bertani broth</td>
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<td>mAbs</td>
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<td>Mitotic Exit Network</td>
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<td>N</td>
<td>Nucleus</td>
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<td>OD</td>
<td>Optical Density</td>
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<td>Open Reading Frame</td>
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<td>polyclonal Antibodies</td>
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<td>PARP</td>
<td>Procyclic Acidic Repetitive Protein</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
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<tr>
<td>PFR-A</td>
<td>Paraflagellar Rod-A gene</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PI</td>
<td>Propidium Iodide</td>
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<tr>
<td>PLE</td>
<td>Phleomycin</td>
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<tr>
<td>P&lt;sub&gt;r&lt;/sub&gt;RNA</td>
<td>rRNA promoter</td>
</tr>
<tr>
<td>PVP</td>
<td>PolyVinylPyrrolidone</td>
</tr>
<tr>
<td>rDNA</td>
<td>ribosomal DeoxyriboNucleic Acid</td>
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<td>RNAi</td>
<td>RNA interference</td>
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<td>RT-PCR</td>
<td>Reverse Transcriptase-Polymerase Chain Reaction</td>
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<td>Standard Deviation</td>
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<td>SCC</td>
<td>Sister Chromatid Cohesion</td>
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<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
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<td>TAC</td>
<td>Tripartite Attachment Complex</td>
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<td>T&lt;sub&gt;brucei&lt;/sub&gt;</td>
<td>Trypanosoma brucei</td>
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<td>Tris-Phosphate Buffer-Tween</td>
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<td>Tetracycline</td>
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<td>Tet OP</td>
<td>Tetracycline Operator</td>
</tr>
<tr>
<td>TetR</td>
<td>Tetracycline Repressor</td>
</tr>
<tr>
<td>VSG</td>
<td>Variant Surface Glycoprotein</td>
</tr>
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<td>WHO</td>
<td>World Health Organisation</td>
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ABSTRACT

The genome of the African trypanosome, *Trypanosoma brucei*, presents an unusual karyotype in which two main classes of chromosomes, large and small minichromosomes, need to be faithfully replicated and segregated during the cell cycle. Although the large and minichromosomes are colocalised and segregated by association with the mitotic spindle, minichromosomes exhibit segregation patterns that differ from those observed for large chromosomes. To address whether this difference is reflected at a molecular level, two different proteins that have highly conserved functions in eukaryotic chromosomes segregation were characterised in this study. The first protein, SMC3, is a component of the chromosome cohesion apparatus that holds sister chromatids together after their replication until segregation at anaphase. The second protein, separase, is a cysteine protease that resolves sister chromatid cohesion at the onset of anaphase and has, in other organisms, additional functions during mitosis. The *T. brucei* cohesin subunit, TbSMC3, localised to the nucleus as a chromatin-bound protein from G1 phase until metaphase and dissociated from chromatin during anaphase until the completion of cell division. On the other hand, cytoplasmic localisation of separase with nuclear exclusion was prevalent until the onset of metaphase when the protein re-localised to the nucleus, thus providing a potential control mechanism to prevent premature cohesin cleavage. Interference with the normal expression of SMC3 and separase by RNA interference resulted in defects in growth rate, cell cycle progression and chromosomes segregation. TbSMC3 depletion produced a lethal phenotype and inhibition of cell cycle progression. Similarly, lethality with severe inhibition of cell cycle progression was the main feature of separase depletion. Using fluorescence *in situ* hybridisation (FISH), it was shown that SMC3 depletion had no visible effect on the symmetric segregation of the minichromosome population, but interferes with the faithful mitotic segregation of large chromosomes. In contrast, separase depletion blocks the segregation of both large and minichromosomes. In separase-depleted mitotic cells, cohesins remained bound to chromatin, which is in contrast to rapid dissociation of cohesins from chromatin in wild-type mitotic cells. The severity of segregation phenotypes after separase depletion was additionally explained by defects in the mitotic spindle assembly. In both SMC3 and separase depleted cells, cytokinesis in the absence of mitosis/karyokinesis was not inhibited in procyclic cells, resulting in the generation of anucleate “zoid” cells. The lethality imposed on trypanosome cells after depletion of both SMC3 and separase proteins indicate that they can serve as potential drug targets for anti-parasite chemotherapy.
1. GENERAL INTRODUCTION

1.1 Trypanosoma brucei and the related diseases

The African trypanosome, *Trypanosoma brucei*, is a unicellular protozoan parasite that causes sleeping sickness and debilitating Nagana disease in humans and animal livestock, respectively. The disease, sleeping sickness, affects mainly sub-Saharan African countries with estimated 300,000-500,000 cases of the disease reported in 2000 according to WHO (http://www.who.int/trypanosomiasis_african/en/), but the actual number is unknown as the infected people live in remote rural areas with inadequate medical surveillance. During that year, 2000, only 27,000 cases were diagnosed and treated from the sleeping sickness disease. In 2007, the number of new diagnosed cases of the disease has fallen to less than 15,000 according to the same source (WHO). Apart from the high mortality in humans associated with the acute cerebral form of the disease, a great economic impact is imposed on local communities because of Nagana disease that affects their cattle livestock. Sleeping sickness is invariably fatal if untreated, and all the available drugs have side effects, are costly and drug-resistances have recently emerged (Smith et al., 1998). Also, any chance to develop a specific vaccine is diminished by the fact that the parasites continuously change their antigenic surface coats in the bloodstream of the mammalian hosts.

Human African trypanosomiasis (HAT) or sleeping sickness is caused by two subspecies of *T. brucei*, namely *T. b. gambiense* and *T. b. rhodesiense*. The former subspecies occurs in Central and West Africa and associated with a chronic form of infection that can persist for several months before any clinical symptoms of the disease appear. The latter one causes the more virulent and acute infection in Southern and East Africa that commonly involves the central nervous system with confusion, bad coordination and sleeping disturbances (Hide, 1999). Beside *T. b. brucei*, the two main subspecies responsible for the Nagana disease in the animal livestock are *T. congolense* and *T. vivax*. *T. brucei* parasites are transmitted to their mammalian hosts by tsetse flies, which inhabit an area of the African continent extending about 10 degrees above and below the equator. Interestingly, the animal subspecies of *T. brucei, T. b. brucei*, is
susceptible to lysis by the normal human serum which in contrast to the human subspecies, *T. b. gambiense* and *T. b. rhodesiense* that are resistant to the trypanolysis activity (Pays et al., 2006). The trypanolytic activity is due to the human-specific serum apolipoprotein L1 (apo-L1) and the resistance to this activity is conferred by a truncated form of the variant surface glycoprotein (VSG) termed serum resistance associated (SRA) protein (De Greef and Hamers, 1994; Vanhamme et al., 2003).

### 1.2 Cellular architecture and life cycle of *Trypanosoma brucei*

*T. brucei* shows the typical features of the eukaryotic cell (*Figure 1.1*). All major organelles such as nucleus, mitochondrion, endoplasmic reticulum (ER) and Golgi apparatus can be seen by the conventional cytological means. The trypanosome cell has an elongated leaf-like shape that is defined by a highly polarized, subpellicular microtubule cytoskeleton (Gull, 1999). The cytoskeleton microtubules have a defined polarity with their minus ends toward the anterior end and plus ends toward the posterior end. The trypanosome cell motility depends on a single flagellum that originates from the basal body and exits the cell body through the flagellar pocket at the posterior end of the cell (Vaughan and Gull, 2003). The origin of the flagellum, the basal body, attaches to the mitochondrion by means of a tripartite attachment complex (TCA) structure (Ogbadoyi et al., 2003). *T. brucei* has a single mitochondrion which is an elongated structure that extends from the posterior to the anterior of the cell (*Figure 1.1*). The morphological characters of the mitochondrion vary between different stages of the trypanosome life cycle. In the bloodstream form, the mitochondrion is much repressed with a simple tubular structure devoid of cristae. This reflects the absence of the mitochondrial metabolic activity in these forms in which the cell relies on glycolysis as the main source for energy generation (Parsons, 2004). In the tsetse fly procyclic form, the mitochondrion is highly active due to the limited supply of glucose in the midgut of the fly. The single mitochondrion of *T. brucei* possesses a small genome called the kinetoplast (Shlomai, 2004). The kinetoplast is a network of topologically interlocked DNA rings including a few dozen maxicircles (~23 kbp) and several thousand minicircles (~ 1kbp) (Luks et al., 2002; Shlomai, 2004). The replication of the kinetoplast is intimately associated with the basal body replication in a microtubule-dependent way (Robinson and Gull, 1991).
Figure 1.1 Cell structure and morphology of *T. brucei*. The simplified sketch illustrates the most prominent features of a trypanosome cell. The thick arrow shows the transverse cut section in the cell body revealing the structure of the cytoskeleton microtubules. The figure is explained in more details in the text and is based on previous detailed drawings of the cellular structure of *T. brucei* (Grunfelder et al., 2003; Matthews, 2005).
The life cycle of *T. brucei* alternates between two different environments in two different hosts, the tsetse fly and the bloodstream of the mammalian hosts (Figure 1.2). During their life cycle, parasites differentiate into distinct life cycle stages that differ in their morphological, structural and biochemical properties (Fenn and Matthews, 2007; Matthews, 2005). The procyclic and epimastigote forms in the tsetse fly and the long slender form in the mammalian host are the proliferative stages of the life cycle. The infective metacyclic form and the short stumpy form are cell cycle-arrested in preparation for transmission to a different host. All trypanosome forms are covered by glycosylphosphatidylinositol (GPI)-anchored proteins which are procyclin in case of procyclic and epimastigote forms, or variant surface glycoproteins (VSGs) in the metacyclic, long slender and stumpy forms. The sequential expression of VSG genes enable the bloodstream parasites to escape the host immune system and to establish a persistent chronic infection in a process known as antigenic variation (Barry and McCulloch, 2001). Upon differentiation to the procyclic form in the tsetse fly midgut, the parasite switch to express an invariant glycoprotein named procyclin to cover its surface (Acosta-Serrano et al., 2001; Roditi et al., 1989). The infective metacyclic trypanosomes express metacyclic VSGs for as long as 7 days after entry into the bloodstream of their hosts, after that they switch to express bloodstream VSGs (Esser et al., 1982). Trypanosomes are extracellular in the bloodstream of their hosts. The host is able to mount an efficient humoral immune response against an existing VSG isotype but by expressing a new VSG variant, the parasite can escape the immune response and new wave of parasitaemia can arise. As the parasite density increases in the bloodstream, the long slender form differentiates to the short stumpy form which is cell cycle-arrested in G1 as adaptation for the transmission to the tsetse fly (Matthews et al., 2004). Once taken by a tsetse fly during the blood meal, the stumpy form transforms to the proliferative procyclic form in the midgut of the fly.
Figure 1.2 The life cycle of *Trypanosoma brucei*. The life cycle of *T. brucei* alternates between its invertebrate host, the tsetse fly and the bloodstream of its mammalian host (Matthews, 2005; Matthews et al., 2004). The parasite experiences many changes to its cellular biology including the surface coat, mitochondrial activity, positioning of their organelles, typically kinetoplast, and its proliferation status. In the bloodstream of the mammalian hosts, trypanosomes proliferate as slender forms which express one major surface protein on their coat termed variant surface glycoprotein (VSG). The activity of the mitochondrion is repressed as parasites directly acquire their nutrition from the host’s blood and the kinetoplast is located at the posterior of the cell. The non-proliferative stumpy forms differentiate to the proliferative procyclic in the midgut of the fly. In the procyclic form, the kinetoplast becomes sub-terminal and the surface coat consists of procyclin. After establishment in the midgut, the procyclic trypanosomes migrate to the salivary gland where they differentiate to the epimastigote forms and the parasites attach to the salivary glands until their final differentiation to the metacyclic forms. These forms re-acquire their VSG coat in preparation for infection of a new host during another blood meal.
1.3 **Genome architecture of *Trypanosoma brucei***

Based on cytophotometry, DNA renaturation, pulsed field gel electrophoresis (PFGE) analysis, and recently the genome sequencing project, the haploid nuclear genome of *T. brucei* is about 35 megabase pairs (Mbp) in size and 25% variation occurs amongst different isolates (Berriman et al., 2005; Borst et al., 1982; Hope et al., 1999; Van der Ploeg et al., 1984a). The nuclear karyotypes of *T. brucei* are classified into three main classes based on their size and mobility on PFGE (Figure 1.3A): large chromosomes of 1-6 Mbp termed megabase chromosomes (MBCs), intermediate 200-900 kilo base pairs (Kbp) sized chromosomes (ICs) and small chromosomes of 50-150 Kbp termed minichromosomes (MCs). The large megabase chromosomes are diploid in the nucleus of *T. brucei* whereas the 1-5 intermediate and ~100 minichromosomes are of uncertain ploidy (Ersfeld et al., 1999; Hope et al., 1999; Melville et al., 1998). Minichromosomes are linear DNA structures and very numerous in the genome of *T. brucei*. Interestingly, the electrophoretic analysis indicates that the most related species of *T. brucei*, *T. b. gambiense*, contain only few if any minichromosomes which have smaller size (~25 kb) compared to ~100kb size of *T. brucei* minichromosomes (Dero et al., 1987; Gibson, 1986). Other kinetoplastid genomes such as *Trypanosoma cruzi* (*T. cruzi*) and *Leishmania major* (*L. major*) are devoid of the two categories of the smaller chromosomes, intermediate- and minichromosomes (El-Sayed et al., 2005; Gibson and Miles, 1986; Ivens et al., 2005). On the other hand, variable minichromosome sets are demonstrated in the genomes of mammalian (Mills et al., 1999; Shen et al., 2000; Yang et al., 2000), plant (Birchler et al., 2008; Han et al., 2007) and drosophila (Sun et al., 1997) cells, which can exist naturally or artificially engineered through the genetic manipulation. In human cells, minichromosomes can occur naturally (Carine et al., 1989) or can be generated de novo either by assembling telomeric repeats and alphoid centromeric DNA, by using telomere-directed fragmentation of human X chromosome or by exposure to irradiation (Harrington et al., 1997; Mascarello et al., 1980; Mills et al., 1999).

Based on the genome sequencing data, the *T. brucei* megabase genome contains about 9000 predicted protein coding genes per haploid genome, including 1700 *T. brucei*-specific genes and ~900 pseudogenes (Berriman et al., 2005). The diploid
megabase chromosomes contain all housekeeping genes and over 20% of the genome presents subtelomeric genes, the majority of which are related to the capability of antigenic variation performed by the parasite (Berriman et al., 2005; Van der Ploeg et al., 1984c). Homologous pairs of megabase chromosomes which often differ in size and numbers are used to assign the name of these chromosomes (as I-XI from smallest to largest chromosome) while letters are used to differentiate alleles of the same chromosome as Ia and Ib (Turner et al., 1997). Also, a considerable size variation occurs between homologues of large chromosomes in the different stocks of *T. brucei*. Based on the old structural map of the megabase chromosome 1 using restriction digests and more recently the genome sequencing project, large chromosomes have unique structure (Figure 1.3B) (Berriman et al., 2005; Melville et al., 1999). Both strands of the megabase chromosome contain long, nonoverlapping large clusters of protein coding genes (Hall et al., 2003). In trypanosomes, the protein coding genes are transcribed into long polycistronic precursor mRNAs. The precursor and polycistronic mRNAs are processed at the posttranscriptional level by the 5’ spliced leader trans splicing and 3’ polyadenylation (Clayton and Shapira, 2007; Horn, 2001; Palenchar and Bellofatto, 2006). The developmentally regulated abundant surface proteins such as VSGs and procyclins are transcribed by RNA polymerase I, a polymerase confined to the transcription of ribosomal DNA (rDNA) in other eukaryotes (Lee and Van der Ploeg, 1997). Most if not all large chromosomes contain VSGs specific transcription units known as expression sites (ESs). Using hybridization studies and genome sequencing data, about 20 expression sites have been identified that are subtelomeric on megabase and intermediate chromosomes (Berriman et al., 2005; Turner et al., 1988; Zomerdijk et al., 1990). Sequencing of the bloodstream-form ESs reveals a variant range of 11 polymorphic genes, called expression site associated genes (ESAGs) with common promoter located upstream (Berriman et al., 2005; Hertz-Fowler et al., 2008; Zomerdijk et al., 1990). VSGs can be encoded by a mosaic of genes or hybrids known as pseudogenes. The majority of VSG genes are oriented away from telomere (subtelomeric) which is consistent with other protozoa due to the efficiency by which these chromosome domains can recombine with each other ectopically (Barry et al., 2003). Also, most VSG genes have one or more upstream 70-bp repeat (Figure 1.3B). In addition to subtelomeric VSG genes, VSG-related genes and pseudogenes are internally located on large chromosomes and lack the upstream 70-bp repeats (Berriman et al., 2005). At the interior boundary of ES is a large region of INGI and RIME repeats.
that are responsible for most of the size differences between megabase chromosome alleles. Also, the observed size polymorphisms of large chromosomes can be attributed to the different VSG gene arrays of subtelomeres which can account for over 75% of chromosome length (Callejas et al., 2006). This structural organisation of large chromosome in trypanosome differs considerably from higher eukaryotes, but similar to other kinetoplastid genomes (Ravel et al., 1996).

In Figure 1.3B, the general structure of minichromosomes is shown. The 50-150 kb linear chromosomes contain the same telomeric repeat sequences (TTAGGG)\text{\textsubscript{n}} as the large chromosomes. The structure of minichromosomes is composed mainly of internal tandem arrays of 177-bp repeats (70-90% of MC structure) (Wickstead et al., 2004). Also, other repeats such as GC-rich and AT-rich repeats occur within the structure of MC (Weiden et al., 1991). Many of these small chromosomes contain silent copies of VSG genes near their telomeres but none of the minichromosomes are found to possess an active VSG-ESs. In order to be transcribed, these telomeric VSGs copies need to be translocated to active ESs on large or possibly intermediate chromosomes by DNA recombination. Apart from silent VSG genes, no other protein-coding genes are assigned to minichromosomes. Due to the predominance of the 177-bp repeats, it is difficult to determine the ploidy of MCs. Also, the only comprehended advantage of maintaining the large numbers of minichromosomes in \textit{T. brucei} genome is to serve as a reservoir of telomeric copies of VSGs with adjacent telomeric homology as favoured substrates for the gene-conversion to the active expression site for the antigenic variation in the early infection stages (Morrison et al., 2005; Robinson et al., 1999). Chromosomes with intermediate mobility on PFGE between megabase and minichromosomes are termed intermediate chromosomes that contain some VSG-ESs, but lack the housekeeping genes (Borst and Ulbert, 2001; Wickstead et al., 2004). The numbers and sizes of these chromosomes vary between different parasite isolates (1-5 with 200-900 Kbp in size) possibly due to the DNA rearrangement that occurs during the antigenic switching (Van der Ploeg et al., 1984a; Van der Ploeg et al., 1984b).

During their presence in the bloodstream of their mammalian hosts, trypanosome parasites are kept under continuous immune attack from the complement system and the host antibodies. For escaping the immune response, trypanosomes
repeatedly change their surface coat made of one major VSG antigen (Donelson, 2003; Pays et al., 2004). When the immune response rises against the antigen, parasites shift to express another variant that cannot be detected by the complement system. In this way, the parasites are able to escape the killing by the host immune system and repopulate the host causing long-lasting chronic infection. The antigenic coat is a homogenous and dense coat made up of $10^7$ copies of a single VSG (Pays et al., 2004). The genome of *T. brucei* contains an estimated 1000 non-expressed VSG genes which constitute ~10% of the total genome size (Berriman et al., 2005; Van der Ploeg et al., 1984c). The parasite expresses only a single VSG at any given time with switching between the different VSGs expression occurs during infection. VSGs expression switching occurs at a rate of $10^{-2}$ to $10^{-7}$ cell$^{-1}$ generation$^{-1}$ with culture-adapted strains exhibiting the lower switch rates (Turner, 1997; Turner and Barry, 1989). In order for a given VSG gene to be expressed, it needs to be contained within a subtelomeric ES on large and intermediate chromosomes (Borst and Ulbert, 2001; Pays and Nolan, 1998). Transcriptional switching of VSG genes depends on either recombination of a new VSG gene into the active ES or transcriptional silencing of an active ES with concomitant activation of a new one (Borst and Ulbert, 2001; Cross et al., 1998). The best studied mechanism is gene conversion or duplicative transposition, in which the active VSG gene in ES is replaced with a duplicated copy of another unexpressed VSG (Robinson et al., 1999). In order to maintain the capability of effective antigenic variation, *T. brucei* needs to maintain its large reservoir of VSG genes on its chromosomes complement through the successive generations, and to inherit an equal complement to its daughter cells. With special emphasis on *T. brucei*, I will first discuss the cell cycle and its regulation, moving to the current understanding of chromosome segregation, finishing with a special emphasis on mitotic proteins involved in these processes such as the cohesin complex and separase as the main focus of my research project.
Figure 1.3

A) 

B) 

1) B-ES [Red] 

M-ES [Red] 

- Telomere [Red] 
- B-YOS [Red] 
- 70-kb repeat [Blue] 
- EUB [Yellow] 
- Repetitive sequences [Light Blue] 
- House-keeping genes [Green] 
- Promoter [Blue Arrow] 
- M-YOS [Red]

2) 

- Telomere [Red] 
- 70-kb repeat [Blue] 
- 177-kb repeat [Light Blue] 
- Ssa1-VOS [Red] 
- Unknown genes [White]
**Figure 1.3** Karyotypes and the main structural features of *Trypanosoma brucei* chromosomes. **A.** The pulsed field gel electrophoresis (PFGE) of *T. brucei* field isolate (TRUE927/4). The identity of chromosomal karyotypes was indicated on the right according to the results of Southern blot hybridization with numerous cDNA probes. Sizes of some chromosomes are indicated in Kbps on the left of the gel. Adapted from Melville et al., 1998 (Melville et al., 1998). **B.** Schematic representation of large (1) and minichromosome (2) structure in *T. brucei*. 1. The main features of megabase chromosomes of *T. brucei* with special emphasis on the telomeric and subtelomeric sequences. Whereas the bloodstream telomere-linked VSG expression sites (B-ES) resides on left telomere and associates with repetitive elements and ESAG genes, the telomere-linked expression site for metacyclic VSGs (M-ES) was on the right end. Two promoters that drive the expression of VSG genes and ESAG genes are also indicated with the unique housekeeping genes. 2. Minichromosomes are distinguished by their shorter length when compared to megabase chromosome. The most significant feature of minichromosomes was the 177-bp repeats that can constitute 70-90% of their sequence (Wickstead et al., 2004). Also, telomeres of some of these minichromosomes are linked to VSG genes although no active expression sites are detected on these chromosomes (Weiden et al., 1991). The diagrams are not drawn to scale.
1.4 The cell cycle of *Trypanosoma brucei*

1.4.1 An overview of the eukaryotic cell cycle

The eukaryotic cell cycle is typically divided into four phases (Figure 1.4) (Lodish, 2008). During $G_1$ (G for gap), the cell grows and prepares its DNA content for replication. When the cell enters the DNA synthesis phase ($S$), each chromosome is replicated into two chromatids. As will be discussed later, the replicated sister chromatids are held together from the time of their synthesis until their segregation in mitosis by a multi-subunit protein complex termed cohesin (Haering and Nasmyth, 2003). After DNA replication, the cell undergoes another phase termed $G_2$ in preparation for the cell division. These three stages are collectively called interphase and account for most of the cell cycle duration. After interphase, the cell enters mitosis ($M$) to segregate its replicated DNA content into the newly formed daughter cells. Mitosis is sub-divided into four stages according to the shape and position of the chromosomes (Figure 1.4). The first stage is called **prophase** during which chromosomes undergo a condensation process. Also during prophase, the nucleolus disintegrates and the nuclear envelope disappears in cells undergoing an open mitosis. In many organisms such as yeast, trypanosomes and many other protozoa, the nuclear envelope remains intact throughout mitosis (closed mitosis). During the next mitotic stage, **metaphase**, the condensed visible chromosomes start to bind to the spindle microtubules emanating from centrosomes at opposite spindle poles. Two opposing forces, the pulling force by the attached microtubules and the stabilizing force by the sister chromatid cohesion lead to chromosomes congression at the metaphase plate. Once all sister chromatids are properly aligned, cells progress to **anaphase** and chromatids are separated to the opposing poles of the cell. During the last mitotic stage, **telophase**, the reformation of the nuclear envelope and DNA de-condensation occurs. Finally, a cleavage furrow appears and the cell proceeds to cytokinesis that completes the formation of the two new daughter cells.
Figure 1.4 Schematic representation of the cell cycle in the animal cell. The duration of different phases of the cell cycle are not drawn to scale. \( G_1 \) (gap phase 1), \( S \) (Synthesis phase), \( G_2 \) (gap phase 2) and \( M \) (Mitosis). More details are inside the text.
1.4.2 **The cell cycle in *Trypanosoma brucei***

Although the cell cycle of *T. brucei* follows the broad scheme of the typical eukaryotic cell cycle, it shows some unique features that are distinct from the classical experimental model such as a mammalian cell (McKean, 2003). *T. brucei* contains a number of single copy organelles such as the mitochondrion, the flagellum, a basal body. These single organelles in trypanosomes are precisely replicated and segregated in order to provide equal entities to their progenies. Also, the cell cycle of *T. brucei* is intimately linked to the parasite differentiation (Matthews, 2005; Matthews et al., 1995). For example, the non-proliferative metacyclic form in the tsetse salivary gland and the short stumpy form in the mammalian bloodstream are arrested in G\(_1\) of their cell cycle and this arrest is only released once the parasites differentiate to the long-slender and procyclic proliferative forms (Fenn and Matthews, 2007; Matthews, 2005).

Trypanosomes undergo the same periodic nuclear (N) events: the G\(_1\), S, G\(_2\) and M phases following the classical scheme of any eukaryotic cell (Figure 1.5) (Woodward and Gull, 1990). But in addition to the nuclear mitotic events, trypanosomes co-ordinately replicate and segregate their single mitochondrial genome, the kinetoplast. Therefore, *T. brucei* exhibits a well-coordinated kinetoplast (K) cycle where the kinetoplast replication (K_S) phase starts before the chromosomes replication (N_S) phase and the kinetoplast segregation (D) phase is completed before the onset of the nuclear segregation (M) phase (Figure 1.5) (Woodward and Gull, 1990). The replication and segregation of the two genomes provides a comprehensive marker to identify the different stages of the cell cycle for any individual trypanosome cell (Sherwin and Gull, 1989). As shown in Figure 1.5, the overall cell cycle of the procyclic form of *T. brucei* is divided into nuclear (N) and kinetoplast (K) events (Woodward and Gull, 1990).
Figure 1.5 The cell cycle of *Trypanosoma brucei*. The diagram is a schematic representation of the major events during the cell cycle of *T. brucei*. It illustrates the different phases of replication and segregation of the nucleus (N) and the kinetoplast (K) during the cell cycle. The total duration of the cell cycle in the procyclic form of *T. brucei* is estimated to be 8.5 hours. S\textsubscript{n}, nuclear S phase; M, nuclear mitosis; C, cytokinesis; S\textsubscript{k}, kinetoplast S phase; D, kinetoplast division; A, kinetoplast segregation. The sketch in the lower panel illustrates the major morphological changes that take place during the cell cycle of *T. brucei* procyclic form based on the information in the upper panel. More information is included in the main text. Adapted with modification from Woodward and Gull, 1990 (Woodward and Gull, 1990).
The cell cycle progression in the bloodstream form is very similar to the procyclic form, although the \textit{in vitro} duration is shorter (6 hr) compared to 8.5 hr in the procyclic form. The first cytological event of the cell cycle is the maturation and elongation of the pro-basal body that occurs during $G_1$ phase. The new daughter flagellum nucleates simultaneously with the formation of a new pro-basal body from the mature basal body and it grows alongside the existing one (Kohl et al., 2003). The nuclear and mitochondrial genomes exhibit separate S phases with the kinetoplast S phase starting before and being shorter than the nuclear S phase. The synchronisation of the nuclear and mitochondrial division cycle indicates a degree of cross-talk between the two organelles (Woodward and Gull, 1990). Early in $G_2$ phase, the kinetoplast segregation (D) takes place before the onset of the nuclear mitosis (M) in a microtubule-dependent way as the tripartite attachment complex (TAC) ensures the movement of the replicated kinetoplast follows the segregation of the basal bodies (Ogbadoyi et al., 2003; Robinson and Gull, 1991). As will be discussed in details later, the mitosis in \textit{T. brucei} is a ‘closed’ process and the nuclear M phase is completed without the disruption of the nuclear envelope. The mitotic spindle is assembled and localised entirely inside the nucleus (Ogbadoyi et al., 2000). After nuclear division (karyokinesis) in the procyclic form, one nucleus is repositioned between the two basal bodies. In the bloodstream forms, the dynamics of positioning of these two organelles is different to the procyclic form and the two nuclei are localised anterior to both basal bodies. Therefore, the order of two organelles (N and K) is KKNN in the bloodstream forms and is KNKN in the procyclic forms (Tyler et al., 2001). The growing new flagellum is connected to the distal tip of the old one through the flagellar connector (FC) and to the cytoskeleton through the flagellum attachment zone (FAZ). The position of the flagellum is an important parameter that determines the geometry of the cell division by impacting on the positioning of the cleavage furrow at the onset of cytokinesis (Kohl et al., 1999). In animal cells, cytokinesis starts before the completion of mitosis and the two events cannot be separated. In \textit{T. brucei}, cytokinesis occurs only after the completion of mitosis and karyokinesis and the two events can be experimentally uncoupled (Hammarton, 2007; Hammarton et al., 2007). Experimental evidence in \textit{T. brucei} has indicated that entry into cytokinesis was more dependent on kinetoplast division and segregation rather than on the completion of mitosis, possibly because kinetoplast segregation is intimately linked to flagella positioning which in turn plays a significant role in defining the initiation point of cytokinesis (Kohl et al., 2003; Ploubidou et al.,
Cytokinesis occurs through the unidirectional ingression of the cleavage furrow that forms along the entire helical axis of the cell from the anterior to the posterior end (Hammarton et al., 2007; Kohl et al., 2003).

1.4.3 Regulation of cell cycle progression

Control of the cell cycle progression requires the participation of different proteins to ensure the coordination of cell cycle events. The cell cycle of the eukaryotic cell is driven by the sequential activation and inactivation of a number of cyclin-dependent kinases (CDKs) and its regulatory subunits, cyclins and also by a number of CDK inhibitors and activators (Nurse, 2002). A number of specific checkpoints operate to ensure that no phase of the cell cycle can be started before the completion of the previous one (Lodish, 2008). The first checkpoint is S/G2 as the presence of unreplicated DNA prevents entry into mitosis by inhibiting the activation of CDK1 by Wee1. The second operating checkpoint is the spindle-assembly checkpoint as the improper assembly of the mitotic spindle prevents the initiation of anaphase and utilises a number of checkpoint proteins such as Mad2, Bub1 and Bub2. The spindle-position checkpoint prevents telophase and mitotic exit until the proper segregation of daughter chromosomes. The last DNA-damage checkpoint is triggered in response to any DNA damage that occurs during the cell cycle. Therefore, the cell cycle progression is delayed if a number of key events have not properly occurred such as DNA replication during the S phase or spindle microtubule attachment to chromosomes during the metaphase. Any error in these checkpoints has dramatic consequences for the cell and can result in aneuploidy, cell death or cancer (Kastan and Bartek, 2004).

With the sequencing of *T. brucei* genome (Berriman et al., 2005), sequence homologues of many CDKs and mitogen activated protein kinases (MAPKs) have been identified, although their function may be divergent from their counterparts in higher eukaryotes (Hammarton, 2007; Hammarton et al., 2003b). Interestingly, some of the most conserved checkpoint components of mammalian cells and yeast cannot be simply characterised in the *T. brucei* genome by sequence-based homology, either because they
are absent or highly diverged in nature. In the procyclic form, blocking of mitosis does not interfere with cytokinesis progression and results in the generation of polyploid and anucleate (zoids) cells as an indication of the absence of the mitosis-to-cytokinesis checkpoint (Ploubidou et al., 1999). Also, some aspects of cell cycle regulation may differ between life cycle stages as observed after RNA interference (RNAi) knockdown of the mitotic B-cyclin known as CYC6 (Hammarton et al., 2003a). In the procyclic form, depletion of CYC6 inhibits mitosis but not cytokinesis producing a population of zoid cells and cells with polyploid nuclear DNA content. This phenotype mirrored the defect observed after the mitotic inhibition by the spindle microtubule-depolymerising drug, rhizoxin, confirming the absence of the mitosis-to-cytokinesis checkpoint in this life cycle form (Ploubidou et al., 1999). On the other hand, CYC6 depletion in the bloodstream forms inhibits both mitosis and cytokinesis but not kinetoplast duplication resulting in cells with multiple kinetoplasts (Hammarton et al., 2003a).

Ten cyclins (CYC2-11) and eleven CDKs termed Cdc-related kinases (CRK1-4 and CRK6-12) have been identified in *T. brucei* with a potential role in the cell cycle regulation (Figure 1.6) (Hammarton, 2007; McKean, 2003; Parsons et al., 2005). When compared to the single-celled fission yeast, *Schizosaccharomyces pombe* (*S. Pombe*), which has only a single CDK (CDC2) and a single mitotic cyclin (CDC13), this number of cyclins and CDKs is considered a lot for the single cell of *T. brucei*. In contrast to the well dissected CDKs of mammalian cells, little is known about the interaction between CRKs and cyclins of trypanosome. The only exception is the kinase dependent activity of CRK3, the trypanosome CDK1 homologue, on the two mitotic cyclins, CYC2/CYC6, for promoting mitosis (Hammarton et al., 2003a; Tu and Wang, 2004). Also, In *T. brucei*, CRK2 has a potential role in the growth of the posterior microtubules and the double knockdown of CRK1 and CRK2 accumulates cells in G1 phase of the cell cycle (Tu et al., 2005; Tu and Wang, 2005). Kinase homologues, other than CDKs, are known to be involved in the cell cycle regulation such as polo-like kinases (TbPLK), an aurora kinase (TbAUK1) and the mitogen activated protein kinase (TbMAPK2) with variable roles in mitosis and cytokinesis initiation, however no specific cell cycle arrest is observed after their depletion from *T. brucei* (Kumar and Wang, 2006; Muller et al., 2002; Tu et al., 2006). Also a 14-3-3 protein and the nuclear scaffolding protein, TRACK1, are required for cytokinesis initiation and cell cycle
progression (Inoue et al., 2005; Rothberg et al., 2006). During mitosis, the anaphase promoting complex/cyclosome (APC/C) poly-ubiquitinates (adds a poly-ubiquitin chain) a number of cell cycle regulators including cyclins, thus signalling them for the degradation by the proteasome (kiss of death). Downregulation of the regulatory subunits, cyclins and consequently the catalytic activity of CDK/CRK promotes anaphase initiation and mitotic exit. Downregulation of the activity of the putative APC components, APC1 and Cdc27, in *T. brucei* enriches the cells at the G2/M transition (Kumar and Wang, 2005). When the proteasome itself is disrupted, the cell cycle is arrested in G2/M transition affecting the initiation of M phase in the bloodstream form, but not the procyclic form, confirming the difference in the cell cycle regulation between the two life cycle forms (Li et al., 2003). Finally, VSG synthesis in the bloodstream form acts as a checkpoint for the initiation of cytokinesis as the inhibition of VSG synthesis and results in the accumulation of 2N2K cells with blocking of any subsequent mitosis (Sheader et al., 2005).
Figure 1.6 Summary of the cell cycle regulation of *Trypanosoma brucei*. The diagram is a summary of the different proteins implicated in the regulation of different phases of the cell cycle of *T. brucei* procyclic form. Experimentally studied and verified cell cycle regulators are showed above the different nuclear division cycle phases (G₁, Sₙ, G₂, M and C). Also the duration of the different phases of the kinetoplast division cycle in relation to the nuclear cycle was indicated (G₁, Sₖ, G₂, D and A). More information is detailed back in the text. M = Nucleus division; D = Kinetoplast division; C = Cytokinesis and A = Kinetoplast segregation.
1.5 Chromosome segregation in *Trypanosoma brucei*

Like any eukaryotic cell, trypanosomes have to faithfully replicate and segregate their genetic content to yield viable daughter cells. Any defect in this process potentially affects the ability of the parasite to persist and continue its developmental cycle in the hosts and between different hosts. Like many protists, *T. brucei* undergoes a closed mitosis and the nuclear envelope remains intact throughout the cell cycle (Ogbadoyi et al., 2000; Solari, 1995). Also, the nucleolus does not disintegrate during mitosis which is similar to the related kinetoplastid *Leishmania*, but differs from other trypanosomatids such as *Blastocrithidia* and *T. cruzi* in which the nucleolus disperses during mitosis (De Souza and Meyer, 1974; Ogbadoyi et al., 2000; Solari, 1983). In *T. brucei*, the segregation of the nuclear genome is dependent on the formation of an intact mitotic spindle inside the mitotic nucleus (Ersfeld and Gull, 1997). The role of the mitotic spindle microtubules in the segregation of the genomes is conserved in many organisms, though the mechanism of chromosomes association and segregation by the spindle varies between the different organisms (Goode, 1975; Heath, 1980; Kubai, 1975). In dinoflagellates, the spindle assembles outside the mitotic nucleus and the association with chromosomes occurs via a specialized structure in the nuclear envelope with the envelope being intact throughout mitosis (Ris, 1975). In some fungi, the spindle assembles inside the nucleus during the onset of the mitosis (Winey et al., 1995). The chromatin structure in *T. brucei* is different from higher eukaryotes in that the condensation to 30 nm fibres is absent and chromosomes do not undergo condensation at the start of mitosis (Hecker et al., 1994; Vickerman and Preston, 1970). In *T. brucei*, like in other eukaryotic cells, the chromatin formation is based on the nucleosomes that contain the canonical core histones H2A, H2B, H3 and H4 (Burri et al., 1994; Hecker et al., 1994). The high divergence of the N-terminal sequences of these core histones and the linker histone H1 which is smaller in size and lacks the conserved globular N-terminal domain of the typical histone H1 probably contributes to the absence of mitotic condensation in trypanosomes (Alsford and Horn, 2004; Burri et al., 1993; Burri et al., 1995), despite the conservation of the putative condensin subunit orthologues (SMC2 and SMC4), which are essential for the chromatin condensation in other organisms (see next section) (Hirano, 2005). Additionally, the structural equivalents of the microtubule organizing centres (MTOC) such as spindle pole bodies
or centrosomes are not characterised in the mitotic nucleus of \textit{T. brucei} (Ogbadoyi et al., 2000).

By using \textit{in situ} hybridisation in combination with immunofluorescence microscopy, the mechanism of chromosome segregation during mitosis in \textit{T. brucei} has been investigated (Ersfeld and Gull, 1997; Gull et al., 1998; Wickstead et al., 2003). During mitosis, chromosome partition occurs by association of the mitotic spindle and both large and minichromosomes segregate with different dynamics (Ersfeld and Gull, 1997; Gull et al., 1998). By using electron microscopy, only a few spindle microtubules terminate in trilaminar putative kinetochores (Ogbadoyi et al., 2000; Solari, 1995). The few kinetochore-like structures and the small number of spindle microtubules are incompatible even with the segregation of 22 megabase chromosomes during mitosis by the conventional single chromosome/single microtubule interaction (Gull et al., 1998). Additionally, the segregation of 100 or more of intermediate and minichromosomes added to the complexity of the equal chromosome = microtubule interaction ratio required for the segregation of other eukaryotic genomes. In mammalian cells, a clear relationship between chromosome size and its mitotic stability has been indicated (Spence et al., 2006). In human cells, the accuracy of segregation of minichromosomes varies in different vertebrate cell lines (Shen et al., 2001). Minichromosomes segregate accurately in human and chicken cell lines with formation of active centromeres, while centromere formation is undetectable and the mitotic segregation is inaccurate in mouse cell line (Shen et al., 2001). Human minichromosome 1 (MC1) remains mitotically stable over 200 generations in the human-CHO hybrids cell line even after growth in the absence of drug selection (Guiducci et al., 1999). In \textit{T. brucei}, the segregation of minichromosomes occurs in association with the mitotic spindle during mitosis (Ersfeld and Gull, 1997). Experimental evidence for the mitotic stability of minichromosomes in \textit{T. brucei} is provided by the drug resistance marker studies in which individual neomycin resistance gene tagged- minichromosomes are stably inherited for more than 130 generations in the absence of drug selection (Wickstead et al., 2003; Zomerdijk et al., 1992). Recently, by mapping etoposide-mediated topoisomerase-II chromosomal cleavage sites, putative centromeric DNA sequences have been identified on large chromosomes, although no such sequences could be detected on intermediate and minichromosomes (Obado et al., 2007). A possible model explaining minichromosomal segregation postulates the lateral association of chromosomes with anti-parallel spindle microtubules (\textbf{Figure 1.7}) (Gull et al., 1998).
Figure 1.7 Model of chromosomal segregation of *Trypanosoma brucei*. This model shows how *T. brucei* segregates their large and small chromosomes population. 

A. In this model, lateral stacking model (Gull et al., 1998), megabase chromosomes segregate by the classical microtubule-kinetochore interaction (1), whereas minichromosomes segregate via the lateral association with interdigitated pole-to-pole microtubules (2). 

B. The replicated product of minichromosomes, sister chromatids, attach laterally to the microtubule plus end (+) move to the minus-end (-) by the minus-end directed motor proteins. Chromosomes stabiliser (?), possibly cohesin, may have a role in this model by stabilising and keeping sister chromatid cohesion to avoid any missegregation until the proper anaphase takes place. Adapted with modification from Gull et al., 1998 (Gull et al., 1998).
This way, it is theoretically possible to segregate this population of minichromosomes using a small number of microtubules. In the same model, large chromosomes are possibly segregated by kinetochores interaction with the spindle microtubules following the classical mitosis in other eukaryotes. After chromatid resolution, individual minichromosomes are transported along the microtubules toward their minus-ends (-) at the spindle poles. The movement of minichromosomes might involve the kinesin- and dynein-like motor proteins (Kline-Smith et al., 2005).

Although this model and the identification of centromeres on large chromosomes contributes toward understanding the mitotic pathway in this kinetoplastid, many of the molecular elements involved in the faithful segregation of chromosomes are yet to be characterised. The genome sequence projects of the kinetoplastids did not provide much detail about the mitotic mechanism in trypanosomes (Berriman et al., 2005; El-Sayed et al., 2005; Ivens et al., 2005). For example, homologues of proteins that constitute kinetochores in yeast, mammals and other model organisms were not identified in *T. brucei* genome either because they are absent or highly divergent (Berriman et al., 2005; Fukagawa, 2004). Therefore the mechanism of chromosome segregation in *T. brucei* may be simpler than in other organisms or the proteins involved are highly diverged. In any case, this makes studying the different molecules involved in this process an interesting area of trypanosome biology and could leads to the identification of parasite-specific drug targets.

In yeast and mammalian cells, the replicated chromosome (sister chromatids) are kept together from the time of DNA replication at S phase until their segregation by the mitotic spindle at anaphase. This sister chromatid cohesion is maintained by the cohesin complex (Haering and Nasmyth, 2003; Nasmyth, 2005). At the onset of anaphase, the spindle assembly checkpoint regulates the coordinated dissociation of sister chromatid cohesion by activating specific protease, separase, that cleaves the cohesin complex leading to the release of sister chromatids (Ciosk et al., 1998; Uhlmann et al., 1999; Uhlmann et al., 2000). The two components of the cohesion machinery, the cohesin complex and separase, are highly conserved in different eukaryotic organisms (Ciosk et al., 1998; Haering and Nasmyth, 2003; Losada and Hirano, 2005; Uhlmann et al., 2000; Wirth et al., 2006).
1.6 The cohesin complex and chromosome cohesion

1.6.1 Definition and structure

Over the course of the eukaryotic cell cycle, chromosomes are faithfully duplicated to sister chromatids and subsequently separated equally to the newly formed daughter cells. Proper segregation of sister chromatids is essential for the development and propagation of living organisms. Failure of proper segregation of chromosomes leads to cellular dysfunction, aneuploidy and, in some organisms, to the development of cancer. The process of sister chromatid cohesion prevents the premature and untimely segregation of sister chromatids until commencement of anaphase (Michaelis et al., 1997). The cohesion process is important not only for the proper alignment of chromosomes on the mitotic spindle, but also for the generation of tension across centromeres to counteract the pulling force of the spindle microtubules, therefore ensuring bipolar microtubule-kinetochore attachment (Tanaka et al., 2000).

The multisubunit protein responsible for the sister chromatid cohesion is known as the cohesin complex. It was first identified in yeast (Michaelis et al., 1997) and is conserved in eukaryotes (Guacci et al., 1997; Losada et al., 1998; Michaelis et al., 1997). As shown in Table 1.1, the cohesin complex consists mainly of two subunits of the Structural Maintenance of Chromosome (SMC) protein family, namely SMC1 and SMC3 (Figure 1.8A) (Michaelis et al., 1997; Nasmyth and Haering, 2005; Strunnikov et al., 1993). In addition to SMC proteins, the complex also contains two other non-SMC protein subunits of the kleisin subfamily termed SCC1/Rad21 (sister chromatid cohesion 1/double-strand break repair 21) and SCC3 (sister chromatid cohesion 3) (Guacci et al., 1997; Michaelis et al., 1997; Nasmyth and Haering, 2005). A fifth subunit, PDS5 (precocious dissociation of sisters), binds to the cohesin complex temporarily to maintain the sister chromatid cohesion during G2 phase and early mitosis (Hartman et al., 2000). A separate complex contains SCC2 and SCC4 proteins and is required for loading of cohesins onto chromosomes before DNA replication (Ciosk et al., 2000).

SMC proteins are highly conserved in the eukaryotes and related proteins have also been identified in prokaryotes (Cobbe and Heck, 2004). Bacterial cells have a
single SMC protein that forms a homodimer, such as MukB in *E. coli* (Hiraga, 1993). Eukaryotic organisms have at least six SMC family members that dimerise with the other regulatory non-SMC subunits to form three functionally distinct SMC protein complexes ([Table 1.2](#)) (Losada and Hirano, 2005). SMC1/SMC3 heterodimers form the core of the cohesin complex (Michaelis et al., 1997), SMC2/SMC4 form the core of the condensin complex responsible for the condensation of mitotic chromosomes (Hirano, 2005). Finally, SMC5/SMC6 form a complex involved in the DNA repair mechanism and the cohesion of highly repetitive DNA sequences (Lehmann, 2005).

SMC proteins have a highly conserved structure as judged by the EM observation of single molecules ([Figure 1.8B](#)) (Melby et al., 1998). SMC proteins consist of an N-terminal ATP-binding domain, two segments of coiled coils separated by a hinge domain and an ATP-hydrolysis C-terminal domain. The hinge is quite flexible as the arms can open up to 180° to separate the head domains. The amino terminus and carboxyl terminus of SMC molecules fold back on themselves, forming antiparallel intramolecular coiled coils and bringing the ATP-binding and hydrolysis domains in close proximity with the hinge region positioned at the opposite end (Haering et al., 2002; Melby et al., 1998). A model has been proposed in which the antiparallel coiled coils from two SMC proteins interact to form a V-shaped heterodimer ([Figure 1.8C](#)) (Anderson et al., 2002; Haering et al., 2002; Melby et al., 1998). In yeast, the C and N-termini of SCC1/Rad21 bind to ATPase heads of the SMC1/SMC3 heterodimer, respectively, to form a ring with the fourth subunit SCC3 binds to SCC1 to reinforce that ring structure (Gruber et al., 2003). The formation of this closed circular cohesin structure is dependent on ATP binding and hydrolysis cycles which play a role in the cohesin interaction with DNA (Arumugam et al., 2003).
Figure 1.8 The structure and configuration of the cohesin complex. A. The cohesin complex proteins SMC1 (green) and SMC3 (blue) folded back and heterodimerise with each other at their respective hinge domain (hinge-mediated dimerisation) bringing their head domains in close proximity to each other. The most ascribed ring structure of cohesin brought about when SCC1 binds to head domains of SMC1 and SMC3 while SCC3 interacts with SCC1 to reinforce the complex (Gruber et al., 2003). B. Molecular structure of the single SMC protein with an arm composed of two coiled coil domains with two ATP binding and hydrolysis head domains, N and C-terminus domains, respectively. Two motifs, walker A and DA box (walker B), at the N and C-termini function in the binding and hydrolysis of ATP, respectively (Arumugam et al., 2003). C. A model of the interaction of two SMC proteins (blue and red) through their coiled coil arms (Coiled coils dimerisation). This intramolecular interaction brings the close association of the two SMC proteins globular head domains at one end and the hinge domain at the other end forming a V-shaped heterodimer (Anderson et al., 2002; Haering et al., 2002; Melby et al., 1998).
Table 1.1 The components of the eukaryotic chromosome cohesion machinery. Different protein homologues implicated directly (as a part of the cohesin complex) or indirectly in loading, establishing or resolving chromosomes cohesion were denoted in different eukaryotic organisms such as human (*Homosapiens*), yeast (*Saccharomyces cerevisiae* and *Scizosaccharomyces pombe*) and *Trypanosoma brucei* (*T. brucei*). *T. brucei* proteome (at GeneDB: [http://www.genedb.org/genedb/tryp/](http://www.genedb.org/genedb/tryp/)) was searched for the components of cohesion machinery using homologues from the other organisms. Where no protein homologue could be identified in the *T. brucei* proteome is denoted by ?.

<table>
<thead>
<tr>
<th></th>
<th><em>Homosapiens</em></th>
<th>Budding yeast</th>
<th>Fission yeast</th>
<th><em>T. brucei</em></th>
<th>References</th>
</tr>
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<tr>
<td><strong>SMC</strong></td>
<td>SMC1α</td>
<td>SMC1</td>
<td>PSM1</td>
<td>SMC1</td>
<td>(Guacci et al., 1997; Haering et al., 2002; Losada et al., 1998; Michaelis et al., 1997)</td>
</tr>
<tr>
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<td>SMC3</td>
<td>PSM3</td>
<td>SMC3</td>
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<tr>
<td><strong>Kleisin</strong></td>
<td>Rad21</td>
<td>MCD1</td>
<td>Rad21</td>
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<tr>
<td><strong>Pds5</strong></td>
<td>PDS5a,PDS5b</td>
<td>PDS5</td>
<td>PDS5</td>
<td>PDS5</td>
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<td><strong>Sec2/Scc4 complex</strong></td>
<td>Delangin</td>
<td>Sec2</td>
<td>Mis4</td>
<td>Sec2</td>
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<tr>
<td></td>
<td>Mau-4</td>
<td>Scc4</td>
<td>Ssl3</td>
<td>?</td>
<td>(Watrin et al., 2006)</td>
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<td>Esco1,Esco2</td>
<td>Eco1</td>
<td>Eco1</td>
<td>?</td>
<td>(Zhang et al., 2008a)</td>
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<tr>
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<td>Cdc5</td>
<td>Plo1</td>
<td>PLK</td>
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<tr>
<td><strong>Shugoshin</strong></td>
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<td>Sgo1</td>
<td>Sgo1</td>
<td>?</td>
<td>(Wang and Dai, 2005)</td>
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<td>Separase</td>
<td>ESP1</td>
<td>Separin/Cut1</td>
<td>Separase</td>
<td>(Nasmyth et al., 2000; Uhlmann et al., 2000)</td>
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<tr>
<td><strong>Securin</strong></td>
<td>PTTG1</td>
<td>Pds1</td>
<td>Cut2</td>
<td>?</td>
<td>(Ciosk et al., 1998; Jallepalli et al., 2001)</td>
</tr>
<tr>
<td><strong>APC/C</strong></td>
<td>APC/C</td>
<td>APC/C</td>
<td>APC/C</td>
<td>APC (7)</td>
<td>(Cohen-Fix et al., 1996)</td>
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<tr>
<td><strong>Subunits</strong></td>
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</tbody>
</table>

28
Table 1.2 The three SMC protein complexes of eukaryotic organisms. The composition of each complex (cohesin, condensin and DNA repair complex) in different organisms is indicated. ? denotes the absence of any putative protein homologue in *T. brucei* that could be identified by the simple BLAST search against the genome/proteome database of *T. brucei*.

<table>
<thead>
<tr>
<th>Protein Complex</th>
<th><em>H. sapiens</em></th>
<th><em>Budding yeast</em></th>
<th><em>Fission yeast</em></th>
<th><em>T. brucei</em></th>
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<td><strong>Cohesin</strong></td>
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<tr>
<td>SMC</td>
<td>SMC1α</td>
<td>SMC1</td>
<td>PSM1</td>
<td>SMC1</td>
<td>(Guacci et al., 1997; Haering et al., 2002; Losada et al., 1998; Michaelis et al., 1997)</td>
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<td>SMC3</td>
<td>SMC3</td>
<td>SMC3</td>
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<td>SCC3</td>
<td>Psc3</td>
<td>SCC3</td>
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<tr>
<td>Pds5</td>
<td>Pds5, Pdsb</td>
<td>Pds5</td>
<td>Pds5</td>
<td>Pds5</td>
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<tr>
<td><strong>Condensin</strong></td>
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<td>SMCM2</td>
<td>Cut14</td>
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<td>SMC2</td>
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<td>SMC4</td>
<td>SMC4</td>
<td>Cut3</td>
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<tr>
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<td>Cnd1</td>
<td>TbCND1</td>
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<td>TbCND3</td>
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<tr>
<td>CAP-H</td>
<td>Brn1</td>
<td>Cnd2</td>
<td>TbCND2</td>
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<td><strong>DNA repair</strong></td>
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<tr>
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<td>SMC5</td>
<td>Spr18</td>
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<td>Rad18</td>
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<td>Nse1</td>
<td>Nse1</td>
<td>Nse1</td>
<td>?</td>
<td>(Shinohara et al., 1992)</td>
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<td>RAD51</td>
<td>Rad51</td>
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</table>
1.6.2  Cohesin interaction with chromosomes

In yeast, cohesin proteins bind chromosomes at both the centromere and along the chromosome arms at specific sequences termed Cohesin Attachment Regions (CARs) (Blat and Kleckner, 1999). Cohesin is spaced at lower densities along the chromosome arms while it is more concentrated at centromeres. The potential tighter cohesion at centromeres acts in counteracting the pulling force of the spindle microtubules enabling the proper alignment of chromosomes during metaphase. Additionally, cohesin is present at the telomeres of yeast chromosomes (Glynn et al., 2004). However, in yeast, the adhesion of highly repetitive chromosomal regions such as ribosomal DNA (rDNA) regions is found to be cohesin-independent (D'Amours et al., 2004; Sullivan et al., 2004). CARs lack any consensus sequences except that they are AT rich and found primarily in the intergenic regions of chromosomes. The lack of consensus sequences of CARs suggests that cohesin binds chromatin in a sequence non-specific manner. The proposed ring configuration of the cohesin complex, lack of any binding specificity to the chromatin and the need to link two sister chromatids together at the same time has led to several models of how cohesin binds to chromosomes (Figure 1.9) (Haering and Nasmyth, 2003; Losada, 2007). The most frequently ascribed and cited model is that a single cohesin ring encircles the two sister chromatids together within the coiled coil arms (Gruber et al., 2003; Haering et al., 2008). An alternative model involves the holding of each sister chromatid by a single cohesin and the two cohesin complexes interact with each other (Huang et al., 2005). In the third proposed two rings model, a single cohesin ring holds one chromatid but attaches to the other chromatid via an anchoring protein (Chang et al., 2005). Most recently, a handcuff model of the sister chromatid cohesion is described in which two cohesin rings hold the sister chromatids (Zhang et al., 2008b). The handcuff is established via SCC1/Rad21 interaction and enforced by SCC3 binding. Although none of these models have been experimentally studied to date, the ring model remained to be the most convincing for two reasons (Haering et al., 2008). The first comes from studying the topological interaction of cohesin with circular minichromosomes (Ivanov and Nasmyth, 2005). The second explains why cohesion is suddenly lost when the cohesin SCC1 subunit is cleaved and the ring is opened by separase at anaphase (Uhlmann et al., 1999).
Figure 1.9 Models of the cohesin complex-mediated sister chromatid cohesion. A. The structure of the cohesin complex and arrangement of its main subunits, SMC1 and SMC3 to form the ring-like structure enforced by SCC1 and Scc3 (Gruber et al., 2003; Haering et al., 2002). B-D Models proposed of how cohesin mediates the sister chromatid cohesion (Haering and Nasmyth, 2003; Losada, 2007). B. Single cohesin ring encircles the two sister chromatids together (Gruber et al., 2003; Haering et al., 2008). C. Each sister chromatid held by a single cohesin ring and the two cohesin rings interacts with each other (Huang et al., 2005). D. A single cohesin ring holds one chromatid but attached to the other chromatid via an anchoring protein (Chang et al., 2005). E. The most recent handcuff model of the sister chromatid cohesion is described in which the two cohesin rings hold the sister chromatids and the handcuff is established by SCC1 and enforced by SCC3 association (Zhang et al., 2008b).
1.6.3 Cohesin and the chromosome cycle

In yeast, cohesin binds chromosomes in late G$_1$ phase, establishes the sister chromatid cohesion during DNA replication at S phase and remains bound during G$_2$ and early mitosis and dissociates from chromosomes at the metaphase-to-anaphase transition (Haering and Nasmyth, 2003). In metazoan cells, the chromosome cohesion cycle follows the same scheme as yeast except most of cohesins dissociates from the chromosome arms before metaphase in a process called ‘prophase pathway’ (Figure 1.10) (Losada and Hirano, 2005; Waizenegger et al., 2000). Cohesin is first loaded onto chromosomes before S phase by a conserved mechanism that involves the action of a cohesin loading complex composed of SCC2 and SCC4 proteins (Ciosk et al., 2000). Cycles of ATP-binding and hydrolysis might have a role in opening the cohesin ring and loading of cohesin to chromosomes before the ring is closed after SCC1 binding. Establishment of cohesion between sister chromatids occurs simultaneously with DNA replication as many replication factors have a role in the cohesion process (Skibbens, 2005). Eco1/Eso1/Ctf7 is an essential gene in the fission and budding yeasts and functions in the formation of the cohesion during DNA replication. It interacts genetically with the sliding clamp PCNA and the clamp loading replication factor RF-C/Ctf8 that enables DNA polymerase to slide along DNA during replication providing a link between the replication process and cohesion (Skibbens et al., 1999). According to one cohesin ring model, the replication machinery passes through the pre-assembled cohesin ring leaving the replication products, the sister chromatids, entrapped in the same ring (Haering et al., 2008; Haering and Nasmyth, 2003). Once the cohesion is established, cohesin is maintained on chromosomes throughout G$_2$ phase where PDS5 has an important role in maintaining sister chromatid cohesion (Hartman et al., 2000).

During mitosis, two different mechanisms are involved in releasing sister chromatid cohesion and resolution of sister chromatids (Waizenegger et al., 2000). In vertebrates, most of cohesins dissociate from chromosome arms during prophase and only a small proportion is left to keep cohesion at centromeres in a process termed the ‘prophase pathway’ (Figure 1.10). This step might be important as it gives more space for the loading of condensin and the re-shaping of chromosomes during the prophase. The prophase pathway is regulated by phosphorylation of the cohesin subunit SCC3 by
two mitotic kinases, polo and aurora B kinases (Sumara et al., 2002). Cohesins left at the centromere are protected from the prophase pathway by a conserved complex consisting of shugoshin/Sgo protein (Japanese for ‘guardian spirit’) and the protein phosphatase 2A (PP2A) (Gregan et al., 2008; Rivera and Losada, 2006; Rivera and Losada, 2008; Tang et al., 2006; Wang and Dai, 2005). Centromeric cohesion counteracts the pulling forces of the spindle microtubules and generates tension between the sister kinetochores of chromatids at centromeres. Tension across centromeres stabilises the microtubule-kinetochore attachment and therefore promotes proper chromosome alignment during metaphase (Tanaka et al., 2000). At the metaphase-to-anaphase transition, once all sister chromatid pairs are aligned properly and attached in a bipolar manner to the spindle microtubules, the spindle checkpoint is deactivated (Tan et al., 2005). Spindle checkpoint proteins such as BUB1 and Mad2 inhibit the activity of APC/C and its activator CDC20 until proper alignment of chromosomes is achieved. APC/C together with CDC20 promotes the ubiquitin-dependent proteolysis of the separase inhibitor, securin, by the 26S proteasome (Ciosk et al., 1998). As will be discussed later, after securin degradation, the active separase cleaves the cohesin subunit SCC1 at two specific sites to release sister chromatid cohesion (Uhlmann et al., 1999; Uhlmann et al., 2000). Phosphorylation of the SCC1 subunit by polo-like kinase CDC5 facilitates its cleavage by separase during anaphase (Alexandru et al., 2001). In vertebrate cells, cohesin reloading onto chromosomes is started in telophase while, in yeast, the cohesin loading is delayed until late G1 (Losada et al., 1998; Sumara et al., 2000; Uhlmann and Nasmyth, 1998).
Figure 1.10 The chromosome cohesion cycle (Haering and Nasmyth, 2003). The cohesin complexes are loaded onto chromosomes at G₁ phase before the DNA replication has started. A complex composed of Scc2-Scc4 proteins is required for the loading of cohesin to chromosomes. Sister chromatid cohesion is established during DNA replication at S phase when the replication machinery pass through the already assembled cohesin ring leaving the replication products, sister chromatids, entrapped in the cohesin ring. Eco1/Ctf7 protein is involved in the cohesion establishment through the interaction with the replication factors. Cohesion was maintained through the G₂ phase and pds5 protein might have an active role in the cohesion maintenance. At the start of mitosis, in the prophase, most of the cohesins were phosphorylated at SCC3 subunits and dissociated from chromosomes by the action of polo and aurora B kinases. Cohesin protector, shugoshin, protects cohesin at centromeres from the prophase phosphorylation until the final dissolution of cohesion takes place at anaphase when the cohesin complex is cleaved at its SCC1/Rad21 subunit by the protease, separase.
1.6.4 Roles of cohesin beyond chromosomes cohesion

The cohesin complex proteins also function in DNA repair and regulation of gene expression (Hagstrom and Meyer, 2003). During S and G2 phases, cohesin is involved in the repair of double-strand breaks (DSBs) by homologous recombination (HR). After DNA damage, cohesin recruits to a region spanning ~100 kbp of the double strand break (DSB) site in yeast (Strom et al., 2004). The recruitment of cohesins is under the control of the cohesin loading factor, SCC2, and other damage repair factors such as Mre11. Cohesin links the two sister chromatids together in the vicinity of the damage site for the repair by HR to take place at the affected chromatid using the non-affected sister as a template (Sjogren and Nasmyth, 2001). The first evidence of cohesin involvement in gene expression regulation comes from studies in Drosophila (Rollins et al., 2004). Mutation of the cohesin loading factors such as SCC2/Nipped-B reduces the expression of certain genes (Rollins et al., 2004). Mutation of human SCC2 and the other cohesion factors associates with a human disease known as Cornelia de Lange syndrome characterised by multiple defects in developmentally regulated transcription (Dorsett, 2007). Also, mutation of the human cohesin establishment factor, Esco2, associates with another disorder known as Roberts's syndrome characterised by chromosomal abnormality of premature centromere separation (Dorsett, 2007). In vertebrates, cohesin is expressed in post-mitotic cells and therefore cohesin may have functions other than sister chromatid cohesion. In these cells, cohesin associates with CCCTC-binding factor (CTCF) which acts as a transcriptional insulator protein (Donze et al., 1999; Rubio et al., 2008; Uhlmann, 2008; Wendt et al., 2008). Therefore, cohesin contributes to gene regulation by enabling the CTCF to insulate the gene promoter from its enhancers thus separating active and inactive chromatin regions. Finally, the different components of the cohesin complex are involved in the mitotic spindle assembly during mitosis in vertebrate and plant cells (Deehan Kenney and Heald, 2006; Gregson et al., 2001; Lam et al., 2005; Wong and Blobel, 2008). In these organisms, the cohesin re-localizes to the spindle poles in the cytoplasm during mitosis and interacts with NuMA, a spindle pole-associated factor required for mitotic spindle organization (Gregson et al., 2001). The depletion of cohesin alone or the co-depletion of cohesin and condensin have cumulative effects on the mitotic
spindle assembly and chromosome structure (Deehan Kenney and Heald, 2006; Gregson et al., 2001).

1.6.5 Cohesin-independent pairing of chromosomes

Many of the early studies on cohesin have also predicted a non-cohesin dependent pairing of sister chromatids. Even loss of the structural cohesins results in only 50-60% of chromosome cohesion defects and the most affected regions were telomeric regions (Michaelis et al., 1997). Other chromosomal loci such as the highly repetitive ribosomal DNA (rDNA) and centromeres utilise not only cohesin but also alternative cohesion structures and cohesin-independent complexes (D'Amours et al., 2004; Sullivan et al., 2004). The focus shifted to CDC14 phosphatase, condensins, SMC5 and SMC6 and the pre-replication complexes (ORC) (D'Amours et al., 2004; Machin et al., 2005; Shamu and Murray, 1992; Shimada and Gasser, 2007; Torres-Rosell et al., 2005). CDC14, a protein phosphatase known for its role in mitotic exit, promotes the condensin enrichment at the rDNA locus for their segregation in a condensin-dependent manner (D'Amours et al., 2004). The SMC5-SMC6 complex has a role in partitioning of chromosomes during anaphase by preventing the formation of sister chromatid junctions (Torres-Rosell et al., 2005). DNA catenation is found to form the basis of linking repetitive DNA regions of chromosomes together and the resolution of this catenation is separase-independent (Uhlmann, 2007). Topoisomerase II resolves this catenation-based cohesion independently of the cohesin cleavage and separase activation (Shamu and Murray, 1992; Wang et al., 2008). Pre-replication complex (ORC2) depletion produces a significant premature sister chromatid separation that is comparable to cohesin defects (Shimada and Gasser, 2007). Also, silencing complexes such as the deacytelase Sir play a role in promoting sister chromatid pairing as sir3 mutant cells precociously separate yeast minichromosomes despite being heavily loaded with cohesins (Chang et al., 2005).
1.7 Separase, a multifunctional cell cycle protease

1.7.1 Structure of separase

Separases from different species except *Drosophila* are large proteins of approximately 150-230 kDa. The protein consists of two major domains with highly specialised functions (Figure 1.11A). A highly conserved C-terminal region contains the protease domain with a Ca$^{2+}$-binding motif and an N-terminal region that is highly variable and that binds securin (Viadiu et al., 2005). This structural feature is interesting because as mentioned earlier securin shows a high variation in its sequences between organisms and therefore is able to bind the highly variable domain of separase. As shown later in the Results section, the conserved protease domain at the C-terminus contains the cysteine (C) and histidine (H) residues that form the signature motif of the CD clan of cysteine proteases (Uhlmann et al., 2000). After cleaving the cohesin complex before anaphase, separase also cleaves itself between the N- and C-terminal regions but the two halves remain associated for a while until the C-terminal part becomes degraded by a destruction pathway (Zou et al., 2002). This represents another way of separase regulation and contributes to switching off separase activity after anaphase. *Drosophila* separase, SSE, has limited homology to other known separases (Jager et al., 2001). It is about one-third the size of other separases and consists only of a divergent endopeptidase domain. Also, it associates with the securin homologue in *Drosophila* termed Pimples (PIM) which in turn forms a complex with a third protein called Threerows (THR). It is proposed that THR is needed for SSE activation after securin/PIM degradation (Jager et al., 2001; Jager et al., 2004). Therefore, in all organisms studied to date, once securin/PIM is degraded, separase becomes active by undergoing conformational change that allows the non-catalytic N-terminal domain to bind and activate the catalytic C-terminal domain (Figure 1.11B).
1.7.2 Regulation of separase activity

Since its first role in separating sister chromatids during mitosis was uncovered in yeast, separase has been extensively studied in many organisms (Ciosk et al., 1998; Funabiki et al., 1996; Uhlmann et al., 2000; Wirth et al., 2006). As mentioned before, sister chromatids remain joined together during the cell cycle until their proper alignment at the metaphase plate and recognition by spindle microtubules. At anaphase onset, separase becomes active and cleaves the SCC1 subunit of the cohesin complex enabling the successful segregation of all chromosomes (Nasmyth, 2002; Nasmyth et al., 2000; Uhlmann et al., 1999; Uhlmann et al., 2000). The role of separase in regulating mitosis is universal as homologues of separase are highly conserved in many eukaryotic genomes (Jager et al., 2001; Nasmyth et al., 2000; Uhlmann et al., 2000; Wirth et al., 2006). The mechanism of separase regulation appears to be conserved in many organisms. To avoid premature sister chromatid separation, separase activity is inhibited by binding to the inhibitory chaperone, securin, and in mammalian cells additionally by CDK1-dependent phosphorylation (Holland and Taylor, 2006; Hornig et al., 2002; Stemmann et al., 2001). The dual ubiquitin-dependent degradation of securin and the CDK1 activator subunit, cyclin B, by APC/C plays an essential role in separase activation at anaphase onset. Interestingly, in higher eukaryotes separase not only cleaves cohesin but also cleaves itself thus promoting its downregulation after anaphase (Papi et al., 2005; Waizenegger et al., 2000; Zou et al., 2002). In yeast and mammalian cells securin not only inhibits separase, but also promotes its nuclear transport and accumulation via differential phosphorylation of securin by CDC28 (Agarwal and Cohen-Fix, 2002; Hornig et al., 2002; Jallepalli et al., 2001; Jensen et al., 2001; Kumada et al., 1998). Although conserved in function in different organisms from yeast to human, securins from different species do show little amino acid sequence conservation (Jager et al., 2001; Viadiu et al., 2005).
1.7.3 Multiple roles of separase in the eukaryotic cell cycle

During the chromosome cycle and at anaphase onset, the protease separase is activated to cleave cohesin and to trigger the sister chromatids separation (Cohen-Fix, 2000; Nasmyth et al., 2000; Uhlmann et al., 2000). Recently, more functions of separase other than sister chromatid separation started to be uncovered in many organisms. The first such role was demonstrated in *Xenopus* where separase triggers the disengagement of the mother and daughter centrioles before mitosis (Tsou and Stearns, 2006). In yeast in addition to splitting sister chromatids, separase has an additional role of linking anaphase to exit from mitosis by driving the cell cycle forward (Stegmeier et al., 2002; Sullivan and Uhlmann, 2003). Separase might be involved in mitotic exit in two different ways. First, separase promotes the release of CDC14 phosphatase from the nucleolus most likely by promoting the phosphorylation of the CDC14 inhibitor, Net1 (Sullivan and Uhlmann, 2003). Activated CDC14 reverses the activity of CDK1 which is the initial step in the cascades of the mitotic exit network (MEN) (Stegmeier et al., 2002). Second, the active separase downregulates the activity of the PP2A phosphatase thus facilitating the phosphorylation and destruction of the CDC14 inhibitor Net1 (Queralt et al., 2006). In this way, separase contributes to the mitotic exit network (MEN) and CDC14 early anaphase release (FEAR) network. In higher eukaryotes, human separase not only cleaves cohesin but also cleaves itself between its N- and C-terminal domains (Papi et al., 2005; Zou et al., 2002). The C-terminal fragment is unstable and is rapidly eliminated from the cell while the N-terminal part persists in the cell. Surprisingly, the remaining N-terminal fragment of human separase contributes to the mitotic entry of the subsequent cell cycle by downregulating the CDK inhibitory kinase, Wee1 (Papi et al., 2005). Therefore, separase is not only involved in anaphase but also has a role in cell cycle progression through the mitotic exit, and this role is extended to promote the mitotic entry during the next cell cycle of higher eukaryotes. In addition to cleaving cohesin and prompting the progression through the different cell cycle stages, more separase-dependent functions are observed during anaphase in yeast. The first evidence comes from the defect in the mitotic spindle assembly observed after artificial cleavage of cohesin SCC1 subunit by Tobacco etch virus (TEV) protease in the absence of separase activity (Uhlmann et al., 2000). Also when the cohesin subunit MCD1/SCC1 is depleted and separase is thermally inactivated, anaphase did not occurs, even in the presence of successful sister chromatid segregation (Jensen et al., 2001).
This suggests that separase has at least one more target than SCC1 cleavage and additional functions during the anaphase. A kinetochore and spindle-associated protein, Slk19, is cleaved by separase at metaphase-to-anaphase transition in the budding yeast (Figure 1.11B) (Sullivan et al., 2001). The cleavage products appear at the spindle midzone with the cells in which Slk19 cleavage is inhibited exhibiting a frequent spindle breakage and a delay in the mitotic exit (Sullivan et al., 2001). Additionally, when separase autocleavage is inhibited by site-specific mutation, cells enter mitosis with a slower rate with a defect in the bipolar mitotic spindle assembly and the congression of chromosomes on these spindles (Papi et al., 2005). This might be caused by the abnormal lower level of CDK activity at the mitotic entry which plays a significant role in the spindle microtubules regulation during mitosis. More recently, the protease activity of separase in yeast was shown to be involved in the spindle elongation during anaphase independently of cleaving the cohesin complex (Baskerville et al., 2008). This protease activity may involve the cleavage and downregulation of other spindle destabilising motor proteins such as Kar3 and Kip3 (Baskerville et al., 2008). This role of separase is consistent with its localisation to the mitotic spindle during anaphase (Jensen et al., 2001).
Figure 1.11 The structure, regulation and function of separase during mitosis. The schematic representation shows the structure, regulation and function of separase during mitosis. A. The structural domains of separase show the non-conserved N-terminal domain (yellow) to which securin binds and inhibits separase, and the highly conserved C-terminal domain (red) that contains the catalytic protease domain (Viadiu et al., 2005). ‘N’ and ‘C’ indicate the N- and C-termini of the protein, respectively. B. Regulation and roles of separase during mitosis. Separase is kept inactive for most of the cell cycle by binding to securin. At anaphase onset, securin is polyubiquitinylated (uuuu) and degraded by APC/C and its activator Cdc20. This brings the catalytic C-terminal domain with the N-terminal domain so separase becomes a catalytically active protease. The protease-dependent activity of separase has two roles during mitosis. First, separase cleaves cohesin at anaphase onset to remove the chromosome-bound cohesins. At the same time, separase has another substrate, Slk19, whose cleavage product plays an active role in stabilising the mitotic spindle. On the other hand, the protease-independent activity of separase promotes the CDC14 release from the nucleolus which reverses the CDK1 activity, thereby promoting the mitotic exit.
1.8 Aim of the study

As mentioned before, the precise mechanisms of chromosome segregation, including megabase and minichromosomes, in *T. brucei* are still poorly understood (Ersfeld and Gull, 1997; Gull et al., 1998). Two of the highly conserved components of chromosome segregation machinery in different eukaryotes studied so far are the cohesin complex and separase proteins. This study set out to explore the expression, localisation and the function of these proteins with special emphasis on the proposed roles in genome segregation of trypanosomes. Although a high degree of functional conservation is expected, it was unclear whether all classes of chromosomes in the *T. brucei* genome are subjected to the same molecular mechanisms of cohesion-dependent mitotic progression. Therefore, the focus of the work is to look at potentially differential sensitivities of different types of chromosomes in response to perturbation of components of the cohesion machinery. In particular, the project focuses on the characterisation of the putative candidate protein of the cohesin complex, cohesin subunit SMC3 and the candidate *T. brucei* separase homologue. All these built up toward discovering potential drug targets for anti-parasite chemotherapy with proteins involved in chromosomes segregation being considered as promising targets for the discovery of anti-trypanosome drugs.
2. MATERIALS AND METHODS

2.1 Identification of the cohesion proteins in Trypanosoma brucei

The Trypanosoma brucei genome database (GeneDB) available at (http://www.genedb.org/genedb/tryp/) was searched using the known sequences of the cohesion proteins of yeast, Saccharomyces cerevisiae to identify cohesion candidate proteins. Sequences with high homology such as cohesin SMC1 and SMC3 as well as other protein sequences derived from the T. brucei genome were used to procure the corresponding homologous sequences from public available databases. Exhaustive reciprocal BLAST searches were carried out using the search engine at NCBI (available at http://blast.ncbi.nlm.nih.gov/BLAST/). In some cases, the BLAST search also incorporated the conserved protein domain search. As necessary, multiple sequence alignment for the retrieved sequences was performed using multiple sequence alignment software, ClustalW (Thompson et al., 1994) which is available at (www.ebi.ac.uk/Tools/Clustalw2/index.html) to find the conserved residues and functional domains. Also, other structure predictions such as coiled coil domain formation (Lupas et al., 1991) (http://www.ch.embnet.org/software/COILS_form.html) were performed.

2.2 Production of TbSMC3 recombinant proteins and generation of polyclonal antibodies

2.2.1 Construction of the protein expression vectors

TbSMC3 (accession no: Tb927.5.3510) was chosen as a marker for the cohesin complex. Three fragments from the open reading frame (ORF) of the TbSMC3 gene were selected for the expression of the corresponding three recombinant peptides (Table 2.1). The three DNA fragments were amplified from the genomic DNA of 427 strain using proofreading Accusure DNA polymerase (Bioline) and 6 different gene specific primers (Table 2.2) that can be used to express three proportionally equal peptide fragments as indicated in Table 2.1 (SMC3.1: 98-280 aa; SMC3.2: 311-500 aa, SMC3.3: 721-920 aa). The three blunt-end PCR products were cloned in-frame into the 6xHis tag protein expression vector, pET100/D-TOPO (Figure 2.1; Invitrogen, USA)
according to the manufacturer’s protocol producing three different protein expression constructs. The expression constructs specifically add an N-terminal polyhistidine tag (6 histidine residues) to the resultant recombinant protein that can bind tightly and specifically to an affinity matrix that contains chelated nickel atoms. The three generated DNA constructs were transformed into TOPO10 *E. coli* cells (Invitrogen, USA) which are deficient in the T7 RNA polymerase gene to avoid any basal transcription in the absence of induction. The positive transformants were chosen to isolate the plasmid DNA from *E. coli* cells using plasmid prep kits (Macherey-Nagel, Germany). All the three constructs were sequenced to ensure the in-frame cloning (sequence results are available on request).
Figure 2.1 The diagram shows the main features of the pET100/D-TOPO vector (Invitrogen, USA). The plasmid has been genetically engineered to be a linearized vector with 3' deoxythymidine (T) overhangs. The (T) overhangs is activated by being covalently bound to the topoisomerase I enzyme. The PCR products will have a 3' A overhangs that can complement the 3' T overhangs of the vector and allow for fast ligation with the already bound topoisomerase I. The vector contains the ampicillin resistance gene which allows the selection of the transformed E. coli cells. The vector has a polyhistidine tag (6xHis) located upstream to the site of insertion of PCR fragment. The polyhistidine tag, under conditions of physiological pH (pH 7.0), will bind specifically to divalent cations (e.g. Co^{2+} and Ni^{2+}) of the metal affinity resins (BD Talon, Clontech USA). Recombinant proteins containing the polyhistidine tag at their N-termini were purified using divalent metal columns where they specifically bind. The expression of the recombinant protein was under the control of the lac operator. The expression of the recombinant protein is induced by the addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG). The vector is ~5800 bps long. The vector diagram was taken from the pET100/D-TOPO vector manual (Invitrogen, USA).
<table>
<thead>
<tr>
<th>Amino acid sequences of the three fragments of TbSMC3 gene (Tb927.5.3510) from T. brucei gene database (<a href="http://www.genedb.org/genedb/tryp/">www.genedb.org/genedb/tryp/</a>) that were chosen for the protein expression. The sequences of the three hypothetical peptide fragments of TbSMC3 protein were indicated in colours as TbSMC3/1 in blue, TbSMC3/2 in red and TbSMC3/3 in green. Amino acid positions were indicated on the right hand side of the sequences.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Table 2.1</strong></td>
</tr>
<tr>
<td><strong>TbSMC3/1</strong></td>
</tr>
<tr>
<td>MYIKNILISG FRSYREQAFE QRSPKNNVI VGKNGAGKSN FFIAIQFVLC EKFMNLSSVE 60</td>
</tr>
<tr>
<td>RKDLFHVSAG RPALPVEI IFDNSGRLY IPGSKAVNVE EVRIRSLVVRQ 120</td>
</tr>
<tr>
<td>TATDIRQLE SAQSTSNTPY YIPEVOQISS LANMSDEERF QLIDKAVGTR YEVRRKQESE 180</td>
</tr>
<tr>
<td>KILEETEVEQH EKIGESIAQL EERLELRSE SDELMSTQEI DKKKKCVVQIC ILMSDLNAAR 240</td>
</tr>
<tr>
<td>EELDRRLDDE NSYMRSRSGRD HYIDEHAEIK ISEAEEIRN 300</td>
</tr>
<tr>
<td><strong>TbSMC3/2</strong></td>
</tr>
<tr>
<td>TLMREKGQVEQNLNGLNSTM KRTESVKTQV LKRGVELNQK IAETNAQNLK KLAAIQQLQI 360</td>
</tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>EELDRRLDDE NSYMRSRSGRD HYIDEHAEIK ISEAEEIRN 540</td>
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</table>

Note: Amino acid positions were indicated on the right hand side of the sequences.
Table 2.2 Primer sequences used to generate the three DNA fragments of TbSMC3 gene (TbSMC3/1, TbSMC3/2 and TbSMC3/3). The sequences of the forward (For) and reverse (Rev) primers were indicated in the 5’ to 3’ directions. The three blunt-end PCR products generated by these primers were cloned in-frame into the 6xHis tag protein expression vector, pET100/D-TOPO producing three different protein expression constructs.

<table>
<thead>
<tr>
<th>Gene fragment</th>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TbSMC3/1</td>
<td>For</td>
<td>GAAGTGCGTATACGGCGTACAC</td>
</tr>
<tr>
<td></td>
<td>Rev</td>
<td>ATTACGTATCTCCGATTTCAG</td>
</tr>
<tr>
<td>TbSMC3/2</td>
<td>For</td>
<td>TTAAATTGTATGTGATCTCTCT</td>
</tr>
<tr>
<td></td>
<td>Rev</td>
<td>TTGACTGCGGGCTTTGTTGT</td>
</tr>
<tr>
<td>TbSMC3/3</td>
<td>For</td>
<td>AAGACGGAGATTCCTCCCAT</td>
</tr>
<tr>
<td></td>
<td>Rev</td>
<td>AGTCCCTCCGTCGAATCTC</td>
</tr>
</tbody>
</table>
2.2.2 Production of the recombinant TbSMC3 proteins

The recombinant proteins were obtained by transformation of *E. coli* strain BL21 DE3 (Novagen) with the three protein expression constructs, pET100/D-TOPO that contains the respective SMC3 PCR fragments (SMC3/1, 2, 3). Bacterial *E. coli* strain, BL21 DE3, used for the expression experiment was treated chemically with CaCl$_2$ to be competent “Ultra-competent” for the transformation reaction (Inoue et al., 1990).

Before the large batch protein expression and purification, a pilot expression was performed to detect if the expected recombinant protein product is found in the soluble or insoluble fraction (inclusion bodies) of the bacterial cell lysate. Plasmid DNA was used for the transformation of the chemically competent *E. coli* (BL21 DE3) cells according to the standard protocol (Inoue et al., 1990). Briefly, 200 µl of the bacterial cell suspension was transferred to pre-chilled falcon tubes. Then 2 µl of the plasmid DNA (with a concentration of 10 ng/µl) was added and gently mixed with the cell suspension. After incubation of the reaction mix on ice for 30 minutes, the cells were heat shocked for 45 seconds at 42°C. The cells were chilled on ice for 5 minutes before 250 µl of SOC broth (20 g Bacto-tryptone, 5 g Bacto-yeast extract, 0.6 g NaCl, 0.5 g KCl, 10 mM MgCl$_2$, 20 mM glucose/ 1000 ml double-distilled H$_2$O) was added to the reaction. The transformation reaction was then incubated for 45 minutes at 37 °C at sloped horizontal position with shaking (200 rpm) for good aeration to recover the bacterial cells. The entire transformation reaction was then transferred to 10 ml Lauria-Bertani (LB) broth (10 g Bacto Tryptone, 5 g Bacto Yeast extract, 10 g NaCl/ 1000 ml ddH$_2$O) containing ampicillin (100 µg/ml). The transformation reaction was grown overnight at 37 °C with shaking before proceeding to the pilot expression.

In the pilot expression, a pre-warmed 10 ml LB broth containing ampicillin (100 µg/ml) was inoculated with 500 µl from the overnight cultures of the three transformation reactions (SMC3/1, 2, 3). The transformation reactions were further incubated for an additional 2 hours with shaking (200 rpm) at 37 °C until an optical density (OD600) of ~ 0.5-0.8 was achieved. The zero time point samples (500 µl) were
collected from each culture before the protein expression. The collected samples were centrifuged at 10,000xg for 10 minute at 4 °C. The cell pellets were then resuspended in 95°C-hot 1X SDS PAGE sample buffer (0.045 mM Tris-HCl; pH 6.8, 10% glycerol, 1% SDS, 0.02 g bromophenol blue, 2.5% β-mercaptoethanol).

Before the induction of the corresponding recombinant protein expression, each cell culture was further subdivided into two cultures before the inducer, IPTG (Isopropyl-β-D-thiogalactopyranoside) was added to the final concentration of 1 mM to only one culture with the other culture left as an induction control. The induced and the control non-induced cultures were further incubated for an additional 4 hours at 37 °C with shaking with small aliquots (500 µl) collected hourly, centrifuged and processed as before. After incubation, the whole cell cultures were centrifuged at 4000xg for 20 minutes at 4 °C. The cell pellets were suspended in ice-cold lysis buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole, pH 8.0). The cell suspension was then treated with lysozyme (Sigma) for 30 minutes on ice and the cells were lysed using sonication (on ice, 5 X 10 sec at 200-300 W). The lysed cells were then centrifuged at 20,000xg for 20 minutes at 4 °C to separate the insoluble from the soluble fractions of the cell lysate. The supernatant was carefully decanted and collected as the soluble fraction while the pellet was suspended in ice-cold lysis buffer as the insoluble fraction. Equal amounts of the soluble and insoluble protein fractions with equivalent volumes of 2X SDS PAGE sample buffer were mixed before being boiled at 95 °C for 5 minutes. All the collected samples, including the zero time point samples, were analyzed by electrophoresis on 12 % Tris/Glycine SDS PAGE gel to detect the successful expression of the target protein and to localise the expressed protein as to the supernatant or to the cell pellet.

After the initial pilot expression was successful to detect the expression of the target protein, large scale expression of the recombinant TbSMC3/1,2,3 proteins was adapted. 300 ml of LB broth containing the transfected BL21 DE3 was incubated at 37 °C with shaking (200 rpm) until an optical density (OD600) of ~0.5-0.6. The inducer (IPTG) was added to induce the expression of the corresponding recombinant protein and the culture was further incubated for another 4 hours. The cells were harvested by
centrifugation and the cell pellets were suspended in ice-cold lysis buffer, treated with lysozyme for 30 minutes on ice before shearing by sonication (on ice, 5 times 10 seconds each at 200-300 W). The whole cell suspension was then centrifuged at 20,000xg for 20 minutes at 4 ºC to separate the soluble from the insoluble fractions. Depending on the localisation of recombinant protein (either in the supernatant or as inclusion bodies in the cell pellet), a suitable protein purification condition was adapted. As the three expressed recombinant peptides (SMC3/1,2,3) were mostly located in the insoluble fraction (inclusion bodies) of the cell lysate, denaturing condition using guanidine hydrochloride \([\text{NH}_2\text{C(NH)}_2\text{NH}_2\text{HCl}]\) was adapted during the preparation and purification of recombinant proteins. The supernatant of the centrifuged cell lysate was discarded and the cell pellet was suspended in 30 ml of the denaturing lysis buffer (6 M guanidine.HCl, 50 mM NaH\(_2\)PO\(_4\), 300 mM NaCl, and 10 mM imidazole, pH 8.0) to solubilise the protein precipitate. The solubilised protein solution was then centrifuged at 20,000xg for 10 minutes at 4 ºC to clarify the protein suspension.

As the recombinant peptides were designed to contain the polyhistidine tag (six histidine residues) at their N-termini, the purification of the corresponding recombinant proteins from the insoluble fraction was done using the metal affinity resins (BD Talon, Clontech USA). Under the conditions of physiological pH (pH 7.0), the modified protein with polyhistidine tag binds specifically and tightly to the chelated cations (e.g. Co\(^{2+}\) and Ni\(^{2+}\)) bound to the metal affinity matrix, whereas most of \(E.\ coli\) proteins will not bind to such a matrix. All the purification steps were done as recommended by the manufacturer and the denaturing conditions were used to solubilise the recombinant proteins during all the purification steps as mentioned before. The elution of the corresponding recombinant protein from the column was done in elution buffer (6 M guanidine.HCl, 50 mM NaH\(_2\)PO\(_4\), 300 mM NaCl, and 250 mM imidazole, pH 8.0).

The three purified recombinant proteins (SMC3/1,2,3) were then dialysed against Tris-Buffered Saline (TBS) buffer (20 mM Tris, 150 mM NaCl, pH 7.6 with HCl) provided with descending urea [(NH\(_2\))\(_2\) CO] concentrations. Urea was first used at 6 M concentration and then decreased to 4 M with the final concentration of 2M was
used to maintain the solubility of the purified protein under the denaturing conditions. The final dialysed protein was then concentrated by applying Polyvinylpyrrolidone [(PVP), Mw 40,000] to the outside of the dialysis tubing in order to remove most of the water from the protein samples. The purified proteins were concentrated to 600-1000 µg ml⁻¹ and the final concentration of each recombinant peptide of TbSMC3/1, 2, 3 was determined by Bicinchoninic Acid Protein Assay (BCA) Kit (Sigma). Also the degrees of purity of each of the purified recombinant peptides were determined by electrophoresis on 12 % Tris/Glycine SDS PAGE gel and Coomassie staining.

2.2.3 Generation and affinity purification of polyclonal antibodies

To obtain polyclonal IgG antibodies against the three TbSMC3 peptides (TbSMC3/1, 2, 3), 2 mg of each peptide was combined together after the protein purification and concentration. The peptide mix was used to immunise rabbits to raise the rabbit polyclonal antibodies (Yorkshire Biosciences). After the first and second bleeding of the rabbits, the raised antibodies were affinity purified specifically against the corresponding recombinant antigen. The purified recombinant peptide is prepared for the affinity purification protocol of the antibodies by additional dialysis against the coupling buffer (200 mM NaHCO₃, 500 mM NaCl, pH 8.3) supplemented with 2 M urea [(NH₂)₂ CO]. After two rounds of dialysis, the purified recombinant proteins were used for the affinity purification of the antibodies. CNBr-activated Sepharose™ 4B (Amersham Biosciences) containing primary amino groups was used as a coupling matrix ligand for the affinity purification protocol as suggested by the manufacturer. Briefly, 1 g of CNBr-activated Sepharose was prepared by washing with several aliquots of 1 mM HCl on a sintered glass filter (porosity G3) and used as the coupling ligand. After binding of the recombinant protein to the coupling ligand matrix, the matrix was rotated for 2 hours at room temperature. The coupling matrix was allowed to precipitate by gravity flow and the supernatant was discarded. The matrix was suspended in 10 ml blocking buffer (200 mM glycine, pH 8.0) and rotated for 20 minutes at room temperature. After precipitation of the matrix and the removal of the
blocking buffer, the matrix was washed 4-5 times with the coupling (200 mM NaHCO₃, 500 mM NaCl, 2M Urea, pH 8.3) and washing (100 mM acetate, 500 mM NaCl, pH 4.0) buffer. The final wash of the matrix was done using 1X phosphate buffered saline (PBS) buffer (137 mM NaCl, 3 mM KCl, 16 mM Na₂HPO₄, 3 mM KH₂PO₄, pH 7.4) supplemented with 500 mM NaCl to minimise the non-specific binding. The polyclonal IgG antibody suspension was added and the matrix was further incubated for 30-60 minutes at room temperature. The coupling matrix was washed twice with PBS containing NaCl before transferring to the purification column. The specifically-bound antibodies were eluted by a low acidic pH glycine buffer (100 mM glycine-HCl, 100 mM NaCl, pH 2.5). The eluted antibodies were promptly neutralised to pH 7 after elution using a basic neutralisation buffer (1 M Tris-HCl, pH 9). The eluted antibody fractions were further dialysed against PBS and the antibacterial compound (0.01 % Na.azide) was added before the permanent storage of antibodies at -80 °C. Unless otherwise specified, the affinity purified antibodies were used at a dilution of 1:1000 for the western blotting and 1:200 for the immunofluorescence microscopy.
Cell Culture and transfection of *Trypanosoma brucei*

Procyclic *T. brucei* cell line 29-13 co-expressing T7 RNA polymerase and Tet repressor was grown in semi-defined (SDM-79) medium (Brun and Schonenberger, 1979) supplemented with 10% fetal bovine serum (FBS) at 28 °C. Where necessary, G418 (15 µg ml\(^{-1}\)) and hygromycin (50 µg ml\(^{-1}\)) were kept added throughout the experiments to maintain the integrated genes for T7 RNA polymerase and tetracycline repressor (*TetR*), respectively (Wirtz et al., 1999). Procyclic 427 trypanosome cells were grown in SDM-79 supplemented with 10% fetal bovine serum (FBS) without any antibiotics. Procyclic 449 cells was grown in SDM-79 medium supplemented with 2.5 µg ml\(^{-1}\) phleomycin to maintain the Tet repressor (*TetR*) gene (Biebinger et al., 1997).

Culture and transfection of *T. brucei* was carried out essentially as described previously (Vassella et al., 2001; Wang et al., 2000). For transfection, trypanosome cultures growing exponentially to the mid-log phase were harvested and washed twice by the ice-cold, filter-sterilised Cytomix buffer (2 mM EGTA, 120 mM KCl, 0.15 mM CaCl\(_2\), 10 mM K\(_2\)HPO\(_4\), 25 mM HEPES, 5 mM MgCl\(_2\).6H\(_2\)O, 0.5 % Glucose, 100 µg/ml BSA, 1 mM Hypoxanthine, pH 7.6). The cell pellet was finally suspended in ice-cold Cytomix buffer to a final cell concentration of 4 X 10\(^7\) cells ml\(^{-1}\). 10 µg of the linearised DNA construct with Not1 was used for the transfection of the trypanosome cell suspension at the final volume of 500 µl. The transfection was done by electroporation using BTX Electro Square Porator, ECM 830 (BTX) with the following parameters (1700 V, 3 pulses, 100 µs pulse length time, 200 ms pulse interval time). All the transfection steps and handling of the cells were carried out on ice. Cells were allowed to recover overnight in SDM-79 medium at 28 °C without any drug selection. Transfection was always done in the late afternoon and the cells were recovered overnight in the absence of any drug selection. In the morning, the transfected cells were diluted to the concentration of 2 X 10\(^5\) cells ml\(^{-1}\) with fresh SDM-79 medium containing the suitable selection antibiotic based on the construct drug resistance marker. The cells were evenly distributed to each well of a 24-well plate, sealed with parafilm before being incubated at 28 °C. 10-15 days later, cells that were not transfected are killed by the antibiotics and all the likely transfected cells begin to grow producing positive clonal cell lines.
For TbSMC3 (GeneDB accession number: Tb927.5.3510), a fragment corresponding to 505 bp of the open reading frame (nt 83-588 bp) was selected as RNAi target by the automated web-based tool, RNAit (Redmond et al., 2003). The corresponding RNAi fragment was amplified from the 427 strain genomic DNA using fragment-specific forward and reverse primers (Table 2.3). A flanking XhoI and HindIII restriction sites were introduced to the termini of the sense and anti-sense primers, respectively, so that the RNAi fragment can be cloned into the corresponding XhoI and HindIII sites of pZJMβ vector (Figure 2.2A) after double-digest of both the PCR insert and the vector with the same restriction enzymes (Wang et al., 2000). After successful cloning, the RNAi fragment will replace the α-tubulin stuffer fragment of pZJMβ plasmid between the two opposing head-to-head T7 promoters (Figure 2.2A) (Wang et al., 2000). So that after the transcription induction, the same DNA fragment will be transcribed twice in opposite directions giving rise to the complementary double-strand RNAs (dsRNAs) which is the starting signal of the RNAi pathway. The PCR reaction containing 10 mM Tris-HCl (pH 8.8 at 25°C), 20 mM (NH₄)₂SO₄, 2 mM MgCl₂, 0.1% (v/v) Tween 20, 200 µM of each dNTP, 1 µg genomic DNA, 1 µM of each primer and 1.25 U Taq DNA polymerase was set up at the following conditions: initial denaturation step of 94 ºC for 2 minutes followed by 30 cycles carried out at 94 ºC for 30 seconds, 55 ºC for 30 seconds and 72 ºC for 60 seconds, with the final extending cycle of 72 ºC for 5 minutes. The PCR insert was initially cloned into pGEM-T easy (Promega) following the protocol provided by the manufacturer. To specifically amplify the corresponding PCR insert in pGEM-T easy, the resulting construct was used to transform the E. coli XL1-Blue cells (Stratagene) that were treated chemically to be ultra-competent for the transformation electroporation (Inoue et al., 1990). After transformation, the positive colonies were selected using the blue/white screening as instructed by the manufacturer (Promega). Small plasmid minipreps were prepared from the resulting white colonies and the plasmid DNA was harvested from the bacterial cells by the alkaline lysis (Sambrook et al., 1989). The PCR insert was digested from pGEM-T easy and subsequently ligated into the corresponding XhoI and HindIII sites of pZJMβ vector (Wang et al., 2000). The construct was used to transform the chemically competent E. coli XL1-Blue cells and the plasmid DNA was purified from the maxiprep (50 ml) bacterial culture by the NucleoBond PC 100 columns (Macherey-Nagel) according to the manufacturer protocol.
Figure 2.2 Schematic representation of the main features of the two RNAi vectors, pZJMβ (Wang et al., 2000) and pALC14 (Bochud-Allemann and Schneider, 2002). A. pZJMβ vector as linearised by NotI restriction digest so the construct can be integrated by homologous recombination into the ribosomal DNA (rDNA) spacer region, a transcriptionally inactive segment of the T. brucei genome (Wirtz and Clayton, 1995). The gene fragment of interest was cloned between the two opposing head-to-head T7 promoters to replace the α-tubulin stuffer fragment of the plasmid after XhoI/HindIII double restriction digest (Wang et al., 2000). The double-stranded RNAs (dsRNAs) were transcriptionally induced by T7 RNA polymerase under the control of tetracycline operators (Tet Op). Selection of the positive transfected trypanosome cells was under the control of a phleomycin resistance gene (BLE). B. The main features of the second RNAi vector, the pALC14, in its linearised form. The same RNAi gene fragment was inserted twice by two separate cloning steps in head-to-head opposite directions and separated by 406 bp stuffer DNA fragment. After transcription, double RNA transcripts separated by the stuffer loop can form the stem-loop structure (Bochud-Allemann and Schneider, 2002). The transcription of dsRNAs was driven by the procyclin promoter (Procyc promoter) rather than T7 promoter under the control of 2 times tetracycline operators (2X Tet Op). Selection of the positive clones was under the control of a puromycin (PURO) resistance gene.
For Tbseparase (Tb927.1.3120), a fragment corresponding to the 5’-part of the coding region of the putative separase gene was selected as RNAi target by the automated web-based tool, RNAit (Redmond et al., 2003). The 537 bp DNA fragment was amplified from the genomic DNA using sense primer carrying flanking BamHI/HindIII sites and antisense primer carrying flanking XhoI/XbaI sites (Table 2.3). So that after double-digest with the corresponding restriction enzymes, the DNA insert can be cloned twice, in opposite directions, into the Stem-loop pALC14 plasmid (Figure 2.2B) (Bochud-Allemann and Schneider, 2002). PCR was performed on the genomic DNA using the corresponding primers (Table 2.3) as mentioned before for TbSMC3 gene except the following conditions are different: initial denaturation step of 94 ºC for 2 minutes followed by 30 cycles carried out at 94 ºC for 40 seconds, 55 ºC for 40 seconds and 72 ºC for 60 seconds, with the final extension cycle of 72 ºC for 5 minutes. The resulting PCR fragment was then first digested with HindIII/XbaI and inserted into the corresponding sites of the Stem-loop pALC14 vector digested with the same restriction sites. The resulting construct carrying the first gene fragment as well as the original PCR fragment were both digested with BamHI/XhoI, allowing the insertion of the same gene fragment into opposite direction. After successful cloning into pALC14, the DNA construct was used to transform XL-1 Blue E. coli cells as mentioned above. The amplified DNA construct was purified from the large maxiprep bacterial culture by the NucleoBond PC 100 columns (Macherey-Nagel).

For the integration of the RNAi constructs into the ribosomal DNA (rDNA) spacer region, linearization of 10 µg of the purified RNAi DNA constructs was done by single restriction digest with NotI followed by transfection into the procyclic 29-13 cell line as mentioned before. 24 hours after transfection, stable transfectants were selected in the presence of 5 µg ml⁻¹ of phleomycin (pZJMβ vector) or 2 µg ml⁻¹ puromycin (pALC14 vector). Clonal cell lines were obtained by the limiting dilution and plating in 24-wells plate. After 10-15 days incubation at 28 ºC, screening of the positive clones was undertaken. For screening, the cloned stable transfectants that reached a constant growth rate after at least three regular sub-culturing passages were cultured in the presence of 1 µg ml⁻¹ tetracycline to monitor the RNAi phenotypes. The growth rate of the induced and non-induced cell lines was monitored by a CASY cell counter and
analyser system (Innovatis AG, Germany) at regular time intervals with starting cell count of $4 \times 10^5$ cells ml$^{-1}$. The mid-phase logarithmic growth of the cells was maintained by diluting the cells to the original $4 \times 10^5$ as necessary. Also after the RNAi induction, the depletion of the corresponding protein or mRNA was monitored by collecting $1 \times 10^7$ Cells from both the induced and non-induced cultures at 24 hours intervals. The corresponding cell lysates and mRNA samples were processed for western blot or PCR as mentioned later in this chapter.

2.5 Ectopic tagging and expression of separase

Full length open reading frame (ORF) of Tbseparase (Tb927.1.3120) was amplified from 449 procyclic strain genomic DNA by the proof reading AccuSure DNA polymerase (Bioline). Gene-specific sense and antisense primers with flanking ApaI and MluI restriction sites, respectively, were used to amplify the corresponding DNA insert (Table 2.3). PCR on the genomic DNA was performed as mentioned before with the following conditions: initial denaturation step of 94 °C for 10 minutes followed by 30 cycles performed at 94 °C for 30 seconds, 60 °C for 30 seconds, 72 °C for 3 minutes followed by the last extending cycle of 72 °C for 5 minutes. The PCR product was A-tailed by Taq DNA polymerase allowing the subsequent cloning of DNA insert into pGEM-T easy vector as suggested by the manufacturer (Promega). After amplification, the DNA insert was released specifically from the pGEM-T easy vector by an ApaI and MluI double restriction digest. For adding the epitope Myc tag to the C-terminus of the protein (C$^{\text{myc}}$ tagging), the entire ORF (3480 kbp) was subsequently cloned into the double c-Myc tag expression vector pHD1484 using ApaI/MluI restriction sites (Figure 2.3) (Colasante et al., 2006). The plasmid contains the hygromycin resistance gene ($\text{HYG}$) for antibiotic resistance selection and the expression of the Myc-tagged protein was under the tetracycline-inducible procyclin promoter (Figure 2.3). The inserted DNA fragment was subjected to sequencing to ensure the correct reading frame of the construct after cloning. The sequencing was done at MWG Biotech Inc. and the DNA sequencing result is available on request. The purified DNA construct was used for transfection of procyclic 449 cell line according to the standard transfection protocol as mentioned above. The selection of the clonal cell lines was done on the following day.
by the addition of 50 µg ml⁻¹ hygromycin. Expression of the Myc-tagged protein was induced by the addition of 1µg ml⁻¹ of tetracycline. 24 hours after the induction, cell samples from both the induced and non-induced cultures were collected and processed for either western blotting or immunofluorescence microscopy as mentioned later to detect the protein expression and subcellular localisation, respectively. To see the effect of separase overexpression on the growth rate, cells were counted every 24 hours following the induction with the cell density kept at 4 X 10⁵ ml⁻¹ by dilution with fresh medium containing the appropriate antibiotics.
Figure 2.3 Schematic representation of the basic features of the Myc tag epitope expression vector, pHD 1484 (Colasante et al., 2006). The gene of interest (target sequence) was inserted between ApaI and MluI sites after restriction digest of the vector with the same restriction enzymes to add the epitope tag (2xMyc) at the C-terminus of the protein. Expression of the target protein was under the power of the procyclin promoter (PARP) and the selection of the positive clonal cell lines was done under the hygromycin resistance (HYG) which expressed by the rRNA promoter (PrRNA). The induction of the expression was done after the addition of tetracycline which freed the tetracycline operator (Tet Op) from its repressor. The construct was integrated at the non-transcribed space (rDNA spacer) between ribosomal DNA (rDNA) genes after linearization by NotI restriction digest.
2.6 Fluorescence-Activated Cell Sorting (FACS) analysis

For FACS analysis, time samples of the positive clonal *T. brucei* cells (2 x 10^7 cells) were collected regularly at 24 hour intervals after tetracycline induction. The harvested cells were centrifuged at 1500xg for 5 minutes at 4 °C and washed once in PBS. The cells were fixed by adding 70% EtOH/H_2O while vortexing the samples at high speed. The cells were then kept on ice for an hour before being fixed overnight at 4 °C. After overnight fixation, cells were centrifuged at 1500xg for 5 minutes at 4 °C and the cells were suspended in the staining solution composed of propidium iodide (Sigma) and Ribonuclease A (RNase A) to the final concentration of 50 and 200 µg ml^{-1}, respectively. Cells were incubated at 37 °C for at least 30 minutes and examined by a FACS Calibur flow cytometer using detector FL3-A (Becton Dickson). The cells were analyzed for their DNA content and cell cycle position as the signal of the FL3-A channel (propidium iodide fluorescence) was recorded against the cell number. 20,000 cells were recorded every time and the DNA content of the peaks was determined by CellQuest software version 3.0.3 (Becton Dickson).

2.7 Immunofluorescence microscopy

For the tabulation of cells with different number of nuclei and kinetoplasts in the individual cells, and the number of cells with different morphologies after RNAi, cells were stained with 4',6-diamidino-2-phenylindole (DAPI). For DAPI staining, ~5 X 10^5 cells were collected from the trypanosome culture, centrifuged at 1500xg for 5 minutes at 4 °C. After the cell pellet was washed once in 1X PBS (137 mM NaCl, 3 mM KCl, 16 mM Na_2HPO_4, 3 mM KH_2PO_4, pH 7.4), trypanosome cells were then fixed in suspension with 3.6% formaldehyde in PBS for 15 minutes at room temperature. The cells were washed twice in PBS before being finally suspended in PBS and allowed to settle on poly-L-lysine coated slides (Sigma) for 20 minutes. Non-attached cells were washed away by brief dipping in PBS before the cells were permeabilised with 0.1% Nonidet P-40 (NP-40) for 5 minutes at room temperature followed by 5 minutes wash in PBS. Slides were removed from PBS and mounted in Vectashield containing DAPI.
Slides were examined under a Nikon Epifluorescence microscope (Nikon) and the data were recorded for a population of 400 cells, performed in duplicate at each time point.

For the immunofluorescence experiments, cells were harvested, washed twice in 1X PBS and fixed in suspension with 3.6% formaldehyde in PBS for 15 minutes at room temperature. After another wash with 1X PBS, the cells were settled on poly-L-lysine coated slides (Sigma) for 20 minutes and the non-attached cells were washed away by brief dipping in 1X PBS before permeabilisation of the cells with 0.1% NP-40 for 5 minutes followed by 5 minutes wash in 1X PBS. After incubation with the primary antibody for 1 hour, the slides were washed three times for 5 minutes each in 1X PBS before a further incubation for 1 hour with the secondary antibody. All the immunofluorescence procedures and antibody incubation was done at room temperature unless stated otherwise.

The various primary antibodies used in this study were affinity-purified anti-SMC3 polyclonal antibodies (pAbs) produced in rabbits (Yorkshire Biosciences, UK), mouse Anti-Myc (clone 9E10, obtained from ECACC) monoclonal antibodies (mAbs), KMX-1, a mouse mAbs against Physarum polycephalum amoebal tubulin that preferentially stains spindle microtubules in *T. brucei* (Sasse and Gull, 1988). Primary antibodies were used at a dilution of 1:200 in PBS. The various secondary antibodies were anti-rabbit FITC-conjugated antibody (Sigma, Germany) and anti-mouse FITC-conjugated antibody (DAKO, Denmark) used at 0.5 µg ml⁻¹. Following incubation with secondary antibody, cells were washed and the nuclei and kinetoplasts were stained by mounting in VectaShield containing 4',6-diamidino-2-phenylindole (DAPI) (Vector laboratories). For preparation of cytoskeletons and extraction of all the detergent-soluble proteins, trypanosome cells were freshly collected and treated with detergent prior to their fixation. For this purpose, cells was harvested and washed once in ice-cold PBS before being allowed to settle on poly-L-lysine coated slides. After removing the non-bound cells by PBS washing, cells were incubated with ice-cold 1X PEM extraction buffer (0.1M Pipes, 2 mM MgSO₄, 1 mM EGTA, 0.1% NP-40, pH 6.9) for 30-60 seconds before being fixed by 3.6% formaldehyde in PBS. After fixation, the
detergent-extracted cytoskeleton preparations were washed in 1X PBS before being processed for the immunostaining as mentioned above for the whole cells. The slides were examined under a Nikon Epifluorescence microscope (Nikon) and the images were acquired with a CCD camera (Rober) controlled by MetaVue software, version 5.0 (Molecular Devices Inc). Images were then processed and pseudocoloured in Adobe Photoshop (Adobe).
2.8 **Protein electrophoresis and western blot**

Trypanosome cells for protein extraction and electrophoresis were washed once with 1X PBS before being dissolved in 95°C-hot 1X SDS PAGE loading buffer (0.045 M Tris-Cl, pH 6.8, 10% glycerol, 1% SDS, 0.02 g bromophenolblue, 2.5% β-mercaptoethanol) before being boiled for 5 minutes in the sample buffer at 95 ºC. 2 x 10^6 cells were loaded into each lane of 10% or 12% Tris/Glycine SDS PAGE gel for electrophoresis and separation of the total protein extract. The separated total proteins were transferred to a Protran® nitrocellulose membrane (Whatman GmbH, Germany) for western blotting. At the same time, the separated protein samples on Tris/Glycine SDS PAGE gel were stained with Coomassie stain [0.2 % (w/v) Comassie Brilliant Blue R250, 50% (v/v) methanol, 10% (v/v) Acetic acid]. After transfer, the nitrocellulose membranes were prepared for western blotting by blocking the membranes for 60 minutes with 5% semi-skimmed milk in TBS buffer and 0.1% Tween (TBS-T). The membranes were probed by the primary antibody for 45 minutes and washed three times, 10 minutes each, with TBS-T buffer. The membranes were further incubated with secondary antibody for another 45 minutes.

Following the washing steps as before, the membranes were processed for the chemiluminescence using western lighting reagents (PerkinElmer). To detect the immunogenic signals, the membranes were exposed to X-ray light sensitive film (Amersham) for 1-5 minutes according to the specific protein abundance. Primary antibodies used were anti-SMC3 polyclonal antibodies (pAbs) and mouse Anti-Myc monoclonal antibodies (mAbs) used at 1:1000 and 1:500 dilution in TBS-T buffer, respectively. Secondary antibodies were ZyMax anti-rabbit horseradish peroxidase (HRP) conjugated antibody (Invitrogen, USA) and anti-mouse HRP-conjugated antibody (Sigma, Germany) used at ~0.5 µg ml⁻¹ dilution in TBS-T buffer.
2.9 Fluorescence In Situ Hybridisation (FISH)

The in situ hybridisation experiments were carried out to detect the potential effect of the depletion of SMC3 and separase gene transcripts on the segregation pattern of the two main chromosomes categories of T. brucei, megabase and minichromosomes. FISH was carried out using two specific DNA probes to assess the segregation pattern of an individual chromosome or chromosomes population. The first specific DNA probe was directed to a fragment of a diploid pair of megabase chromosome 1 (Chr.1) which has a size of ~ 1.1 Mb (Berriman et al., 2005; Hall et al., 2003). By using FISH, Chr.1 appears as two and four visible dots in the case of interphase and mitotic stage, respectively (Ogbadoyi et al., 2000). The DNA probe is targeted to the tubulin gene cluster, α-tubulin (Tb927.1.2400) and β-tubulin (Tb927.1.2350) genes, which spans approximately 60 Kb of Chr.1. The second DNA probe was directed to small minichromosomes where the highly repetitive 177-bp repeat element was used as a DNA marker to visualise the segregation pattern of the minichromosomes population (Sloof et al., 1983; Weiden et al., 1991). FISH on an individual minichromosome is not possible due to the absence of a sufficiently large DNA sequence that could serve as a specific probe against a single minichromosome.

Both α- and β-tubulin genes were amplified from the 449 strain genomic DNA by a standard PCR reaction using a pair of specific sense and antisense primers (Table 2.3). PCR products were cleaned up on the NucleoSpin Extract II column (Macherey-Nagel, Germany) before being cloned into pGEM-T easy vector (Promega) for the amplification. The 177-bp repeat from the minichromosome was amplified from the genomic DNA with two specific primers (Table 2.3) and also purified on the NucleoSpin Extract II column (Macherey-Nagel, Germany). The two clonal tubulin genes (α and β) were labelled by nick translation using Digoxigenin-11-dUTP (Roche Diagnostics, USA). A nick translation reaction containing 1 µg of plasmid DNA, 1 mM dNTPs mix, 1 mM DIG-11-dUTP, 0.002 U DNase I and 10 U E. coli DNA polymerase I in 1X NT polymerase buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM DTT) was incubated at 16 ºC for 2 hours (all components were from Fermentas except DIG-11-dUTP which was from Roche). The average size of the DNA probe was monitored by agarose gel electrophoresis. When the optimal size of the probe which is around 300-
500 bp was reached, the reaction was stopped by 10 mM EDTA before being heated to 95 °C, otherwise the reaction continued to be incubated until the DNA was trimmed to the proper size. The minichromosomal 177-bp repeat probe was labelled by PCR using a Biotin-11-dUTP (Roche Diagnostics, USA). PCR containing 10 ng of a pre-amplified 177 bp DNA fragment and 10 mM Tris-HCl (pH 8.8 at 25°C), 20 mM (NH₄)₂SO₄, 0.1% (v/v) Tween 20, 2 mM MgCl₂, 0.2 mM of dNTP without dTTP, 0.13 mM dTTP, 0.07 mM BIO-dUTP, 5 units Taq DNA polymerase was set up at the following conditions: 1 cycle of denaturation carried out at 94 °C for 2 minutes followed by 30 cycles at 94 °C for 1 minute, 50 °C for 1 minute, 72 °C for 2 minutes and the last extension cycle of 72 °C for 10 minutes. Labelled DIG- and BIO-DNA probes were purified on NucleoSpin Extract II column (Macherey-Nagel, Germany) before being tested for their hybridisation efficiency. The labelled probes were diluted empirically for each individual application and co-precipitated using herring sperm DNA (100 µg ml⁻¹) and yeast tRNA (100 µg ml⁻¹) and dissolved in hybridisation buffer [50% formamide (Sigma) deionised with mixed BED resin (Sigma), 2X standard saline citrate (SSC; 1.75 g NaCl, 0.8 g sodium citrate/100 ml ddH₂O; pH 7.0) and 50 mM sodium phosphate buffer, pH 7.0].

All the FISH procedures were carried out as described before (Ersfeld and Gull, 1997) with some modifications. Trypanosome cells were harvested and washed twice in PBS before being fixed in suspension with 3.6% formaldehyde in PBS for 15 minutes. After fixation, the cells were washed twice with PBS and allowed to settle down on poly-L-lysine coated slides (Sigma) for 1 hour at room temperature. The cells were permeabilised by 0.1% NP-40 in PBS for 5 min before being washed twice in fresh PBS. The cells were digested with 10 µg ml⁻¹ RNaseA (Sigma) in PBS for 30 minutes at room temperature to suppress the nonspecific hybridisation. After washing twice in PBS, the cells were prehybridised for 60 minutes at room temperature in hybridisation buffer. Following prehybridisation, cells were hybridised with the corresponding DNA probe, slides sealed with Gene Frame (ABgene, UK) and denatured at 85°C for 5 minutes and incubated overnight at 37 °C. After hybridisation, the slides were washed with 50% formamide, 2X SSC for 30 minutes at 37 °C followed by 10 minutes in 2X SSC at 50 °C and 30 minutes in 0.2X SSC at 50 °C and finally in 4X SSC for 10 minutes at the room temperature.
For visualisation of DIG-labelled probe, cells were incubated with the primary sheep anti-digoxigenin Fab fragment (Boehringer Mannheim) at 0.3 µg/ml in blocking solution composed of PBS/1% BSA (Sigma) for 60 minutes at room temperature. After three washes in TBS-T buffer, cells were incubated with the secondary anti-sheep IgG FITC-conjugate (Vector laboratories) diluted to 5 µg ml\(^{-1}\) in blocking solution for 45 minutes. For visualisation of BIO-labelled probe, cells were washed three times in TBS-T before being incubated with streptavidin-Cy3 conjugate (Sigma) in blocking solution for 45 minutes at room temperature. After washing as above, cells were mounted in Vectashield containing 4',6-diamidino-2-phenylindole (DAPI) (Vector laboratories). The slides were examined by Nikon Epifluorescence microscope (Nikon) and the images were acquired with a CCD camera (Roper) controlled by MetaVue software, version 5.0 (Molecular Devices Inc). Images were then processed and pseudocoloured in Adobe Photoshop.

### 2.10 RNA extraction and reverse transcriptase-PCR (RT-PCR)

Total RNA was extracted from the induced and non-induced cell lines using total RNA extraction kits according to the protocol provided by the manufacturer (Qiagen). To eliminate any remaining genomic DNA contaminants, the extracted total RNA was subjected to two rounds of RNase-free DNase digest (Qiagen). Final purified RNA was dissolved in RNase-free H\(_2\)O, and their concentration was measured and adjusted by the spectrophotometer before being stored at – 80 °C. First stranded cDNA was then synthesized from RNA using Ominsplot® RT kits (Qiagen) and dT\(_{18}\)-oligo primer to a final concentration of 10 pMol/µl. Reverse transcription (RT) reactions were done following the exact protocol provided by the manufacturer (Qiagen). Identical RT reactions were set up for each RNA sample without the reverse transcriptase (RT) enzyme to act as a control for the absence of the genomic DNA contamination. Standard PCR reactions were then performed on the generated cDNA template to amplify the target DNA fragment using a pair of gene-specific primers that differs from the primer
pair used in generating the original RNAi construct (Table 2.3). PCR reactions on the cDNA samples were done using proof reading AccuSure DNA polymerase (Bioline) under the following conditions: initial denaturation at 94 °C for 10 minutes followed by 30 cycles carried out at 94 °C for 30 seconds, 55 °C for 40 seconds, 72 °C for 40 seconds and the last extending cycle of 72 °C for 10 minutes. As a loading control, 100 bp PCR fragment was amplified from the same cDNA samples using PFR-A gene specific primers (Table 2.3) under the same PCR reaction conditions.

2.11 Statistical analysis

All the statistical analysis was carried out using Excel (Microsoft). Error bars were obtained by calculating the standard deviations (± SD) from at least two independent experiments. The chi-squared test was used to compare the frequency of cells with or without mitotic spindles in 1N2K trypanosome populations after SMC3 and separase depletion.
Table 2.3 Sequences of the primers used in this study. Sense and antisense primer sequences used in making the RNAi constructs of both TbSMC3 (1) and TbSeparase (2), ectopic c-Myc epitope tagging of TbSeparase (3), cDNA production of both TbSeparase (4) and PFR-A genes (5), generation of DNA probes of both α- and β-Tubulin gene (6 and 7, respectively) and generation of 177-bp fragment probe (8). All the primer sequences are described in the 5’ to 3’ directions. Bold and underlined regions are the restriction sites for the enzymes XhoI in 1-For; HindIII in 1-Rev; BamHI/HindIII in 2-For; XhoI/XbaI in 2-Rev; ApaI in 3-For; MluI in 3-Rev, respectively.

<table>
<thead>
<tr>
<th>Gene name</th>
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<th>Sequence (5’-3’)</th>
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</thead>
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</tr>
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<td></td>
<td>1-Rev</td>
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<td>TbSeparase (RNAi)</td>
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<td>CGGATCCAAGCTTTGTCTAAACGAGCGGAAGTG</td>
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<td></td>
<td>2-Rev</td>
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<tr>
<td>TbSeparase (C-myc tagging)</td>
<td>3-For</td>
<td>AGAGGGCCCATGAGGCCGACTCAGTTCCG</td>
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<td></td>
<td>3-Rev</td>
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</tr>
<tr>
<td>TbSeparase (RT-PCR)</td>
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<td></td>
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3. RESULTS: Functional characterisation of cohesin SMC3 and separase proteins in *Trypanosoma brucei*

3.1 SMC proteins in *Trypanosoma brucei*

3.1.1 Cohesin and condensin components are conserved

The genome database of *T. brucei* (http://www.genedb.org/geneddb/tryp/) was searched using well-characterized sequences of the different components of SMC protein complexes. Different SMC protein sequences from yeast and human were used to identify potential *T. brucei* candidate orthologues using BLAST searches of the parasite database (http://www.genedb.org/geneddb/tryp/blast.jsp). Sequences with high similarity obtained from the *T. brucei* genome were used for a reciprocal BLAST search using PSI-BLAST (Altschul et al., 1997) against the protein database (available at http://blast.ncbi.nlm.nih.gov/blast.cgi). High confidence BLAST hits were then used to classify the different *T. brucei* cohesin and condensin homologues. Four homologues of candidate subunits of SMC complexes, cohesin and condensin, were identified in the trypanosome genome as SMC1, SMC2, SMC3 and SMC4 (*Table 3.1*). Using a similar approach, non-SMC subunits of the core cohesin and condensin complexes were also identified in the genome of *T. brucei* (*Table 3.1*). On the other hand, no candidate genes of the SMC5-SMC6 complex and their subunits such as Nse1 could be identified in the *T. brucei* genome. This could indicate the absence of a role for this complex in the DNA repair pathway in trypanosomes or, a high degree of divergence of functionally equivalents proteins.
Orthologues of the three SMC complexes (cohesin, condensin and DNA repair) subunits were identified in *T. brucei* genome database using experimentally-characterised sequences from other eukaryotic organisms and BLAST search at (http://blast.ncbi.nlm.nih.gov/BLAST/). In *T. brucei*, sequences with E-values higher than $10^{-5}$ or no BLAST hits after several queries were indicated by question mark (?). All the accession numbers are from GeneDB (http://www.genedb.org/genedb/tryp/) of *T. Brucei*.

<table>
<thead>
<tr>
<th>SMC complex</th>
<th><em>T. brucei</em></th>
<th>GeneDB accession number</th>
</tr>
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<td></td>
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</tr>
<tr>
<td></td>
<td>TbSMC3</td>
<td>Tb927.5.3510</td>
</tr>
<tr>
<td></td>
<td>TbRad21</td>
<td>Tb927.7.6900</td>
</tr>
<tr>
<td></td>
<td>TbSCC3</td>
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<td></td>
<td>TbPds5</td>
<td>Tb11.02.4280</td>
</tr>
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<td><strong>Condensin</strong></td>
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<tr>
<td></td>
<td>TbCnd1</td>
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<td></td>
<td>TbCnd2</td>
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<td>NSE1</td>
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Table 3.1 Orthologues of the three SMC complexes (cohesin, condensin and DNA repair) subunits were identified in *T. brucei* genome database using experimentally-characterised sequences from other eukaryotic organisms and BLAST search at (http://blast.ncbi.nlm.nih.gov/BLAST/). In *T. brucei*, sequences with E-values higher than $10^{-5}$ or no BLAST hits after several queries were indicated by question mark (?). All the accession numbers are from GeneDB (http://www.genedb.org/genedb/tryp/) of *T. Brucei*. 
3.1.2 Cohesin complex candidate proteins are highly conserved in *T. brucei*

Candidate protein sequences of the cohesin complex SMC subunits such as SMC1 and SMC3 and non-SMC subunits such as SCC1/RAD21, SCC3 and PDS5 were obtained from the *T. brucei* genome. To confirm their identities, all the cohesin complex SMC and non-SMC candidate genes of *T. brucei* were compared against their orthologues of yeast and human (Table 3.2; Appendix 3). As indicated, *T. brucei* homologues have molecular masses that corresponded well to their counterparts of yeast and human. By the phylogenetic analysis, all *T. brucei* cohesin candidate proteins were most closely related to SMC1, SMC3, SCC1, SCC3 and PDS5 from different eukaryotic organisms (Appendix 3). To determine their amino acid sequence identities and similarities to the other protein homologues, protein sequences from *T. brucei* were aligned individually against their orthologues in kinetoplastids (*T. cruzi* and *L. major*) and other eukaryotes (*S. pombe*, *S. cerevisiae* and *Homo sapiens*) as indicated in Table 3.3. TbSMC1 showed identity of 42-52% to kinetoplastid orthologues and 25-29% to eukaryotic homologues. The identities of TbSMC3 were 45-53% and 26-31% to kinetoplastid and eukaryotes, respectively. Non-SMC subunits such as TbSCC1/RAD21, TbSCC3 and TbPDS5 showed identities of 38-44%, 35-46% and 45-48% to their kinetoplastid homologues and of 31-33%, 20-22% and 22-23% to their eukaryotic homologues, respectively (Table 3.3). In summary, all the identities (%) of *T. brucei* proteins to their homologues were above 25% except for the least two candidate genes, SCC3 and PDS5.
Table 3.2 Candidate SMC and non-SMC proteins from *T. brucei* were listed together with the corresponding homologous proteins from other organisms, human (*H. sapiens*) and yeast (*S. cerevisiae* and *S. pombe*). Protein molecular masses were shown in kilodaltons (kDa) and all the database accession numbers were underlined. All the accession numbers were from UniProt protein database (http://beta.uniprot.org/) except for *T. brucei* candidate proteins which were from *T. brucei* GeneDB.

<table>
<thead>
<tr>
<th>Gene</th>
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<td>PDS5</td>
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<td></td>
<td>Q29RF7</td>
<td>Q9HFF5</td>
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Table 3.3 Candidate proteins of the cohesin complex that retrieved from the GeneDB of *T. brucei* were compared against protein homologues from their related kinetoplastid parasites (*T. cruzi* and *L. major*), yeast (*S. pombe* and *S. cerevisiae*), and human (*H. sapiens*). Protein amino acid sequences were retrieved either from GeneDB in case of kinetoplastids (*T. brucei, T. cruzi* and *L. major*) or from UniProt database (http://beta.uniprot.org/) in the case of other organisms. Sequences were aligned and compared for their identical (Id) and similar (Si) residues using ClustalW (Thompson et al., 1994) available for the accession at EBI (http://www.ebi.co.uk/tools/clutalw/).

<table>
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<tr>
<th></th>
<th>TbSMC1</th>
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<td>Id</td>
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<td><em>S. pombe</em></td>
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<tr>
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<td>53</td>
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</table>
SMC proteins from different organisms have a conserved structure that is predicted to perform a functional configuration (Haering et al., 2002; Melby et al., 1998). The large protein of SMC family (130-150 kDa) has an N-terminal ATP-binding domain, two coiled coil domains separated by the hinge domain and ATP-hydrolysis C-terminal domain. Two functional motifs termed Walker A and B (DA box) are located at the N- and C-terminal head domains of SMC protein, respectively. The two Walker motifs function in ATP binding to head domains of SMC protein forming ABC-like ATPase domain (Lowe et al., 2001; Weitzer et al., 2003). A third C-terminal amino acid leucine, serine, glycine, glycine (LSGG) motif, and termed signature motif plays a role in the ATP-hydrolysis and stabilisation of the DNA-cohesin interaction (Arumugam et al., 2003; Hirano and Hirano, 2004). The current model of a single SMC protein configuration has indicated the folding of the two coiled coils in antiparallel direction around the flexible hinge domain forming V-shaped heterodimer, thereby bringing the two ATPase heads in close proximity (Melby et al., 1998).

Conserved domains of the SMC protein family were identified when *T. brucei* candidate SMC protein, TbSMC3, was aligned against sequences of *L. major*, yeast and human (Figure 3.1). The conserved consensus sequences identified in TbSMC3 protein were shown to correspond to the Walker A and B motifs and the amino acid (LSGG) signature motif. When the different cohesin SMC and non-SMC subunit proteins from *T. brucei* were subjected to coiled coils prediction (http://www.ch.embnet.org/software/coils-form.html) according to (Lupas et al., 1991), it was found that only TbSMC1 and TbSMC3 can potentially form coiled coil domains around a short unstructured region that corresponds to the position of the hinge region in the typical SMC protein (Gruber et al., 2006) (Figure 3.2). In contrast, the kleisin candidate proteins of trypanosomes, TbRad21 and TbSCC3, failed to adopt any structural coiled coils. The implication was that large subunits of SMC protein complexes such as SMC1 and SMC3 with their associated partners in *T. brucei* are most likely to adopt a similar functional configuration to their counterparts in other organisms, although no experimental studies were conducted to test this prediction.
Figure 3.1 SMC3 protein homologue of *T. brucei* was aligned and compared against that of *L. major*, *S. cerevisiae*, *S. pombe* and *Homo sapiens*. Sequence alignment was carried out using ClustalW (Thompson et al., 1994) at EBI (http://www.ebi.co.uk/tools/clustalw/). Sequence alignment output was then box-shaded using BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html). Residues where 50% or more are identical are shaded in black whilst residues which are 50% or more similar are shaded in gray. The highly conserved ATP-binding motif (Walker A) at the N-terminus and Walker B motif (DA-box) at the C-terminus which play a significant role in ATPase activity of SMC proteins are shown above sequences. Also the ATPase-hydrolysis motif (LSGG) associated with the C-terminus was indicated.
**Figure 3.2** Coiled coils prediction for the different SMC candidate proteins of *T. brucei*. The cohesin complex candidate protein sequences, TbSMC1, TbSMC3, TbRad21 and TbSCC3, were subjected to coiled coils prediction (Lupas et al., 1991) at Coils (http://www.ch.embnet.org/software/coils-form.html). *T. brucei* main cohesin subunits, TbSMC1 and TbSMC3, were found to form two long coiled coil domains around a short hinge domain in the middle which is the characteristic structural feature of SMCs protein family. As expected, two non-SMC subunits, TbRad21 and TbSCC3, showed low prediction for the coiled coils formation.
3.1.3 Separase is highly conserved in *Trypanosoma brucei*

Separase is a clan CD cysteine peptidase responsible for cleaving the cohesin SCC1 subunit at the metaphase-to-anaphase transition to initiate successful sister chromatid resolution (Nasmyth et al., 2000; Uhlmann et al., 1999). Separases from different organisms except *Drosophila* are large proteins of 150-230 kDa with two major domains, a highly conserved C-terminal domain containing the protease activity and a highly variable N-terminal domain that binds to the separase inhibitor, securin (Viadiu et al., 2005). The protease domain at the C-terminus contains the conserved cysteine (C) and histidine (H) residues which adopt a signature motif of CD clan proteases that has an essential role for the separase catalysis (Figure 3.3) (Barrett et al., 1998; Uhlmann et al., 2000). The two catalytic residues are invariably surrounded by glycine (G) or serine (S) residues and preceded by amino acid sequences that predicted to form hydrophobic parallel β sheets (Viadiu et al., 2005) (Figure 3.3A). Mutation of the catalytic histidine or cysteine residues abolished the ability of the enzyme to cleave its targets represented by SCC1/MCD1 of the cohesin complex *in vitro* (Uhlmann et al., 2000). *T. brucei* protein homologue of separase was annotated in the GeneDB, which was most closely related to separases from different species by phylogenetic analysis (Appendix 4). Catalytic residues, along with the hydrophobic parallel β sheets were also identified in the putative candidate separase homologue of *T. brucei* after alignment with sequences from other species (Figure 3.3A). The presence of the catalytic dyad preceded by the glycine (G) residues and hydrophobic sheets implies a conserved proteolytic function for *T. brucei* separase homologue during the cell cycle.

*T. brucei* separase homologue has a molecular mass of 126 kDa and is therefore much smaller than yeast and mammalian separases (>210 kDa). Mechanisms of separase regulation are similar in model organisms where its function has been studied in details (Ciosk et al., 1998; Cohen-Fix, 2000; Uhlmann et al., 2000; Wirth et al., 2006). Separase is kept inactive by binding to securin until metaphase-to-anaphase transition (Jallepalli et al., 2001). Securin binds separase at the variable N-terminal domain which consists of 26 Armadillo (ARM-repeats) in the human protein (Viadiu et al., 2005). Secondary structure and motif analysis showed that, like human separase, separase homologue from *T. brucei*
contains N-terminal ARM-repeats (Appendix 5). Four different secondary structure prediction servers (3D-PSSM, mGenTHREADER, HHpred and Phyre), which employed different algorithms, all predicted superhelical structures of ARM-type repeats in the N-terminus of *T. brucei* separase. This could imply that the trypanosome separase homologue could provide an interaction interface for an as yet unidentified securin homologue. By using the BLAST search and sequence-sequence homology approach, I failed to retrieve any putative candidate protein that could serve as securin candidate protein in *T. brucei*. This is most likely due to the high divergence at the level of the primary sequences of securins from different organisms (Jager et al., 2001; Viadiu et al., 2005). In addition to securin, separase activity is also inhibited at metaphase by cyclin-mediated phosphorylation in human cells (Holland and Taylor, 2006; Stemmann et al., 2001). Separase activation at the metaphase-to-anaphase transition requires the degradation of securin and the removal of the inhibitory phosphate group. When separase of *T. brucei* aligned with human and yeast separases, four putative phosphorylation sites (P) were found to be conserved in the trypanosome separase (Figure 3.3A).

Proteolysis of SCC1 by separase before anaphase is sufficient for the sister chromatid segregation and failure to cleave SCC1 in yeast associates with karyokinesis failure and anaphase arrest (Uhlmann et al., 1999). The consensus recognition sequence for separase is present in all SCC1 homologues and is composed of the motif SxExxRx. Whereas two conserved recognition sites (site A and B) are present in most of SCC1 homologues, only one of the two sites is universally conserved in all SCC1 proteins identified so far. In the kinetoplastids (*T. brucei* and *L. major*), the SCC1 homologue contained both recognition sites corresponding to site A and B of other species (Figure 3.3B). While sites A and B are conserved in *T. brucei*, site B is less conserved in *Leishmania* with serine (S) residue is replaced by asparagine (N) one. Phosphorylation of the conserved serine (S) residue in the recognition site of SCC1 is an essential prerequisite for efficient cleavage at the conserved arginine (R) residue by the corresponding separase (Alexandru et al., 2001). The conserved serine (S) and arginine (R) residues are present in the putative separase cleavage sites of *T. brucei* SCC1 homologue (Figure 3.3B). In yeast, phosphorylation of cohesin SCC1 subunit at ten different serine (S) residues by CDC5 kinase boost the efficient proteolysis of SCC1 by separase (Alexandru et al., 2001; Uhlmann et al., 1999). In the *T. brucei* SCC1 homologue, positional conserved serine (S) residues to the yeast homologue were also identified (data not shown).
Figure 3.3 Cohesion cleavage machinery was highly conserved in *T. brucei*. A. Sequence alignment in the highly conserved C-terminal region of separase. *T. brucei* separase (Tb927.1.3120) is aligned and compared against separases from different organisms such as *T. cruzi* (Tc00.1047053508405.80), *Homosapiens* (Q14674) and *S. cerevisiae* (Q03018). The conserved histidine (H) and cysteine (C) residues predicted to form the catalytic dyad were indicated underneath, whereas the hydrophobic parallel β strands on the N-terminal side of these catalytic residues were boxed underneath. P represents the potential four phosphorylation sites from all separases studied so far. All the accession numbers were from UniProt protein database available at (http://beta.uniprot.org/uniprot/) except for trypanosome sequences which were from GeneDB (http://www.genedb.org). B. Comparison of the putative cleavage recognition sites of SCC1 homologues of kinetoplastids (*T. brucei* and *L. major*) against experimentally characterised recognition sites of proteins from other organisms such as *S. cerevisiae*, *S. pombe* and *Candida albicans* (*C. Albicans*). The putative consensus separase recognition sites (SxExxRx) were indicated.
3.2 Expression and localisation of *T. brucei* cohesin SMC3 protein

3.2.1 Recombinant expression of TbSMC3 protein in *E. coli*

After cloning of three SMC3 gene fragments (TbSMC3/1, 2, 3) into the bacterial protein expression vector, pET100/D-TOPO, expression constructs were used for the expression of the corresponding recombinant three peptides in *E. coli* cells (BL21 DE3) inclusion bodies. Different induction time samples as well as different fractions (soluble and insoluble) of the bacterial cell lysate after the sonication-based fractionation were tested to optimise the protein expression. Successful recombinant protein expression in *E. coli* was detected by Coomassie staining and examining the Coomassie-stained SDS PAGE gel (Figure 3.4). The presence of specific protein bands from culture samples induced by IPTG when compared to the samples from the non-induced culture indicated a successful induction of the protein expression. Molecular masses of the three expressed recombinant peptides corresponded to their predicted molecular masses (Figure 3.4; Table 3.4).

All the three recombinant peptides were found to be mostly insoluble and therefore make it easy to adopt the same protocol during the protein purification procedure (Figure 3.4; Table 3.4). The purification of the histidine-tagged peptides by metal affinity chromatography was done under denaturing conditions using guanidine hydrochloride. After purification, the purity and approximate concentration of each of the recombinant protein was determined by SDS PAGE gel and Coomassie staining (Figure 3.4D; Table 3.4). Finally, ~6 mg of the three recombinant peptides (TbSMC3/1, 2, 3), 2 mg each, were combined and used as an antigen for rabbit immunisation.
Table 3.4 The three TbSMC3 gene fragments (TbSMC3/1, 2, 3) and peptides used for the recombinant protein expression. Amino acid (aa) residues composition of each peptide is indicated with the theoretical molecular masses as determined at ExPASy Proteomics (http://www.expasy.ch/tools/pi_tool.html) was shown. Peptide localisation after expression and the final protein concentration after purification are also indicated.

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<th>Amino acid residues</th>
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<th>Fraction localisation</th>
<th>Protein concentration</th>
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</thead>
<tbody>
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<td>22.2</td>
<td>Insoluble</td>
<td>100 µg/ml</td>
</tr>
<tr>
<td>TbSMC3/3</td>
<td>200</td>
<td>23.1</td>
<td>Insoluble</td>
<td>100 µg/ml</td>
</tr>
</tbody>
</table>
Figure 3.4 The expression profiling of the three recombinant TbSMC3 peptides (TbSMC3/1, 2, 3). A, B, C were SDS-PAGE gel electrophoresis of the bacterial *E. coli* cell lysate used for profiling the expression of the three recombinant TbSMC3 peptides (TbSMC3/1,2,3), respectively after pilot expression in *E. coli* cells. Standard protein marker showed on the left side with the molecular bands indicated. Various samples on the gel are: Zero-time point sample collected before induction of protein expression (0h), samples collected 1 hour from the culture with (+) or without (-) IPTG induction and samples collected 2 hours with (+) or without (-) IPTG induction, respectively. Soluble (S) and insoluble (Ins) fractions prepared from either IPTG-induced (+) and non-induced (-) cultures were also electrophoresed and examined for the potential protein expression. Arrows (→) indicates bands corresponding to the inclusion bodies over-expressed proteins in different *E. coli* cell fractions after IPTG induction. D. The final purified and dialysed three TbSMC3 peptides (1, 2, and 3) were tested on SDS-PAGE gel to estimate the relative purity before proteins can be used to immunise rabbits.
3.2.2 Characterisation of anti-TbSMC3 polyclonal antibodies

In order to check the specificity of the antibodies to their respective antigens, the polyclonal antibodies were tested by western blotting against each of three recombinant peptides (TbSMC3/1, 2, 3) (Figure 3.5). Each antiserum reacted with its precursor recombinant peptide indicating successful immunisation (Figure 3.5, Lane 1). In order to check for the native TbSMC3 protein-specific recognition by these antibodies, they were tested against fractionated whole trypanosomes cell lysates. Positive bands were obtained in the whole cell lysates with a molecular mass (~136 kDa) corresponding to the native TbSMC3 molecular mass (Figure 3.5, Lane 3). The unpurified antiserum showed non-specific bands in these trypanosome lysates. To alleviate this, the antibodies were affinity-purified against the three precursor recombinant peptides (TbSMC3/1, 2, 3). After affinity purification, two of the three antibody fractions (Anti-TbSMC3/1, 3) reacted specifically and produced a single band on the fractionated trypanosome cell lysate (Figure 3.5, Lane 4). Although it reacted specifically to its peptide, the affinity purified anti-TbSMC3/2 antibody only reacted weakly to the whole cell lysate of trypanosomes, and the antibody failed to detect its antigen in situ by using the immunofluorescence microscopy (data not shown). Therefore, anti-TbSMC3/2 antibody was excluded from any further use and only the two other antibodies (Anti-TbSMC3/1, 3) were applied for all the downstream applications. The two antibodies were mixed at equimolar ratios and used for western blotting and immunofluorescence microscopy. In all immunoblotting reactions, the pre-immune sera of rabbits did not react with a band corresponding to the molecular mass of TbSMC3 (Figure 3.5, Lane 2).
Figure 3.5
Figure 3.5 Western blot results of the three purified anti-SMC3 antibodies where, A) Anti-SMC3/1 Abs, B) Anti-SMC3/2 Abs and C) Anti-SMC3/3 Abs. The non-purified antibody was tested against their precursor peptide (Lane 1) and the whole trypanosome cells extract (Lane 3). After the affinity purification, the antibody was also tested against the whole trypanosome cell extract (Lane 4). In every case, the pre-immune sera were tested against the trypanosome extract (Lane 2). Note that two antibodies, anti-SMC3/1&3, reacted specifically to the whole trypanosome extract and give specific bands corresponding to the theoretical molecular size of the native TbSMC3 (136.3 kDa), whereas the anti-SMC3/2 antibody only reacted weakly to the native trypanosome extract as this was also confirmed by the immunofluorescence results (data not shown).

3.2.3 TbSMC3 is expressed in bloodstream and procyclic forms of T. brucei

Cohesin subunits are known to be expressed in different types of tissues and cells even those which considered being in a non-differentiating status (Zhang et al., 2007). One example is the Drosophila neurons that no longer proliferate and so have no sister chromatids in their nuclei, although the cells are positive for cohesins (Pauli et al., 2008; Schuldiner et al., 2008). In T. brucei, some developmental stages are non-proliferating such as the stumpy form which has its cell cycle arrested at G1 phase in preparation for the fly transmission (Matthews, 2005; Matthews et al., 2004). The expressions profile of cohesin TbSMC3 in different forms of T. brucei was investigated by using reverse-transcriptase PCR (RT-PCR) performed on mRNAs from trypanosome populations of different forms such as long slender form (LSF), short stumpy form (STF) and procyclic form (PCF). The TbSMC3 gene expression result has indicated that the gene was expressed in different forms with a paraflagellar rod A (PFR-A) gene used as a reference gene for the template loading control (Figure 3.6A). As a negative control, RT-PCR performed on RNA samples in the absence of RT enzyme demonstrated the absence of any genomic DNA contamination (data not shown). Cohesin SMC3 expression in the stumpy form may be due to an active cohesion activity or as a result of contamination by bloodstream form cells. By using western blotting, anti-TbSMC3 antibody recognised two equivalent native protein pools from the cellular extracts of the bloodstream and the procyclic forms (Figure 3.6B). In the bloodstream form cells, the western blot data can be used as an expression reference for the cohesin protein, especially the immunofluorescence microscopy proved technically inefficient in these cells due to the dense VSG coat on their membranes when compared to the procyclic cells.
Figure 3.6 Expression profile of TbSMC3 in the different forms of *T. brucei*. A. PCR performed on cDNA samples from the different forms such as procyclic (PCF), long slender (LSF) and stumpy form (STF). PCR on the genomic DNA of 449 cells was used as a control (C). DNA ladder (L) is 1Kb marker. As a template loading control, PCR performed on the same samples using paraflagellar rod A (PFR-A) gene specific primers as a reference gene. As a negative control, RT-PCR performed on RNA samples in the absence of RT enzyme was used (data not shown) B. Western blot (WB) of anti-TbSMC3 antibodies on the total cell extract of bloodstream form (BSF) and procyclic form (PCF) showed antibodies to immunoreact with a native protein of molecular mass between 100-150 kDa (right). Equal cell extract loading done by the Coomassie staining of SDS PAGE gel (left).
3.2.4 TbSMC3 localised in the nucleus during the cell cycle of *T. brucei*

Affinity purified anti-TbSMC3 polyclonal antibody raised against the recombinant TbSMC3 protein was used to determine the subcellular localisation of the cohesin protein in *T. brucei* (Figure 3.7). On a western blot, the anti-TbSMC3 antibody detected a single band at ~136 kDa in *T. brucei* whole-cell lysate (Figure 3.7A). On immunofluorescence microscopy, anti-TbSMC3 staining signal was localised in the nucleus of trypanosome cells (Figure 3.7B). The nuclear signal was persistent through the entire cell cycle, although at different intensities in the different cell cycle stages. The strongest signals were in 1N2K (G₂ and early mitosis) and dividing (2N2K) cells (Figure 3.7B; panels 2&4), while early interphase (1N1K) and anaphase (2N2K) cells showed the lowest signals (Figure 3.7B; panels 1&3). In yeast, cohesin subunits bind chromatin from late G₁ phase until the onset of anaphase (Losada, 2008; Uhlmann, 2007). Another cohesin subunit of *T. brucei*, TbSCC1, follows the same chromatin association pattern as the protein expression was detectable from late G₁ phase to metaphase (Gluenz et al., 2008; Sharma et al., 2008). On the other hand, the TbSMC3 nuclear signals are persistent and did not disappear even at anaphase (Figure 3.7B). As experimentally-characterised, the cohesin SCC1 subunit is cleaved at anaphase by separase (Uhlmann et al., 1999). Therefore, the cleaved SCC1 subunit is no longer detectable at anaphase and was probably re-synthesised in the subsequent cell cycle. On the other hand, the main cohesin subunits such as SMC3 might remain intact after its removal from chromatin. We hypothesised that although removed from chromosomes; TbSMC3 protein remained intact and persisted in the nucleus even in a non-chromatin-bound status during the closed mitotic cycle of *T. brucei.*
Figure 3.7 Immunolocalisation of cohesin TbSMC3 subunit in *Trypanosoma brucei*. 
A. Western blot on total protein from trypanosome cell culture lysate (2 x 10^6 cells ml⁻¹). The blot is probed with α-TbSMC3 polyclonal antibodies (pAbs) and showed immunogenic band of ~ 136 KDa. B. Immunolocalisation of TbSMC3 during the cell cycle of *T. brucei*. 427 procyclic trypanosome cells were formaldehyde-fixed and immunostained with α-TbSMC3 antibody. Cells of the different cell cycle phases such as 1N1K, 1N2K and 2N2K (arrows denote kinetoplasts) showed nuclear staining signals were recorded. Anti-SMC3 signals were in green and DNA-stained DAPI signals in red. Merged Anti-SMC3 and DAPI signals as well as the phase-contrast images of the cells were shown. Scale bar represents 2 µm.
3.2.5 TbSMC3 dissociates from nuclear DNA during specific stages of cell cycle

To address the hypothesis of temporal TbSMC3 association with chromatin during the cell cycle of *T. brucei*, cells were detergent-extracted prior to fixation to remove any non-chromatin bound, soluble SMC3 protein. Asynchronous cells treated this way exhibited differential staining for TbSMC3 during different cell cycle stages (Figure 3.8A). In these cells, SMC3 signals were detected only in interphase and early mitotic cells. No nuclear signal was detected in cells during post-metaphase until the completion of cytokinesis and cellular division (Figure 3.8A). An intense SMC3 staining was present in 1N1K interphase cells (upper panel). An intermediate, punctuated staining was observed in 1N2K cells with a spherical nucleus, representing prophase and metaphase cells (second panel). A residual staining was still visible in 1N2K cells that have oval nuclei and are in late metaphase or early anaphase (third panel). The signal was completely absent from late anaphase and dividing 2N2K cells (fourth and fifth panel). Quantitative analysis of the extracted, anti-SMC3-immunostained cells revealed the presence of the nuclear signals (SMC3-positive) in 99% of 1N1K cells, 83% of 1N2K cells and only 4% of 2N2K cells. The absence of the nuclear signals (SMC3-negative) was recorded in 1% of 1N1K, 17% of 1N2K, while most (96%) of 2N2K cells (when the cohesin was expected to be released from chromatids) were SMC3 negative (Figure 3.8C). From the total of 400 cells counted for the nuclear signals of TbSMC3, 75% were TbSMC3-positive and 25% were TbSMC3-negative. During their cell cycle, trypanosome cell fractions were assorted into G₁ (40%), S (18%), G₂ (22%), M (8%) and cytokinesis (12%), by using nuclei (N) and kinetoplasts (K) configuration in combination with BrdU incorporation (Sherwin and Gull, 1989; Woodward and Gull, 1990). Therefore, cohesin-positive cells (75%) were most likely to be in S, G₂ and partially G₁ and M phases, while 25% of cohesin-negative cells were in cytokinesis and partially G₁ and M phases. Accordingly, *T. brucei* cohesins were most likely loaded into chromatin from mid G₁ until late mitosis. TbSMC3 interaction is similar to cohesin dynamics in mammalian cells where about one third (35%) of nuclear cohesin bound very stable to chromosomes during DNA replication until anaphase while the remaining pool (65%) moves on and off chromatin throughout the rest of the cell cycle (Gerlich et al., 2006). This could imply that, although TbSMC3 is equally expressed during the entire cell cycle, the active loading and binding to chromatin starts from late G₁ phase until late mitosis. This expression pattern is similar to the cohesion cycle in yeast (Uhlmann, 2007) and also to TbSCC1 expression in procyclic (Gluenz et al., 2008) and bloodstream (Sharma et al., 2008) cells. Also, in western blots, detergent soluble and insoluble SMC3 fractions were detected in cell extracts prepared from asynchronous cultures (Figure 3.8B).
Figure 3.8 Differential sensitivity of cohesin subunit, TbSMC3, to NP-40 detergent-extraction in trypanosome cells. A. The nuclear signal of TbSMC3 persists only in interphase and early mitotic cells, but is not detectable in late mitotic cells. An intense staining is present in 1N1K interphase cells (top panel). An intermediate, punctuated staining is observed in 1N2K cells with a circular nucleus, representing prophase and metaphase cells (second panel). A residual staining is still visible in a 1N2K cell (third panel, left cell) that has an oval nucleus and is in late metaphase or early anaphase, but has disappeared from late anaphase (1N2K with elongated nucleus) and dividing cells (2N2K, fourth and fifth panel). B. In vitro NP-40 cell fractionation and SMC3 detection by western blotting has showed signals in the detergent-insoluble (Insol) and detergent-soluble (Sol) fractions. W, whole cells solubilised in SDS PAGE sample buffer. C. Quantitation of SMC3 positive and negative cells in different cell cycle phases such as 1N1K, 1N2K and 2N2K after in situ NP-40 extraction and immunofluorescence analysis (n=400 cells, S.D. of two independent counts are indicated). Scale bar represents 2 µm.
3.3 RNAi-based knockdown of SMC3 gene and its effect on the growth rate of procyclic form of *T. brucei*

RNAi is the method of choice for downregulation of endogenous gene expression in *T. brucei* (Ullu et al., 2002). Here, RNAi was used to specifically suppress the expression of the endogenous TbSMC3 gene in procyclic *T. brucei*. After transfection and selection of clonal cell lines, cells were induced with tetracycline to analyse any RNAi-induced phenotypes. First, the growth rate of the induced cells was compared against the control non-induced cells. Up to 24 hours post-induction, the growth rate of the induced cells followed that of the non-induced ones (Figure 3.9A). 48 hours after RNAi induction, cessation of growth was evident in the induced culture. On day 4 post-induction, complete growth cessation was recorded with a substantial number of dead cells accumulated in the culture induced for TbSMC3 depletion (Figure 3.9A). The phenotypic analysis of the growth rate was done simultaneously with analysis for the depletion of the corresponding TbSMC3 protein. Western blotting data on total cell lysates extracted from the induced (+) and non-induced (-) cells showed a progressive decrease in the levels of the endogenous TbSMC3 protein expression starting from day 2 post-induction (Figure 3.9B). On day 4 post-induction, depletion of more than 95% of the protein level was evident compared to the control non-induced cells. *In situ* depletion of TbSMC3 protein from clonal cells after tetracycline induction was confirmed by the immunofluorescence microscopy and also showed the disappearance of the SMC3 nuclear staining signal from the induced (+ Tet) cells (Figure 3.9C). In summary, RNAi-induced depletion of TbSMC3 showed that the protein is, as expected, essential for cell survival in *T. brucei*. 
Figure 3.9
**Figure 3.9** RNAi-mediated knockdown of TbSMC3 in the procyclic form of *T. brucei*.  
**A.** 29-13 procyclic cells transfected with TbSMC3-pZJMβ RNAi construct were grown in the presence (induced) or the absence (non-induced) of tetracycline. The in vitro growth rate of the cells was monitored daily for five successive days after tetracycline induction. **B.** After RNAi induction, the depletion of the corresponding native TbSMC3 protein was monitored by western blot using anti-SMC3 antibody for 4 successive days in the induced (+) and the non-induced (-) culture. Protein equal loading control was confirmed by the coomassie staining of parallel SDS PAGE samples. **C.** *In situ* depletion of TbSMC3 protein from trypanosome cells after RNAi induction was confirmed by microscopic analysis of control (- Tet) and TbSMC3-depleted (+ Tet) cells on day 4 post-induction. TbSMC3 staining signal can be easily seen to have disappeared from the nucleus of the induced cell (arrow). Scale bar: 2 µm.

### 3.4 The effect of RNAi-mediated knockdown of TbSMC3 on the cell cycle progression of *T. brucei*

To determine how the growth defect phenotype observed after TbSMC3 knockdown was related to any potential cell cycle progression defects, FACS analysis was performed on cells after RNAi induction. Cells from both the induced (+ Tet) and non-induced (- Tet) cultures were stained with propidium iodide and processed for FACS for 4 successive days post-induction. The most evident defect in the cell cycle progression was observed on day 3. The defect included a decrease in both G₁ and G₂/M peaks with a corresponding increase in cell population peak with < 2N DNA content (**Figure 3.10A**). This population represented anucleate cells (zoids) and their accumulation was confirmed by microscopic analysis. These zoids usually arise by cytokinesis in the absence of karyokinesis. It was already known that the procyclic cells of *T. brucei* could proceed normally with their kinetoplast segregation and cytokinesis in the absence of mitosis (Hammarton et al., 2003a; Ploubidou et al., 1999). FACS data after TbSMC3 depletion represented a defect in the cell cycle progression with fewer cells in G₁, S and G₂/M peaks favoured by accumulation of cells in G₀ peak (zoids). Statistically at day 3 post-induction, as calculated by CellQuestPro software, the non-induced culture (- Tet) has 48% of their cells in G₁, 15% in S, 32% in G₂/M phases while only 2% were zoids (**Figure 3.10B**).
Figure 3.10 Flow cytometry profiling of TbSMC3-RNAi depleted cells of *Trypanosoma brucei*. A. FACS analysis of the cellular DNA content of the induced (+ Tet) and the non-induced (- Tet) cells stained with propidium iodide. Overlaid FACScan peaks of the induced (+ Tet) and non-induced (- Tet) cells on day 3 after RNAi induction showed a large proportion of cells with a DNA content <2C with a reduction in the percentage of cells in all phases of the cell cycle after TbSMC3 depletion. B. The same profiling summarized the percentage of cells in the respective cell cycle phases displayed on A as determined by CellQuestPro software.
On the other hand and after SMC3 depletion, the percentage of zoids increased to 20% concurrently with a decrease in G1 cells to 38%, S cells to 11% and G2/M cells that represented 24% of cell population. The proportion of zoids that arise from the non-induced culture was most likely to be caused by a low level leakiness of the RNAi-induction system (pZJMβ) in the absence of any targeted induction.

To verify the FACS data observation, DAPI-stained cells from the RNAi induced (+ Tet) and non-induced (- Tet) cultures were microscopically examined for their nuclei (N) and kinetoplast (K) configuration for 2 successive days after RNAi induction (Figure 3.11). As in FACS, a progressive increase in the number of zoids was reported after TbSMC3 depletion which can be up to 7% on day 2 post-induction. Also there was a slight increase in cells with a non-configured number of nuclei and kinetoplasts (xNxK) that could not be detected by FACS. Statistically, the non-induced culture (- Tet) has 63% of the population with 1N1K, 20% with 1N^s2K (spherical nucleus), 10% with 1N^d2K (dumbbell-shaped nucleus), 5% with 2N2K, 2% with xNxK and no zoids were recorded. The induced (+ Tet) culture, on the other hand, has 60% of cells with 1N1K, 15% with 1N^s2K, 10% with 1N^d2K with a substantial number of zoids (7%) and xNxK cells (5%). On day 3 and 4 after RNAi induction, the microscopic analysis of N and K configurations, like FACS analysis, was challenging due to the accumulation of the dead cells in the induced RNAi culture, which makes further statistical scoring unreliable.
Figure 3.11 Frequency distribution of nuclei (N) and kinetoplasts (K) configuration of TbSMC3-RNAi depleted cells for 48 hours after RNAi induction. Cells from the induced (+ Tet) and non-induced (- Tet) cultures were stained with DAPI and 200 cells were scored under the epifluorescence microscope for the configuration of nuclei (N) and kinetoplasts (K) in different cells such that 1N1K, 1N^s2K (spherical nucleus), 1N^d2K (dumbbell-shaped nucleus), 2N2K, 0N1K (zooids) and cells with multiple configuration (xNxK). The histogram was presented as the percentage of cells (%) versus time course (in hours) after RNAi induction. Error bars indicate ± S.D, n = 3.
The effect of TbSMC3 silencing on large and minichromosomes

Given the unusual karyotype of *T. brucei*, the segregation patterns of large and minichromosomes in TbSMC3-depleted cells were assessed. Fluorescence *in situ* hybridisation (FISH) was employed using two specific chromosomal probes directed to the minichromosome population (177 bp repeats) and the megabase chromosome number 1 (Chr.1, tubulin gene). Cell progression through mitosis was monitored by staining of nuclear and kinetoplast DNA as a cell cycle marker (Sherwin and Gull, 1989). By applying FISH on wild-type (WT) 427 and non-induced RNAi trypanosome cells, Chr.1 can be seen as 2 dots in G1 cells, representing the two alleles in the diploid genome, while the minichromosomes were visualised as a chromosomal population cluster (Appendix 1). This is due to the absence of a single, large enough minichromosome-specific markers that can be used to label a single minichromosome. As cells progress through anaphase, Chr.1 was seen as 4 dots (representing the four chromatids of the replicated diploid chromosome) before anaphase initiation (Appendix 1). Successful anaphase was indicated by well-separated chromatids represented by 2 dots near each nuclear pole (Figure 3.12; upper panel). In the same cells and during anaphase, the replicated minichromosome population was segregated as two compact clusters of DNA that aligned along the nuclear axis (Figure 3.12; upper panel), previously shown to be the position of the mitotic spindle (Ersfeld and Gull, 1997).

On the other hand, TbSMC3-depleted cells showed, after 2 days of RNAi induction, a mixture of normal and aberrant chromosome segregation patterns in mitotic cells (Figure 3.12; lower three panels). For megabase chromosomes, the symmetrical pattern of Chr.1 segregation was disturbed and an unequal distribution of chromatids was frequently observed (Figure 3.12, lower panels). The most frequently observed defect was a 3:1 ratio of unequally segregated chromatids of large chromosomes, instead of 2:2 normal patterns. In other cases some chromatids lagged behind in the centre of the dividing nucleus with already fully segregated minichromosomes indicating altered dynamics of large chromosome segregation (Figure 3.12, last panel). In some cells, 4:0 ratios were also observed indicating a complete failure of chromosomal segregation during anaphase.
Figure 3.12 Immunofluorescence analysis of chromosomal segregation defects in TbSMC3-depleted cells. Fluorescence in situ Hybridisation (FISH) was used to assess the segregation of large chromosomes and minichromosomes in SMC3-depleted trypanosome cells. Large chromosomes were represented by a probe detecting the α/β -tubulin locus on chromosome 1 (Chr.1, green signal), minichromosomes by the 177 bp-repeat probe (MC, red signal). Cells were also stained with DNA-staining DAPI (blue). Panels showed FISH analysis on day 2 either in the absence (non-induced) or presence (induced) active RNAi-induction. Scale bars represent 2 µm.
For minichromosomes, cells with aberrant segregation patterns were occasionally observed as fragmented clusters accompanied by missegregation of large chromosome. Statistical analysis was performed by counting FISH signals from a total of 400 mitotic cells from both induced (+) and non-induced (-) cultures each day over a period of 4 days and compared to 427 wild-type (WT) trypanosome cells. After RNAi induction and SMC3 depletion, the proportion of unequally segregated chromatids of Chr.1 rose from 22% on day 1 to more than 50% on day 3 when compared to WT and non-induced cells (Figure 3.13A). On the other hand, minichromosome segregation patterns of TbSMC3-depleted cells did not visibly diverge from that of WT and non-induced cells and only a maximum of 5% of mitotic cells were defective in minichromosome segregation (Figure 3.13B). A possible interpretation of these data was that TbSMC3 depletion only affects segregation of large chromosomes with little or no evident adverse effect on minichromosome segregation. However, we cannot discount the possibility that minichromosome segregation could be essentially random in TbSMC3-depleted cells, because we only monitored their segregation pattern as a whole population and not, in contrast to large chromosomes, at an individual level. The appearance of normal minichromosome segregation patterns was in contrast to the drastic minichromosomal segregation defects that were caused by disrupting the mitotic spindle using anti-tubulin drugs (Ersfeld and Gull, 1997) or by RNAi knockdown of separase homologue in *T. brucei* (Chapter 3.9). Taken together, large chromosome segregation defects along with the disruption of the cell cycle progression were most likely responsible for the growth defect and cellular death observed after TbSMC3 depletion.
Figure 3.13 Quantitative analysis of the proportion of the counted mitotic cells that showing either normal (equal/segregated) or defect (unequal/non-segregated) segregation of large- (Chr.1) and minichromosomes (MC) in TbSMC3-depleted cells. A. Histogram of the proportion of the counted mitotic cells that showing either normal (equal) or defect (unequal) segregation of Chr.1 in the induced (+) and non-induced (-) RNAi cells as defined in the materials and methods. B. Also histogram of the same mitotic cells but this time counted for either normal (segregated) or defect (non-segregated) minichromosomes population (cluster) using the same method defined in the materials and methods. The non-transfected procyclic 427 cells were scored for the pattern of chromosomal segregation and the data were used as a control. The counting data were obtained from more than 400 individual cells for 4 days, performed in triplicate for both large and minichromosomes segregation. Error bars indicate ± S.D, n = 3.
3.6 Differential localisation of separase during the cell cycle

To investigate the chromosomal cohesion machinery further, I extended my research to investigate the role of separase, the protease responsible for resolving the cohesin-mediated chromosome cohesion, in *T. brucei*.

A single putative separase gene has been identified in *T. brucei* on the basis of sequence homology (Mottram et al., 2003). However, there was no experimental evidence that the identified putative gene was the orthologue of separase (Jager et al., 2001; Mottram et al., 2003). First, the subcellular localisation of *T. brucei* separase (TbSep) was investigated. The gene was expressed in procyclic trypanosomes as an ectopically C-terminal cMyc-tagged protein (TbSep\(^{cMyc}\)) by using a Tet-inducible construct. After expression induction with tetracycline, western blotting using anti-Myc monoclonal antibodies detected a single immunogenic band on whole cell lysates with a molecular mass of approximately 130 kDa (*Figure 3.14A*). This molecular mass corresponded well to the native *T. brucei* separase homologue of 126 kDa, demonstrating the successful expression of TbSep\(^{cMyc}\) (*Figure 3.14A*). Immunofluorescence microscopy-based studies revealed the predominant cytoplasmic localisation of TbSep\(^{cMyc}\) in trypanosome cells during the cell cycle (*Figure 3.14B*). The fluorescence signal was excluded from the nucleus during interphase (1N1K) and late mitotic phases (2N2K). However, protein re-localisation occurred during mitosis, when the fluorescence signal was first observed in the nucleus of metaphase (1 oval shaped nucleus, 2K) cell and with full nuclear occupancy occurred in the early anaphase cell (1 bi-lobe nucleus, 2K) cells (*Figure 3.14B*).

To exclude the possibility of any over-expression artefacts, the expression of the tagged protein was monitored during a time interval course of induction over 14 hours (*Figure 3.15*). Western blot analysis indicated that protein expression started at 3 hours post-induction (*Figure 3.15A*). Even before protein expression was detectable by western blotting after 1.5 hour; nuclear exclusion was observed in interphase cells by the immunofluorescence microscopy (*Figure 3.15B*). The nuclear exclusion in these
cells was persistent during the entire course of the expression, irrespective of the protein expression level assessed by western blotting (Figure 3.15). On the other hand, intranuclear localisation was evident in mitotic cells throughout the induction course as evident from the microscopy analysis (Figure 3.15C). These data indicated that this differential localisation was not related to any ectopic overexpression of separase. In human cells, similar nuclear exclusion of separase was observed when the cells were not in mitosis (Sun et al., 2006).
Figure 3.14 Expression and localisation of cMyc-tagged separase (Sep$^{cMyc}$) during the cell cycle of *Trypanosoma brucei*. A. Western blot of total protein of trypanosomes cell extract probed with anti-cMyc monoclonal antibody showed a single immunogenic band of ~126 KDa at 24 hours after Tet induction (+ Tet). B. Immunofluorescence localisation profile of cMyc-tagged separase in procyclic trypanosomes across the cell cycle. During most stages of the cell cycle separase is excluded from the nucleus (panels 1,2,5). As the cells progress into metaphase as denoted by the well-segregated kinetoplasts and oval nucleus (panel 3), the protein starts to appear in the nucleus (3) with complete nuclear occupancy in anaphase (4). After completion of karyokinesis, but before cytokinesis, the protein is again excluded from the nucleus (5). DAPI-stained DNA and FITC/DAPI merge were also showed. Scale bars = 2 µm.
Figure 3.15 Time course induction of TbSep\textsuperscript{cMyc} expression in \textit{Trypanosoma brucei}. \textbf{A.} Total protein content prepared from the whole cell extract at different time intervals (1.5, 3, 6, 10 and 14 hours) was probed by anti-Myc monoclonal antibodies. Single specific immunogenic band with molecular mass of \textasciitilde126 KDa was first observed at 3 hrs post-induction. \textbf{B} & \textbf{C} were the immunolocalisation profiling of TbSep\textsuperscript{cMyc} expression in the interphase and mitotic cells, respectively, at the same time intervals mentioned above. DNA stained with DAPI (red) and cMyc-tagged protein with FITC (green). Scale bars = 2 \textmu m.
3.7 Separase overexpression and its effect on the growth rate and cell cycle progression

After induction of TbSep$^{cMyc}$ expression for a prolonged period of time, the excess protein was found to adversely affect the growth rate and cell cycle progression in these cells (Figure 3.16). First, progressive increase of TbSep$^{cMyc}$ expression level in the induced cells (+) compared to non-induced cells (-) over the time course was confirmed by western blot (Figure 3.16A). When the cells harbouring the cMyc expression construct of separase were induced with tetracycline and allowed to grow for 5 days, no detectable differences between the growth rate of the induced and non-induced cultures was observed for the first 2 days (Figure 3.16B). On day 3, the induced cells (+ Tet) started to grow slower when compared to non-induced cells (- Tet) and on day 4 post-induction, there was a complete cessation of growth in the induced cells.

After TbSep$^{cMyc}$ overexpression, a decrease in the $G_1$ peak of the induced (+ Tet) cells when compared to non-induced (- Tet) cells was evident by FACS analysis (Figure 3.16C). Also there was a slight increase in the zoid peak. This was in contrast to the RNAi-based depletion of TbSMC3 (Chapter 3.4) and TbSep (see below) where a considerable increase in the number of anucleate zoids was recorded. This may be due to the inability of the affected cells to complete cytokinesis after a possible cell cycle block and could therefore explain the absence of large number of zoids after the protein overexpression.
Figure 3.16 Separase overexpression affected the growth rate and the cell cycle progression. Cells harbouring cMyc expression construct of separase were induced with 1µg ml$^{-1}$ tetracycline and allowed to grow for 5 days post-induction. A. Total cell extracts of cells from both the induced (+) and the non-induced (-) cultures were probed with anti-Myc mAbs for 4 successive days post-induction. B. Cell density ($\times 10^6$) from the induced (+ Tet) and non-induced (- Tet) cultures was plotted against time (in days) as a growth curve. C. On day 3 post-induction, the induced (+ Tet) and non-induced (- Tet) cells were stained by propidium iodide and processed for FACS analysis as mentioned in materials and methods section.
3.8 Depletion of separase by RNAi and its effect on the growth rate and cell cycle progression

3.8.1 Separase depletion produced a lethal phenotype

A double-stranded stem-loop RNA transcript specific for the separase homologue of *T. brucei* was expressed from a tetracycline-inducible stem-loop vector, pALC14 RNAi construct (Bochud-Allemann and Schneider, 2002). Inhibition of the growth rate of the induced (+ Tet) culture was observed as early as 24 hours after induction when compared to the non-induced (- Tet) culture (Figure 3.17A). On day 3, cell growth ceased completely and the non viable cells were substantially accumulated in the induced culture on day 4 post-induction. The growth impairment observed after RNAi induction was prompt and started even quicker than that observed for the TbSMC3-RNAi knockdown. To confirm depletion of the corresponding transcript of TbSep, RNA samples were extracted from both induced (+) and non-induced (-) cultures over 4 days post-induction and also from procyclic 427 cells as a control. The level of RNA transcripts was monitored by reverse transcriptase-PCR (RT-PCR). PCR performed on cDNA templates generated from extracted RNAs showed a gradual decline of the level of TbSep transcript from induced cells starting from day 1 post-induction (Figure 3.17B). On day 3 and 4 post-induction, no detectable TbSep transcripts were observed when the induced (+) culture was compared to non-induced (-) one. PCR performed on the same RNA samples using paraflagellar rod-A (PFR-A) gene specific primers was used as a loading control assuming approximately equal transcription of this gene coding for a structural component of the flagellum (Deflorin et al., 1994). Also, RT-PCR reactions were performed on all the extracted mRNA samples in the absence of the reverse transcriptase activity which revealed the absence of any possible background genomic DNA contamination (data not shown).
Figure 3.17 RNAi-based knockdown of separase homologue produced a lethal phenotype in the procyclic form of *Trypanosoma brucei*. **A.** Mid-log phase culture of 29-13 procyclic cells transfected with pALC14-TbSep stem-loop construct was grown in SDM-79 medium with (induced) or without (non-induced) 1µg ml\(^{-1}\) tetracycline. The in vitro growth rate of the cells (x10^6) was monitored daily for 4 successive days after tetracycline induction and plotted against time (in days) and showed nearly complete growth cessation after day 3 of induction. **B.** RT-PCR analysis of separase mRNA/cDNA showed efficient depletion after 3 – 4 days post-induction. Transcript levels of the flagellar protein PFR-A were used as a template control.
3.8.2 Interference with the cell cycle progression after separase depletion

FACS analysis was employed to assess the effect of separase silencing on cell cycle progression over a period of 48 hours after RNAi induction (Figure 3.18). In accordance with the rapid cessation of the growth rate, a defect in the cell cycle progression became evident in the induced (+ Tet) cells after 24 hours (Figure 3.18A). A decrease in the G1 (2n) population was seen after 24 hours of RNAi induction and the defect became more pronounced after 48 hours (Figure 3.18A). In addition, after 48 hours, a decrease in the G2/M population and a dramatic increase in the number of cell with DNA content smaller than 2n, most likely representing zoids, was observed. The accumulation of anucleate zoids and the decrease of G1 and G2/M populations suggested a failure of karyokinesis but continuation of cytokinesis.

The inhibition of cell cycle progression was further confirmed by the cytological analysis of DAPI-stained cells from both induced (+ Tet) and non-induced (-Tet) cultures at 24 and 48 hours of RNAi induction (Figure 3.18B). In accordance with the rapid impairment of growth rate and cell cycle progression, a difference in the nuclear/kinetoplast configuration between the two cultures was observed after 24 hours. The most significant difference was the increasing number of zoids and mitotic cells characterised by dumbbell-shaped nuclei (1N^d2K). This was accompanied by a decrease in the number of interphase (1N1K) and early mitotic cells with spherical-shaped nuclei (1N^s2K). Also, a considerable increase in the number of cells with multiple nuclei and kinetoplasts was reported (Figure 3.18B). After 48 hours, the non-induced (- Tet) culture has 62% of the population with 1N1K, 15% with 1N^s2K (spherical nucleus), 11% with 1N^d2K (dumbbell-shaped nucleus), 6% with 2N2K (dividing nucleus) and 2% of cells are zoids. The induced (+ Tet) culture, on the other hand, showed an increase of zoids to 20%, 1N^d2K cells to 24% with a corresponding decrease of 1N1K cells to 23%. The emergence of large number of zoids after separase depletion is similar to cell cycle defects observed after the depletion of a number of cell cycle regulators in procyclic *T. brucei* (Hammarton, 2007). Also, the accumulation of metaphase cells as a result of depletion of a putative separase homologue provided evidence that this protein is indeed the functional separase in *T. brucei* involved in promoting metaphase-to-anaphase progression.
Figure 3.18
Figure 3.18 Cell cycle analysis and phenotypes after RNAi-knockdown of separase in *Trypanosoma brucei*. A. Trypanosome cells from both induced (+ Tet) and non-induced (- Tet) cultures were stained with propidium iodide and analysed for their DNA content by FACS. Indicated was the FACS analysis on 24 and 48 hours post-induction with different peaks representing different DNA content (<2n, G₁, S, G₂/M and >2n). B. The induced (+ Tet), non-induced (- Tet) and 427 WT cells were stained with DAPI and categorised according to their nuclei (N) and kinetoplasts (K) configuration at 24 and 48 hours post-induction.

3.9 Separase is essential for the segregation of both large and minichromosomes

3.9.1 Partition of large and minichromosomes was inhibited after separase depletion

Being essential for resolving the cohesin complex and sister chromatid separation, the hypothesis is that knockdown of TbSep expression would have overlapping effects in comparison to RNAi depletion of TbSMC3. However given the multifunctionality of separase activity in other organisms, additional phenotypes were potentially expected after protein depletion in *T. brucei*.

As described for TbSMC3-RNAi, FISH was used to assess the effect of separase depletion on chromosomal segregation patterns in *T. brucei*. In contrast to TbSMC3-depleted cells, trypanosome cells deficient of separase were unable to segregate both types of chromosomes, large and minichromosomes (Figure 3.19). Non-induced cells during anaphase have two dots at each nuclear pole, representing the four replicated chromatids while the minichromosomal population segregated as two equal clusters (Figure 3.19, two upper panels). In separase-depleted cells, the tubulin gene marker for Chr.1 frequently showed a non-symmetrical distribution between the dividing nuclei (Figure 3.19, three lower panels). This abnormal segregation can be seen in different patterns. First, Chr.1 marker was scattered along the nuclear axis in association with fragmented clusters of minichromosomes population (Figure 3.19,
The second abnormally deviated pattern was 3:1 ratio instead of the normal 2:2 one (Figure 3.19, panel 4). In more pronounced cases, cells with 4:0 FISH dots for large chromosome marker were microscopically spotted indicating complete failure of large chromosome segregation, even though minichromosomes were successfully partitioned (Figure 3.19, panel 5). In contrast to the data obtained for the TbSMC3-RNAi, symmetrical segregation patterns of minichromosomes were often disrupted and fragmented resulting in unequal distribution to daughter nuclei (Figure 3.19, panels 3 and 4). These asymmetrical distributions were observed in approximately 50% of anaphase cells. In the remaining 50%, the segregation patterns appeared symmetrical. But given the technical limitations to visualise an individual minichromosome by FISH, it was probable that the segregation anomalies affected a larger population of minichromosomes than was observable by the microscopic examination.

For the first 24 hours, no detectable differences for the segregation of both classes of chromosomes were observed between induced and non-induced cultures (Figure 3.20). After 48 hours of RNAi induction, almost 50% of induced cells have uneven segregation patterns for large chromosomes, compared to only 7% of non-induced cells (Figure 3.20A). For minichromosomes, 48% of the induced (+) cells failed to segregate their minichromosomes after separase depletion when compared to only 4% of non-induced cells (Figure 3.20B). After 48 hours, the RNAi culture contained large number of dead cells and therefore any further statistical analysis was not possible. 427 cells were also examined for their chromosomal segregation patterns and were used as an experimental control in parallel to the induction experiment. In these cells, only 1-2% and 0-1% abnormal segregation patterns was reported for large and minichromosomes, respectively. These numbers were lower than those reported for the non-induced cells indicated a certain degree of leakiness of the RNAi construct in these cells in the absence of RNAi induction.
Figure 3.19 Immunofluorescence analysis of chromosomal segregation defects in separase-depleted cells. Fluorescence in situ hybridisation (FISH) was used to assess the segregation of large chromosomes and minichromosomes in separase-depleted cells 2 days after RNAi-induction. Labels are as described in Figure 3.12. Note the aberrant patterns of minichromosomes distribution in the dividing nuclei (panel 3 and 4 from top, red signals) and large chromosome distribution (panels 3, 4, 5, green signals). Scale bar represents 2 µm.
Figure 3.20 Quantitative analysis of chromosomal segregation defects in separase-depleted cells. Quantitative analysis of the proportion of the counted mitotic cells that showed either normal (equal) or defective (unequal) segregation of Chr.1 (A) and normal (segregated) or defective (abnormal-segregated) minichromosomes (B). After RNAi, cells from the induced (+) and non-induced (-) culture were scored for pattern of chromosomal segregation. Wild-type procyclic 427 cells were scored and used as a control. Data were obtained from 400 cells for each time point, performed in triplicate for both large and minichromosomes segregation. Error bars represents ± S.D, n = 3.
3.9.2 Cohesin-chromosome resolution in *T. brucei* was dependent on separase activity

The reported missegregation of chromosomes in cells depleted of separase can be attributed to the failure to remove the cohesin complex from chromatid pairs before anaphase. Therefore one would expect persistence of cohesin association with chromatin in the absence of a functional separase activity. To examine this, trypanosome cells depleted of separase by RNAi were subjected to detergent extraction as described before (Chapter 3.2.5). When separase-depleted cells were detergent-extracted and subjected to the immunostaining with anti-SMC3 antibodies, immunofluorescence microscopy analysis revealed the persistence of the staining signal in both early and late anaphase cells (Figure 3.21A). This was in contrast to what was observed for WT 427 detergent-extracted cells where the chromatin-bound, detergent-resistant TbSMC3 signals were not detectable in these stages of the cell cycle (Figure 3.8A).

A substantial difference was seen between detergent-extracted, separase-depleted (+ Tet), non-depleted (- Tet) and WT 427 cells by analysing anti-SMC3 fluorescence signals (Figure 3.21B). In WT 427 cells, positive anti-SMC3 signals were reported in 83% of 1N2K cells, while 17% of these cells were SMC3-negative. In 2N2K cells (when the cohesin was expected to be released from chromatids), the percentages of positive and negative signals were 4% and 96% in these cells, respectively. Similar results were reported for RNAi cells in the absence of induction (- Tet). On the other hand, after RNAi induction (+ Tet) and separase depletion, only 47.5% of 2N2K cells were SMC3-negative, while 52.5% of these cells were SMC3-positive after detergent extraction (Figure 3.21B).
Figure 3.21
**Figure 3.21** The cohesin-chromosome interaction after separase deficiency. **A.** Procyclic trypanosome cells depleted of separase protein by RNAi were detergent-extracted before being formaldehyde-fixed and immunostained by α-SMC3 antibodies. Immunofluorescence analysis showed two cells in a clear anaphase stage with persistent nuclear signals corresponding to SMC3 nuclear localisation. FITC signal was in green, DAPI in red and the two signals were merged while the scale bar = 2 µm. **B.** Positive and negative SMC3 staining signals were scored in the different cell categories (1N1K, 1N2K and 2N2K) of 427, RNAi induced (+ Tet) and RNAi non-induced (- Tet) cells. Standard deviation (± SD) was represented as error bars of two experimental replicates.
3.9.3 Differential sensitivity of mitotic spindles formation to separase and SMC3 depletion in Trypanosoma brucei

If the role of separase in T. brucei was restricted to resolving the cohesin-chromosome tie before anaphase, the impact of separase depletion on minichromosomes propagation should be comparable to the apparent symmetric segregation patterns observed after TbSMC3 depletion. But the noticeable differences between TbSMC3- and TbSep-depleted cells regarding the segregation of the minichromosomes population pointed out for an extending role of separase in trypanosome cells. In yeast and mammalian cells, in addition to its role in cohesin cleavage, separase is essential for the formation of a stable mitotic spindle via a cohesin-independent pathway (Baskerville et al., 2008; Jensen et al., 2001). To test whether separase depletion had an effect on spindle formation in T. brucei, trypanosome cells were immunostained with the anti-β-tubulin antibody (KMX-1), a monoclonal antibody that preferentially stains the spindle microtubules (Sasse and Gull, 1988). In T. brucei, the mitotic spindle is assembled inside the nucleus (Vickerman and Preston, 1970). When wild-type (WT) T. brucei cells were stained with KMX-1, essentially all the 1N1K cells were without mitotic spindles while 1N2K cells are equally divided between 50% spindle-positive and 50% spindle-negative cells (Tu et al., 2006). The spindle-positive cells are believed to be in mitotic status while spindle-negative cells are from the time of kinetoplast replication until mitosis initiation (Ploubidou et al., 1999).

Using KMX-1 on WT 427 cells, rhomboid-shaped spindles converging at opposite poles of the nuclei (Figure 3.22A; upper panel) were observed in 46% of 1N2K cells. At 24 and 48 hours of RNAi induction and separase depletion, a significant difference in the number of cells with assembled spindles was observed when compared to WT 427 cells (p < 0.01) (Figure 3.22B). On the other hand, there was no significant change in the number of cells with spindles 24 hours after TbSMC3 depletion compared to WT cells (p > 0.01). However, at 48 and 72 hours of RNAi induction, the depletion of TbSMC3 adversely affects the spindle formation in these cells when compared to WT 427 cells (p < 0.01). By comparing the two depletion events, mitotic spindle defects were more pronounced after separase knockdown. The percent of 1N2K cells with spindles (plus spindle) was reduced 47% and 58% at 24 and 48 hours
following separase depletion, respectively (Figure 3.22B). TbSMC3 depletion, on the other hand, only produced 4% and 18% reduction in the number of the spindle-positive cells at the same time intervals (Figure 3.22B).

Due to the difference in the RNAi-inducible system used and the efficiency of depletion observed between the two proteins (as mentioned before), the adverse effect of SMC3 depletion on the spindle formation at 72 hours was compared to separase depletion at 48 hours. Despite the time factor and its relation to the depletion efficiency, a highly significant difference in the mitotic spindle defects between the two depletion events was recorded (p < 0.01). These data were compatible with the differential effects observed for the TbSMC3 and separase disruption on the mitotic pathway of T. brucei. Also, the defect in the spindle assembly observed after separase depletion is compatible with the view that, similar to its functional diversity in other cellular systems, the role of separase is not restricted to cohesin resolution in order to initiate chromatid segregation. The T. brucei separase homologue most likely is involved in the assembly and maintenance of the mitotic spindle.
Figure 3.22

A

- Tet

+ Tet

B

Separase RNAi

C

SMC3 RNAi

% of cells

% of cells

0 10 20 30 40 50 60

0 10 20 30 40 50 60

4 hr - 24 hr - 48 hr - 72 hr +

4 hr - 24 hr - 48 hr - 72 hr +

Plus spindle

Minus spindle

Plus spindle

Minus spindle
**Figure 3.22** Mitotic spindle defects in separase-depleted cells. 

**A.** Cells from RNAi non-induced (- Tet; top panel) and induced (+ Tet, lower panel) cultures were formaldehyde-fixed and immunolabelled with anti-β tubulin monoclonal antibody KMX-1 (green) and DAPI (red). In the normal early mitotic cell (1oval nucleus, 2K), nucleus have a well-developed rhomboid-shaped mitotic spindle. In separase-depleted cells such early spindle configurations are often not detectable, scale bars refer to 2µm. 

**B.** Tabulation of 1N2K mitotic cells after separase depletion by RNAi as either positive (plus) or negative (minus) for the mitotic spindle after KMX-1 staining. Wild-type 427 cells were used as a positive WT control. Error bars are standard deviations (±SD) from two independent experiments (n=2) where about 200 cells were counted in each experiment.

**C.** As in **B** where a similar analysis after SMC3 depletion by RNAi was performed except that the analysis was extended for 72 hours instead of 48 hours in case of separase RNAi.
4. DISCUSSION AND CONCLUSION

4.1 General discussion

Mechanisms regulating mitosis and cell cycle progression are still poorly understood in the early branching eukaryote, *T. brucei*. The sequenced genome of *T. brucei* has offered us the opportunity to directly target and characterise candidate proteins involved in the regulation and mechanics of these processes. Using approaches such as gene deletion, RNAi and protein-protein interaction mapping, the function of many of these proteins have been revealed (Hammarton, 2007). Among the well experimentally-characterised proteins are the mitotic cyclins (cyclins 1-6), cyclin-related kinases (CRK1, 2 and 3), anaphase promoting complex (APC) and centrins (Hammarton et al., 2004; Kumar and Wang, 2005; Li and Wang, 2003; Shi et al., 2008; Tu and Wang, 2004).

Sequence-homology exploration of the *T. brucei* genome using eukaryotic protein sequences of known functions revealed only very few proteins that are involved in mitotic processes such as building kinetochores, checkpoints pathways and chromosomes segregation (Berriman et al., 2005), either because the rest of these proteins are either absent from the parasite genome or have highly divergent sequences (Hammarton, 2007). This may be due to the early branching and divergence of *T. brucei* as many structures such as kinetochore have been identified, although their protein constituents remain unknown and a sequence-homology approach did not reveal any candidate proteins (Ogbadoyi et al., 2000; Solari, 1980; Urena, 1986). Another category of proteins can clearly be assigned to known protein families, but are sufficiently divergent to prevent any functional assignment. An example of such proteins is represented by the expanded family of kinesin-type motor proteins identified in the *T. brucei* genome (Wickstead and Gull, 2006).

Nevertheless, a small number of highly conserved proteins with likely roles during mitosis have been identified in the parasite genome. One example is the cohesin complex that is responsible for sister chromatid cohesion prior to chromosome segregation (Haering and Nasmyth, 2003). Cohesin subunits, SMC1, SMC3, SCC1 and
SCC3 are highly conserved with clear homology to their counterparts in other organisms. Another example is the putative separase protein of *T. brucei* which is also highly homologous to other eukaryotic separases especially at the level of its catalytic C-protease domain (Jager et al., 2001; Uhlmann et al., 2000; Wirth et al., 2006). The *T. brucei* putative separase possesses a highly conserved protease domain, whose proteolytic activity is responsible for cleaving cohesin (Mottram et al., 2003). The functional conservation of one cohesin complex component in *T. brucei* was recently confirmed by experimental characterisation of cohesin TbSCC1 (Gluenz et al., 2008). RNAi-mediated depletion and site-directed mutagenesis of TbSCC1 resulted in karyokinesis failure and cell cycle progression defects.

In yeast, expression of the small cohesin subunit SCC1/MCD1 is tightly regulated during the cell cycle: it is repressed during G1, induced early in S phase (or very late in G1), and is much downregulated at anaphase when the protein cleaved by separase (Guacci et al., 1997; Michaelis et al., 1997; Uhlmann et al., 1999). The larger cohesin subunits SMC1, SMC3 and SCC3 are present at constant levels throughout the cell cycle, but bind chromatin only in the presence of the enforcing SCC1 subunit (Ciosk et al., 2000; Gruber et al., 2003; Guacci et al., 1997; Michaelis et al., 1997; Toth et al., 1999; Valdeolmillos et al., 2004). In *T. brucei* procyclic and bloodstream cells, a similar pattern was observed for the cohesin subunit, TbSCC1 (Gluenz et al., 2008; Sharma et al., 2008). TbSCC1 is expressed prior to DNA synthesis in late G1, remains in the nucleus throughout S- and G2-phases of the cell cycle and is repressed at anaphase.

Here in this study, the functional characterisation of one of the large cohesin complex subunits, TbSMC3, was performed. Immunofluorescence analysis and western blotting data confirmed the endogenous expression of TbSMC3 in *T. brucei*. As anticipated, the protein was localised to the nucleus of trypanosome cells. But in contrast to the cell cycle-dependent expression profile of TbSCC1, TbSMC3 was present and detectable throughout the cell cycle. Additionally, we did not observe much fluctuation in the level of TbSMC3 in the nuclei of whole fixed cells at different cell cycle phases. However, *in situ* analysis of detergent-extracted cells indicated that TbSMC3 signals were strongly detectable in the nuclei of interphase and early mitotic
cells, decreased in intensity toward anaphase and became undetectable in anaphase and dividing cells. Accordingly, two fractions, detergent-soluble and insoluble, of the TbSMC3 protein were observed in asynchronous trypanosome populations. The cell cycle-dependent, transient chromatin-binding patterns of cohesin subunits in *T. brucei* are similar to other cellular systems. In mammalian cells, using photobleaching and quantitative live-cell imaging, two distinct pathways of cohesin association with chromatin were identified during the cell cycle (Gerlich et al., 2006). The first nuclear cohesin pool bound very stably to chromosomes after DNA replication and persisted until chromosome segregation at anaphase. The second pool of cohesins exchanged on and off chromatin during the entire interphase but was absent from metaphase cells. This transient interaction is the hallmark of chromosome passenger proteins (Beaudouin et al., 2006; Kimura and Cook, 2001; Phair et al., 2004). In *T. brucei*, which undergoes a closed mitosis, TbSMC3 persists in the nucleus throughout the cell cycle either in a non-chromatin associated status (soluble pool) or as chromatin associated (insoluble pool), compatible with a generic model of cell cycle-dependent cohesin-chromatin interaction (Gerlich et al., 2006; Guacci et al., 1997; Michaelis et al., 1997). In contrast, the separase-cleaved SCC1 subunit needs to be replenished either at a transcriptional or translational level and therefore is likely to be the critical component for the cohesin assembly on chromosomes.

After TbSMC3 localisation studies, the functional role of the cohesin protein in *T. brucei* was characterised by RNAi-based inducible depletion. The effects on chromosome segregation and cell cycle progression after the protein depletion were analysed. Yeast and human cells deficient in any of the cohesin complex subunits frequently fail to complete metaphase chromosome alignment, show chromosome segregation defects and are unable to complete cytokinesis (Hauf et al., 2001; Uhlmann et al., 1999). Knockdown of the cohesin subunit of SMC3 triggers dysfunctional mitosis with chromosomal instability and aneuploidy in human and zebrafish cells (Ghiselli, 2006). Also in humans, mutations of the cohesin SMC3 and SMC1A proteins cause Cornelia De Lange Syndrome as a result of chromosome missegregation due to apparent defects in the cohesins binding to chromatin (Deardorff et al., 2007). In *Xenopus*, cohesin depletion by SMC proteins-specific antibodies causes misalignment of chromosomes, defective spindle attachment and missegregation during anaphase.
(Deehan Kenney and Heald, 2006). After RNAi-mediated downregulation of TbSMC3 expression in *T. brucei*, I studied the possible differential effects on the segregation of large and minichromosomes as well as the general cell cycle progression phenotypes. As anticipated, the function of the cohesin complex was conserved with regard to large chromosomes. Cells with TbSMC3 deficiency demonstrated missegregation of large chromosomes. The observed growth inhibition and lethal phenotype after TbSMC3 depletion is most likely due to the loss of essential housekeeping genes maintained on these large chromosomes. On the other hand, the analysed population of minichromosomes did not show much deviation to the normal symmetrical segregation patterns of control non-induced cells. Given the unusual mode of segregation of these highly repetitive small chromosomes, I considered the possibility that the cohesion of minichromosomes might be independent of the SMC3-containing cohesin complex. This result is based on the microscopic analysis of cells where the entire population of minichromosomes was visualised by the fluorescent *in situ* hybridisation (FISH). Therefore, I cannot strictly rule out the missegregation of few individual minichromosomal chromatids which cannot be detected by this approach. Unfortunately, post-RNAi analysis of the minichromosome population patterns in cloned cells by pulsed-field gel electrophoresis (PFGE) was not possible, due to the overriding lethal effect of SMC3 depletion as a result of the loss of essential large chromosomes.

In yeast, the segregation of highly repetitive chromosomal regions such as ribosomal DNA (rDNA) and telomeres occurs late in mid-anaphase, long after cleavage of the cohesin complex has taken place (D'Amours et al., 2004; Sullivan et al., 2004; Torres-Rosell et al., 2004). The cohesion of these genomic regions is apparently not dependent on the cohesin complex and the disjunction of these chromosomal loci is regulated by mechanisms other than cohesin cleavage and separase activation. Here, an alternative mechanisms involving the FEAR network, CDC14 phosphatase, topoisomerase II and the SMC2-4 condensin complex, orchestrate the timely resolution of these chromosomal regions before mitotic exit (D'Amours et al., 2004; Lam et al., 2006; Sullivan et al., 2004). Additionally, DNA-repair SMC proteins, SMC5 and SMC6, ensure the correct segregation of chromosomes, especially those loci with highly repetitive nature, by preventing the formation of sister chromatids junction (Torres-
Rosell et al., 2005). The FEAR network, regulated by the non-proteolytic function of separase, is required for releasing CDC14 from the nucleolus and supporting the resolution of rDNA and telomeric regions (D'Amours et al., 2004). The role of condensin in rDNA segregation is demonstrated by condensin subunit mutants, which failed to support CDC14-induced rDNA segregation (D'Amours et al., 2004). Topoisomerase II is involved in the resolution of highly catenated sister chromatids in the absence of the cohesin cleavage and separase activation (DiNardo et al., 1984; Shamu and Murray, 1992; Uemura et al., 1987; Uhlmann, 2007). Indeed, topoisomerase II is essential for segregation of late-separating regions and the segregation of nucleolar DNA failed in top2 mutants (Sullivan et al., 2004). The importance of condensins for chromosome segregation during anaphase by promoting DNA de-catenation by topoisomerase II has been demonstrated in many organisms (Cervantes et al., 2006; D'Ambrosio et al., 2008; Strunnikov et al., 1995). In vertebrates, it is unclear whether these additional pathways of chromosome segregation are operating, given the presence of considerable amounts of repetitive sequences in their genomes. Here, only few loci, such as centromeric regions, show late cohesin-independent segregation (Batzer and Deininger, 2002).

While genes coding for the SMC5 and SMC6 proteins could not be annotated in the T. brucei genome, genes coding for the core condensin complex (SMC2 and SMC4) and their associated proteins were identified. Whereas no functional studies on the condensin complex have yet been conducted on trypanosomes, RNAi-based silencing of nuclear topoisomerase II homologue (TbTOP2) leads to pleomorphic nuclear abnormalities such as absent, enlarged or fragmented nuclei (Kulikowicz and Shapiro, 2006). Unfortunately, no further analysis of the possible chromosomal segregation defects were conducted after TbTOP silencing which might be due to the unusual T. brucei topoisomerase (presence of mitochondrial and nuclear homologues). In future, it will be interesting to elucidate any involvement of cohesin-independent pathways such as the roles of the condensin complex and topoisomerases in the resolution of the T. brucei genome, specifically at those loci with a highly repetitive nature such as minichromosomes.
As demonstrated by vertebrate genomes, only few genomic regions show cohesin-independent cohesion and resolution, despite the prevalence of repetitive sequences. This might discount the principle of repetitiveness as a contributing factor in the cohesin-independent pathways of chromosomes cohesion and resolution. So what other theories might explain the absence of cohesin's activity from certain genomic parts? The size of the chromosome involved might play a role in determining its cohesion and segregation. In yeast, when the cohesin-free rDNA region transferred from large to small chromosome, no chromosomal loss was detected (Freeman et al., 2000). This indicates that rDNA regions bear unique features and that the chromosome size exerts no effects on their segregation. The remaining interpretation is the heterochromatic (transcriptionally-inactive) nature of rDNA regions as the absence of any RNA polymerase transcription activity might permit unusual modes of cohesion, condensation and segregation. Minichromosomes of *T. brucei* are transcriptionally inactive (Ersfeld et al., 1999; Wickstead et al., 2004). A possible explanation for the functional redundancy of the cohesin role in minichromosomes segregation could be the transcriptional inactive status of these chromosomes. In several organisms it has been shown that the cohesin complex, beside its role in chromosome segregation, also participates in regulating gene expression during interphase as a transcriptional insulator by isolating the active promoters from their enhancers (Donze et al., 1999; Rubio et al., 2008; Uhlmann, 2008; Wendt et al., 2008). As such a role is not required for the transcriptionally silent chromosomes, trypanosomes could utilize alternative mechanisms to replace the mitotic functions of cohesin in linking replicated minichromosomes. Large chromosomes, which carry all essential housekeeping genes, have to be linked by cohesins because any error in their segregation will have a lethal effect on trypanosome cells. For minichromosomes, the only proposed role is to expand the VSGs repositories in the *T. brucei* genome through duplicative VSG gene conversion to ESs on large chromosomes (Alsford et al., 2001; Robinson et al., 1999). Cohesin-independent mechanisms were more likely to play the main role in the mitotic progression of these transcriptionally-silent small chromosomes. In the future, it will be interesting to reveal any direct association of the cohesin complex proteins and chromosomes of *T. brucei* particularly minichromosomes and to look for any alternative mechanisms behind their segregation.
At a cellular level, TbSMC3 depletion resulted in a defect in karyokinesis progression without affecting the mitotic exit and cytokinesis. The cell cycle progression defect after TbSMC3 deficiency was characterised by a decrease in the G\textsubscript{1} and G\textsubscript{2}/M populations with a sharp increase of anucleate zoids as evident from FACS data. Microscopic analysis of cells for nuclei and kinetoplasts configurations revealed the emergence of zoids in cell populations depleted of SMC3 protein. Despite the slight difference between the two cultures (the induced and non-induced ones) as evident from the microscopic data of cells, this could not explain the rapid lethality imposed on cells after SMC3 expression disruption. However, the microscopic analysis was done only for the first two days following RNAi induction because I found it very difficult to do any microscopy on cells starting from day 3 when the accumulation of dead cells hampered any further analysis. At the same time, FACS data on day 3 were most likely to explain the lethal phenotype observed when the anucleate cells (zoids) accumulated substantially in the induced culture. This was similar to what has been described for the depletion of TbSCC1 in procyclic cells where cytokinesis still occurred in the absence of karyokinesis, producing zoids subpopulation (Gluenz et al., 2008). Similar phenotypes were obtained using the microtubule assembly inhibitor, rhizoxin, and after the mitotic cyclin, CYC6, was depleted from the procyclic form cells (Hammarton et al., 2003a; Ploubidou et al., 1999; Robinson et al., 1995). Also knockdown of the cdc2-related kinase (CRK) homologue, which plays a role in the cell cycle progression, produced zoids in addition to cells with one enlarged nucleus and one kinetoplast (Tu and Wang, 2004). Absence of the mitosis-to-cytokinesis checkpoint is the hallmark of procyclic form cells undergoing cellular division as initiation of cytokinesis is neither dependent on completion of mitosis nor completion of nuclear DNA replication (Hammarton, 2007; Hammarton et al., 2003a; Ploubidou et al., 1999). Another explanation for the different phenotypic outcomes in the two life cycle stages may be attributed to the different geometry of the organelles position in the two cell types. Before cytokinesis, the order of nuclei and kinetoplasts in the dividing cell is KKNN in the bloodstream form and KNKN in the procyclic form (Tyler et al., 2001). Thus in the bloodstream form, the blocked or delayed cytokinesis may be due to a physical barrier caused by the undivided nucleus that prevent the cleavage furrow ingression, rather than by any active checkpoint surveillance. In C. elegans, cells depleted of the cohesin
SMC1, SMC3 or SCC1 proteins are deficient in their mitosis but could proceed normally with their cytokinesis (Mito et al., 2003). In contrast to these data, procyclic trypanosome cells depleted of the anaphase promoting complex (APC) by RNAi accumulate at the G2/M transition (1N2K) with short spindles indicating anaphase arrest, though cells are also inhibited from undergoing cytokinesis (Kumar and Wang, 2005). This was expected as APC depletion not only blocks separase activation and cohesion resolution, but also blocks any possible role for separase in the activation of CDC14 release and the mitotic exit network (Stegmeier et al., 2002; Sullivan and Uhlmann, 2003). This is in contrast to SMC3 function which is a structural component of the cohesin complex without any predicted regulatory roles during mitosis.
My investigation on chromosome cohesion in *T. brucei* was extended by studying the localisation of the candidate separase protein and by the phenotypic analysis of its depletion by RNAi. Immunolocalisation of the separase homologue in *T. brucei* revealed a nuclear exclusion of the protein for most of the cell cycle. Only when the cells entered metaphase, the protein re-localised to the nucleus and remained there until the completion of anaphase. In organisms that undergo a closed mitosis, such as *T. brucei*, selective nuclear exclusion could present a strategy to prevent the uncontrolled activity of this protease. However, the question raised now is how this differential localisation of separase could be achieved in trypanosomes. In other organisms, separase is kept inactive by its binding to the inhibitory chaperone, securin, until metaphase-to-anaphase transition when securin is targeted for degradation by the anaphase promoting complex (APC/C) (Ciosk et al., 1998; Jallepalli et al., 2001). Any sequence homology inference about the presence of a putative securin homologue in *T. brucei* genome is very difficult due to the highly divergent securin sequences identified in different species (Jager et al., 2001; Viadiu et al., 2005). In terms of size and possible regulation, the separase homologue of *T. brucei* has a molecular mass of 126 kDa, and is smaller than yeast and mammalian separase (>210 kDa). By secondary structure analysis and motif predictions, *T. brucei* separase homologue, like larger separases, was found to possess N-terminal armadillo (ARM) repeats that mediate securin-separase interaction in human cells ([Appendix 5](#)) (Viadiu et al., 2005). However, Drosophila separase is only 73kDa and completely lacks ARM repeats, but has been shown to associate with the securin homologue, PIM (Heeger et al., 2005; Jager et al., 2001; Jager et al., 2004; Leismann et al., 2000). Securin not only inhibits separase, but also promotes its activity by mediating its nuclear transport and accumulation inside the mitotic nucleus (Agarwal and Cohen-Fix, 2002; Hornig et al., 2002). In human cells, separase activity itself is additionally inhibited at metaphase by cyclin B1-mediated phosphorylation (Holland and Taylor, 2006; Stemmann et al., 2001). The nuclear exclusion of separase, as a possible mechanism of separase activity regulation, has been reported in many cellular systems. In human cells, separase is excluded from nucleus in interphase cells providing an additional pathway of preventing premature cohesin cleavage (Sun et al., 2006). In fission yeast cells, which undergo a closed mitosis, cytoplasmic localisation of separase has been observed and separase is imported into the nucleus in a securin-dependent manner late in G\textsubscript{2} phase and during mitosis (Kumada et al., 1998). In the budding yeast, separase accumulates in the nucleus only during
mitosis, also in a securin-dependent way (Agarwal and Cohen-Fix, 2002; Jensen et al., 2001). In these organisms, the nuclear separase is probably exported to the cytoplasm after the mitotic exit. Alternatively, separase may be retained in the cytoplasm in the early stages of the cell cycle by a cytoplasmic component. Studies on the separase homologue, Cut1, in fission yeast have pointed to the existence of such cytoplasmic retention factor (Kumada et al., 1998). The presence of such a regulatory factor could also explain the temporal lag between separase and securin nuclear localization when securin is mostly nuclear at S phase, whereas separase first appears in the nucleus of yeast in late G2 (Jensen et al., 2001; Yamamoto et al., 1996). In future, it will be interesting to investigate whether trypanosomes possess an equally complex level of separase regulation similar to yeast and mammalian cells or whether a more simple mechanism of regulation, e.g. sole reliance on the differential nuclear exclusion in a closed mitosis, has evolved. Also, the potential identification of a securin homologue in trypanosomes will need the development of forward genetics or an in vitro biochemical assay.

When the tagged version of *T. brucei* separase is overexpressed by prolonged expression of the ectopic gene, a lethal phenotype had emerged. The lethal phenotype resulting from the protein overexpression may be attributed to the unregulated accumulation of the excess protein into the nucleus. Most likely, the excess protein resulted in premature cleavage of TbSCC1 cohesin subunit by overwhelming any additional putative modes of separase inhibition during interphase and early mitosis. In human cells, overexpression of separase in excess of securin induces premature loss of sister chromatid cohesion by overriding the separase inhibition by both securin and the cyclin B1-mediated inhibition by phosphorylation (Holland and Taylor, 2006). At the level of the cell cycle regulation, however, no major block was observed in the cell cycle progression after separase overexpression which in contrast to the protein downregulation (see next paragraph). In yeast and after separase overexpression, cells are able to drive the mitotic cyclin destruction and the mitotic exit independent of the anaphase initiation (Tinker-Kulberg and Morgan, 1999).
A functional analysis of the *T. brucei* separase by RNAi revealed a much more comprehensive effect on cell cycle progression and chromosome segregation than TbSMC3 depletion. First, and consistent with its proposed function, SMC3 nuclear signals persisted in detergent-extracted cells after depletion of separase by RNAi (Figure 3.21) when compared to the WT trypanosome cells (Figure 3.8). The most likely explanation is that the under-normal separase activity is no longer able to initiate the dissociation of the cohesin complex from chromatin by cleaving the SCC1 cohesin subunit. Similar to the lethal effect observed after its overexpression, separase depletion produces a lethal phenotype. These data are in contrast to the RNAi data that were published as a result of chromosome 1 wide RNAi screen in bloodstream cells (Subramaniam et al., 2006). Given the central role of separase during mitosis in eukaryotes, it is extremely unlikely that the essential molecular pathway that been described here for procyclic cells does not operate in bloodstream cells. Moreover, in procyclic and bloodstream form cells, expression of a separase-resistant dominant negative non-cleavable SCC1 mutant also produced a lethal phenotype (Gluenz et al., 2008).

At the cellular level, separase depletion induces a block in the cell cycle progression evident 24 hours after RNAi induction. The most significant defect was a decrease in both G1 and G2/M cells with a sharp increase in the number of zoids. Another feature of the separase-depleted population was the accumulation of cells arrested at metaphase with elongated and enlarged nuclei and well-segregated kinetoplasts. The resulting phenotype represented a block in mitotic progression while cytokinesis can be initiated and completed in separase-deficient cells. This phenotype is reminiscent to the RNAi-based depletion of the mitotic cyclin, CYC6, in the procyclic form but not the bloodstream form (Hammarton et al., 2003a). Also, procyclic trypanosomes expressing mutated, separase-resistant TbSCC1 protein are blocked in their cell cycle with accumulation of large numbers of zoids and cells with enlarged nuclei (Gluenz et al., 2008). Thus in the procyclic form, cytokinesis initiation is not dependent on the chromosome cohesion pathway. Also, in the mammalian cells, but not in yeast, separase depletion blocks sister chromatid separation but does not affect the progression of other aspects of mitotic exit, cytokinesis, or chromosome replication (Kumada et al., 2006; Wirth et al., 2006). In contrast to the separase, RNAi data in *T.*
the depletion of the anaphase promoting complex (APC), a separase activator in other organisms, from *T. brucei* procyclic cells enriched the cells at the G₂/M transition with accumulation of cells possessing two kinetoplasts and single enlarged nucleus indicating a block in both mitosis and cytokinesis (Kumar and Wang, 2005). My explanation is that the down-regulation of APC by RNAi is most likely not enough to fully suppress the activity of cyclin-dependent kinase (CDK) homologues of *T. brucei*. Sustained high levels of CDK therefore blocks all aspects of mitotic exit and cytokinesis and cells arrest at the metaphase-to-anaphase transition. On the other hand, when separase activity is downregulated by RNAi, APC is still active and can drive all aspects of cell cycle progression including cytokinesis initiation in the absence of mitosis. In budding yeast, separase has a second role in driving the cell cycle forward by linking anaphase to the mitotic exit (Stegmeier et al., 2002; Sullivan and Uhlmann, 2003). The non-proteolytic function of separase contributes to the activation of a phosphatase CDC14 as a signal for exiting mitosis by counteracting the activity of CDK during the mitotic exit (Stegmeier et al., 2002; Sullivan and Uhlmann, 2003). In *T. brucei*, it is unclear whether separase is involved in promoting the mitotic exit in a similar way to other eukaryotes such as yeast or whether the defect in cell cycle progression after separase depletion is a consequence of the absence of non-proteolytic regulatory functions. More future research may uncover this aspect of the cell cycle regulation in *T. brucei*.

When chromosome segregation patterns were assessed after separase depletion, a much more comprehensive effect was observed in comparison to SMC3 depletion. As expected, the majority of cells deficient in separase have abnormal nuclear DNA staining patterns. A substantial number of cells examined were found to possess elongated nuclei with fragmented nuclear DNA staining and two well segregated kinetoplasts indicating failed mitosis. In contrast to TbSMC3 depletion, both large and minichromosomes segregation were affected. Symmetrical well-segregated patterns observed for minichromosomes after SMC3 depletion were absent from a large proportion of the separase-deficient cells. This would explain the prompt growth and cell cycle progression defect observed in these cells. Also, the fragmented patterns of minichromosomes population during anaphase in the separase-depleted cells clearly demonstrate that gross segregation defects at the level of these small chromosomes can
be detected by the fluorescent in situ hybridisation (FISH). As a cohesin-cleaving protease, separase depletion and mutation affects the chromosome segregation and inhibits anaphase in cellular systems such as mammalian (Chestukhin et al., 2003; Gimenez-Abian et al., 2005; Kumada et al., 2006; Wirth et al., 2006) and yeast cells (Baskerville et al., 2008; Jensen et al., 2001).

The differential phenotypic defects of chromosomes segregation observed after separase and SMC3 depletion would have been surprising, if the role of separase in *T. brucei* was restricted to SCC1 cleavage and cohesion resolution. This is because of the minimal defects inflicted on the segregation of minichromosomes population after cohesin SMC3 depletion. However, data from yeast and vertebrate cells showed that separase performs several functions in addition to the cohesin cleavage and cell cycle progression (Queralt and Uhlmann, 2005). In Xenopus, separase is involved in centriole disengagement prior to mitosis (Tsou and Stearns, 2006). In yeast, under conditions of SCC1/MCD1 depletion and thermal inactivation of separase, anaphase did not occur, despite the normal resolution of sister chromatid cohesion (Jensen et al., 2001). In yeast and human cells, separase is also essential for the assembly, establishment and elongation of the mitotic spindle (Baskerville et al., 2008; Khmelinskii and Scheibel, 2008; Papi et al., 2005). In yeast, it was found that separase targets and cleaves the kinetochore-associated subunit, SLK19, which is necessary for mitotic spindles stabilisation during anaphase (Sullivan et al., 2001). Therefore, multilayer deficiencies would be expected after the separase downregulation in these organisms. In *T. brucei*, and after separase depletion, the number of cells able to assemble normal mitotic spindles decreased progressively without alteration of the general cell morphology. As the faithful segregation of both large and minichromosomes are both dependent on the formation of an intact mitotic spindle during anaphase (Ersfeld and Gull, 1997; Gull et al., 1998), the observed missegregation of both classes of chromosomes can be explained by the general mitotic spindle assembly defect caused by the depletion of separase. Chromosome missegregation was exaggerated by failure to resolve chromosome-cohesin interaction as demonstrated earlier in separase-depleted cells. Similar spindle defect phenotypes associated with compromised DNA segregation were also observed after RNAi silencing of a number of cell cycle regulators in *T. brucei*. Similar to separase depletion, a spindle assembly defect was observed after the RNAi-
induced depletion of aurora-B kinase homologue (TbAUK1) of *T. brucei* in both procyclic and bloodstream forms (Li and Wang, 2006; Tu et al., 2006). Also downregulation of the anaphase promoting complex (APC) components, APC1 and CDC27, in *T. brucei* procyclic form arrested the cells with two kinetoplasts and enlarged nuclei that contain abnormal short mitotic spindles (Kumar and Wang, 2005). Therefore, the formation and assembly of normal mitotic spindles is likely to be linked to the normal function of the cell cycle regulators in *T. brucei* as seen after RNAi silencing of AUK1, APC and now separase.

On the other hand, and after SMC3 depletion, the number of mitotic cells able to assemble normal mitotic spindles is comparable to the wild type trypanosome cells. This could explain why SMC3-depleted cells are able to segregate their minichromosome population during mitosis. In contrast to separase, researches looking for the functional involvement of the cohesin components in the spindle machinery are scarce and often contradicting each other. In vertebrates and plant cells, cohesin is involved in the mitotic spindle assembly during mitosis (Deehan Kenney and Heald, 2006; Gregson et al., 2001; Lam et al., 2005; Wong and Blobel, 2008). The cytoplasmic localisation of the cohesin complex in these organisms allow the interaction of the cohesin subunits with the spindle pole-associated factor, NuMA, required for the mitotic spindle organization during metaphase (Gregson et al., 2001). However the transient depletion of cohesins from vertebrate cells did not lead to visible impairment of the normal spindle assembly and formation (Kong et al., 2009). In *T. brucei*, it is most likely that the relationship between the normal spindle assembly and function and the normal activity of different cohesin subunits does not exists. *T. brucei* undergoes closed mitosis with restricted nuclear localisation of its cohesin SMC3 subunit. The common feature of the cohesin involvement in the spindle formation in other organisms is its cytoplasmic localisation which allows the direct interaction of the protein with the spindle components. This condition is not achievable in the case of *T. brucei* because of the closed nature of its mitosis.
4.2 Conclusion and future prospective

In conclusion, two of the main putative *T. brucei* proteins involved in the chromosomal cohesion and segregation machinery in other organisms, cohesin SMC3 and separase, were characterised in this study (Table 4.1). The *T. brucei* cohesin subunit, TbSMC3, localised to the nucleus as a chromatin-bound protein during most of cell cycle phases except during anaphase and cell division. On the other hand, separase cytoplasmic localisation with nuclear exclusion was prevalent until metaphase and anaphase when the protein re-localised to the nucleus. This cell cycle-dependent nuclear exclusion of separase represents, to our knowledge, the first example of such a mechanism to operate in kinetoplastids and provides a tractable system to investigate the regulation of differential nuclear access in this organism. Interference with the normal expression of both proteins, SMC3 and separase, brings about variable defects in the growth rate, cell cycle progression and mitosis. TbSMC3 depletion associated with a lethal phenotype, a moderate block of the cell cycle progression and defect in large chromosomes segregation. On the other hand, the lethal phenotype with severe inhibition of cell cycle progression associated with the blockade of large and minichromosomes segregation are more evident after separase depletion. The severity of phenotypes that resulted after separase expression interference when compared to SMC3 silencing was later explained by the apparent defect in the mitotic spindle assembly which is less evident in case of SMC3. In both cases, cells can still drive cytokinesis in the absence of mitosis resulting in variable generation of zoids. A clear indication of the absence of the mitosis-to-cytokinesis checkpoint in the procyclic form is evident from this study which is similar to a deficiency in other cell cycle regulators. This provides more evidence that the cell cycle regulation in *T. brucei* is divergent from other eukaryotes with some regulatory pathways being missing from the *T. brucei* genome (Berriman et al., 2005; Hammarton, 2007). The lethality imposed on trypanosome cells after depletion of both SMC3 and separase proteins indicates that they can serve as potential drug targets for anti-parasite chemotherapy.

Notwithstanding these findings, many questions which are raised by this study need to be dealt with in any future prospective studies (Box 1). For example, it will be interesting to elucidate the possible interaction patterns between the components
of the cohesin complex such as SMC3 and different chromosomes of *T. brucei* particularly minichromosomes. It is also important to characterise the potential roles of other components known to be involved in chromosomes resolution during anaphase in other organisms, such as the condensin complex, CDC14 and topoisomerase II, and to investigate the possible unique pathways that might exist in *T. brucei*.

Regarding TbSMC3 and its depletion effect on the minichromosomes segregation, as mentioned before, it was challenging to analyse the segregation defect at the level of a single minichromosome as the case for large chromosome. This brings up the first question, are the cohesin complex proteins actively involved in linking minichromosomes? This could be resolved by looking for any direct association of the cohesin complex proteins and the different regions of *T. brucei* genome particularly minichromosomes. In yeast, chromosome-cohesin interaction patterns were studied by using the chromatin immunoprecipitation (ChIP) and affinity tags directed against cohesin subunits (Rubio et al., 2008; Zhang et al., 2008b). In *T. brucei*, the technique is modified and recently applied to study the specific DNA-protein interactions in these parasites (Ruan et al., 2004; Ruan et al., 2007). In contrast to large chromosomes, the *in situ* hybridisation remained technically limited for the analysis of any segregation defect at the level of a single minichromosome. This is because of the limitations related to finding a single, large-sized specific locus, such as tubulin locus, which can be used to tag an individual MC and be visualised by the classical FISH approach. *In vivo* and *in vitro* green fluorescent protein (GFP) tagging of chromosomes has been applied successfully to localise a particular DNA sequence in the nucleus at any given time in the two main proliferative developmental forms of *T. brucei* (Landeira and Navarro, 2007; Navarro and Gull, 2001). This is based on a tetracycline-inducible system (Wirtz et al., 1999) and GFP fused to the *lacI* repressor that binds to the *lac* operator sequences inserted into the chromosome (Robinett et al., 1996; Straight et al., 1996). In the future, a similar approach could be adapted to tag a single minichromosome and can be used to analyse any potential segregation defects after TbSMC3 and separase knockdown. Also the research focus could be switched to characterise the roles of any other potential cohesin-independent pathways, such as the condensin complex and topoisomerase II, in the segregation of *T. brucei* genomic loci particularly those with high repetitive nature such as minichromosomes.
The selective nuclear exclusion of separase has provided an interesting pathway of separase regulation in trypanosomes, but how is this regulation achieved at the molecular level? Is this regulation depends on the protein-protein interaction (binding to securin and/or inhibitory phosphorylation) like other organisms? Except cleaving cohesin, does the separase contributes to the mitotic exit kinetics in trypanosomes by targeting other regulatory subunits such as releasing CDC14 from its inhibitory protein, Net1? Also it was unknown if the chromosome segregation defect after separase knockdown is solely due to inhibiting the cohesin cleavage or, as in yeast, separase could target other spindle motor associated proteins. These aspects of separase kinetics require further research through analysis of RNAi cells, complementation studies, post-translational modification assays and the protein-protein interaction studying of the Myc-tagged protein.
Table 4.1 Characterisation and different RNAi phenotypic effects of the two cohesion proteins, SMC3 and separase in *Trypanosoma brucei*.

<table>
<thead>
<tr>
<th>Characterisation</th>
<th>Cohesin (TbSMC3)</th>
<th>Separase (TbSep)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Localisation</td>
<td>Nuclear</td>
<td>Cytoplasmic (with nuclear re-localisation during metaphase and anaphase)</td>
</tr>
<tr>
<td>Growth phenotype</td>
<td>Growth inhibition 48 hours after RNAi induction</td>
<td>Growth inhibition 24 hours after RNAi induction</td>
</tr>
<tr>
<td>Cell cycle progression</td>
<td>Mild inhibition with zoids formation</td>
<td>Severe inhibition</td>
</tr>
<tr>
<td>Large chromosome segregation</td>
<td>Severe defect (Up to 50% segregation defect)</td>
<td>Severe defect (Up to 60% segregation defect)</td>
</tr>
<tr>
<td>Minichromosomes population segregation</td>
<td>Mild defect (up to 5% segregation defect)</td>
<td>Severe defect (more than 50% segregation defect)</td>
</tr>
<tr>
<td>Mitotic spindle assembly</td>
<td>Mild inhibition of spindle assembly</td>
<td>Severe inhibition of spindle assembly</td>
</tr>
</tbody>
</table>
Is the cohesin proteins interact with and involved in the segregation of all classes of *T. brucei* chromosomes?
Most likely the cohesion and segregation of large chromosomes is dependent on an intact cohesin complex, different approaches such as ChIP and GFP chromosomal tagging are required to resolve the minichromosomes segregation dilemma.

Are the other cohesin-independent mechanisms of chromosome cohesion and segregation functional in *T. brucei*?
RNAi-depletion of topoisomerase II produces nuclear defect phenotypes in *T. brucei* (Kulikowicz and Shapiro, 2006). However it was not known if topoisomerase II, condensin, and possibly CDC14, are also involved in the cohesion and segregation of some of *T. brucei* genome loci specially minichromosomes as described in yeast (D'Amours et al., 2004; Sullivan et al., 2004).

Are the separase regulation pathways similar to yeast and other eukaryotes, or is a more simple kinetic functional in trypanosomes?
Identification of separase-interacting proteins such as securin by the sequence homology is challenging in *T. brucei*, as other organisms, due to the highly divergent sequences of these proteins. It would be necessary to identify any separase-interacting proteins by using approaches such as biochemical assay (protein co-immunoprecipitation) and forward genetics.

Are the mitotic progression defects observed after separase knockdown a result of failure to promote the sister chromatid separation only, or exaggerated by a defect in the mitotic exit network (MEN) as reported in yeast?
The mitotic exit defect in trypanosome after separase depletion might be a consequence of two concomitant deficiencies in separase proteolytic and non-proteolytic activities which can affect chromosome segregation, normal spindle assembly and also the MEN network with all these exaggerate the cell cycle progression defect.
5. REFERENCES


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Toth, A., R. Ciosk, F. Uhlmann, M. Galova, A. Schleiffer, and K. Nasmyth. 1999. Yeast cohesin complex requires a conserved protein, Eco1p(Ctf7), to establish


6. **APPENDICES**

**Appendix 1**

FISH-based analysis of chromosomal segregation patterns of WT 427 trypanosome cells. Tubulin gene cluster (green) was used as a probe to mark the mitotic segregation of large chromosome number 1 (Chr.1), while minichromosomal 177 bp repeats (red) was used to tag minichromosome populations. The cell cycle positional phase was determined by the DNA-based staining (blue) of nucleus (n) and kinetoplast (k). During early interphase (1), Chr.1 was represented by two dots of the non-replicated chromosome while MCs was a single stained cluster. After DNA replication (2), 4 FISH dots represented the replicated 4 chromatids of the single diploid chromosome and MCs appeared as elongated cluster. In the early mitotic cell (3), the four chromatids of Chr.1 and MCs cluster moved a part as early sign of partition. Cell in the early anaphase (4) with clearly segregated large and minichromosomes to opposite nuclear poles. Late in anaphase (5), chromosomes have moved a part by a proportional distance. Last panel (6) represented a cell clearly completed its karyokinesis with both chromosomal markers resided at the far ends of the spindle poles.

**Appendix 2**

List of all biological solutions and reagents used throughout this study.

**Appendix 3**

Maximum likelihood tree of cohesin SMC protein sequences. Selected SMC proteins of the cohesin complex from kinetoplastids and eukaryotic organisms in which all cohesin SMC protein sequences are available were used to build up the phylogenetic tree. The maximum likelihood phylogram was constructed from the concatenated alignment of SMC protein sequences using PhyML service at [www.phylogeny.fr](http://www.phylogeny.fr) (Dereeper et al., 2008). High accuracy protein sequences alignment was done using MUSCLE at [http://www.drive5.com/muscle/](http://www.drive5.com/muscle/) (Edgar, 2004) before being used as an entry to build up the consensus tree. Scale bar denoted the number of accepted substitutions in distance units and bootstrap percentages support for the nodes were indicated. *T. brucei* proteins were in red and the kinetoplastid-specific sequences were denoted by brackets. Lm (*Leishmania major*); Tb (*Trypanosoma brucei*); Tc (*Trypanosoma cruzi*); Sc (*Saccharomyces cerevisiae*); Dm (*Drosophila melanogaster*); Xl (*Xenopus laevis*); Hs (*Homo sapiens*).
Appendix 4
Maximum likelihood phylogram of eukaryotic separases. The same consensus tree was built as before for separase protein sequences from kinetoplastids (*T. brucei*, *T. cruzi* and *L. major*) along with other separases from eukaryotic organisms. Note the *Drosophila* separase homologue (**SSE**) which is highly diverged from its related protein sequences. Scale bar denoted the number of accepted substitutions in distance units and bootstrap percentage support values for the nodes are indicated.

Appendix 5
N-terminal domain of *T. brucei* separase adopts secondary structure similar to armadillo (ARM)-repeats family. A. Prediction of proteins that adopt secondary structures similar to N-terminal region of separase proteins. The first 800 amino acid residues (N-terminal domains) were analysed by structure prediction servers including the 3D-PSSM (Kelley et al., 2000) ([http://www.sbg.bio.ic.ac.uk/~3dpssm](http://www.sbg.bio.ic.ac.uk/~3dpssm)), mGenTHREADER (McGuffin and Jones, 2003) ([http://bioinf.cs.ucl.ac.uk/psipred/psiform.html](http://bioinf.cs.ucl.ac.uk/psipred/psiform.html)) and I-TASSER (Zhang, 2008) ([http://zhang.bioinformatics.ku.edu/I-TASSER/](http://zhang.bioinformatics.ku.edu/I-TASSER/)). The calculated E-values along with the top scoring hits obtained with the two methods are shown. All the assigned proteins are belonging to the Armadillo repeat superfamily according to pFam database (PF00514), except the shaded boxes which indicates proteins of clathrin adaptor core family (PF00637). B. 3D structure model of *T. brucei* separase protein and the best hit model#1 of human importin-β as output from I-TASSER server. C. Predicted secondary structures for the amino acid sequences of *T. brucei* separase. Amino acid residue numbers were given in the first line. Secondary structures were predicted using two structure prediction servers, HHpred (Soding et al., 2005) ([http://toolkit.tuebingen.mpg.de/hhpred](http://toolkit.tuebingen.mpg.de/hhpred)) and Phyre (Kelley and Sternberg, 2009) ([http://www.sbg.bio.ic.ac.uk/phyre/](http://www.sbg.bio.ic.ac.uk/phyre/)). Server name was indicated on the right-hand side, while the tendency of every residue to adopt secondary conformation was indicated under the sequences either as a helix (H, red), an extended β-sheet (E, blue) or a coil (C, yellow). The boundaries of ARM-repeats were indicated by vertical lines as output from the I-TASSER server. In conclusion, *T. brucei* separase up to 800 residues was found to adopt a superhelical structure similar to protein family of ARM-type repeats.
Appendix 1
## Appendix 2

<table>
<thead>
<tr>
<th>Buffer Name</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS (phosphate Buffered Saline)</td>
<td>137 mM NaCl, 3 mM KCl, 16 mM Na$_2$HPO$_4$, 3 mM KH$_2$PO$_4$, pH 7.4</td>
</tr>
<tr>
<td>TBS (Tris-Buffered Saline)</td>
<td>20 mm Tris, 150 mM NaCl, pH 7.6 with HCl</td>
</tr>
<tr>
<td>SOC broth (Super Optimal broth with catabolite repression)</td>
<td>20 g Bacto-tryptone, 5 g Bacto-yeast extract, 0.6 g NaCl, 0.5 g KCl, 10 mM MgCl$_2$, 20 mM glucose/ 1000 ml ddH$_2$O</td>
</tr>
<tr>
<td>Lauria-Bertani (LB) broth</td>
<td>10 g Bacto Tryptone, 5 g Bacto Yeast extract, 10 g NaCl/ 1000 ml ddH$_2$O</td>
</tr>
<tr>
<td>1x SDS PAGE sample buffer</td>
<td>0.045 mM Tris-Hcl, pH 6.8, 10% glycerol, 1% SDS, 0.02 g bromophenolblue, 2.5% β-mercaptoethanol</td>
</tr>
<tr>
<td>Cell lysis buffer</td>
<td>50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole, pH 8.0</td>
</tr>
<tr>
<td>Denaturing lysis buffer</td>
<td>6 M guanidine.HCL, 50 mM NaH$_2$PO$_4$, 300 mM NaCl, and 10 mM imidazole, pH 8.0</td>
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<tr>
<td>Protein elution buffer</td>
<td>6 M guanidine.HCL, 50 mM NaH$_2$PO$_4$, 300 mM NaCl, and 250 mM imidazole, pH 8.0</td>
</tr>
<tr>
<td>Affinity matrix coupling buffer</td>
<td>200 mM NaHCO$_3$, 500 mM NaCl, pH 8.3</td>
</tr>
<tr>
<td>Affinity matrix blocking buffer</td>
<td>200 mM glycine, pH 8.0</td>
</tr>
<tr>
<td>Affinity matrix washing buffer</td>
<td>100 mM acetate, 500 mM NaCl, pH 4.0</td>
</tr>
<tr>
<td>Affinity matrix elution buffer</td>
<td>100 mM glycine-HCl, 100 mM NaCl, pH 2.5</td>
</tr>
<tr>
<td>Antibody neutralisation buffer</td>
<td>1 M Tris-Hcl, pH 9</td>
</tr>
<tr>
<td><strong>Cytomix buffer</strong></td>
<td>2 mM EGTA, 120 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄, 25 mM HEPES, 5 mM MgCl₂.6H₂O, 0.5 % Glucose, 100 µg/ml BSA, 1 mM Hypoxanthine, pH 7.6</td>
</tr>
<tr>
<td>--------------------</td>
<td>----------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>1X SDS PAGE sample buffer</strong></td>
<td>0.045 mM Tris-Hcl, pH 6.8, 10% glycerol, SDS, 0.02 g bromophenolblue, 2.5% β-mercaptoethanol</td>
</tr>
<tr>
<td><strong>SDS PAGE electrophoresis buffer</strong></td>
<td>25 mM Tris, 192 mM Glycine, 0.1% (w/v) SDS</td>
</tr>
<tr>
<td><strong>SDS PAGE gel staining solution</strong></td>
<td>0.2% (W/V) Coomassie Brilliant Blue r250, 50% Methanol, 10% acetic Acid</td>
</tr>
<tr>
<td><strong>SDS PAGE gel de-staining solution</strong></td>
<td>40% Methanol, 10% Acetic Acid</td>
</tr>
<tr>
<td><strong>SDS PAGE gel clearing solution</strong></td>
<td>5% Methanol, 7.5% Acetic Acid</td>
</tr>
<tr>
<td><strong>Western blotting transfer buffer</strong></td>
<td>0.05 MES, 0.05 Tris Base, 3.4 mM SDS, 1.025 mM EDTA, pH 7.3; 10% Methanol</td>
</tr>
<tr>
<td><strong>Western blotting blocking buffer</strong></td>
<td>1X TBS, 5% Semi-skimmed milk</td>
</tr>
<tr>
<td><strong>PEM extraction buffer</strong></td>
<td>0.1M Pipes, 2 mM MgSO₄, 1 mM EGTA, 0.1% NP-40, pH 6.9</td>
</tr>
<tr>
<td><strong>FACS staining solution</strong></td>
<td>200 µg ml⁻¹ Ribonuclease A, 50 µg ml⁻¹ Propidium iodide in 1X PBS</td>
</tr>
<tr>
<td><strong>Nick translation reaction</strong></td>
<td>50 mM TrisHCl (pH 7.5), 10 mM MgCl₂, 1 mM DTT, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM dTTP, 1 mM DIG-11-dUTP, 0.002 U DNaseI, 10 U DNA polymeraseI, 0.25 µg template DNA.</td>
</tr>
<tr>
<td><strong>Standard sodium saline (SSC)</strong></td>
<td>1.75 g NaCl, 0.8 g sodium citrate/100 ml ddH₂O; pH 7.0 with HCl</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>FISH hybridisation buffer</strong></td>
<td>50% formamide, 2X SSC, 10% dextran sulphate, 50 mM sodium phosphate buffer, pH 7.0</td>
</tr>
</tbody>
</table>
| **FISH washing buffers**        | **First:** 2X SSC, 50% formamide (37°C)  
|                                 | **Second:** 2X SSC (50°C)  
|                                 | **Third:** 0.2X SSC (50°C)  
|                                 | **Four:** 4X SSC (22°C)  |
| **Antibiotics**                 | Hygromycin 50 µg ml⁻¹  
|                                 | Neomycin (G418) 15 µg ml⁻¹  
|                                 | Phleomycin 5 µg ml⁻¹  
|                                 | Puromycin 2 µg ml⁻¹  
|                                 | Tetracycline 1 µg ml⁻¹  |
Appendix 4

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### Appendix 5

#### A)

<table>
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<tr>
<th>Query sequences</th>
<th>3D-PSSM</th>
<th>mGenTHREADER</th>
<th>I-TASSER</th>
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<tbody>
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<td><strong>E-value</strong></td>
<td><strong>Protein</strong></td>
<td><strong>E-value</strong></td>
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<td>1.80e-01</td>
<td>Human transportin</td>
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<td>human importin-β</td>
<td>2.96e-02</td>
<td>Human PP2A subunit</td>
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<tr>
<td>Homosapiens separase</td>
<td>human PP2A subunit</td>
<td>3.01e-02</td>
<td>Mouse α-adaptin</td>
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<td>human PP2A subunit</td>
<td>7.25e-02</td>
<td>Human transportin</td>
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<tr>
<td>S. pombe Cut1</td>
<td>human PP2A subunit</td>
<td>2.16e-01</td>
<td>Mouse α-adaptin</td>
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</table>

#### B)

![Image 1](image1.png)

![Image 2](image2.png)
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<th>Sequence</th>
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</tbody>
</table>
APPENDIX REFERENCES


**Membership of Professional Associations**

1- Member of British Society of Parasitology (BSP)
2- Member of American Society of Parasitology (ASP)
3- Member of Egyptian Society of Parasitology (ESP)