An Essential Cell Cycle-regulated Nucleolar Protein Relocates to the Mitotic Spindle Where It Is Involved in Mitotic Progression in *Trypanosoma brucei*

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Trypanosoma brucei and TbNOP66 are two novel nucleolar proteins isolated in *Trypanosoma brucei*. They share 92.6% identity, except for an additional C-terminal domain of TbNOP86 of 182 amino acids in length. Both proteins are found in *Trypanosoma* brucei, but similarity to other eukaryotic proteins could not be found. TbNOP86 and TbNOP66 are expressed at similar level in procyclic and bloodstream forms, although the relative level of expression of TbNOP66 is 11 times lower. TbNOP86 undergoes post-translational modifications, as it is found predominantly at 110 kDa compared with the predicted 86 kDa. Immunofluorescence of overexpressed ty-tagged TbNOP86 and TbNOP66 showed that both proteins accumulated in the nucleolus of G1 cells. This was confirmed by the co-localization of an endogenous TbNOP86-myc with the nucleolar protein Nop140. TbNOP86-ty localization is cell cycle-regulated, because it co-localizes with the mitotic spindle in mitotic cells. TbNOP86 is required for mitotic progression in both life stages as depleted cells are enriched in the G2/M phase. In procyclic cells, a reduced growth rate is accompanied by an accumulation of zoids (0N1K), 2N1K, and multinucleated cells (xNyK). The 2N1K cells are blocked in late mitosis as nucleolar segregation is completed. TbNOP86 depletion in bloodstream form caused a drastic growth inhibition producing cells bearing two kinetoplasts and an enlarged nucleus (1N*2K), followed by an accumulation of 2N2K cells with connected nuclei and xNyK cells. These studies of TbNOP86 provide a more comprehensive account of proteins involved in mitotic events in trypanosomes and should lead to the identification of partners with similar function.

Trypanosoma brucei is an ancient protozoan parasite and an agent of important diseases in human (African sleeping sickness) and cattle (nagana). The cell division cycle in *T. brucei* displays unique characteristics compared with other eukaryotes (1), and cellular morphology is determinant in the cell cycle progression (2). Indeed, cell division requires replication and segregation of the nucleus, kinetoplast (representing the mitochondrial DNA), flagellum, and basal body. The sequential G1, S, G2, and M phases that constitute the eukaryotic nuclear cell cycle is present in trypanosomes (3). Briefly, G1 cells have one nucleus and one kinetoplast (1N1K). The kinetoplast and nuclear DNA have a discrete S phase such that segregation of the replicated kinetoplast is completed before onset of mitosis. This results in 1N2K cells in late G2, followed by mitosis (M phase) resulting in 2N2K cells and then cytokinesis producing two 1N1K cells.

Mitosis must ensure faithful transmission of the nuclear genetic material to daughter cells. A more detailed model of the G2/M transition is starting to emerge in trypanosomes, a model that contrasts with the general eukaryotic model wherein numerous cellular checkpoints tightly coordinate mitotic progression and cytokinesis. Compared with other eukaryotes, cell cycle regulation in *T. brucei* appears simpler, as only a limited number of conserved mitotic proteins involved in the G2/M phase have been identified: cyclin homologs (CYC6 (4) and CycB2 (5)), ccdc2-related kinase (CRK3) (6), aurora kinase homologue (TbAUK1) (7) and homologues of the anaphase-promoting complex/cyclosome (APC1 and CDC27) (8). Different cell cycle mechanisms operate as the parasite alternates between the procyclic (insect stage) and bloodstream form (mammalian stage). In procyclic cells, there is a dissociation of mitosis and cytokinesis such that entry into cytokinesis does not rely on mitosis or nuclear synthesis but mainly on basal body segregation (9). Inhibition of mitosis by RNAi2 (CYC6 (4) and CRK3 (6)) or with anti-microtubule agents (2) results in the generation of anucleated cells (zoids) (0N1K) and cells with an enlarged nucleus (1N*1K, 1N* being a replicated DNA delayed segregation of the replicated kinetoplast is completed before onset of mitosis). This suggests novel cell cycle checkpoints and contrasts with bloodstream cells, which cannot enter cytokinesis under the same conditions (6). Accordingly, the study of mitotic proteins in *T. brucei* has highlighted distinctive stage-specific phenotypes following mitotic disorders. Depletion of the mitotic cyclin CYC6 caused enrichment of 0N1K in procyclic forms and cells with one nucleus and multiple kinetoplasts.

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**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The work described in this study uses *T. brucei* 427 strains co-expressing T7 RNA polymerase and tetracycline repressor (22). The *T. brucei* 427 host cell line 29-13 procyclic forms were cultured at 27 °C in SDM79 medium (Sigma) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Invitrogen), and 5 μg.ml⁻¹ hemin. G418 (10 μg.ml⁻¹) and hygromycin B (25 μg.ml⁻¹) were added to the culture medium to maintain the selection of T7RNAP- and TetR-expressing cells. *T. brucei* 427 host cell line 90-13 bloodstream forms were cultured at 37 °C in Iscove’s modified Dulbecco’s medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 36 mM sodium bicarbonate, 136 μM-l⁻¹ hypoxanthine, 39 μg.ml⁻¹ thymidine, 110 μg.ml⁻¹ sodium pyruvate, 28 μg.ml⁻¹ bathocuprone, 0.25 mM β-mercaptoethanol, 2 mM l-cysteine, and 62.5 μg.ml⁻¹ kanamycin (23). The bloodstream form cell line uses G418 (2 μg.ml⁻¹) as a single marker for co-expression of T7RNAP and TetR.

**Preparation of the Insoluble Protein Fraction and Characterization of NOP86**—Bloodstream form total cell extracts were prepared as described previously (24), with an additional Triton X-100 treatment (25). The insoluble protein fraction was loaded on a 10% SDS-polyacrylamide gel. The 110-kDa Coomassie Blue-stained band was digested in the gel with trypsin, and the peptide mixture was analyzed by on-line capillary high-performance liquid chromatography coupled to a nanospray ion trap mass spectrometer (26).

**Transfection**—Transfection of procyclic cells was performed essentially as described previously (27). Cells transfected with pLew100 or p2T7ti vectors were selected with 5 μg.ml⁻¹ phleomycin and cloned by limiting dilutions in microtiter plates. To induce the expression of epitope-tagged protein or RNAi, stable transfectants were cultured with 1 μg.ml⁻¹ tetracycline. Transfectants expressing endogenous TbNOP86-myc were selected with 1 μg.ml⁻¹ puromycin. Transfection of bloodstream cells was carried out at 37 °C. Briefly, 2 × 10⁶ cells were harvested, washed twice with Cytomix buffer, pH 7.6 (2 mM EGTA, 120 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄/KH₂PO₄, pH 7.6, 25 mM Hesper, 0.5% glucose, 1 mM hypoxanthine, 100 μg.ml⁻¹ bovine serum albumin, 5 mM MgCl₂) and suspended in 0.4 ml of Cytomix with 10 μg of linearized DNA in a 2-mm electroporation cuvette (setting: 1.25 kV, 50 microfarads and 25 ohms). Cells were immediately suspended in 24 ml of Iscove’s modified Dulbecco’s medium, transferred to a 24-well plate, and incubated at 37 °C for 24 h. Transfectants were selected with 2.5 μg.ml⁻¹ phleomycin and cultured with 1 μg.ml⁻¹ tetracycline for RNAi induction.

**Plasmid Constructs**—*T. brucei* TbNOP86 and TbNOP66 are annotated on GeneDB as Tb09.160.1160 and Tb09.160.1180, respectively. According to an earlier annotation release, we have used an ATG located 9 bp downstream of the actual one. Constructs for the expression of TbNOP86 and TbNOP66 ty-tagged proteins were cloned in the pLew100 vector (28), digested with HindIII-BamHI. TbNOP66-ty and TbNOP66-ty were amplified by PCR from *T. brucei* total genomic DNA using Pfx polymerase (Invitrogen). 5’ primers contained a HindIII restriction site (underlined); 3’ primers contained the gene encoding the ty-1 tag (29) (boldface) and a BamHI restriction site (underlined). The ty sequence (EVHT-NODPLD) was modified at the nucleotides level to eliminate the BamHI site. TbNOP86-ty was amplified using 5’ primer 5’-aagtgtaaggagctTTAGACCGGAATACACATG (named P5’) and 3’ primer 3’-aagtgtaaggagctTTAGTCGAAGGG-

(1NxK) in bloodstream forms (4). CRK3-depleted procyclic cells were enriched in 1N*1K and 0N1K as compared with the bloodstream-depleted cells enriched in 1N2K and multinucleated cells (xNyk) (6). Moreover, mitotic proteins were shown to have differential activities in the two life stages of the parasite, because APC1 and CDC27 depletion arrested procyclic cells in metaphase and bloodstream cells in anaphase (8). Functional genomic studies in *T. brucei* have also shown that non-mitotic proteins could play a role in mitotic progression. For example, knockdown of TbAGO1, which itself is required for RNAi, produced abnormal mitotic phenotypes (10).

Previous studies have provided some information about the dynamic morphology of the *T. brucei* nucleus during mitosis (11). The specific stages of mitosis are not well characterized, although a general progression similar to eukaryotic mitosis is observed. In contrast to higher eukaryotes, there is no chromatin condensation, and the mitotic spindle forms within the nucleus without disruption of the nuclear envelope. Chromosomes are segregated by interaction with the mitotic spindle (12), but precise details of the underlying mechanisms remain poorly understood, because no typical centromeric proteins or kinetochore proteins have yet been identified.

The nucleolus is a key sub-nuclear organelle that coordinates the synthesis and assembly of ribosomal subunits (reviewed in Ref. 13). It is also an important pool of dormant proteins, some of which are key regulators of the cell cycle. In contrast to higher eukaryotes, mitotic segregation of the nucleolus in lower eukaryotes proceeds without disassembling nucleolar structure. Accordingly, the nucleolus of trypanosomes, with the exception of *Trypanosoma cruzi* (14), remains intact throughout the mitotic cycle. The nucleolus is segregated along the mitotic spindle, acquiring a bar-shaped form early in mitosis before splitting into two equal clusters at a stage that resembles anaphase in a standard mitotic model (11). Few nucleolar proteins are described in *T. brucei*; most of them are involved in ribosomes biogenesis (NOG1 (15), NOP44/46 (16), and Nopp140 (17)) or VSG expression (ESAG8) (18). Protein kinase CK2, involved in cell cycle progression, was also shown to accumulate in the nucleolus where it interacts with NOG1 (19) and NOP44/45 (20). Nucleolar proteomics have revealed that the human nucleolus is composed of >700 proteins (21). The *T. brucei* genome data base (GeneDB) predicts 15 nucleolar proteins and ~200 ribosomal proteins, leaving considerable work to be done to identify these uncharacterized nucleolar proteins.

In this study, we have identified and characterized TbNOP86, an essential protein involved in mitotic progression in *T. brucei*. TbNOP86-ty localization is cell cycle-regulated, because the G₁ nucleolar protein co-localizes with the mitotic spindle, acquiring a bar-shaped form early in mitosis. TbNOP86-deficient cells are blocked at the G₂/M phase and display distinct phenotypes associated with the development stage of the parasite.

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ATCTTGTTAGTATGGACCTCAGATGCCTGTGTGCA-CAGATGCATC (named P86ty-3'). Tbnop66-ty was amplified using the following set of primers: (P5') and 3' -agatgtaggtgggttcACATGGAAGATGTTGTTAGTGGAC-CTCCTCCAACCTCTCTCCCTTCAC (named P66ty-3').

The construction of the expression vector for Tbnop86-myc integration into the Tbnop66 locus required a two-step cloning into the pMOTag23M vector (30): we cloned the first 300 bp of Tbnop86 3'-untranslated region amplified with 5'-agaatgtaggtgggttcACATGGAAGATGTTGTTAGTGGAC-CTCCTCCAACCTCTCTCCCTTCAC (named P66ty-3'). The construction of the expression vector for Tbnop86-myc integration into the Tbnop66 locus required a two-step cloning into the pMOTag23M vector (30): we cloned the first 300 bp of Tbnop86 3'-untranslated region amplified with 5'-agaatgtaggtgggttcACATGGAAGATGTTGTTAGTGGAC-CTCCTCCAACCTCTCTCCCTTCAC (named P66ty-3').

Secondary antibodies used were horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit IgG (1:5,000 and 1:10,000, respectively, Sigma). Peroxidase activity was revealed with 3,3'-diaminobenzidine tetrahydrochloride (Sigma). The relative level of protein was estimated with Image software (National Institutes of Health).

Immunofluorescence and Microscopy—Trypanosomes were centrifuged from log phase cultures for 5 min, washed in PBS, allowed to settle on poly-l-lysine-coated slides, and fixed 1 h with methanol at −20 °C. After a brief wash in PBS at room temperature, fixed cells were incubated at room temperature with primary and secondary antibodies (diluted in PBS) for 1.5 h and 30 min, respectively, and washed three times for 5 min with PBS after each of the incubations. Primary antibodies were undiluted monoclonal mouse anti-ty (BB2), monoclonal mouse anti-myc (1:20, Santa Cruz Biotechnology), polyclonal rabbit anti-Nop1 (1:1,000), a monoclonal mouse anti-human β-tubulin (β-tub, 1:1,000, Sigma), an undiluted monoclonal mouse anti-ty (BB2) (29), and a monoclonal mouse anti-myc (1:200, Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were fluorescein isothiocyanate-conjugated goat anti-mouse and goat anti-rabbit IgG 1:100 (Molecular Probes), and Texas Red-conjugated goat anti-mouse and goat anti-rabbit IgG 1:100 (Molecular Probes). Finally, cells were incubated 7 min with 1 μg.ml⁻¹ DAPI and mounted in Vectashield (Vector Laboratories, UK). Cells were analyzed on a Zeiss UV microscope, and images were captured by a MicroMax-1300Y camera (Princeton Instruments) and MetaView software (Universal Imaging Corp.).

Flow Cytometry—Propidium iodide (PI) staining was performed as described previously (6). Briefly, cells were fixed overnight at 4 °C in PBS/70% ethanol (v/v). Cells were washed once in PBS and resuspended in 500 μl of PBS supplemented with PI (20 μg.ml⁻¹) and RNase DNase-free (10 μg.ml⁻¹) (Roche Applied Science). Cells were stained for 30 min at room temperature and analyzed with a BD Biosciences FACSCanto flow cytometer using Diva software.

RESULTS

Tbnop86 and Tbnop66 Proteins Share a Similar Molecular Organization—Highly purified insoluble proteins from T. brucei bloodstream forms were separated by SDS-PAGE. A 110-kDa protein was identified following in-gel digestions coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Fig. 1A, asterisk). The 110-kDa protein is encoded by Tb09.160.1160 and Tb09.160.1180 in the T. brucei GeneDB, renamed Tbnop86 and Tbnop66, respectively, based on their cellular localization (NOP for nucleolar protein, see Fig. 4) and their expected molecular mass. Both genes, located in tandem on chromosome 5, are present as one copy per haploid genome (Fig. 1A). The 110-kDa protein was identified following in-gel digestions coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Fig. 1A, asterisk). The 110-kDa protein is encoded by Tb09.160.1160 and Tb09.160.1180 in the T. brucei GeneDB and was used as one copy per haploid genome (Fig. 1A). It is interesting to note that the two sequences flanking Tbnop66 (annotated Tb09.160.1190 and Tb09.160.1170 in GeneDB), likely correspond to intergenic regions and are identical. Tbnop86 and Tbnop66 share similar molecular organization.
having four short divergent domains (named D1–D4) embedded in a common core region of 579 amino acids (Fig. 1). The common core regions are homologous with an overall identity of 92.6%. TbNOP86 has an additional specific domain of 183 amino acids at the C-terminal region. The start codon used throughout this report corresponded to an earlier GeneDB annotation and is located 3 amino acids downstream of the actual annotated start codon. TbNOP86 and TbNOP66 are highly acidic proteins (predicted isoelectric points of 4.4 and 4.7, respectively), because they are rich in glutamic acids (Glu) (~12%). The TbNOP86-specific domain is composed of ~11% aspartic acid (Asp), which is twice the number found in the common core region, explaining the lower predicted pI of TbNOP86. Known motifs were identified with PredictProtein: several putative protein kinase C and casein kinase II (CK2) phosphorylation sites, two coiled-coil domains, and a putative nuclear localization signal (NLS-1) were noted. We identified a second putative NLS (NLS-2) based on the arginine and lysine content and sequence alignment with a few known NLS sequences in trypanosomes.

Genomic Organization of TbNOP86 and TbNOP66 Orthologues in Trypanosomatidae—A data base search with TbNOP86 showed no significant sequence homology to known proteins. Nevertheless, TbNOP86 and TbNOP66 orthologues were found in all available Trypanosomatid genomes (Fig. 2). Discrimination between orthologues was difficult, because some orthologous genes encoded characteristics of both TbNOP86 and TbNOP66. For example, T. brucei gambiense (Tbgamb.21611) encodes tbNOP66 D4 and does not have the TbNOP86-specific domain (both characteristics of TbNOP66) but shares D1, D2, and D3 with TbNOP86. Therefore, the identification of orthologues was determined based on the presence or absence of the TbNOP86-specific domain. TbNOP66 orthologues were only found in African trypanosomes (T. brucei gambiense and T. congolense), whereas TbNOP86 orthologues were present in all trypanosomatids studied (T. brucei gambiense, T. congolesense, T. vivax, T. cruzi, and Leishmania), with one copy per haploid genome (two genes are annotated in T. cruzi as the genome project reference strain is a hybrid).
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Expression of TbNOP86 and TbNOP66 in Procyclic and Bloodstream Forms—The expression of TbNOP86 and TbNOP66 in T. brucei 427 was tested by immunoblot analysis of total cell extracts from procyclic and bloodstream forms (Fig. 3). Blots were analyzed with a rabbit polyclonal antibody raised against both proteins (rNOP). TbNOP66 and TbNOP86 are predicted to be 66- and 86-kDa proteins, respectively. Accordingly, TbNOP66 corresponds to the weak band present at 66 kDa, and it is expressed at a similar level in both life stages, as indicated by the relative intensity of the band compared with the internal marker β-tubulin. TbNOP86 corresponds to the weak band at the expected 86-kDa position (asterisk) and the major band detected at 110 kDa (confirmed with ty-tagged and myc-tagged TbNOP86, see Fig. 4, A and B). TbNOP86 is also expressed at a similar level in both life stages. However, relative protein quantification comparing the 66-kDa band to the 110-kDa band indicated that the level of expression of TbNOP86 is ~11-times higher than TbNOP66. This 11-fold difference in protein expression was also observed at the transcript level as confirmed with reverse transcription-PCR and Northern blot experiments performed on procyclic total mRNA (data not shown).

Nucleolar Localization of TbNOP86 and TbNOP66 in Procyclic Cells—We first fused a ty peptide tag to the C terminus of TbNOP86 and used the tetracycline-inducible system to assess a specific sub-nuclear localization in procyclic T. brucei 427 (Fig. 4A). Western blot analyses were performed on total cell extracts with the BB2 antibody directed against the ty epitope for recognition of the tagged proteins. TbNOP86-ty was detected ~110 kDa in the tet-induced population. Blots performed with rNOP and the internal marker β-tubulin showed that the overall level of expression of TbNOP86 in the induced cell line (TbNOP86 plus TbNOP66-ty) was ~1.5-fold above the TbNOP86 endogenous level in the non-induced population. The localization of TbNOP86-ty was assessed with immunofluorescence (IF) analysis performed on methanol-fixed cells co-stained with BB2 and Nopp140 and counterstained with DAPI. Nopp140 was used as a nucleolar marker, because it was shown to specifically stain the nucleolus using methanol fixation (17). Still, the nucleolus is visible in the phase-contrast image as a dark dot within the nucleus. A merged image of BB2 (green) and DAPI (blue) revealed that TbNOP86-ty is detected as a single dot within the nucleolus in G1 cells (dark region of the DAPI-stained nucleus). As expected, Nopp140 was also shown to stain the nucleolus. A merged image of BB2 and Nopp140 confirmed TbNOP86-ty nucleolar localization, although both proteins only partially overlapped. No fluorescence was detected with BB2 in the non-induced cells (data not shown). To show that the nucleolar localization was not due to overexpression or the ty tag, we made a construct for the integration of an myc-tagged TbNOP86 directly into the TbNOP6 locus (Fig. 4B). Western blots performed with the anti-myc antibody showed that TbNOP86-myc was detected around the predicted 110–116 kDa. However, no fluorescence was detected with BB2 in the non-induced cells (data not shown).
kDa. Blots with rNOP confirmed the endogenous expression of TbNOP86-myc, because no overexpression was detected compared with the non-transformed cells. As for the overexpressed TbNOP86-ty, endogenous TbNOP86-myc was shown to accumulate in the nucleolus (DAPI plus Myc) and to partially co-localize with the nucleolar marker Nopp140 (DAPI plus Myc plus Nopp140). We assessed TbNOP66 localization with TbNOP66-ty-expressing cells (Fig. 4C). TbNOP66-ty was detected around the expected 66.7 kDa (expected size for TbNOP66 plus the ty tag). Immunofluorescence revealed that TbNOP66-ty is also localized in the nucleolus (DAPI plus BB2).

The rabbit (rNOP) and a mouse (mNOP, data not shown) antiserum recognized TbNOP86 and TbNOP66 on Western blots. Immunofluorescence experiments showed they were valuable tools for the localization of overexpressed TbNOP86-ty (see Fig. 9B) and TbNOP66-ty (data not shown). However, they were unsuitable for IF performed on non-transformed cells, because we mostly observed a weak nuclear and nucleolar signal (supplemental data, Fig. S1, mNOP) and a cytoplasmic and weak nuclear signal (supplemental data, Fig. S1, rNOP).

Overexpression of nucleolar TbNOP86-ty induced decreased growth and morphological phenotypes after 6 days of tetracycline induction, a time frame that correlated with the cytoplasmic accumulation of the protein (supplemental data, Fig. S2). No phenotypes were associated with endogenous expression of TbNOP86-myc or TbNOP66-ty overexpression (data not shown).

TbNOP86 Depletion Causes Reduced Growth Rate and Mitotic Defects in Procyclic Cells—Inactivation of TbNOP86 synthesis was performed using RNAi. We attempted to knock down TbNOP86 and TbNOP66 simultaneously using a common 470-bp fragment cloned into the p2T7ti vector (Fig. 5). Western blot analysis with rNOP and β-tubulin showed that we were not able to fully inactivate TbNOP86 synthesis, because 70% depletion was the lowest level obtained after 8 days of tetracycline induction (Fig. 5A, inset). Growth curves showed that a 70% decrease in TbNOP86 level led to a significant growth rate reduction with a doubling time of 29.5 h (H11001 tet) compared with 16.8 h for non-induced cells (H11002 tet) and 12.9 h for non-transformed cells. The intermediate growth curve of the non-induced population suggested that there was a leak in the RNAi system within these cells. FACS analysis of the DNA content showed an accumulation at the G2/M phase, which corresponded to the 4N population (where N represents the haploid DNA content) (Fig. 5B). From day 1, we detected a significant accumulation of polyploid cells (with distinct 6N and 8N populations) and possibly 0N1K cells (accumulation of cells downstream of the 2N population at days 3 and 6). These changes in the distribution of cell morphologies were confirmed by visualization of the DNA content at different time points (Fig. 5C). DAPI-stained cells allowed the visualization of the kinetoplast DNA (k) and the nuclear DNA (n). 0N1K and 2N1K cells started to accumulate at day 1 along
with a decrease in 1N2K cells. The accumulation of multinucleated cells (xNyK) at day 3 suggested that the 2N1K cells likely re-enter multiple rounds of nuclear S phase and mitosis.

Because both TbNOP86 and TbNOP66 were targeted for RNAi, TbNOP66 down-regulation occurred during the 8-day period of RNAi (data not shown), but the phenotypes described above are associated with TbNOP86 depletion. Indeed, we performed specific depletion of TbNOP66 using its 3′-untranslated region and showed that it did not result in any growth or morphological phenotypes (supplemental data, Fig. S3).

**TbNOP86 Depletion Causes Growth Arrest and Mitotic Defect in Bloodstream Cells**—In the bloodstream form, TbNOP86 knockdown within 24 h of RNAi induction as showed by Western blotting (Fig. 6a, inset). Cells stopped proliferating and died within 4 days of RNAi. FACS analysis showed that at time 0 h, the total population distribution of viable non-aggregated G1, S, and G2/M cells was 62.9%, 13.5%, and 20.9%, respectively (Fig. 6B, time 0 h and lower right panel). Within the first 24 h of induction, FACS analysis showed an enrichment of G2/M cells (increase of 10%), and a reduction of S-phase (decrease of 7%) and G1 cells (decrease of 16%). At 24 h, polyploid cells appeared and represented ~20% of the total cell population. As for procyclic cells, the accumulation at the G2/M phase is likely a consequence of nuclear mitosis inhibition. However, cytokinesis is tightly coupled to mitosis in bloodstream forms; therefore, disturbed mitosis could conceivably block cytokinesis within 24-h post-induction. Based on DNA configuration, the morphological distribution of TbNOP86-depleted cells correlated with the FACS results (Fig. 6C). We observed an accumulation of 1N2K cells (G2/M phase) within 12 h of induction and a decrease in the number of 1N1K cells. The 2N2K and xNyK cells increased dramatically. TbNOP66 could not be detected in (−tet) and (+tet) population, because lysates from only 1 × 10^6 cells/lane were loaded on gels for Western blot analysis.

**TbNOP86 Involvement in Mitosis Is Developmentally Regulated**—Mitotic defects in TbNOP86-depleted cells resulted in distinctive stage-specific phenotypes (Fig. 7). In procyclic cells, the major phenotypes were 2N1K, 0N1K, and xNyK cells (Fig. 7A). The two nuclei in 2N1K cells were always connected. All nuclei in the 2N1K and xNyK cells exhibited a darkly stained nucleolus (Fig. 7A, left panel). Immunofluorescence performed on RNAi-induced cells with the nucleolar marker Nopp140 confirmed that nucleolar segregation occurred in the 2N1K and xNyK cells (right panel). As segregation of the nucleolus occurred in anaphase (when the two daughter cells inherit equal nucleolar material), 2N1K cells exhibiting two nucleoli are likely blocked in a later stage of mitosis.

In bloodstream forms, the accumulation at the G2/M phase is first due to an accumulation of 1N2K cells, but more precisely 1N*2K cells bearing an enlarged nucleus and two segregated kinetoplasts (Fig. 7B, left panel). Later the cells are blocked in late mitosis with 2N2K cells exhibiting con-
connected nuclei. Compilation of abnormal phenotypes showed that the DAPI staining of 1N1K cells remained stable throughout the induction period (right panel). However, the percentage of 1N2K cells with an enlarged nucleus (1N*2K) increased to 70% compared with control cells, and the number of 2N2K cells with two connected nuclei increased to ~60%. Overall, a similar phenotype to procyclic cells is generated: xNyK cells with connected nuclei and the number of K inferior or equal to N.

**TbNOP86 Nucleolar Distribution Is Disrupted during Mitosis**—The distribution of TbNOP86-ty was assessed during the complete cell cycle of *T. brucei* with BB2 staining (Fig. 8). As seen in Fig. 4A, TbNOP86-ty is distributed in the nucleolus in G1 phase cells (Fig. 8A). In late G2 (1N2K), when the cell has not yet entered mitosis, we still observed one stained dot in the nucleolus (Fig. 8B). In early mitotic cells, TbNOP86-ty co-localized with the rhomboid-shaped mitotic spindle clearly visible in phase contrast (Fig. 8C). TbNOP86-ty also co-localized with the mitotic spindle in mid-mitotic cells (Fig. 8D). In 2N2K cells, TbNOP86-ty is redistributed as a dot in the nucleolus (Fig. 8E).

Although phase-contrast images are indicative, we performed additional IF on mitotic cells to rule out the possibility that TbNOP86-ty is confined in the nucleolus during mitosis (Fig. 9). Contrary to what we observed in G1 cells, co-staining of BB2 with Nopp140 performed at three stages of mitosis showed that TbNOP86-ty did not co-localize with the nucleolar marker (Fig. 9A). In early mitotic cells, the nucleolus (Nopp140) is localized within the rhomboid mitotic spindle (upper panel). Nopp140 is then distributed at the extremity of BB2 staining, likely corresponding to the mitotic spindle (middle panel). At a later stage of mitosis, when the two nuclei are segregated but still connected by the mitotic spindle, TbNOP86-ty still localized on the mitotic spindle, whereas the nucleolar marker is segregated to each nucleus (lower panel). All co-localization of TbNOP86-ty with the mitotic spindle were confirmed by merged of BB2 staining with phase-contrast images (data not shown). Co-staining of rNOP with the β-tubulin antibody KMX-1 (35) confirmed that TbNOP86-ty co-localized with the mitotic spindle in mitotic cells (Fig. 9B).
We have provided evidence indicating that TbNOP86 is essential for the faithful progression of mitosis in *T. brucei*. Flow cytometry analysis coupled to studies of morphological phenotypes showed that TbNOP86-depleted cells accumulate at the G2/M phase in both procyclic and bloodstream cells. In the procyclic form, the accumulation of 0N1K and 2N1K cells is indicative of cells undergoing cytokinesis but with an incomplete mitosis. This is the same cell phenotype observed in previous studies on mitotic inhibition (CRK3 (6) and CYC6 (4)) or with the microtubule inhibitor rhizoxin (2). The accumulation of 2N1K cells takes place after nucleolar segregation, suggesting an arrest in late mitosis, possibly in late anaphase or telophase as nucleolar segregation occurs in anaphase. In the bloodstream form, cells arrested at the G2/M phase first accumulated at the 1N*2K stage. The enlarged nucleus in these cases points to a block in early mitosis. The 1N*2K cells were able to progress into mitosis but were blocked again in late mitosis, as suggested by the appearance of interconnected nuclei. We cannot exclude the possibility that an accumulation of 1N*2K cells also occurred in procyclic cells, because the RNAi system was somewhat leaky. Still, stage-specific arrest related to TbNOP86 depletion would support observations made in recent studies showing that the checkpoint mechanism differs between procyclic and bloodstream forms (APC1/CDC27 (8) and TRF (36)).

In procyclic cells, mitotic arrest did not prevent cytokinesis (2N1K and 0N1K), and cells managed to divide with a decreased growth rate. This contrasts with the situation in bloodstream forms where depletion of TbNOP86 occurred rapidly and caused a rapid growth arrest. Still, TbNOP86 depletion must have triggered the activation of a specific cell cycle checkpoint in both life stages as the completion of mitosis was blocked. However, the cell cycle arrest was overcome as cells re-entered successive S-phases and mitoses with a significant accumulation of xNyK cells. Similar accumulation of xNyK has been obtained through the RNAi of proteins involved in mitosis (TbAUK1 (37)) and cytokinesis (polo-like kinase (38), RACK1 homologue (39), and MOB1 (40)).

The number of kinetoplasts in the xNyK cells generated from TbNOP86 knockdown was always less than or equal to the number of nuclei. We believe that down-regulation of TbNOP86 further inhibited the kinetoplast cycle. TbNOP66 is not an essential protein, and its depletion in procyclic cells did not induce any morphological or growth phenotype.

We showed that TbNOP86-ty and TbNOP66-ty localize in the nucleolus of procyclic G1 cells. It has been shown previously that nucleolar antibodies behave differently following different fixation procedures (17). Still, we believe that the nucleolar localization of TbNOP86-ty and TbNOP66-ty is accurate, because it was observed with both methanol and formaldehyde fixations (data not shown). The nucleolar localization is not reporter gene-dependent, because the ty tag was shown to be neutral with respect to the localization of the tagged proteins. The nucleolar accumulation of TbNOP86-ty is not an artifact due to overexpression, because endogenous expression of TbNOP86-myc into the *TbNOP86* locus also resulted in nucleolar accumulation.

As for other nucleolar proteins characterized in *T. brucei* (NOG-1 (15) and ESAG (18)), TbNOP86 and TbNOP66 do not contain any RGG boxes in their amino acids sequences, a char-
characteristic motif common to those nucleolar proteins participating in binding to nucleic acids (T. brucei Nopp140 (17), Nopp44/46 (41), and fibrillarin). TbNOP86 was first characterized as a 110-kDa protein present in the insoluble protein fraction of T. brucei (confirmed with mNOP on soluble/insoluble protein extracts, data not shown). Because the nucleolus is not membrane bound, it is possible that a strong association with insoluble nuclear remnants could explain the insoluble state of TbNOP86, as seen with other nucleolar proteins such as Nopp140 (17).

Due to low sequence conservation, we could not find any significant homology with known eukaryotic proteins. At this point we do not know whether TbNOP86 has a homologue outside the Trypanosomatidae, making it difficult to specifically address the role of TbNOP86 in mitotic progression. TbNOP86 and TbNOP66 both lack any motifs that would suggest a role in processing such as RNase or helicase domains. Important interactions with other proteins are indicated, because several putative kinase C and casein kinase II (CK2) phosphorylation sites and two coiled-coil domains are predicted in both protein sequences. Interestingly, the protein kinase CK2 from T. brucei was shown to be localized in the nucleus and nucleolus (19).

It is not surprising that a protein involved in mitosis has a precise nuclear localization. We have provided evidence that TbNOP86-ty localization is cell cycle-dependent. Because TbNOP86-ty is predominantly found in the nucleolus in G1 cells, it is released from the nucleolus as cells enter mitosis wherein TbNOP86-ty co-localizes with the mitotic spindle at all stages of mitosis. TbNOP86-ty likely travels back to the nucleolus after mitosis is completed, because it is still localized on the mitotic spindle in late mitotic cells. To our knowledge, the T. brucei aurora kinase homologue (TbAUK1) involved in spindle formation (7, 37) and the Leishmania major Kin-13 kinesin involved in mitotic progression (42), both nuclear proteins, are the only other proteins shown to co-localize with the mitotic spindle in trypanosomatids (aside from β-tubulin (35)). Indeed, there is an obvious lack of homologues

FIGURE 8. TbNOP86-ty localization is cell cycle-regulated in the procyclic form. A, nucleolar localization of TbNOP86-ty in G1 (1N1K) and B, late G2 cells (1N2K). In early (C) and mid-mitotic cells (D), TbNOP86-ty re-localized to the mitotic spindle as seen by the merged images of phase and BB2 staining. E, in 2N2K cells, TbNOP86-ty is redistributed to the nucleolus. The left image in each panel corresponds to phase contrast, the second panel is DAPI staining, third is BB2 IF, fourth is a merged image of DAPI and BB2, and fifth is a merged image of phase and BB2. Bar, 5 μm.

FIGURE 9. TbNOP86-ty co-localization experiments at different stages of mitosis. A, cells expressing TbNOP86-ty were co-stained with BB2 and the nucleolar marker Nopp140. TbNOP86-ty did not co-localize with Nopp140 during mitosis (merged of BB2, Nopp140, and DAPI). Early (upper panel), mid (middle panel), and late (lower panel) mitotic cells are shown. Bar, 2.5 μm. B, cells expressing TbNOP86-ty were co-stained with rNOP and KMX-1 (specific for the β-tubulin in the mitotic spindle). As shown in Fig. 8 (c and d), TbNOP86-ty co-localized with the mitotic spindle at mitosis. Bar, 4 μm.
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involved in mitosis in the trypanosomatid databases (43), because no centromeric or kinetochore proteins have yet been identified.

We do not believe that TbNOP86 is involved in nucleolar segregation, because orthologues are found in T. cruzi species, which disassembles its nucleolus during mitosis (14). TbNOP86 depletion in the procyclic form does not play a role in entry into cytokinesis. Moreover, most of the proteins known to play a role in cytokinesis in T. brucei are excluded from the nucleus (TbNRK, found in the basal bodies, (44), MOB-1 in the cytoplasm (40), RACK1 in the perinuclear region and in the cytoplasm (39), and PLK1 in the flagellum attachment zone (38)). In humans and yeast, proteins sequestered in the nucleolus (39), and PLK1 in the flagellum attachment zone (40), RACK1 in the perinuclear region and in the cytoplasm (40), TbNOP86 is released from the nucleolus where it localizes with the mitotic spindle in mitotic cells.

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REFERENCES

1. McKean, P. G. (2003) Curr. Opin. Microbiol. 6, 600–607
2. Robinson, D. R., Sherwin, T., Ploubidou, A., Byard, E. H., and Gull, K. (1995) J. Cell Biol. 128, 1163–1172
3. Woodward, R., and Gull, K. (1990) J. Cell Sci. 95, 49–57
4. Hammarton, T. C., Clark, J., Douglas, F., Boshart, M., and Mottram, J. C. (2003) J. Biol. Chem. 278, 22877–22886
5. Li, Z., and Wang, C. C. (2003) J. Biol. Chem. 278, 20652–20658
6. Tu, X., and Wang, C. C. (2004) J. Biol. Chem. 279, 20519–20528
7. Tu, X., Kumar, P., Li, Z., and Wang, C. C. (2006) J. Biol. Chem. 281, 9677–9687
8. Kumar, P., and Wang, C. C. (2005) J. Biol. Chem. 280, 31783–31791
9. Ploubidou, A., Robinson, D. R., Docherty, R. C., Ogobayo, E. O., and Gull, K. (1999) J. Cell Sci. 112, 4641–4650
10. Durand-Dubief, M., and Bastin, P. (2003) BMC Biol. 1, 2
11. Ogobayo, E., Ersfeld, K., Robinson, D., Sherwin, T., and Gull, K. (2000) Chromosoma 108, 501–513
12. Ersfeld, K., and Gull, K. (1997) Science 276, 611–614
13. Nazar, R. N. (2004) IUBMB Life 56, 457–465
14. Elias, M. C., Marques-Porto, R., Freymuller, E., and Schenkin, S. (2001) Mol. Biochem. Parasitol. 112, 79–90
15. Jensen, B. C., Wang, Q., Kifer, C. T., and Parsons, M. (2003) J. Biol. Chem. 278, 32204–32211
16. Jensen, B. C., Brekken, D. L., Randall, A. C., Kifer, C. T., and Parsons, M. (2005) Eukaryot. Cell 4, 30–35
17. Kelly, S., Singleton, W., Wickstead, B., Ersfeld, K., and Gull, K. (2006) Eukaryot. Cell 5, 876–879
18. Hoek, M., Engstler, M., and Cross, G. A. (2000) J. Cell Sci. 113, 3959–3968
19. Jensen, B. C., Kifer, C. T., Brekken, D. L., Randall, A. C., Wang, Q., Drees, B. L., and Parsons, M. (2007) Mol. Biochem. Parasitol. 151, 28–40
20. Park, J. H., Brekken, D. L., Randall, A. C., and Parsons, M. (2002) Mol. Biochem. Parasitol. 119, 97–106
21. Andersen, J. S., Lam, Y. W., Leung, A. K., Ong, S. E., Lyon, C. E., Lamond, A. I., and Mann, M. (2005) Nature 433, 77–83
22. Wirtz, E., Leal, S., Ochatt, C., and Cross, G. A. (1999) Mol. Biochem. Parasitol. 99, 89–101
23. Baltz, T., Baltz, D., Giroud, C., and Crockett, J. (1985) EMBO J. 4, 1273–1277
24. Seyfang, A., and Duszenko, M. (1993) Eur. J. Biochem. 214, 593–597
25. Bakalaras, N., Santarelli, X., Davis, C., and Baltz, T. (2000) J. Biol. Chem. 275, 8863–8871
26. Blenc, J. F., Lalanne, C., Plomion, C., Schmitter, J. M., Bioulac-Sage, P., Balabaud, C., Bonneu, M., and Rosenbaum, J. (2005) Proteomics 5, 3778–3789
27. Brincaud, F., Baltz, D., and Baltz, T. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7963–7968
28. Wirtz, E., and Clayton, C. (1995) Science 268, 1179–1183
29. Bastin, P., Bagherzadeh, Z., Matthews, K. R., and Gull, K. (1996) Mol. Biochem. Parasitol. 77, 235–239
30. Oberholzer, M., Morand, S., Kunz, S., and Seebeck, T. (2006) Mol. Biochem. Parasitol. 145, 117–120
31. Ngo, H., Tschudi, C., Gull, K., and Ullu, E. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 14687–14692
32. Wang, Z., Morris, I. C., Drew, M. E., and En gland, P. T. (2000) J. Biol. Chem. 275, 40174–40179
33. LaCount, D. J., Bruse, S., Hill, K. L., and Donelson, J. E. (2000) Mol. Biochem. Parasitol. 111, 67–76
34. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
35. Sasse, R., and Gull, K. (1988) J. Cell Sci. 90, 577–589
36. Li, B., Espinal, A., and Cross, G. A. (2005) Mol. Cell Biol. 25, 5011–5021
37. Li, Z., and Wang, C. C. (2006) Eukaryot. Cell 5, 1026–1035
38. Kumar, P., and Wang, C. C. (2006) Eukaryot. Cell 5, 92–102
39. Rothberg, K. G., Burdette, D. L., Pfannstiel, J., Jetton, N., Singh, R., and Ruben, L. (2006) J. Biol. Chem. 281, 9781–9790
40. Hammarton, T. C., Lillico, S. G., Welburn, S. C., and Mottram, J. C. (2005) Mol. Microbiol. 56, 104–116
41. Das, A., Peterson, G. C., Kanner, S. B., Frevert, U., and Parsons, M. (1996) J. Biol. Chem. 271, 15675–15681
42. Dubessay, P., Blaineau, C., Bastien, P., Tasse, L., Van Dijk, J., Crobu, L., and Pages, M. (2006) Mol. Microbiol. 59, 1162–1174
43. Berriman, M., Ghedin, E., Hertz-Fowler, C., Blandin, G., Renaudel, H., Bartholomeu, D. C., Lennard, N. J., Caler, E., Hamlin, N. E., Haas, B., et al. (2005) Science 309, 416–422
44. Pradel, L. C., Bonhivers, M., Landrein, N., and Robinson, D. R. (2006) J. Cell Sci. 119, 1852–1863
45. Kaiser, B. K., Zimmerman, Z. A., Charbonneau, H., and Jackson, P. K. (2002) Mol. Cell Biol. 13, 2289–2300
46. Trautmann, S., Rajagopalan, S., and McCollum, D. (2004) Dev. Cell 7, 755–762
47. Raemaekers, T., Ribbeck, K., Beaudouin, J., Annaert, W., Van Camp, M., Stockmans, I., Smets, N., Bouillon, R., Ellenberg, J., and Carmeliet, G. (2003) J. Cell Biol. 162, 1017–1029