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# Validating 3-Phosphoinositide Dependent Protein Kinase-1 as a Drug Target in African Trypanosoma

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***Author's contribution***

*The author NKC performed the analysis, wrote the protocol and wrote the first draft of the manuscript. The findings and conclusions in this report are those of the author.*

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## ABSTRACT

**Introduction:** Human African trypanosomiasis is a parasitic disease that is transmitted to the mammalian host through the bite of an infected tsetse fly of the genus *Glossina* species. The disease is caused by two species, *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense,* disease outcome is dependent on the infective species. Currently, treatment of African trypanosomiasis is dependent on drugs that are highly toxic and drug resistance has been observed. Therefore, there is a need for development of new drugs and vaccines. The genome of *T. brucei* has been completely sequenced and analysis of the kinome has revealed 156 eukaryotic protein kinases. One such kinase is a homolog of the mammalian 3-phosphoinositide dependent protein kinase-1 (PDK-1), a member of the AGC family of protein kinases which shares 35% similarity with the human PDK-1.
**Methods:** In this study, RNA interference of 3-phosphoinositide dependent protein kinase-1 was carried out to investigate the effect of gene knock down on *Trypanosoma brucei brucei*.

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**Results:** *Trypanosoma brucei* 3**-**phosphoinositide-dependent protein kinase-1 is important in *T. brucei brucei* cell proliferation. Further investigations using DAPI and fluorescence microscopy showed that gene knockdown had an impact on cell phenotype.
**Conclusion:** RNA interference was effective in knocking down genes corresponding to *Trypanosoma brucei* 3**-**phosphoinositide-dependent protein kinase-1. Findings of the study demonstrate that TbPDK-1 is important in cell proliferation in blood stream form *Trypanosoma brucei brucei.* These results validate TbPDK-1 as a potential drug target for African trypanosomes.

*Keywords: Trypanosomiasis; TbPDK-1; RNAi; gene knock down; protein kinase.*

## ABBREVIATIONS

*HAT: Human African Trypanosomiasis; PDK-1: 3-phosphoinositide-dependent protein kinase-1; TbPDK-1: Trypanosoma brucei 3-phosphoinositide-dependent protein kinase-1; AGC: PKA, PKC and PKG kinases; CAMK: calcium/calmodulin-dependent protein kinases; CMGC: CDK, MAPK, GSK3 and CLK kinases; TK: tyrosine kinase; TKL: tyrosine kinase like; STE: homologs of yeast Sterile 7, Sterile 11, and Sterile 20 kinases; PKC: Protein kinase C; RNAi: RNA interference; PBS: Phosphate buffered saline; BSF: Blood Stream Form; DAPI: 4',6-diamidino-2-phenylindole.*

## 1. INTRODUCTION

African trypanosomiasis is a vector-borne parasitic disease caused by parasites of the genus *Trypanosoma.* It is transmitted to the mammalian host through the bite of an infected tsetse fly. There are three subspecies of *Trypanosoma brucei; Trypanosoma brucei brucei, Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense,* which can cause varying trypanosomiasis infection [1]. *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* infect humans causing Human African trypanosomiasis (HAT) while *Trypanosoma brucei brucei* infects cattle causing Nagana [1,2]. There are over 50 million people at risk of catching African trypanosomiasis in sub-Saharan Africa; with the countries at most risk being, Democratic Republic of the Congo, Angola, Central African Republic, Congo and South Sudan [1]. The disease has an overwhelming impact on human health as well as the economy in affected areas [3].

Protein kinases play a critical role in cellular processes including cell proliferation, glucose uptake and storage, transcriptional control and other signal transduction pathways [4]. Complete genome and kinome sequencing of *T. brucei* has allowed for functional analysis of the various protein kinases found in trypanosomes [5,6]. *Trypanosoma brucei* has over 156 eukaryotic protein kinases that are thought to be catalytically active and can be grouped into seven major eukaryotic kinase groups on the basis of sequence similarity: AGC, CAMK, CMGC, TK, TKL, STE and others [4].

One such protein kinase is 3-phosphoinositide-dependent protein kinase-1 (PDK-1), a member of the AGC family of protein kinases [7,8]. In mammals, PDK-1 plays an important role in phosphorylating the activation loop, a conserved serine/threonine residue, of members of the AGC family as well as auto-phosphorylating its own active site [9].

A homolog of mammalian PDK-1 has been identified in *T. brucei*, geneDB accession number Tb 09.160.3480, which shares 35% similarity with the human PDK-1. The divergence from the human PDK-1 is promising in the development of drugs against the parasite. Homologs of other AGC family members that require phosphorylation by PDK-1 have been identified in *T. brucei* including a PKC homolog, geneDB accession number Tb927.6.2250, a RAC serine/threonine kinase, and a PKB homolog [10].

This study aimed to investigate the role of the PDK-1 homolog in *T. brucei* and to determine whether it is a potential drug target; through gene knockdown.

## 2. MATERIALS AND METHODS

### 2.1 Construction of RNA Interference Strains

To investigate the effect of specific gene knock down in organisms, RNA interference (RNAi) was performed as previously described [11].

To determine the role of *T. brucei* PDK-1, an RNAi fragment corresponding to the gene was constructed and analysed. The genome sequence corresponding to Tb09.160.3480 taken from GeneDB was analysed using the RNAit software (TrypanoFAN, University of Cambridge, UK) to determine the most suitable region for construction of RNAi strains.

Using CLC genomics benchwork (CLC bio, QIAGEN), primers that correspond to the 5' ends of the sense and anti-sense region were designed. Primers for the sense sequence were designated Oligo3612 with a 5' to 3' sequence of GATCCTCGAGGGTACCG, while the anti-sense sequence, Oligo3613, had a 5' to 3' sequence of GATCTCTAGAGGATCCAC.

To build the construct, genomic DNA from *T. brucei brucei* wild type strain 927 was used to amplify a 453bp PDK-1 homolog region selected for RNAi analysis, through PCR. In order to ensure the correct sequence had been amplified by the selected primers, a sample of the PCR product was sent for sequence analysis. Following DNA extraction, the plasmid vector pGEM-T Easy (Promega) was used to amplify the 453bp TbPDK-1 RNAi insert. The A-tailed PCR product was ligated into the pGEM vector overnight at 4ºC at the restriction sites BamHI and KpnI**.** *Ecoli* DH5α cells (Invitrogen) were transformed with the ligation reaction. For colour screening, IPTG and X-gal were spread on LB agar plates before plating out the transformation reaction. Colony PCR of the white colonies was set up with primers specific to TbPDK-1 homolog to ensure the correct insert was cloned into the pGEM vector. Overnight cultures of the positive colonies were set up and plasmid DNA purification was carried out as per manufactures' instructions (QIAprep miniprep kit, QIAGEN). The plasmid vector pRPa$^{i}$, also known as pGL1875, was used to construct the RNAi fragment [12]. The TbPDK-1 sense insert was cloned by digesting p GL1875 with high fidelity BamHI and KpnI while the anti-sense insert was cloned by digesting the plasmid with XhoI and XbaI. The plasmid was linearised with AscI (Fig.1).

## 2.2 Transfection of 2T1 Blood Stream Form *T.b.b* and RNAi Knockdown

To investigate whether Tb09.160.3480 (TbPDK-1) gene knock down has an impact on the parasite, blood stream form 2T1 cells were transfected with the TbPDK1 RNAi construct, according to published methods. A screen of the *T. brucei* RRNA spacer loci revealed a region with strong repression and high level expression upon induction of transgene regulation. These clones had an incomplete hygromicin-resistant marker inserted in place of the reporter cassette and the resulting strain is termed Blood stream form 2T1 [12].

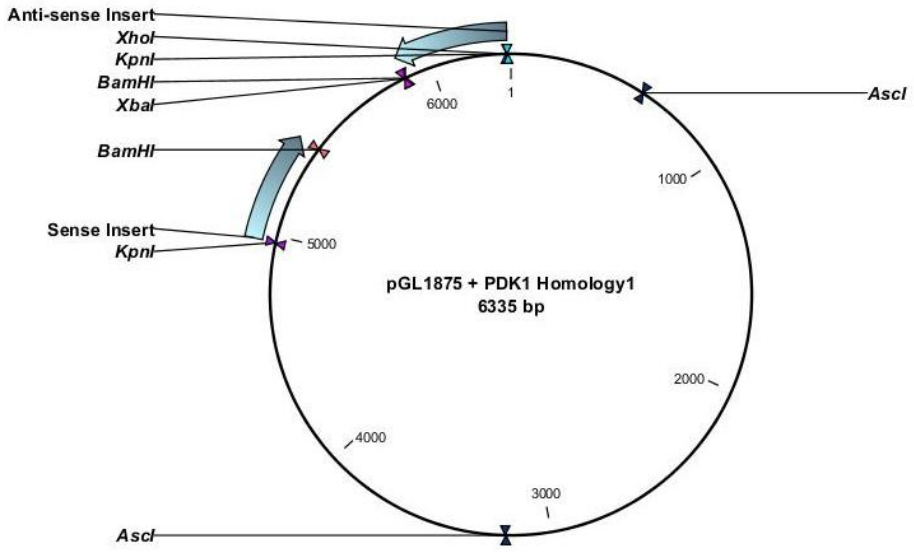


**Fig. 1. Plasmid vector pRPa$^{i}$ (pGL1875) with the restriction sites used to clone the TbPDK-1 RNAi sense and anti-sense inserts. The restriction site (AscI) used to linearise the plasmid is shown**

Blood stream form 2T1 *T. brucei* were grown in HMI9 media to a density of $1\times10^7$ cells per RNAi construct. Cells were centrifuged at 1000 g for 1 minute, the supernatant was discarded and the pellet was resuspended in 100µl nucleofactor and 10µl plasmid containing the RNAi construct was introduced into the cells by electroporation with the X-001 programme (Amaxa Nucleofector, Lonza). Cells were resuspended in 30 ml HMI9 media, dilutions of 1:10, 1:100 and 1:1000 were made from the 30ml neat solution. For each dilution, quadruplicate 24 well plates were set up with 1ml of the sample in each well. The plates were incubated at 37ºC with 5% carbon dioxide for 6 hours after which 0.5ml hygromycin was added to a concentration of 2.5µg/ml. The plates were incubated for a further 5 days in 5% carbon dioxide at 37ºC.

Analysis of transfected cells revealed two clones which were assigned the stabulate/clone numbers 15059, from the 1:10 dilution plate, and 15060, from the neat plate. To analyse the effect of gene knock down on the parasites, each clone was grown in triplicates to a density of $1\times10^5$ cells for the uninduced and tetracycline induced cells. The selectable marker hygromycin was added to a concentration of 2.5µg/ml and tetracycline was added to a concentration of 1ug/ml to the samples to be analysed for RNAi knock down. When cells grew to a density of $1\times10^6$ cells/ml, they were diluted 1:10 in HMI9 media plus hygromince and tetracycline to the induced samples if applicable.

A cell count was carried out for each clone every 24 hours for five days and data was analysed through growth curve analysis and DAPI. A positive control was set up using CRK3 RNAi construct which has previously been shown to have an effect on parasite growth after tetracycline induction [13].

### 2.3 DAPI Analysis

To analyse phenotypic changes that resulted from the gene knock down, DAPI (4',6-diamidino-2-phenylindole) a fluorescent dye that strongly binds DNA was used (Vector laboratories). Analysis involved looking at cell changes from time point zero every 24 hours. A density of $5\times10^5$ cells was used for each analysis. Cells were centrifuged at 1500 g for 5 minutes and the supernatant was discarded. Cells were resuspended in the remaining media and gently spread over the slide. Slides were left to air dry and after drying, were fixed overnight in methanol at -20ºC. Methanol was allowed to evaporate off the slides and cells were rehydrated with PBS for 10 minutes. Vectashield fluorescence mounting media with DAPI was applied to the slides and a slide cover was placed on the slide and left to dry.

The slides were imaged using fluorescence microscopy and analysed with the OpenLab software (PerkinElmer). DAPI settings on the microscope and Openlab were used to visualise cell DNA while phase contrast was used to analyse cell shape and orientation. Cell cycle stages at different time points, zero to 48 hours, were analysed by counting 200 cells per slide for the tetracycline induced clones as well as the uninduced clones and data was compared for significant changes in the induced and uninduced clones. Cell cycle stages were pooled in four categories; 1 nucleus 1 kinetoplast (1N1K), one nucleus two kinetoplast (1N2K), two nuclei two kinetoplast (2N2K) and others.

## 3. RESULTS

### 3.1 TbPDK-1 Knock Down has an Effect on Cell Growth

The effect of the knock-down was analysed through a parasite count every 24 hours for five days after tetracycline induction and growth of the knock-down clones was compared to the uninduced clones. The growth curve for TbPDK-1 RNAi knockdown shows that knockdown had an effect on cell proliferation for both clone's 15059 and 15060, the knock-down effect was observed for both clones (Fig. 2a and 2b). Compared to the uninduced clones, proliferation of the tetracycline induced cells significantly reduced 24 hours after induction, illustrating that TbPDK-1 is important for cell proliferation.

### 3.2 Phenotypic Changes Observed Through DAPI Analysis

To further determine if TbPDK-1 knock-down had an impact on cell proliferation, phenotypic changes were investigated using DAPI analysis at 24 hour intervals post tetracycline induction.

Growth curve and DAPI analysis showed that TbPDK-1 knock-down had an impact on parasite proliferation and phenotype. DAPI analysis of cell cycle stages at different time points, zero to 48 hours, was carried out by counting 200 cells for the tetracycline induced clones as well as the

uninduced clones. The data shows that TbPDK-1 plays a role in cell cycle stages as illustrated in Fig. 3a), 3b), 3c) & 3d). For clone 15059 a 200 cell count at 48 hours of the tetracycline induced culture showed cell cycle stages in the pooled categories had 52% 1N1K cells, 9.5% 1N2K cells, 9% 2N2K cells and 27.5% cells were pooled as others compared to the uninduced culture showing that 76% cells had 1N1K, 16.5% cells had 1N2K, 6.5% cells had 2N2N and 1.5% cells were pooled as others. This data was similar with clone 15060 which showed cell cycle stages of the tetracycline induced culture to be in the following categories; 55.5% 1N1K cells, 10% 1N2K cells, 7% 2N2K cells and 27.5% cells were pooled as others. The uninduced culture had 87% 1N1K cells, 16.5% 1N2K cells, 4.5% 2N2K cells and 1% pooled as others. The induced cultures had a higher percentage of cells categorised as others representing cells that do not confer to normal *T. brucei* cell cycle stages including; indistinguishable organelles and cells, multi-nucleated cells, diffused nuclei, poorly segregated kinetoplast and other phenotypes.

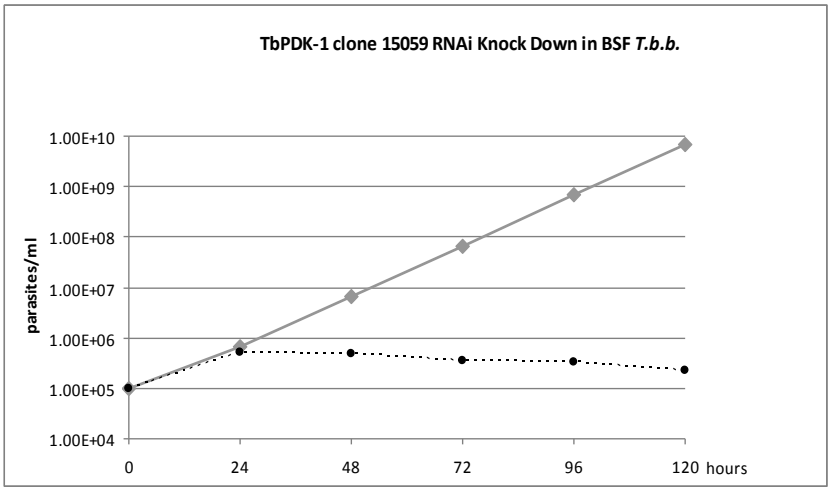


**Fig. 2a. Growth curve analysis of TbPDK-1 Clone 15059 RNAi knock-down. The tetracycline induced clone shows a reduction in cell proliferation after 24 hours induction (Solid line represents uninduced clones while the dotted line represents tetracycline induced clones)**

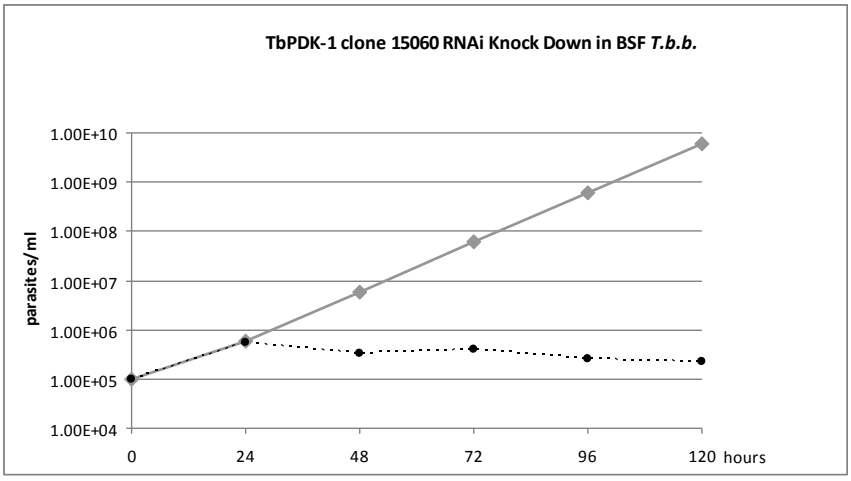


**Fig. 2b. Growth curve analysis of TbPDK-1 clone 15060 RNAi knock-down. The tetracycline induced clone shows a reduction in cell proliferation after 24 hours induction (Solid line represents uninduced clones while the dotted line represents tetracycline induced clones)**

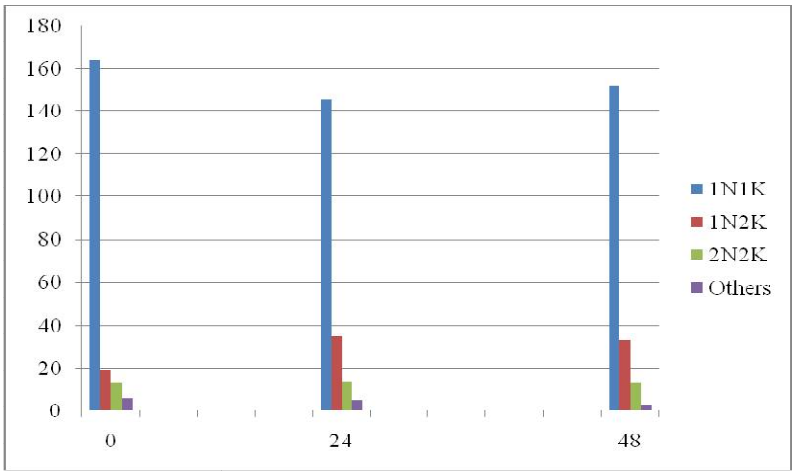


**Fig. 3a. An illustration of *T. brucei* cell cycle stages observed after counting 200 cells from uninduced clone 15059**

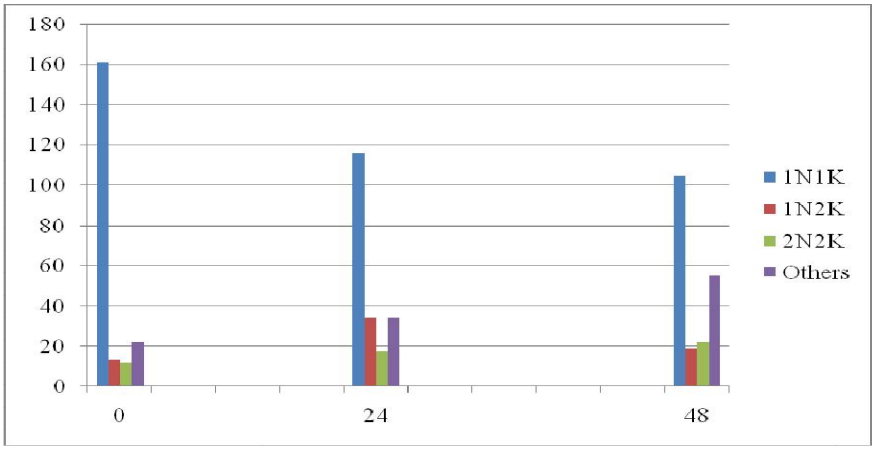


**Fig. 3b. An illustration of *T. brucei* cell cycle stages observed after counting 200 cells from tetracycline induced clone 15059**

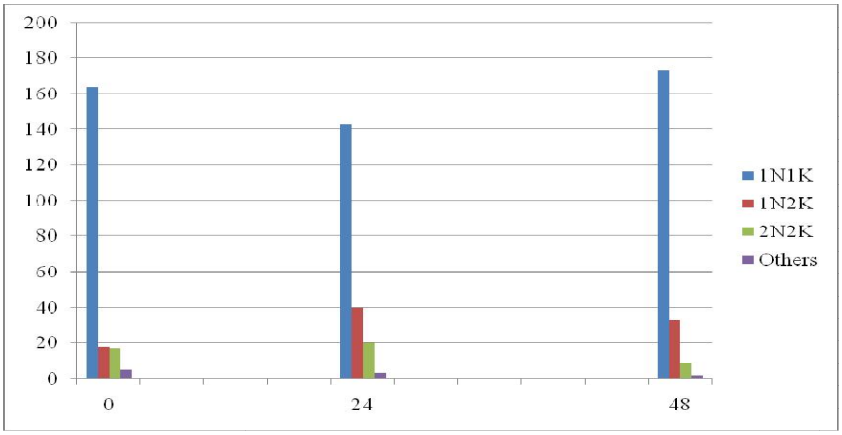


**Fig. 3c. An illustration of cell cycle stages observed in *T. brucei* after counting 200 cells from the uninduced clone 15060**

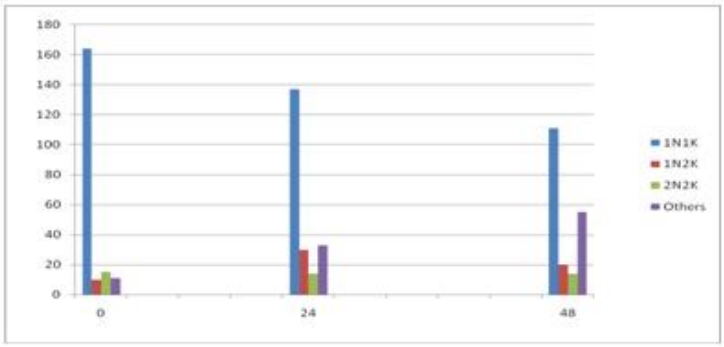


**Fig. 3d. An illustration of *T. brucei* cell cycle stages observed after counting 200 cells from tetracycline induced clone 15060**

## 4. DISCUSSION

*Trypanosoma brucei* infection has a devastating impact on human health, livestock as well as the economy in affected areas [1]. Current treatment regimen involves highly toxic drugs and resistance has been observed [14]. Understanding the mechanisms by which the parasite causes infection and the molecular mechanisms involved in cellular processes and cell proliferation is important in the development of novel drugs and vaccines. This has been facilitated by the genome wide sequencing of *T. brucei* which also allows for *T. brucei* to be used as an important eukaryotic model organism in investigating cellular processes [15].

The identification of 156 eukaryotic protein kinases in *T. brucei* coupled with the possibility to successfully knock down and analyse gene function through RNAi is promising in the challenge to identify new drug targets [4,6,15].

Numerous studies have been carried out on the human PDK-1 which highlight that PDK-1 is the master regulator of the AGC family of protein kinases [7,8]. It has been shown that PDK-1 plays an important role in a number of cellular processes and pathways by activating its substrates through phosphorylation of a conserved serine/threonine activation site [7]. Use of RNAi for gene knock down has shown that TbPDK-1 is important in cell proliferation in blood stream form *T. brucei brucei.* This is evident through comparing cell proliferation in the uninduced clones and tetracycline induced clones, Fig. 2a. and 2b. Twenty four hours after tetracycline induction, there is no significant difference between induced and uninduced clones. However, there is a significant drop in the number of parasites after 48 hours in the tetracycline induced clones compared to the uninduced clones. Analysis of parasites through DAPI shows phenotypic changes in the induced clones with an increase in the number of cells with a phenotype other than the normal phenotype (Fig. 3). Phenotypes observed in the induced parasites include diffused nuclei, poorly segregated nuclei and undistinguishable cells/phenotypes; which is not characteristic of the wild type phenotype. TbPDK-1 gene knock down has validated the role of the gene in *T. brucei* cell viability; demonstrating the potential of PDK-1 as a drug target for African trypanosomes.

## 5. CONCLUSION

RNAi was effective in knocking down genes corresponding to *Trypanosoma brucei* 3-phosphoinositide-dependent protein kinase-1. Findings of the study demonstrate that TbPDK-1 is important in cell proliferation in blood stream form *Trypanosoma brucei brucei.* These results validate TbPDK-1 as a potential drug target for African trypanosomes.

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## COMPETING INTERESTS

Author has declared that no competing interests exist.

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