Overexpression of Trypanosoma Brucei Polo Like Kinase TbPLK Inhibits Cytokinesis by Modulating Endogenous Protein Expression

Mohamed Bessat
Department of Parasitology/Faculty of Veterinary Medicine/Alexandria University, Alexandria, Egypt
mnooh2000@yahoo.com

***Abstract*—The single polo like kinase, TbPLK, regulates cytokinesis in *Trypanosoma brucei*. TbPLK is temporally and spatially regulated through the cell cycle of *T. brucei*. Temporally, TbPLK emerges in S-phase, maintains its expression in G2/M phase and vanishes in late mitosis through G1 phase. Spatially, TbPLK first appears at posterior end of FAZ, where it co-localizes with basal body, then progresses to the dorsal point of cell at FAZ till reaches the anterior tip of the cell. Even more the ectopic expression of TbPLK is detrimental to growth of procyclic *T. brucei* cells. Thus TbPLK seems to be a subject to tight control in trypanosome cells. Here, and by using growth curve and flow cytometry assays, cells with TbPLK ectopic expression were found with inhibited growth and cytokinesis with accumulation of cells with multiple nuclei and kinetoplasts. Cell growth and cell cycle progression, however, progress normally when kinase-dead mutant of TbPLK is overexpressed. Careful examination of endogenous TbPLK expression profiles after the two overexpression events, wild-type and kinase-dead mutant, reveals that endogenous protein is stabilized only with overexpression of wild-type protein. Therefore, the stabilized endogenous protein in the background of the overexpressed hyper-catalytic active TbPLK inhibits growth and cell division.**

***Index Terms*—Trypanosoma brucei, polo like kinase, cell cycle, overexpression**

I. Introduction

Trypanosoma brucei is a eukaryotic pathogen of kinetoplastid protozoa that causes the African trypanosomiasis, sleeping sickness in humans and Nagana in cattle. *T. brucei* cell divides by the longitudinal binary fission from anterior to posterior end of the cell. The cell division process is coherently organized to provide daughter cell with a copy each of duplicated single copy organelles such as nucleus, flagellum, mitochondrion and basal body [1], [2]. The flagellum attachment zone (FAZ) originates from the basal body at the posterior end of the cell and extends in a helical pathway along the dorsal side toward the anterior end where it connects tip of flagellum to the cell cortex [2], [3]. When *T. brucei* cell divides, a newly formed FAZ extends from daughter basal body following the path of the old FAZ providing the positional cues to the cleavage furrow and cytokinesis initiation site [3], [4]. The longitudinal division process of trypanosome is in stark contrast to that of metazoans, in which cytokinesis depends mainly on the formation of the actomyosin contractile ring and the membrane abscission [5]. The cell division process is also distinct between the two life cycle stages, the procyclic and bloodstream forms [1], [6]. In the bloodstream form, cytokinesis is mainly primed by the completion of mitosis [7], [8]. In the procyclic form, on the other hand, cells with inhibited mitosis are capable of cell division with production of nucleate cells or zoids [9]. Thus, studying the molecular control of cell division in *T. brucei* and proteins that regulate this process constitutes an interesting aspect of trypanosome biology. *T. brucei* maintains a number of highly conserved proteins that regulate cell division in other eukaryotes. The unique aspect of *T. brucei* cytokinesis is apparently regulated by polo homologue, polo-like kinase (PLK). *T. brucei* genome expresses only a single PLK homologue, TbPLK with a conserved structure possessing two polo boxes at the C-terminus and a kinase domain at the N-terminus [10]. This is different from metazoans where several PLK homologues are expressed and only similar to the single PLK homologue of budding yeast, CDC5 [11], [12]. Indeed, TbPLK maintains all the functional capabilities to replace the deletion mutant of *S. cerevisiae* CDC5 [9]. In yeast, CDC5 is a nuclear protein that functions to regulate G2/M transition, the metaphase-anaphase transition, anaphase release and mitotic exit [11], [13]. However, TbPLK is excluded from the nucleus throughout the cell cycle of *T. brucei* and regulates only cytokinesis with no apparent control over mitosis [9]. Instead, the protein associates with the basal body and its related bilobed structure involved in Golgi duplication when it emerges in S-phase [14], [15]. During G2 and early mitosis, TbPLK translocalizes along the growing tip of the replicating FAZ at the dorsal mid-point toward the anterior end of the cell [9], [15] before dissipating into cytoplasm at anaphase [16]. The FAZ localization of TbPLK is dependent on its two polo boxes with no involvement of its kinase activity [17], [18]. Therefore and consistent with its unique localization, no mitotic functions were recorded for TbPLK with roles only limited to regulating basal body duplication, kDNA segregation, Golgi duplication, flagellum inheritance and cytokinesis [9], [14], [19], [20]. Inhibiting the TbPLK

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activity by drug inhibitor or through RNAi depletion upon its emergence at S-phase specifically inhibits cytokinesis initiation within the same cell cycle [16].

In procyclic form *T. brucei*, ectopic expression of Ty-tagged TbPLK, but not kinase-dead variant, inhibited growth and cytokinesis due to defects in basal body duplication and kinetoplast segregation [19]. In a similar line of evidence, altered TbPLK activity through ectopic expression of wild-type TbPLK tagged with triple hemagglutinin (HA) tag, but not kinase-dead mutant, inhibited cell growth with accumulation of cells with multiple nuclei/kinetoplasts due to defective cytokinesis [18]. More intriguingly, both growth and cell cycle progression defects were relieved when the endogenous TbPLK was simultaneously depleted from same cells with overexpressed wild-type (not kinase-dead) protein through 3'-UTR RNAi knockdown [18]. Very similar data were obtained when TbPLK and its kinase-inactive mutant that are tagged with fluorescent (eYFP) protein were overexpressed in the presence or absence of endogenous protein knockdowns (Sun and Wang, unpublished data). It is hard to explain the resulting overexpression phenotypes in the light of their resemblance to those arisen from RNAi-induced depletion of native protein [9]. It could be that some interference between activity and/or expression levels of two proteins (ectopic and endogenous) which may held accountable for the observed growth and cell cycle defects, but no further studies were carried out to confirm these speculations.

Here in this report, I carried out detailed independent analysis of effects of TbPLK overexpression in procyclic *T. brucei* cells. Cell growth, cell cycle progression and protein expression profiles were compared after ectopic expression of wild-type kinase-active and kinase-dead mutant of TbPLK. I showed that only overexpression of catalytically active protein inhibits cell growth and cytokinesis. The expression kinetic of two ectopic proteins is, however, very similar with expression levels peak at G2 and early mitosis and proteins are degraded at anaphase. Nevertheless, I found out that the expression profile of endogenous TbPLK differs when wild-type versus kinase-inactive variant were overexpressed with endogenous protein is highly stabilized with former while echoes that of native protein with later. Data suggest that the slightest alteration of expression and activity of TbPLK is strong enough to elicit defects measurable to its depletion and that a physiologically balanced expression and turnover mechanism is very essential for the TbPLK function in initiating cytokinesis in the protozoan parasite of *T. brucei*.

II. Results

A. TbPLK Overexpression Inhibits Cytokinesis in T. Brucei

To get more insight about the cause of growth inhibition after protein overexpression, TbPLK was tagged at its C-terminus with eYFP and then was expressed from the exogenous inducible Tet system regulated by the tetracycline-inducible T7 promoter [21]. Upon Tet induction, the expression of TbPLK-eYFP fusion protein was detected with western blotting and the protein was localized to FAZs of cells as confirmed by the fluorescence microscopy (data not shown) similar to localization of endogenously tagged TbPLK either with eYFP or 3HA tags [15]. When the growth of cells was carefully monitored, the growth rate of the Tet-induced cells (when TbPLK is overexpressed) was lagging behind the control cells by 5-20% through the first two days of induction (Fig. 1A).

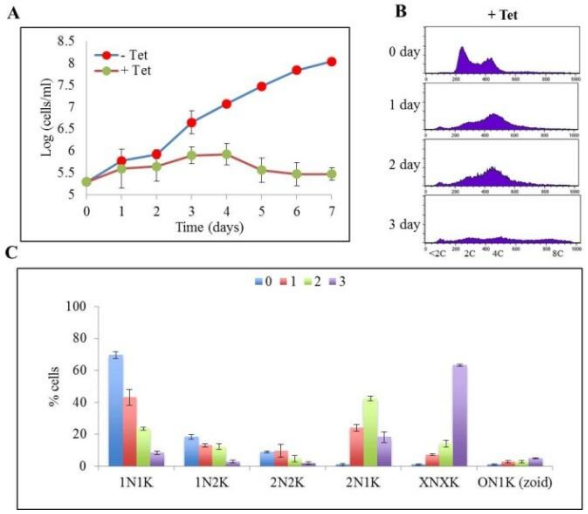

Figure 1. Overexpression of TbPLK.eYFP inhibits cell growth and cytokinesis of *T. brucei*. (**A**) 20-13 procyclic form cells harboring pLew100.PLK.eYFP were monitored for their growth by hemocytometer for seven successive days with (+ Tet) or without (- Tet) tetracycline induction. Error bars are derived from the standard deviation (±SD) of two independent experiments. (**B**) Cell samples from the induced cultures were stained with propidium iodide (PI) and were analyzed by flow cytometry for 4 days beginning at day 0 of Tet-induction. Data are presented as DNA FL2-A peaks (x) against cell counts (y). C (<2, 2, 4, 8) indicates the position of nuclear DNA contents. (**C**) Cell samples from the induced culture at days 0, 1, 2 and 3 of Tet-induction, were collected, stained with DAPI and examined under the epifluorescence microscope to determine configurations of nucleus (N)/kinetoplast (K) in individual cells. Error bars represent the ±SD of two independent experiments were >200 cells were recorded in each experiment.

On day 3, however, there was a complete cessation of growth of cells with overexpressed TbPLK with growth inhibition continued for the rest of the experiment. The overexpression-induced growth inhibition is similar to that resulting from the depletion of the endogenous protein by RNAi [9], except that the former has more immediate effect on the cell growth than the later. In our efforts to further characterize the resulting growth inhibition phenotype, a flow cytometric analysis demonstrated a cell cycle defect in cells with overexpressed protein as there was a drastic inhibition of 2C cells which is followed by accumulation 4C cells 2 days into Tet induction (Fig. 1B). Cells with abnormal DNA contents continued to accumulate in the induced culture with appreciable percentage of cells even developed 8C on day 3 of induction experiment. This is an early indication of continued DNA synthesis and nuclear divisions in the absence of cell divisions in the

light of appearance of 8C cells which later were characterized as multinucleate cells (see below).To build on our initial assumption that TbPLK overexpression ultimately led to inhibited cell division with continued DNA synthesis and mitosis, cell samples duplicate to that of flow cytometry were stained with DAPI and were categorized under the microscope for their NK configurations. Over the course of overexpression induction, there was a significant decrease in the number of cells with 1N1K from 72.5% to <7% of cell population on day 3 of the induction experiment (Fig. 1C). This is compensated by an increase in the cell population with multiple nuclei and kinetoplasts (xNxK) from 1.5% to 62.5%. In between the two events, there was a static decrease in dividing cells (1N2K and 2N2K) and a slight increase in cells with 0N1K (zoids). Aberrant cells with 2N1K morphology were also accumulated over the first two days, but were later diminished as these were apparently replaced by xNxK cells driven by the continuous kinetoplast segregation. When combined together, the data suggest that cells with TbPLK overexpression have their cytokinesis stalled with no effect on nuclear and kinetoplast duplication and division cycles.

B. *The Kinase Activity of TbPLK is Involved in its Overexpression Cell Cycle Defects*

Why the overexpressed TbPLK.eYFP affects growth and cell division in procyclic form *T. brucei*? TbPLK tagged at C-termini with eYFP is known to be catalytically active [17], [18]. It could be the imbalanced kinase activity that over-phosphorylates certain substrates and consequently interfere with the proper cell cycle progression. In human cells, cytokinesis inhibition and formation of multinucleate cells following the overexpression of the PLK1 suggesting that the precise level of kinase activity is important for the correct execution of cytokinesis [22]. To test if hypercatalytic kinase activity of TbPLK has any role in the observed cell cycle defects, I choose to perform analysis similar to the overexpression of wild-type TbPLK.eYFP, but this time on its kinase-dead variant. TbPLK.N169A, in which the asparagine residue is substituted by alanine, is known to be catalytically inactive [17], [19]. When TbPLK.N169A tagged with eYFP was ectopically expressed in procyclic form *T. brucei* cells, the protein was detected at the dorsal midpoint of cell in equivalent FAZ localization, but even though and unlike wild-type TbPLK, appreciable amount of kinase mutant protein was diffused to cytoplasm and nuclei of cells (data not shown; [17]). Despite its aberrant localization, no growth defect was observed when cell proliferation was monitored for 7 days (Fig. 2A). Flow cytometry analysis of cell samples on days 3, 5 and 7 after induction has also indicated the absence of any defects in the cell cycle progression (Fig. 2B). The lack of any cell cycle defects after the kinase mutant overexpression was further confirmed by analyzing nuclei and kinetoplasts in induced cell culture that showed the absence of any aberrant N/K configurations (Fig. 2C). Therefore, it is concluded the lack of any dominant-negative effect of kinase-inactive mutant of TbPLK, and that excess kinase activity is involved in growth and cytokinesis inhibition after wild-type protein overexpression.

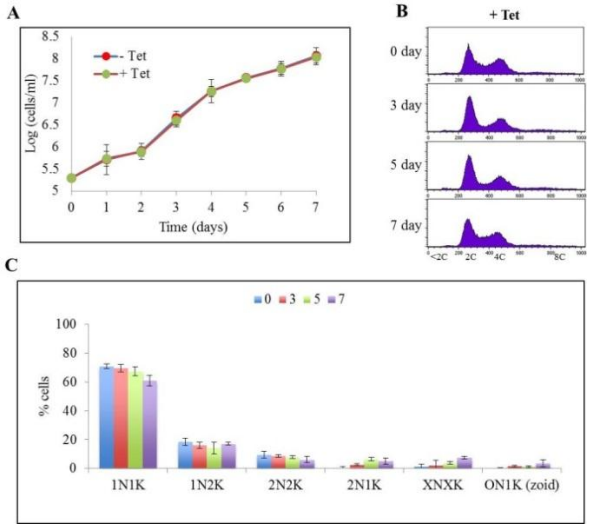

Figure 2. Overexpression of TbPLK kinase-dead mutant did not affect cell growth and the cell cycle progression of *T. brucei*. (**A**) Monitoring the growth rate of cells expressing kinase-inactive mutant, pLew100.PLK.N169A.eYFP, in the absence (- Tet) or presence (+ Tet) of tetracycline induction. (**B**) Flow cytometry analysis of cell samples from the culture expressing TbPLK.N169A.eYFP at 0, 3, 5 and 7 days of Tet-induction (+ Tet). (**B**) DAPI analysis of kinase-dead mutant expressing cells at days 0, 3, 5 and 7; as similar to analysis performed in **Fig. 1C**.

Because our above analysis of wild-type and mutant protein overexpression was conducted on asynchronous cell population and over the course of extended period of time, I need to learn what happen over shorter time duration. Therefore, two cell lines expressing wild-type (PLK.eYFP) and kinase-inactive variant (PLK.N169A.eYFP) were synchronized to S-phase by hydroxyurea treatment for 16 hours. While hydroxyurea synchronization was active, fusion protein expression was induced by tetracycline for the same time duration. When released, proliferation rates of two cell lines were monitored for 12 hours. While growth of cells overexpressing wild-type protein only briefly increased for first 3 hours of release, it never reached doubling point for the rest of experiment (Fig. 3A). Cells with kinase-dead mutant, on other hand, were steadily proliferating till they reached doubling point at 5-6 hour, which is the time point when cells are likely to be executed cytokinesis (Fig. 3A). When cell samples duplicate to that of growth curve analysis were stained with PI and analyzed by flow cytometry, data were obtained that confirm the differential growth of two cell lines. As shown in Fig. 3B (left panel), cells with ectopic kinase-dead mutant expression went from absolute S-phase arrest to G2/M through first 3 hours of release after which dividing cells began to accumulate (3-4 hours) indicating the appearance of G1 cells. At 5-hour time point and through the rest of experiment, there was steady increase in G1 cell population. While similar shift from S-phase to G2/M phase during first 3 hours was observed with cells ectopically expressing wild-type TbPLK, majority of cell population was stalled in G2/M

peak for the rest of release time (Fig. 3B, right panel). As total duration of synchronization and release experiment was 28 hours, data corresponded very well to those from asynchronous population in which 24-hours of wild-type protein overexpression was enough to arrest cells at G2/M phase (Fig. 1B).

When flow cytometric peaks were quantified, S-phase cells of PLK.N169A.eYFP were reduced from 98% to its lowest point at 3-5 hours and then slightly elevated thereafter while G1 cells were steadily increased beginning at 2-3 hours (Fig. 3C, upper panel). G2/M cell population, on the other hand, reached its peak at 3-4 hours-time point and was decreased thereafter. With cells overexpressing PLK.eYFP, there was an inverting relationship between S-phase and G2/M phase cells for first 3 hours while G1 cells level remained low for the total duration of release. At 3-4 hours-time point, G2/M population reached its peak and that persisted for the rest of experiment (Fig. 3C, lower panel). These data are consolidating our previous conclusion that kinase activity of TbPLK is involved in growth and cell cycle defects of protein overexpression.

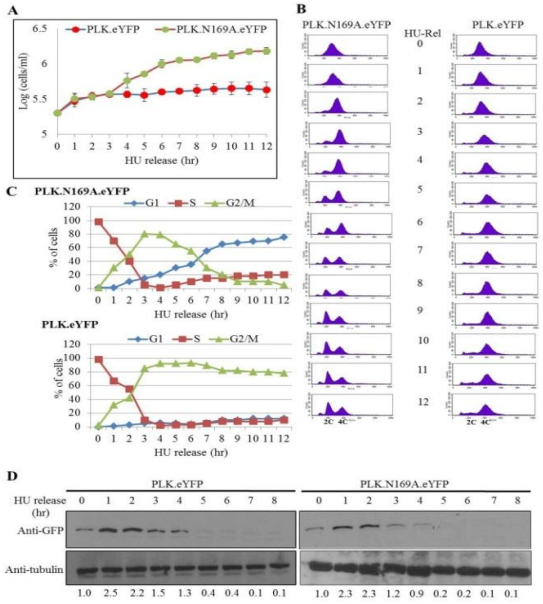

Figure 3. The kinase activity is indispensable for the growth and cell cycle defects of TbPLK.eYFP overexpression. In (**A**), cells harboring either pLew100.PLK.eYFP or the kinase-dead mutant pLew100.PLK.N169A.eYFP were synchronized to late S-phase, released; and in the same time duration, the exogenous eYFP-tagged protein expression in the two cell lines was induced with 1.0 µg/ml tetracycline. The growth rate of two cell lines was monitored hourly for the total duration of 12 hours. PI-stained cell samples were analyzed by the flow cytometry and the FL2-A DNA profiles are presented in (**B**), while the quantitative analysis of percentages of cells in G1, S or G2/M phases are demonstrated in (**C**). (**D**) Cells expressing either pLew100.PLK.eYFP or pLew100.PLK.N169A.eYFP that are synchronized in late S-phase with HU and released were analyzed for eYFP-fusion protein expression by Western blotting at 1-hour time intervals for the total of 8 hours. Blots were stained with anti-GFP (JL-8) antibody and were detected with HRP-conjugated anti-mouse IgGs. The equalized samples loading were determined by anti-tubulin staining of the stripped blots. In two experiments, fusion protein expression levels (relative to tubulin intensity) were determined by the ImageJ software, and values are presented underneath each individual blot. Values at 0-hour time point were set at 1.0.

C. *Ectopically Expressed Wild-type And Kinase-inactive Mutant TbPLK Proteins Are Degraded at Anaphase*

So that imbalanced catalytic activity of overexpressed protein is involved in the abnormal growth and cell cycle phenotypes, but what about the expression levels of these ectopically expressed proteins? In most species studied to date, PLKs are regulated by degradation [12]. In budding yeast, CDC5 degradation depends on a complex of APC/C-CDH1 and an N-terminal degradation motif [23], [24]. Similarly, Human PLK1 degradation was found to be dependent on the activity of APC/C complex and occurs in synchrony with turnover of other cell cycle regulators at anaphase [25]. *In vivo* and *in vitro* studies of TbPLK in *T. brucei* have showed that protein was downregulated after anaphase and through G1 phase until its emergence at S-phase [15]. The highly conserved degradation machinery of APC/C appears to be extended to involves TbPLK as protein sequence possesses a canonical APC/C degron motif (D-box) in the linker region between catalytic domain and polo box regulatory domain (unpublished data). To test if imbalanced amount of overexpressed protein, in addition to its imbalanced kinase activity, might plays a role in the observed abnormal phenotypes, expression profiles of both kinase-active and kinase-dead TbPLK were monitored for 8 hours after hydroxyurea-induced S-phase arrest and release. As expected, the two ectopically expressed proteins were detected at S-phase (after HU-release) with peaking of expression at 1-2 hours which corresponds to early mitosis of *T. brucei* cell cycle (Fig. 3D). To our surprise, expression levels of two proteins were specifically downregulated by more than 50% at 4 to 5 hour transition time point (Fig. 3D). This specific time point fits very well to late anaphase and exiting of mitosis [26]. The apparent degradation was progressively continued for the rest of experiment. Fluorescence microscopy analysis of eYFP signals in synchronized-released cells also confirmed the disappearance of PLK and PLK.N169A fluorescence signals from majority of anaphase cells (data not shown). This is unexpected because expression of two ectopic proteins in this case was not subject to endogenous control as it was regulated by exogenous Tet-controllable system, which was continuously switched on by the persistent presence of tetracycline in the induced culture medium.

D. *The Overexpressed TbPLK Modulates the Endogenous Protein Expression*

As mentioned before, growth and cell cycle defects are only evident after ectopic expression of TbPLK in the background of endogenous protein, and that these defects were fully relieved when endogenous protein was simultaneously depleted from cells overexpressing kinase-active, but not kinase-dead TbPLK [18]. Comparative analysis of kinase-active versus kinase-dead variants of TbPLK in this study has indicated that dominant-negative effect is only achieved with catalytically active protein, even though two proteins showed closely similar expression-degradation profiles.

The most common factor in both cases is the endogenous protein. This may indicates that interference between endogenous TbPLK and its ectopic kinase-active version, but not kinase-dead, is responsible for the above-mentioned results. To test on this, expression profile of endogenous TbPLK was monitored in the background of ectopic overexpression of two protein variants. To do this, the endogenous locus of TbPLK in two cell lines (PLK.eYFP and PLK.N169A.eYFP) was modified to express triple hemagglutinin-fusion protein through epitope tagging. Immunofluorescence analysis confirmed the correct targeting of PLK.3HA to FAZs; and growth and cell cycle analysis indicated the absence of unintentional defects from constitutive fusion protein expression (data not shown). In the absence of induction and ectopic protein expression, immunoblotting analysis showed the degradation of endogenous fusion protein after 4 hours of hydroxyurea S-phase arrest and release (Fig. 4, upper panel). However after induction and overexpression of wild-type PLK.eYFP, there was stabilization of endogenous protein throughout entire release time (Fig. 4, middle panel). This stabilization of endogenous fusion protein was never evident after ectopic overexpression of catalytic inactive PLK.N169A.eYFP (Fig. 4, lower panel). When cumulated together, data suggest that interference between activity and expression of endogenous and ectopic TbPLK may accounts for growth defects and cytokinesis inhibition registered with the later.

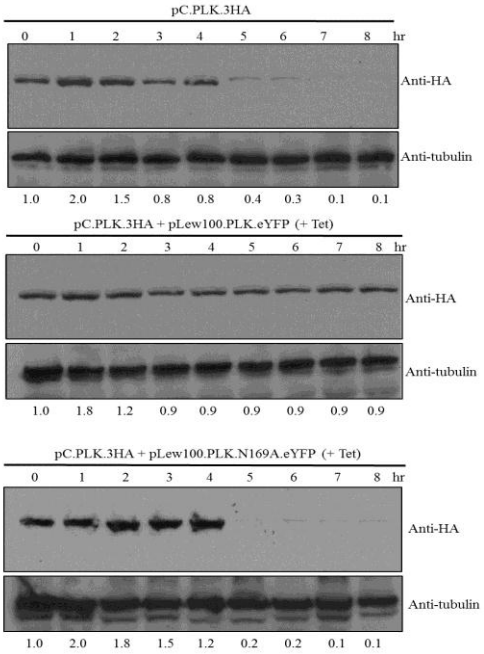

Figure 4. The overexpressed TbPLK stabilizes the expression of the endogenous protein. Western blotting analysis of endogenous TbPLK.3HA expression in the absence (upper panel) or the presence of either catalytic active (middle panel) or catalytic-inactive (lower panel) TbPLK overexpression. Cells were synchronized at S-phase, released and time point cell samples were fractionated on SDS-PAGE and were blotted to PVDF membranes. Blots were immuno-detected by HRP-conjugated anti-HA IgG antibody; were stripped and re-probed again with anti-tubulin for the equal loading controls. Levels of 3HA-fusion proteins were calculated as in **Fig. 3D**, and values are shown underneath individual lanes.

III. Discussion and Conclusion

In this report, I have examined the consequences of overexpressing either wild-type or kinase-inactive TbPLK in *T. brucei* cells. After dissecting data, three major conclusions can be reached out from this analysis. The first such conclusion is that only the overproduced wild-type protein is capable of dominant negative effect by inhibiting growth and cytokinesis. The second dependent conclusion is that kinase activity is involved in the observed phenotypes. Finally, the endogenous TbPLK is stabilized only in response to overproduction of wilt-type protein. Nevertheless, the observed impairment of growth and cell cycle progression after expression of ectopic wild-type protein echoed that of endogenous protein depletion [9]. This brings up question of whether postulated function of TbPLK in cytokinesis requires activity of kinase, or that protein has to be degraded in order for successful initiation of cytokinesis. In proliferating mammalian cells, severe impairment in cell division was recorded in response to both overexpression and functional depletion of PLK1 [22], [27]. It seems that *T. brucei* is no exception, with perturbing both activity and expression level of TbPLK either by ectopic overexpression or functional depletion are leading to the same phenotypic outcomes.

When compared side-by-side, only fully active TbPLK is capable of exerting dominant negative effect over endogenous protein as demonstrated by growth and cytokinesis inhibition. This corresponds very well to previously shown data in *T. brucei* from ectopic expression of catalytic active and inactive TbPLK fused to various epitope tags [18], [19]. However, our present data opposed those reported from other eukaryotic organisms in which the overexpressed kinase-dead mutants of PLK affect growth and cell cycle progression in a similar way to overexpression of wild-type protein. In mammalian cells, overexpression of wild-type and kinase inactive PLK1 resulted in impaired cytokinesis and the formation of multinucleated cells [22]. Similar data were reported in budding yeast where overexpression of both wild-type and a catalytically inactive form of CDC5 inhibited cytokinesis and resulted in the formation of multinucleate cells [28]. This even extended to other kinases as similar results were obtained with overexpression of catalytically active and inactive Aurora-A and Aurora-B kinases in mammalian cells [29], and TbAUK1 of *T. brucei* [30]. To this end, the question remains of why these differential phenotypes of overexpression two protein forms (active and inactive) were seen between *T. brucei* and other eukaryote polo like kinases. Clues to answer this question can be obtained by comparing protein kinetics in different overexpression cases. In human cells, localization studies have indicated that the myc-tagged wild-type and kinase-inactive (K82R) Plk1 proteins exhibited intracellular localization patterns similar to those of endogenous protein [22], [31]. In yeast cells, wild-type or kinase-inactive (N209A) CDC5 fused with GFP also displayed similar localization to endogenous CDC5 protein [32]. Because two protein forms have similar effects, and also

turned to be share similar localization patterns, authors of these studies have suggest that resulting phenotypes may be caused by titration of some proteins interacting with Plk1, and not by excess catalytic activity [22]. This is different in the case of TbPLK in *T. brucei*. While wild-type TbPLK localization was restricted to FAZ, that of overexpressed kinase-dead mutant was extended to cytoplasm and nucleus with some, albeit weaker, FAZ signal [17]. This means less competition between endogenous and ectopic mutant protein on FAZ which may explains the absence of any dominant negative phenotype with mutant (kinase dead) protein expression.

In synchronized population of *T. brucei* cells, both catalytic active and inactive ectopic TbPLK were downregulated at anaphase. This is similar to anaphase downregulation of endogenous TbPLK [15], which is also confirmed in our present study. On the other hand, the most plausible explanation of differential growth and cell cycle defects of wild-type and kinase-inactive TbPLK comes from studying endogenous protein profiles in both ectopic expression cases. In the absence of any ectopic protein expression, level of endogenous TbPLK was significantly downregulated at anaphase of synchronized cells, most likely by APC/C mediated degradation as recorded in other cellular systems [24], [25]. Very similar expression-degradation profile was also reported after induction and ectopic expression of kinase-inactive variant of TbPLK. The ectopic expression of fully active TbPLK, on the other hand, associated with inhibited degradation and stabilization of endogenous protein. Thus, TbPLK in either ectopic or endogenous form has to be degraded specifically at anaphase for cell cycle and cytokinesis to be ensued and that stabilization of either protein form is enough to inhibit protein function. This is similar to human PLK1 where cells with a non-degradable protein form are delayed in cytokinesis [25]. Several studies using mammalian and yeast cellular systems had pointed to interference between ectopic PLK and its endogenous native form [32]-[34], even though they used truncated forms of protein in their ectopic expression experiments. In these studies, overexpression of polo box domain (PBD) inhibits the proper localization and function of the endogenous protein. Their explanation is that steric hindrance competition between ectopic and endogenous proteins in the cell over targeting substrates may lead to inhibition of endogenous protein function. In our case, this is the first time to show that full length ectopic PLK is capable of interfering with endogenous protein through the inhibited degradation and subsequent protein stabilization, a conclusion that has never been observed in any of studied cellular systems thus far. It can be conceivably postulated that the fully active TbPLK, which share the exact localization of endogenous protein at FAZ, is overproduced to the extent of saturating the degradation machinery at this specific cellular location and inhibiting the subsequent endogenous protein degradation. Because kinase-dead TbPLK mutant is mostly delocalized from FAZ, there is no interference with endogenous protein and that two proteins were actively degraded at anaphase. I concluded that the full activity of TbPLK is necessary for *T. brucei* cells to reach cytokinesis and that premature inactivation of protein through RNAi-depletion [9], [16] or its stabilization beyond that point both inhibit cytokinesis.

In all, the present conclusions support the evidence that balanced catalytic activity and expression levels are all important factors that are required for the proper functions of PLK during cell cycle, and in *T. brucei* that alteration of endogenous protein activity by ectopically overexpressed, catalytically active protein was enough to inhibit cytokinesis.

IV. Experimental Procedures

A. Trypanosome Cell Culture

29-13 *T. brucei* procyclic form cells were maintained in Cunningham's medium at 26°C. Fetal bovine serum (HyClone) was supplemented to 10% final concentration. To maintain the genetic background of cells, G418 at 15μg ml-1 and hygromycin B at 50μg ml-1 were constantly added to the culture medium to preserve the constant expression of T7 RNA polymerase and tetracycline repressor, respectively. The growth of cells was maintained at the mid-log phase by regular subculturing in fresh medium with antibiotics.

B. Ectopic and Endogenous Epitope Tagging of TbPLK in T. brucei

For ectopic expression of TbPLK tagged with enhanced yellow fluorescent protein (eYFP), full-length sequence representing the entire ORF of TbPLK was amplified by PCR from 29-13 genomic DNA and cloned into pLew100.eYFP.Phleo plasmid that had 3HA replaced with eYFP sequence [15]. The NotI-linerized pLew100.PLK.eYFP plasmid was transfected into 29-13 cells by electroporation. Stable transfectants were selected under 2.5 μg/ml phleomycin. Single clonal cells expressing TbPLK.eYFP were obtained by limiting dilution and the eYFP-recombinant protein expression was induced by 1 μg/ml tetracycline and was detected by western blotting (see below).

For epitope tagging of the protein at the endogenous loci with triple hemagglutinin (3HA) tag, the 3' terminal fragment (400 bp) of TbPLK ORF sequence without the stop codon was amplified by PCR and cloned into pC.eYFP.Bla in which eYFP modifed to 3HA sequence [16]. The resulting pC.PLK.3HA was linearized with SfoI and then transfected into 29-13 cells stably expressing TbPLK.eYFP. After simultaneous selection under 2.5 μg/ml phleomycin (for pLew100.PLK.eYFP) and 10 μg/ml basticidin (for pC.PLK.3HA), single clonal cells were obtained. The simultaneous expression of differentially tagged TbPLK in the same cells was confirmed by using anti-GFP and anti-3HA in a western blotting assay.

C. Western Blotting

Cell samples were collected by centrifugation, washed once in in phosphate-buffered saline (PBS), and total cell lysates were extracted by boiling for 5 minutes SDS-PAGE laemmli sample buffer. After electrophoresis on

SDS-PAGE, total proteins were transferred to PolyVinylidene DiFluoride (PVDF) membrane (Bio-Rad). Immunoblot membranes were blocked in TBST (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% Tween 20) containing 5% semi-skimed milk for 60 minutes at room temperature. After blocking, immunoblot membranes were probed with primary anti-green fluorescent protein (GFP) MAb JL-8 (Clontech) diluted to 1:5,000 in TBST for 60 minutes. After three washes in TBST for 10 minutes each, membranes were stained with secondary anti-mouse horseradish peroxidase (HRP)-conjugated immunoglobulin G (Promega) diluted to 1:10,000 in TBST for 60 minutes. Memranes were again washed three times in TBST before developed to X-ray film using chemiluminescence (ECL) substrates. For loading controls, membranes were stripped with western stripping buffer (invitrogen), blocked and immunostained as above using mouse anti-tubulin (Sigma) and HRP-conjugated anti-mouse IgG.

D. Fluorescence Microscopy

Cells expressing the eYFP-fusion protein were harvested by centrifugation at 3,000 rpm for 3 minutes at room temperature. Cells were washed twice in PBS before fixed in suspension by the formaldehyde fixation solution (3.7% formaldehyde in PBS) for 15 minutes at room temperature. The fixed cells were washed once more with PBS and were attached to poly-L-lysine-coated cover slips for 20 minutes. The non-attached cells were removed by PBS wash. Cover slips were mounted to the slides in VectaShield medium (Vector Labs) containing 4',6'-diamino-2-phenylindole (DAPI) and examined with an ECLIPSE Ti inverted epifluorescence microscope (Nikon).

E. Immunofluorescence Microscopy

Cells were harvested, washed and fixed in suspension with 3.7% formaldehyde as described above. After adhering to poly-L-lysine-treated cover slips, fixed cells were permeabilized with 0.1% NP-40 in PBS for 5 minutes and washed with PBS. The fixed and permeabilized cells were blocked in 3% BSA in PBS for 60 minutes at room temperature. The blocked cells were stained with primary antibody for 60 minutes. After washing three times in immunostaining wash buffer (0.1% Triton-X100 in PBS), signals were detected by incubation with secondary antibody for 60 minutes. Cells were washed as above with the last wash in PBS only without Triton-X100. The immunostained cells were mounted in VectaShield mounting medium with DAPI and examined with epifluorescence microscope.

F. Cell Cycle Synchronization of T. brucei Cells

Cells expressing fusion proteins and grown at mid-log phase were incubated with 0.3 mM hydroxyurea at 26℃ for 16 hours for enrichment in the late S phase [35]. After hydroxyurea was washed off from cells by double washing in fresh medium, cells were released for synchronized cell cycle progression for an additional 8 hours. Cell samples were collected at an-hour time intervals, washed once in PBS and processed for fluorescence microscopy and immunofluorescence staining (as mentioned above) or for the flow cytometry (see below).

G. Flow Cytometry

The flow cytometry analysis was done as essentially described before [36].

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Mohamed S. N. Bessat was born in Egypt at August, 25th 1974. He obtained his BSc of Veterinary Sciences from Faculty of Veterinary Medicine, Alexandria University, Egypt at 1997. From the same institute, he obtained his MSc of Veterinary Parasitology at 2003. In 2005, he awarded a governmental-funded scholarship to study for his PhD in Biological Sciences from the University of Hull in UK and had been awarded doctor of philosophy at 2009. In 2010, he travelled to UCSF for postdoctoral training in Pharmaceutical Chemistry.

