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## Validation of spermidine synthase as a drug target in African trypanosomes

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Short title. Spermidine synthesis in *Trypanosoma brucei*

## Synopsis

The trypanocidal activity of the ornithine decarboxylase (ODC) inhibitor difluoromethylornithine (DFMO) has validated polyamine biosynthesis as a target for chemotherapy. As DFMO is one of only two drugs used to treat patients with late-stage African trypanosomiasis, the requirement for additional drug targets is paramount. Here we report the biochemical properties of *Trypanosoma brucei* spermidine synthase (TbSpSyn), the enzyme immediately down-stream of ODC in this pathway. Recombinant TbSpSyn was purified and shown to catalyse the formation of spermidine from putrescine and decarboxylated S-adenosylmethionine. To determine the functional importance of TbSpSyn in bloodstream form parasites, we used a tetracycline-inducible RNA interference (RNAi) system. Down-regulation of the corresponding mRNA correlated with a decrease in intracellular spermidine and cessation of growth. This phenotype could be complemented by expressing the spermidine synthase gene from *Leishmania major* in cells undergoing RNAi, but could not be rescued by addition of spermidine to the medium due to the lack of a spermidine uptake capacity. These data therefore genetically validate TbSpSyn as a target for drug development and indicate that in the absence of a functional biosynthetic pathway, bloodstream form *T. brucei* cannot scavenge sufficient spermidine from their environment to meet growth requirements.

**Keywords.** RNA interference, complementation, polyamine, biosynthesis, *Trypanosoma brucei*.

### Abbreviations

DFMO, difluoromethylornithine; HAT, human African trypanosomiasis; Arg, arginine; Met, methionine; ODC, ornithine decarboxylase; dcSAM, decarboxylated S-adenosylmethionine; SAMdc, S-adenosylmethionine decarboxylase; SpSyn, spermidine synthase; BSF, bloodstream form; RNAi, RNA interference; PAPT, putrescine aminopropyltransferase

## Introduction

As a result of coordinated surveillance and treatment programs, the number of people infected annually by the protozoan parasite *Trypanosoma brucei*, the causative agent of human African trypanosomiasis (HAT), has fallen to 70,000 [1]. However, in some regions, mortality rates for HAT exceed HIV/AIDS and malaria. Current treatments for the late-stage of the disease rely on the drugs melarsoprol and difluoromethylornithine (DFMO). However their use is problematic. With no realistic prospect of a vaccine, new drugs against “one of the great neglected diseases” are a priority [2].

One area of parasite biology that has attracted attention in terms of drug development is polyamine biosynthesis [3-6]. Polyamines such as putrescine and spermidine, are aliphatic organic compounds containing two or more amino groups. In most eukaryotes, these molecules play pivotal roles in several processes, including protein/nucleic acid synthesis and cell proliferation/differentiation, and have been implicated in the development of certain cancers [7]. Trypanosomes are unusual in that spermidine is also used in the synthesis of trypanothione, a glutathione:spermidine dithiol conjugate that plays a central role in several detoxification processes and supplies reducing equivalents used in nucleic acid synthesis [8, 9]. As this thiol is both unique and essential to trypanosomatids, any process involving trypanothione is considered a potential target for drug intervention.

The building blocks for polyamine synthesis are arginine (Arg) and methionine (Met) (Figure 1). In most eukaryotes, Arg is hydrolysed to form ornithine and urea, with ornithine metabolised further to putrescine, reactions catalysed by arginase and ornithine decarboxylase (ODC) respectively. Putrescine then reacts with decarboxylated S-adenosylmethionine (dcSAM) to form spermidine, a process mediated by spermidine synthase (SpSyn); the dcSAM is derived from Met as a result of the activity of S-adenosylmethionine decarboxylase (SAMdc) (Figure 1). The trypanocidal activity of various inhibitors which target components of this pathway has been reported [10-14]. DFMO, one of two drugs available to treat late-stage HAT, inhibits ODC activity. The interaction between *T. brucei* ODC and DFMO has been extensively studied and the selective killing activity of the drug has been determined

[15, 16]. The basis for this toxicity stems from the stability of the parasite ODC (half-life ~18 h) compared to that of the mammalian host ODC (half-life ~20 mins) [15, 17]. When DFMO binds covalently to the mammalian enzyme, the complex is rapidly degraded and replaced with newly synthesised ODC. In contrast, the DFMO-ODC molecule remains within the parasite and is only slowly replaced. Thus the level of active protein is decreased leading to a cessation of putrescine formation. Eventually, the parasite stops growing and the non-dividing cells are cleared by the host immune system. Although the activities of other *T. brucei* enzymes involved in polyamine synthesis have been detected [12, 18], their biochemical roles have yet to be fully analysed. Here, using an inducible RNA interference (RNAi) system, we demonstrate that spermidine synthase (EC 2.5.1.16), a key component of polyamine synthesis, is essential to infective *T. brucei*, therefore validating it as a potential target for chemotherapeutic intervention.

## Experimental

### Parasites.

Bloodstream forms (BSF) of the *T. brucei* single marker cell line (SMB) that constitutively express T7 RNA polymerase and the tetracycline repressor protein [19] were grown at 37°C under a 5% CO<sub>2</sub> atmosphere in modified Iscove's medium [20] containing 2 µg ml<sup>-1</sup> G418. Transformed SMB cells were maintained in this growth medium, supplemented with 2.5 µg ml<sup>-1</sup> hygromycin. Tetracycline-free fetal calf serum (10%) (Autogen Bioclear) was used in the growth media. RNA was extracted from parasites using the RNeasy<sup>®</sup> mini kit (Qiagen).

### Biochemical properties.

*TbSpSyn* was amplified from parasite genomic DNA (Table 1 for primers). The fragment was digested with *Bam* HI + *Hin* dIII and cloned into the corresponding sites of the expression vector pTrcHis-C (Invitrogen). In this system the expressed protein contains an amino terminal histidine tag and an epitope detectable with the anti-Xpress monoclonal antibody (Invitrogen). The insert was sequenced using a dye terminator cycle kit (Applied Biosystems) and an ABI Prism 3730 Sequencer. *Escherichia coli* BL21+ transformed with pTrcHis-TbSpSyn were grown in NZCYM broth (Sigma) containing ampicillin and protein expression induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG). Recombinant His-tagged proteins were affinity purified on a Ni-NTA matrix column under native conditions as recommended by the manufacturer (Qiagen). The cell lysis, column wash and elution steps were all carried out in the presence of protease inhibitors (Roche). Fractions were analysed by SDS-PAGE and protein concentrations were determined by BCA protein assay system (Pierce).

TbSpSyn activity was followed by monitoring spermidine formation in the presence of putrescine and dcSAM (modified from [21]). A standard 1 ml reaction contained 100 mM potassium phosphate pH 7.2, 1 mM DTT, 1 mM putrescine, 10 µM dcSAM and 100 µg TbSpSyn. Reactions were incubated at 37°C for 30 min and then terminated by placing the tubes at 95°C for 5 min. Polyamines were derivatised with dansyl chloride then analysed by high performance liquid chromatography (HPLC).

### RNA interference.

An 860 bp DNA fragment corresponding to an internal sequence from *TbSpSyn* was amplified from genomic DNA (Table 1 for primers), digested with *Bam* HI + *Xho* I and cloned into the corresponding sites of the vector p2T7<sup>Ti</sup> [22]. In this vector, the inserted DNA is flanked by two opposing T7 promoters, with each promoter under the control of a tetracycline operator. Constructs were linearised with *Not* I, electroporated into BSF parasites and transformants cloned as described [23]. Induction of RNAi was initiated by adding tetracycline (1 µg ml<sup>-1</sup>) to the culture.

### Complementation vector.

The complete open reading frame of *LmSpSyn* (CAC44919) was amplified from *Leishmania major* genomic DNA (Table 1 for primers), the resultant 903 bp fragment digested with *Sbf* I + *Asc* I, then cloned into the corresponding sites of the *T. brucei* constitutive expression vector pTUB-EX [24]. Constructs were digested with *Not* I + *Xho* I, electroporated into the RNAi-*TbSpSyn* cell line and clones selected. Comparison between *TbSpSyn* and *LmSpSyn* revealed that the two genes were 65% identical and had no conserved stretches >20 bp. Therefore RNAi targeted at the *TbSpSyn* mRNA should not affect the *Leishmania* transcript.

### Detection of the growth inhibition phenotype.

BSF trypanosomes, transformed with the RNAi construct were seeded at 1 x 10<sup>5</sup> cells ml<sup>-1</sup> and incubated at 37°C in the presence of tetracycline (1 µg ml<sup>-1</sup>). Every 24 hours parasite growth was monitored microscopically and the culture diluted back to 1 x 10<sup>5</sup> cells ml<sup>-1</sup>. Control cultures incubated in the absence of tetracycline were grown in parallel. Under these conditions, untreated cell lines grew with a doubling time of approximately 7 – 8 h.

### Analysis of polyamines.

BSF trypanosomes (5 x 10<sup>7</sup>) were lysed in 10% trichloroacetic acid, sonicated and the extract clarified. Polyamines in the lysate were then derivatised as follows: Supernatant (100 µl) was incubated with 200 µl saturated sodium carbonate and 400 µl dansyl chloride (10 mg ml<sup>-1</sup> in acetone) (Sigma) at 60°C for 1 h in the dark. Excess dansyl chloride was removed by addition of 100 µl L-proline (100 mg ml<sup>-1</sup>) and

incubated at 60°C for a further 30 min. Derivatised polyamines were then extracted into toluene and the organic phase dried under argon. Pellets were resuspended in acetonitrile and analysed by HPLC.

### **Spermidine transport studies**

[<sup>14</sup>C] Spermidine trihydrochloride was supplied by GE Healthcare UK Ltd. BSF *T. brucei* or *T. cruzi* epimastigotes, in the exponential phase of growth, were pelleted, washed 3 times in assay buffer (90 mM Tris.HCl pH 7.5, 3.1 mM KCl, 96.9 mM NaCl, 5 mM MgCl<sub>2</sub>, 2 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM glycerol), then resuspended at 2 x 10<sup>8</sup> cells ml<sup>-1</sup>. To initiate uptake, radiolabelled spermidine (0.5 µCi) was added to 50 µl parasite suspension at a final concentration of 45 µM and the reaction incubated at room temperature for an appropriate time. The assay was stopped by the addition of 200 µl 4% (w/v) paraformaldehyde in PBS. The cells were pelleted, washed 3 times in 1 x PBS then lysed in 250 µl 2 % (w/v) SDS in a scintillation vial. 3 ml scintillation fluid (Ecoscint A, National Diagnostics) was added to vials, which were incubated overnight. Incorporated radioactivity was then measured using a Beckman liquid scintillation spectrometer. As control, paraformaldehyde was added to trypanosomes before adding the radiolabelled spermidine. Each time point was performed in triplicate.

### **High Performance Liquid Chromatographic analyses.**

All HPLC equipment and software were from Dionex (UK). Separations were carried out utilising a GENESIS AQ 4 µm column (150 x 4.6 mm) (GraceVYDac) eluting with 20 mM ammonium formate pH 2.7/acetonitrile (15:85) passing through a fluorescence detector (Dionex model RF 2000), with an excitation wavelength of 340 nm and emission wavelength of 510 nm, at a flow rate of 1.0 ml min<sup>-1</sup>. Peak identity was confirmed by measuring the retention time and comparison with commercially available spermidine or putrescine. A calibration curve of spermidine or putrescine was generated with Chromeleon (Dionex software) using known amounts of the standard (0 – 1000 µM) in acetonitrile.



## Results

### Isolation of a *T. brucei* SpSyn gene

A DNA sequence (GeneDB acc. no. XM\_822031) in the *T. brucei* database was identified as containing an ORF related to spermine/spermidine synthases (Pfam PF01564). Based on this, we amplified an 894 bp DNA fragment with potential to encode a 33 kDa protein (designated TbSpSyn). Pairwise alignments revealed that TbSpSyn had 39-43% sequence identity to spermidine synthases of mammalian, plant and fungal origin (Figure 2). The similarity extended across the entire length of the trypanosomal protein and included the putrescine aminopropyltransferase (PAPT) family signature motif ([Val/Ala/Ile]-[Val/Ala/Leu]-[Leu/Ile/Val]-[Leu/Ile/Val]-Gly-Gly-Gly-X-[Gly/Cys]-X-X-[Le/Ile/Val/Ala]-X-Glu) (Prosite PS01330).

### Biochemical properties.

To investigate the activity of the putative spermidine synthase, *TbSpSyn* was cloned into pTrcHis-C and expressed in *E. coli* (Experimental). After induction with IPTG, a 37 kDa band was detected in the soluble fraction of bacterial extracts that could be readily purified by one round of affinity chromatography (Figure 3A). Enzyme activity was assayed by monitoring the production of spermidine from putrescine and dcSAM. After performing each reaction, the total polyamine content was derivatised with dansyl chloride and then analysed by HPLC coupled to a fluorescence spectrophotometer. Standard curves were generated by plotting different [spermidine] against the HPLC-derived area under the peak. From this, the polyamine levels in each assay could be determined. Initial reactions using 1 mM putrescine and 10  $\mu$ M dcSAM in the presence of recombinant TbSpSyn, demonstrated that the parasite enzyme could mediate the formation of spermidine. To further investigate these interactions, assays were carried out using various concentrations of putrescine (12.5 - 1000  $\mu$ M) at a fixed concentration of dcSAM (10  $\mu$ M). Double reciprocal plots of 1/TbSpSyn activity versus 1/[putrescine] were linear (Figure 3B) and extrapolation allowed the apparent  $K_M$  value for putrescine ( $205 \pm 65 \mu$ M) and  $V_{max}$  ( $11.9 \pm 1.4$  nmol spermidine formed  $\text{min}^{-1} \text{mg}^{-1}$ ) to be calculated. To determine whether TbSpSyn activity could be saturated by dcSAM, reactions were carried out using a fixed concentration of putrescine (1000  $\mu$ M) and variable concentrations of dcSAM (0.025

- 10  $\mu$ M) (Figure 3C). Again, TbSpSyn demonstrated Michaelis-Menten kinetics with the parasite enzyme having an apparent  $K_M$  for dcSAM of  $0.09 \pm 0.01$   $\mu$ M.

### **Investigating the functional significance of *T. brucei* SpSyn using RNAi**

Using a tetracycline-inducible RNAi system, we set out to determine if TbSpSyn was required for parasite viability. A DNA fragment (860 bp) was generated using PCR, cloned into p2T7<sup>Ti</sup> [22] and the RNAi construct was transformed into BSF *T. brucei*. To examine whether down-regulation of *TbSpSyn* affected the growth of bloodstream form *T. brucei*, the cumulative cell density of tetracycline-treated parasites was compared against untreated cultures (Figure 4A). In the absence of tetracycline, the RNAi cell lines grew at the same rate as parental cultures. Addition of tetracycline had a dramatic effect (Figure 4A). Within 72 hours there was a significant decrease in the growth rate, with a reduction in the endogenous *TbSpSyn* mRNA (Figure 4B) and a 60% decline in spermidine levels (Table 2). Over the next 48 hours, the cell density remained static. Similar results were observed with two independent clones.

To conclusively demonstrate that the observed growth deficiency was due specifically to a reduction in the *TbSpSyn* transcript, a complementation strategy was used. This involved inserting the *L. major SpSyn* gene into the tubulin locus of the *T. brucei* RNAi cell line using the constitutive expression vector pTUB-EX [24]. In these experiments, parasites induced to undergo down-regulation of *TbSpSyn* while expressing *LmSpSyn* grew at a rate comparable to non-induced controls (Figure 4A). RNA hybridisation confirmed that the level of endogenous *TbSpSyn* had fallen and that expression of the complementing *LmSpSyn* mRNA was occurring (Figure 4B).

### **Spermidine uptake by bloodstream form *T. brucei*.**

Attempts to complement the RNAi-mediated growth defect by adding spermidine to the medium, an approach that has proved successful in analysing *Leishmania* polyamine biosynthesis null mutants [25-28], failed. Interestingly, exogenous spermidine displayed significant trypanocidal activity at concentrations above 10  $\mu$ M (data not shown). This had been previously noted and attributed to polyamine oxidase activity in the heat-inactivated fetal calf serum that is used to supplement the parasite growth medium [29, 30]. At concentrations where no significant toxicity was

observed ( $<5 \mu\text{M}$ ), addition of spermidine to the medium failed to complement for the RNAi-mediated growth defect, even though this level is  $>15$ -fold higher than that found in blood plasma (300 nM) [31]. One possible explanation for this may be that *T. brucei* lack a high affinity spermidine transporter related to that in *L. major* and *T. cruzi* [32, 33]. To determine if BSF *T. brucei* can scavenge spermidine from its environment, uptake assays were performed using radiolabelled polyamine (Experimental section). In these experiments, *T. brucei* displayed no capacity to take up spermidine, even when assays were carried out for one hour (Figure 5). In parallel assays, *T. cruzi* was shown to transport spermidine. Therefore BSF *T. brucei* is incapable of scavenging spermidine and as such, must rely on *de novo* synthesis to acquire this polyamine.

## Discussion

Polyamines are ubiquitous in nature and play crucial roles in several biological processes. All eukaryotes and eubacteria have evolved uptake and/or biosynthetic strategies to acquire them and it has been proposed that inhibitors targeting these systems may be of importance in treating infectious diseases and certain cancers [3-7]. Here, we demonstrate that spermidine synthase in bloodstream form *T. brucei* represents a valid target for chemotherapy.

To confirm that the genome database annotation was correct, we performed SpSyn assays using purified protein generated by expression in *E. coli*. These experiments demonstrated that the recombinant enzyme had apparent  $K_M$  values for putrescine and dcSAM of 205  $\mu\text{M}$  and 0.09  $\mu\text{M}$  respectively (Figure 3). An earlier study investigating the biochemical properties of TbSySyn, partially purified from the parasite, noted that this enzyme functioned as a homodimer of 74 kDa, with apparent  $K_M$ 's for putrescine and dcSAM similar to our data [18]. It was also observed that activity could be markedly reduced by dicyclohexylamine and cyclohexylamine, compounds that act as competitive inhibitors with respect to putrescine. In many respects, these biochemical properties are typical of PAPT's from other sources (see <http://www.brenda.uni-koeln.de>). For example, the  $K_M$  for putrescine of human spermidine synthase (HsSpSyn) is 80  $\mu\text{M}$  [34]. However, TbSpSyn does appear to have a higher affinity for dcSAM compared to the human enzyme, with HsSpSyn exhibiting a  $K_M$  for dcSAM (7  $\mu\text{M}$ ) 70-fold higher than its trypanosomal counterpart [34]. This difference may represent a property exploitable in terms of inhibitor design.

Different trypanosomatid parasites use various mechanisms to acquire polyamines. *Leishmania*, for example, possess a polyamine biosynthetic pathway, plus putrescine/spermidine transporters [25-28, 32], whereas *Trypanosoma cruzi* contains a partial biosynthetic pathway and relies on uptake to meet its polyamine requirement [35-37]. To determine the importance of SpSyn to BSF *T. brucei* and investigate whether a biosynthetic and/or transporter mechanism is used to acquire spermidine, we used RNAi to down-regulate expression of *TbSpSyn* (Figure 4A and B). This experiment clearly demonstrated that TbSpSyn is essential for the growth of BSF *T. brucei*, and that expression of the *L. major SpSyn* gene in these cells can complement

the resulting growth-deficient phenotype (Figure 4A). We were unable to complement the RNAi-mediated growth defect by addition of spermidine to the medium suggesting that for the *T. brucei* sub-species used here, *T. brucei brucei*, scavenging alone does not fulfil the parasite's requirements for spermidine. One possible explanation may be the lack of a high affinity putrescine/spermidine transporter related to that reported in *L. major* and *T. cruzi* [32, 33]. Indeed, comparison of the syntenic regions of the trypanosomatid genomes failed to identify the gene encoding this carrier protein in the *T. brucei brucei* database. If this protein is absent, then, based on ODC gene deletion experiments, putrescine uptake must occur through other carrier(s) [38-40]. The absence of an effective spermidine permease in *T. brucei*, as demonstrated here (Figure 5), may reflect an adaptation to the host bloodstream environment where the plasma concentration of spermidine is low (~300 nM) compared with the levels detected in mammalian cells (mM) where intracellular parasites such as *Leishmania* and *T. cruzi* reside [31, 41].

The regulation of the intracellular polyamine pools in many eukaryotes is accomplished by a series of complex regulatory and degradation pathways with disruption of these systems promoting unwanted side effects such as uncontrolled cell proliferation or apoptosis [42, 43]. Analysis of polyamine levels in trypanosomes undergoing RNAi-mediated depletion of TbSpSyn revealed these cells had decreased spermidine content but, unexpectedly, there had been no significant build up of putrescine (Table 2). Over expression studies of ODC, SpSyn and SAMdc in *Leishmania donovani* also showed that even when enzyme activities were >25 fold higher than wild type, there was little change to the polyamine content [44]. This strongly implies that trypanosomatids have the ability to control metabolic flux through the polyamine biosynthetic pathway. Considering that the parasite enzymes are stable, compared to their mammalian counterparts, and that regulation of gene expression in trypanosomatids does not generally occur at the transcriptional level [15, 45], the processes involved in this regulatory system may be unusual. Recently, a novel mechanism for regulating SAMdc activity in *T. brucei* has been reported [46, 47]. An inactive version of SAMdc, known as the prozyme, forms a heterodimeric complex with the functional enzyme, that stimulates activity by >3 orders of magnitude. It will be of interest to determine whether any other enzyme in the

trypanosomatid polyamine biosynthetic pathway displays an unusual regulatory mechanism.

The success of DFMO, one of two drugs used to treat late stage HAT, has already shown that compounds targeting polyamine synthesis can be used to treat this disease. This study demonstrates that TbSpSyn, the enzyme immediately downstream of ODC in this pathway, is essential in BSF *T. brucei*. This, coupled with the parasites inability to scavenge sufficient spermidine from its environment, validates TbSpSyn as a potential target for future drug development.

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## Tables

	gene	primer	sequence (5' to 3')
RNAi	TbSpSyn	TbSpSyn-1	<i>gga</i> tcctGCTGACGGATGGTTTCGTGA
		TbSpSyn-2	<i>ctc</i> gagTGAGATGGCGGGCAAAGCGTG
Protein expression	TbSpSyn	TbSpSyn-3	<i>gga</i> tcctCTGGACCCGGTCTTCTTGCTGA
		TbSpSyn-4	<i>aag</i> cttTCAAGAATAGTTTTGGTTGAG
Complementation	LmSpSyn	LmSpSyn-1	<i>cct</i> gcaggAGCGACTTGTCGATCAGATTT
		LmSpSyn-2	<i>ggc</i> gcgccTCTCTACTCGTTCAGGTGAGC

**Table 1. Oligonucleotides used in this study.** The sequences in lower case italics correspond to restriction sites incorporated into the primers to facilitate cloning.

cell line	Polyamine (nmol (10 <sup>8</sup> ) <sup>-1</sup> cells)		S/P ratio
	Putrescine (P)	Spermidine (S)	
<b>wild-type</b>			
culture 1	6.75 ± 0.15	10.44 ± 0.15	1.5
culture 2	10.60 ± 0.80	17.90 ± 1.30	1.7
culture 3	6.25 ± 0.55	9.45 ± 0.55	1.5
<b>RNAi-cell line</b>			
culture 1	9.20 ± 0.60	5.10 ± 0.30	0.6
culture 2	12.45 ± 0.35	7.25 ± 0.35	0.6
culture 3	5.25 ± 0.35	2.85 ± 0.25	0.5

**Table 2. Polyamine levels in parasites undergoing RNAi.** Parasites from three independent wild-type and tetracycline-treated (96 hr) RNAi-TbSpSyn cultures were harvested, the polyamines extracted, derivatised with dansyl chloride then analysed by HPLC, with each sample run in duplicate. The putrescine and spermidine levels were determined using standard curves generated in parallel. All samples were checked by TLC to determine derivatisation. Values are the means ± standard error.

## Figure Legends

### Figure 1. Polyamine biosynthetic pathway in trypanosomatids.

In one arm of the pathway, putrescine is synthesised from arginine via ornithine by the enzymes arginase (1) and ornithine decarboxylase (2), the latter being the target for the trypanocidal agent difluoromethylornithine (DFMO). In the other arm of the pathway, methionine is converted to S-adenosylmethionine (SAM) and onto decarboxylated S-adenosylmethionine (dcSAM) by the activities of S-adenosylmethionine synthetase (3) and S-adenosylmethionine decarboxylase (4) respectively. Spermidine synthase (5) then catalyses the formation of spermidine from putrescine and dcSAM. In trypanosomatids, spermidine is required for biosynthesis of the unique thiol trypanothione.

### Figure 2: Sequence analysis of *T. brucei* spermidine synthase.

Alignment of spermidine synthase sequences from *T. brucei* (accession number XP\_827124), *T. cruzi* (XP\_811725), *L. major* (CAC44919), *Arabidopsis thaliana* (CAB61614), human (AAA36633), *Saccharomyces cerevisiae* (yeast) (AAC17191) and *Plasmodium falciparum* (AAN35885). Residues in common with the *T. brucei* sequence are highlighted in light grey; dashes represent gaps in the sequence to optimize alignment. Amino acids that form the characteristic PAPT family signature motif (Prosite Documentation PDOC01033) are indicated by #. Residues that are believed to interact with the substrates dcAdoMet (His<sup>74</sup>, Asp<sup>98</sup>, Asp<sup>118</sup>, Asp<sup>149</sup>, Gly<sup>150</sup>, Asp<sup>168</sup> and Pro<sup>175</sup>) (dark grey) and putrescine (Trp<sup>19</sup>, Gln<sup>64</sup>, Tyr<sup>73</sup>, Asp<sup>168</sup>, Thr<sup>169</sup>, Asp<sup>171</sup>, Glu<sup>203</sup>, Leu<sup>207</sup>, Asn<sup>208</sup>, Gln<sup>209</sup>, Tyr<sup>238</sup>, Pro<sup>239</sup> and Ile<sup>243</sup>) (black) are highlighted [48, 49].



**Figure 3. Biochemical properties of TbSpSyn.**

A. 10% SDS-PAGE gel stained with coomassie blue. Lane 1, crude extract loaded on a Ni-NTA column and flow through (lane 2). The column was washed extensively with 10 mM imidazole (lane 3) and the recombinant protein eluted with 200 mM imidazole (lane 4). Markers (M) are in kiloDaltons. The 37 kDa band (indicated) corresponds to recombinant TbSpSyn. B. Interaction of TbSpSyn with putrescine. TbSpSyn activity was monitored by following the production of spermidine from putrescine (12.5-1000  $\mu$ M) and dcSAM (10  $\mu$ M) using 100  $\mu$ g TbSpSyn. C. Interaction of TbSpSyn with dcSAM. TbSpSyn activity was monitored by following the production of spermidine from putrescine (1000  $\mu$ M) and dcSAM (0.05-10  $\mu$ M) in the presence of 100  $\mu$ g TbSpSyn. In panels B and C, reactions were dansylated and spermidine levels determined by HPLC coupled to a fluorescence detector. Activity is expressed as nmol spermidine formed  $\text{min}^{-1} \text{mg}^{-1}$ .

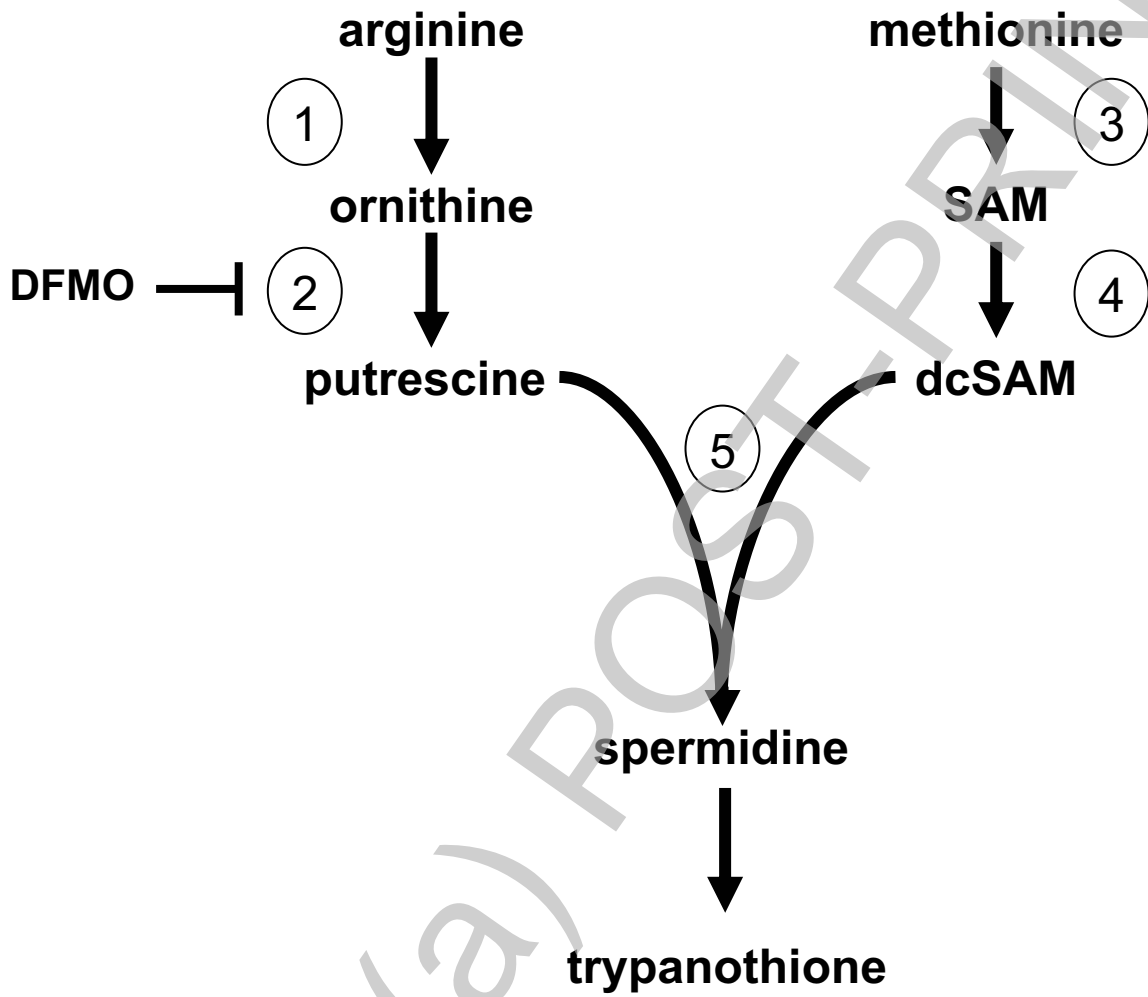
**Figure 4. Spermidine biosynthesis is essential for bloodstream form *T. brucei*.**

A. The growth of tetracycline-treated (+ tetracycline) and non-treated (- tetracycline) BSF *T. brucei* was followed in the parental (SMB) and RNAi cell lines. The growth defect could be complemented by expressing the *L. major* spermidine synthase gene in the RNAi cell line (RNAi + Comp). The data are expressed as cumulative cell number  $\text{ml}^{-1}$ . B. Down-regulation of gene expression by RNAi. Blots containing 15  $\mu$ g of *T. brucei* total RNA from tetracycline-induced (96 hr) (+) and non-induced (-) BSF cell lines were hybridised with radiolabelled probes as indicated. n.d. – not done. Equal loading was judged by ethidium bromide staining of rRNA.

**Figure 5. Bloodstream form *T. brucei* lack a spermidine uptake capacity.**

BSF *T. brucei* or *T. cruzi* epimastigotes ( $2 \times 10^8$  parasites  $\text{ml}^{-1}$  per assay) were incubated with [ $^{14}\text{C}$ ] spermidine for the times indicated. Cells were prepared and uptake of the radiolabel measured by scintillation counting (Experimental section). All data are means from 3 experiments  $\pm$  standard deviation.

**Figure 1**



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Figure 2

- see also: 1042/BJ20071185

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T.brucei -----MPGPGLLADGWFREENG-QWPGQAMSFKVEEVLHDAPTKFQHLISIFETD-RGPWGTVMTLTGCIQLTDFDEFVYHEMLSHTPLC 83
T.cruzi -----MPGSELISGGWFREEND-QWPGQAMSLRVEKVLVDAPTKFQHLTI FESD-RGPWGTVMALDGCIQVTDYDEFVYHEVLGHTSLC
L.major -----MPGPGLLPDGFREEST-MWPGQAQGLKVEKVLVDQPTFQHLTVFESD-RGPWGTVMTLTGAIQLTDFDEFVYHEMLANLSLA
Arabidopsis MDAKETSATDLKRPREEDDNGGAATMETENG DQKKEPACFSTVIPGWFSEMSM-WMPGEAHSKVEKVLV FQKSDYQDVIVFQS-RATYGVKVLVDGVIQLTERDECAYOEMITHLPLC
Human -----MEPGPDGPAASGPAAREGWFRETC-SLWPGQALSLOVEQLLHRRSRYQDILVFRS-RKTYGNVLVLDGVIQLTERDEFVYQEMIANLPLC
yeast -----MAQEITHPTIVDGFREISDTMWPGQAMTLKVEKVLHHEKSKYQDVLIFKS-RTTYGNVLVLDNVIQATERDEFAYOEMIAHLALN
Plasmodium ----MDKLI SNNK LKLSVLLGGLCSLAYYHLKNKFHLSQFCFSKKWFSEFSI-MWPGQAFSLKIKKILYETKSKYQNVLVFES-RTTYGVKVLVLDGVIQLTEKDEFAYHEMMTHVPMT

##### # # #
T.brucei AHPDPVDVLIIGGGDGGVMREVLRHG-----TVKRCVLVDIDGDVIEASKKYFPQISSGFS DPRADV RGDGVAFVREAASESFDYIITDITDPDGPAAELFGEKFYRDVLRILKPRGIC 198
T.cruzi SHPKPERVLIIGGGDGGVLEVLRHG-----TVEHCDLVDIDGEVMEQSKQHFQISRSLTDP RAVRVDGCLAFVRQTPDNTYDVIITDITDPAGPASELFGAEFYKHVLRILKPDGIC
L.major CHHKPERVLVIGGGDGGVVREVL RHKSEKDG VVQSVELVDIDGAVMQQSKKHFPQVACGFANPCVTATVGDGAAFVRNV PDSVYDVIITDITDPKGPASELFGADFYTNVLRILRPGGVV
Arabidopsis SIPNPKKVLVIGGGDGGVLRVARHA-----SIEQIDMCETIDKMVVVDSKQFFPDVAIGYEDPRVNLVIGDGVAF LKNAAEGSYDVIIVDSSDPIGPAKELFEKPPFQSVARALRPGGVV
Human SHPNPRKVLVIGGGDGGVLRVVKHP-----SVESVVQCEIDEDVIQVSKKFLPGMAIGYSSSKVTLHVGDGCFEFMKQND-AFDYIITDSSDPMGPAESLFKESYYQLMKTALKEDGVL
yeast SHPNPKKVLVIGGGDGGVLRVVKHD-----SVEEAWLCEIIDEAVIRLSKEYLPEMAASYSHPKVKTHIGDGFQFLRDYQN-TFDYIITDSSDPEGPAETLFQKEYFQLLSALTEKQVI
Plasmodium VSKEPKNVLVVGGDGGIIRELCKYK-----SVENIDICEIIDEVIEVSKIYFKNISCGYEDKRVNVFIEDASKFLENVTN-TYDYIIVDSSDPIGPAETLFNQNFYEKIYNALKPNGYC

T.brucei CNOGESVWLNRLNLIEMADFIKNKVG FASVKYAMIYTPTYPCGSIIGSLICSKV-AGVDVTQPVRPVES----MPFAGELKYYSDSYHKAAFVLPFRFARHLNQNS 298
T.cruzi CNOGESIWLNLLELIEKMSRFIR-ETGFASVQYALMHVPTYPCGSIIGTLICSKK-AGVDVTKPLRPVED----MPFAKDLKYYDSEHAKASFALPRFARHINNSE
L.major CNOGESVWLNRLNLIEMADFIKNKVG FASVKYAMIYTPTYPCGSIIGTLVCAKS-ADTDVTVPMRPVES----LGFADQLKYYSSDHYHKAAFVLPFRFAAHLNE
Arabidopsis CTCAESLWLNRLNLIEMADFIKNKVG FASVKYAMIYTPTYPCGSIIGTLVCAKS-ADTDVTVPMRPVES----LGFADQLKYYSSDHYHKAAFVLPFRFAAHLNE
Human CCOGCEWLNRLNLIEMADFIKNKVG FASVKYAMIYTPTYPCGSIIGTLVCAKS-ADTDVTVPMRPVES----LGFADQLKYYSSDHYHKAAFVLPFRFAAHLNE
yeast TCOAESMWLNRLNLIEMADFIKNKVG FASVKYAMIYTPTYPCGSIIGTLVCAKS-ADTDVTVPMRPVES----LGFADQLKYYSSDHYHKAAFVLPFRFAAHLNE
Plasmodium VAOCESLWLNRLNLIEMADFIKNKVG FASVKYAMIYTPTYPCGSIIGTLVCAKS-ADTDVTVPMRPVES----LGFADQLKYYSSDHYHKAAFVLPFRFAAHLNE
    
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Stage 2

**Figure 3**

**A**

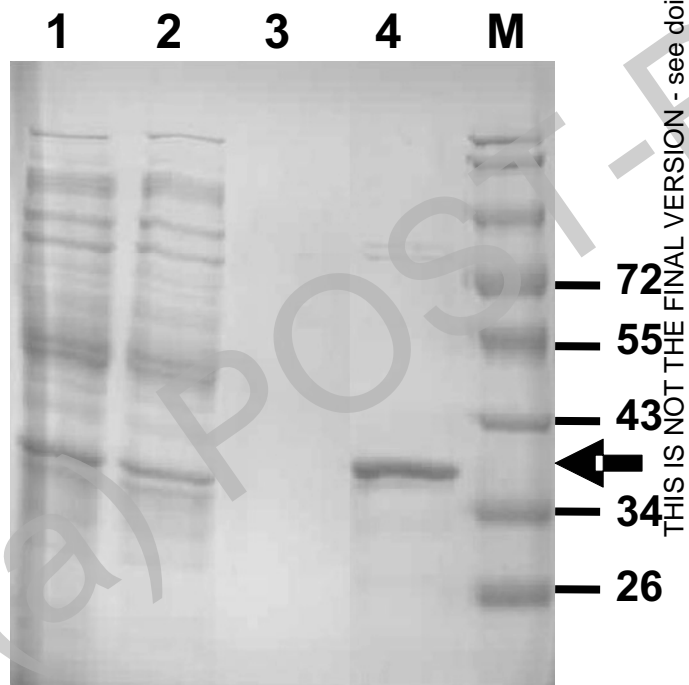


Figure 3

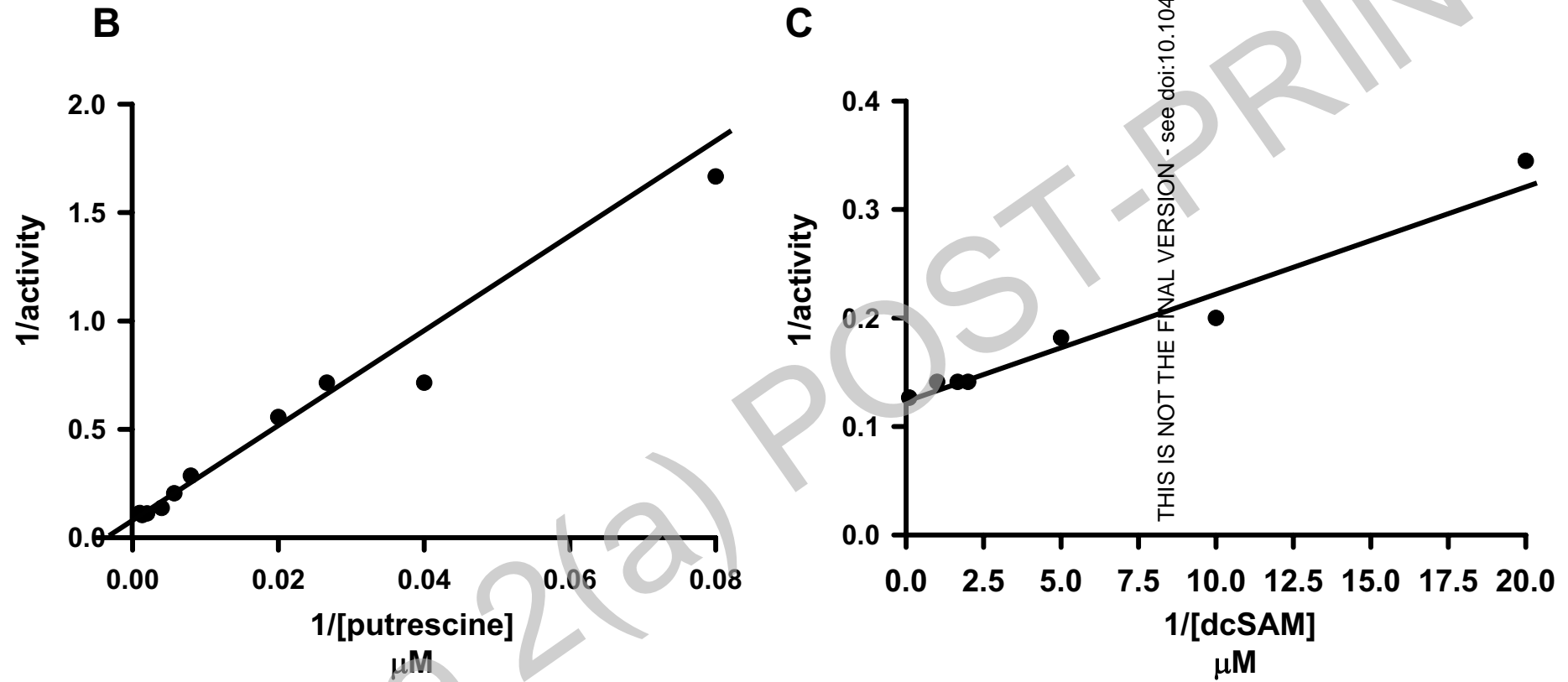
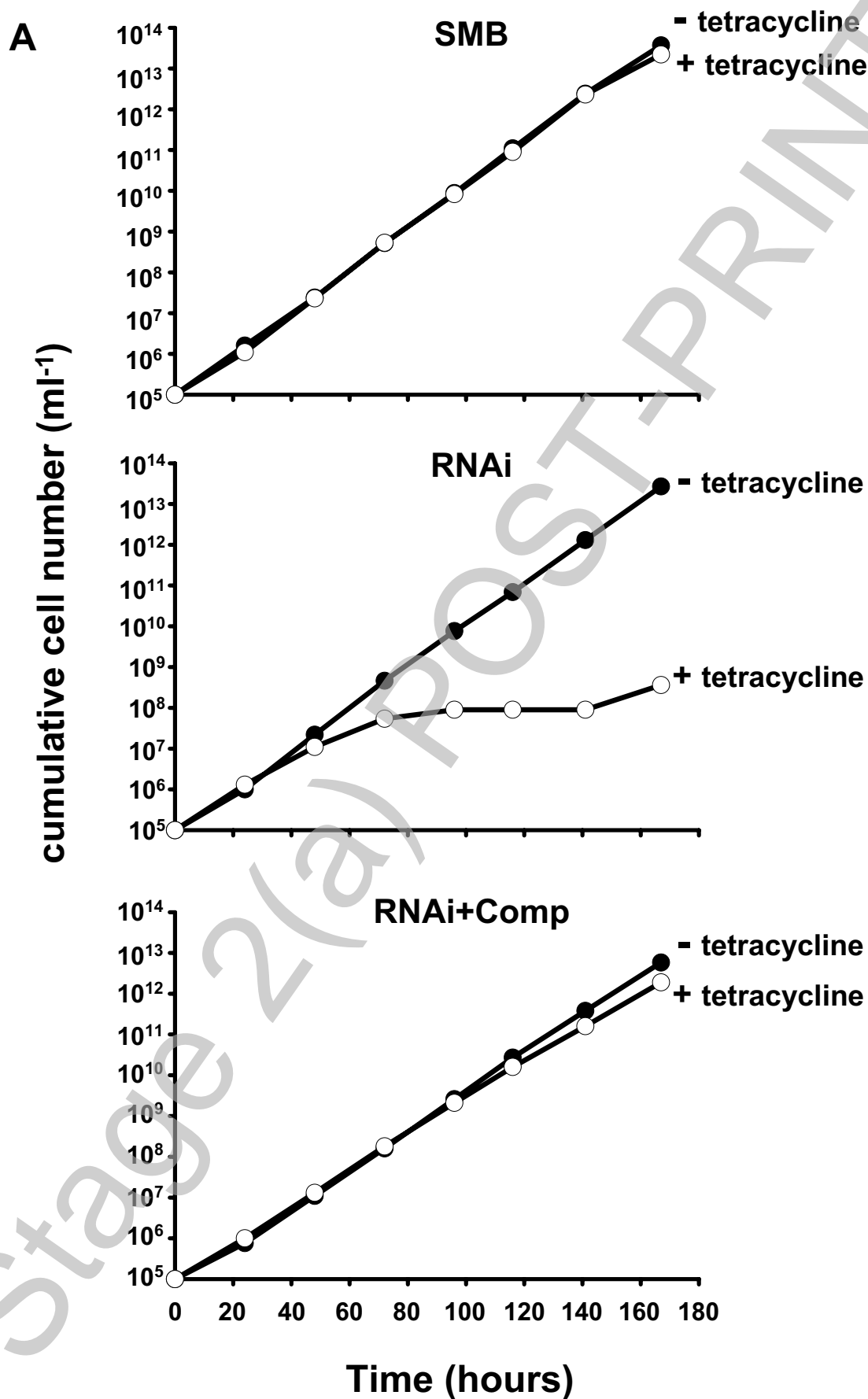
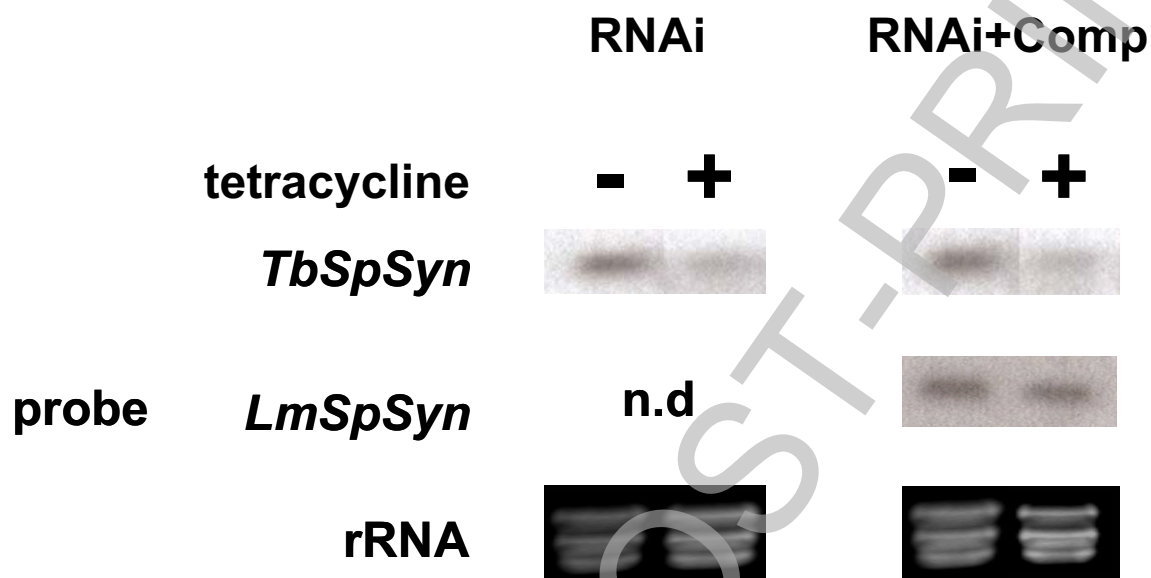


Figure 4



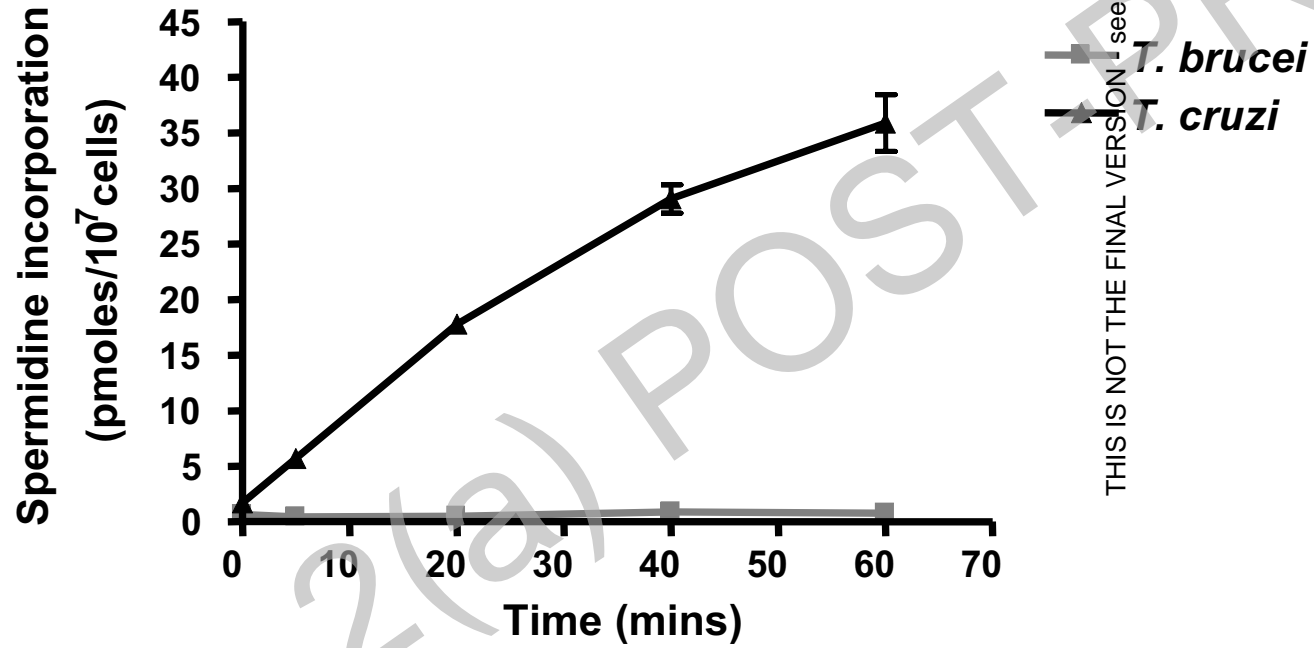
**Figure 4**

**B**



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Figure 5



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Stage 2(a) POST-PRINT