

# Distinct donor and acceptor specificities of *Trypanosoma brucei* oligosaccharyltransferases

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Asparagine-linked glycosylation is catalysed by oligosaccharyltransferase (OTase). In *Trypanosoma brucei* OTase activity is catalysed by single-subunit enzymes encoded by three paralogous genes of which *TbSTT3B* and *TbSTT3C* can complement a yeast  $\Delta stt3$  mutant. The two enzymes have overlapping but distinct peptide acceptor specificities, with *TbSTT3C* displaying an enhanced ability to glycosylate sites flanked by acidic residues. *TbSTT3A* and *TbSTT3B*, but not *TbSTT3C*, are transcribed in the bloodstream and procyclic life cycle stages of *T. brucei*. Selective knockdown and analysis of parasite protein N-glycosylation showed that *TbSTT3A* selectively transfers biantennary  $\text{Man}_5\text{GlcNAc}_2$  to specific glycosylation sites whereas *TbSTT3B* selectively transfers triantennary  $\text{Man}_9\text{GlcNAc}_2$  to others. Analysis of *T. brucei* glycosylation site occupancy showed that *TbSTT3A* and *TbSTT3B* glycosylate sites in acidic to neutral and neutral to basic regions of polypeptide, respectively. This embodiment of distinct specificities in single-subunit OTases may have implications for recombinant glycoprotein engineering. *TbSTT3A* and *TbSTT3B* could be knocked down individually, but not collectively, in tissue culture. However, both were independently essential for parasite growth in mice, suggesting that inhibiting protein N-glycosylation could have therapeutic potential against trypanosomiasis.

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## Introduction

The protozoan parasite *Trypanosoma brucei* is the causative agent of human African trypanosomiasis or sleeping sickness. The life cycle of *T. brucei* includes a bloodstream (trypomastigote) form in the mammalian host and a procyclic form in the midgut of the tsetse fly insect vector. In both forms, the cell surface of *T. brucei* is covered by a coat of glycosylphosphatidylinositol-anchored glycoproteins, the variant surface glycoproteins (VSGs) in the bloodstream (Cross, 1975) and the procyclins in the insect (Mowatt and Clayton, 1987; Richardson *et al*, 1988; Roditi *et al*, 1989). These proteins are N-glycosylated, a posttranslational modification that modulates folding, stability and activity of proteins that is essential in most eukaryotes (Varki, 1993; Sharon and Lis, 1995; Helenius and Aebi, 2004; Ohtsubo and Marth, 2006). N-glycosylation is catalysed by oligosaccharyltransferase (OTase) in the lumen of the endoplasmic reticulum (ER) (Helenius and Aebi, 2004) in which the transfer of oligosaccharide from a lipid-linked oligosaccharide (LLO) donor to selected asparagines in the sequons (N-x-S/T; x≠P) (Gavel and von Heijne, 1990) of translocated proteins occurs. Although OTase in higher eukaryotes is a multiprotein complex of up to eight subunits (Kelleher and Gilmore, 2006), the genome of *T. brucei* predicts that it lacks all except for the catalytic STT3 subunit, for which there are three complete paralogous genes (Berriman *et al*, 2005). Some of the additional subunits of the multiprotein complex OTases increase the diversity of efficiently glycosylated protein substrates. Ribophorin I (Ost1p) acts as a chaperone to enhance glycosylation of specific membrane proteins (Wilson and High, 2007), and the oxidoreductase activity of Ost3/6p increase the glycosylation efficiency of defined sequons (Schulz and Aebi, 2009; Schulz *et al*, 2009). In mammals, the presence of either STT3-A or STT3-B in the OTase complex also influences glycan substrate specificity and glycosylation rate, and both OTase isoforms act sequentially to maximise the efficiency of the N-glycosylation process (Kelleher *et al*, 2003; Ruiz-Canada *et al*, 2009). Although some organisms have extended the range of OTase activity by adding subunits to the core catalytic STT3, it is possible that *T. brucei* has instead duplicated and diversified a single-subunit OTase, as seems to be the case for the related kinetoplastid parasite *Leishmania major* (Nasab *et al*, 2008; Hese *et al*, 2009). This hypothesis is supported by the observation that, though in higher eukaryotes OTase has high specificity for the mature  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  glycan, which it transfers to many

different glycosylation sequons, *T. brucei* selectively transfers Man<sub>5</sub>GlcNAc<sub>2</sub> to one sequon and Man<sub>9</sub>GlcNAc<sub>2</sub> to another in the VSG221 protein (Jones *et al*, 2005; Manthri *et al*, 2008). This suggests that a mechanism exists for glycan donor- and peptide acceptor-specific glycosylation in this organism, although the generality of that mechanism beyond VSG has not been tested.

It has been reported that the single-subunit STT3 OTases from *T. cruzi* and *L. major* can complement an *STT3* deletion in yeast (Castro *et al*, 2006; Nasab *et al*, 2008; Hese *et al*, 2009). We considered, therefore, that yeast could be a useful *ex vivo* model system to investigate the potential substrate specificities of the *T. brucei* OTases and with which to compare the effects of individual TbSTT3 knockdowns in *T. brucei* itself.

## Results

### **TbSTT3B and TbSTT3C complement a $\Delta stt3$ yeast mutant with different efficiencies and different acceptor sequon specificities**

There are three putative intact *STT3* genes in *T. brucei* (*TbSTT3A*, *TbSTT3B* and *TbSTT3C*). Although all three TbSTT3 proteins were efficiently expressed in wild-type yeast cells at similar levels (Supplementary Figure S1A), only expression of TbSTT3B and TbSTT3C, but not of TbSTT3A, could support yeast growth in the absence of endogenous STT3 (Figure 1A). The  $\Delta stt3 + TbSTT3B$  cells grew almost as well as wild type at 23°C, but showed a mild temperature-sensitive growth phenotype at 37°C (Figure 1B); whereas  $\Delta stt3 + TbSTT3C$  cells had a severe growth defect even at 23°C. Owing to the inability of TbSTT3A to support yeast  $\Delta stt3$  growth, we determined its function in trypanosomes by an mRNA silencing approach (see below).

The sequences of TbSTT3B and TbSTT3C share 95% identity, whereas TbSTT3A share only 79% identity with them both (Supplementary Figure S2). To investigate whether the minor sequence differences between TbSTT3B and TbSTT3C influence peptide acceptor selection in yeast, we used a mass spectrometry-based method (Schulz and Aebi, 2009) to analyse the site-specific glycosylation occupancy of cell wall proteins in our *ex vivo* system. We analysed yeast cells with and without yeast STT3 and with different *TbSTT3* genes (Figure 1C; Supplementary Tables SI and SII). Yeast cells with wild-type OTase and in addition a *T. brucei* OTase did not show a reduction in glycosylation at any normally used site, suggesting that expression of *T. brucei* OTases did not interfere with the normal yeast N-glycosylation machinery. In  $\Delta stt3 + TbSTT3B$  cells, most sequons were glycosylated with comparable efficiency to wild-type yeast, whereas  $\Delta stt3 + TbSTT3C$  cells showed lower efficiency at more sites, correlating with the growth phenotypes of these cells (Figure 1A). Furthermore, TbSTT3B and TbSTT3C did indeed have different activities towards some glycosylation sequons, as some sites were more efficiently glycosylated by TbSTT3B and others by TbSTT3C (Figure 1C and D). As for other OTases (Kasturi *et al*, 1995; Petrescu *et al*, 2004) TbSTT3B and TbSTT3C showed a slight preference for threonine over serine in the +2 position of glycosylated sequons (Figure 1E). Significantly, cells expressing TbSTT3B and TbSTT3C partially glycosylated some sites never used by yeast OTase (Figure 1C and F) and the occupancy of these sites was increased in cells lacking yeast OTase activity. This

effect is not solely a result of OTase dosage in cells over-expressing TbSTT3B or TbSTT3C, as the newly glycosylated site was different in each case (i.e. N417 of Tos1p by TbSTT3B and N87 of Ccw14p by TbSTT3C).

We also analysed the sequences surrounding the glycosylated sequons ( $\pm 5$  amino acids) and noticed a tendency towards efficient occupancy of acidic sequences by TbSTT3C. These data will be discussed in more detail later.

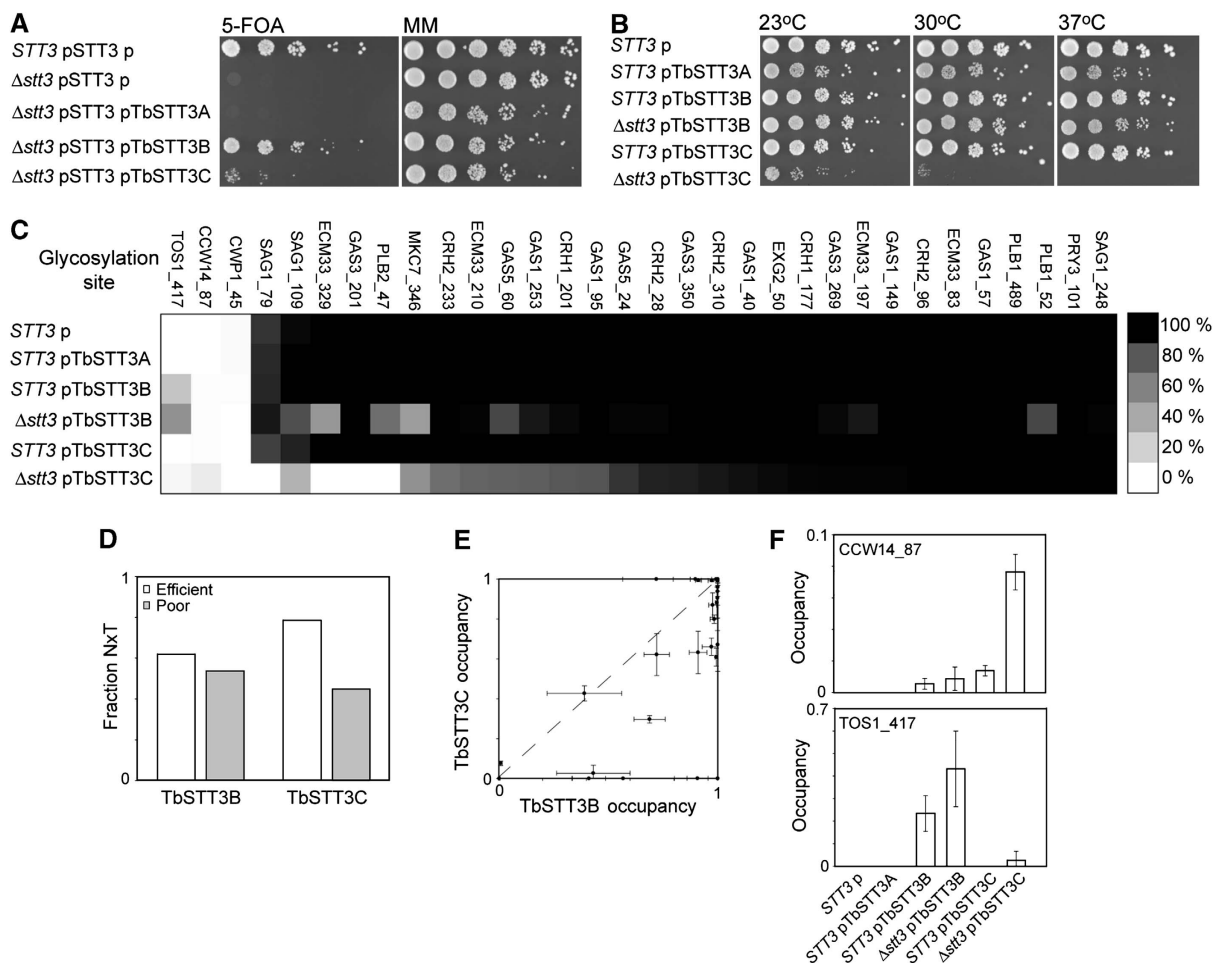
### **Only TbSTT3A and TbSTT3B transcripts are found in bloodstream and procyclic form *T. brucei***

We used semi-quantitative RT-PCR to assess the relative levels of the *TbSTT3* mRNA transcripts in bloodstream and procyclic form trypanosomes (Supplementary Figure 1B). We found that the *TbSTT3A* and *TbSTT3B* genes were expressed in both life cycle stages, with *TbSTT3A* more highly expressed in the bloodstream form, whereas transcripts for the *TbSTT3C* gene were not detected in either life cycle stage of the parasite.

### **The *TbSTT3A* gene encodes a Man<sub>5</sub>GlcNAc<sub>2</sub>-specific OTase responsible for all paucimannose and complex N-glycans in *T. brucei* glycoproteins**

We studied the roles of the *TbSTT3* genes in trypanosomes using inducible RNA interference (RNAi) and glycoprotein structure analysis. Our principal reporter molecule is the VSG coat glycoprotein of the parasite that, in the variant 221 clone used in our studies, contains two N-glycosylation sites (see Figure 2A, inset): one at Asn263 occupied by small, biantennary, endoglycosidase-H (EndoH)-resistant paucimannose and complex structures, which originate from the transfer of Man<sub>5</sub>GlcNAc<sub>2</sub> from the Man<sub>5</sub>GlcNAc<sub>2</sub>-PP-Dol precursor, and one at Asn428 occupied by conventional EndoH-sensitive triantennary oligomannose structures, which originate from the transfer of Man<sub>9</sub>GlcNAc<sub>2</sub> from Man<sub>9</sub>GlcNAc<sub>2</sub>-PP-Dol (Jones *et al*, 2005; Manthri *et al*, 2008). Thus, analysis of VSG221 N-glycosylation allows us to simultaneously assess the effects of genetic or chemical perturbations on both mechanisms of protein N-glycosylation in this organism (Jones *et al*, 2005; Urbaniak *et al*, 2006; Manthri *et al*, 2008; Stokes *et al* 2008). Cell-surface VSG can be purified in a soluble form (sVSG221) after osmotic shock, a process that releases VSG from the parasite surface by the action of endogenous GPI-specific phospholipase C that cleaves the dimyristoylglycerol lipid component of the VSG GPI anchor (Cross, 1975, 1984; Cardoso de Almeida and Turner, 1983; Ferguson *et al*, 1985). This cell surface-derived sVSG221 is amenable to glycoform analysis as an intact glycoprotein by electrospray-mass spectrometry (ES-MS) and as Pronase glycopeptides by ES-MS and ES-MS/MS (Jones *et al*, 2005; Urbaniak *et al*, 2006; Manthri *et al*, 2008; Stokes *et al* 2008). In addition, the N-glycosylation status of both cell surface-derived sVSG221 and of newly synthesised (presumably ER-associated) VSG221, which is not released by osmotic shock (Ferguson *et al* 1986), can be assessed by EndoH and PNGaseF digestion and analysis by SDS-PAGE and Coomassie blue staining or anti-VSG221 western blot, respectively.

The three intact *TbSTT3* genes are arrayed in tandem on chromosome 5. One whole *TbSTT3A,B,C* array was replaced by homologous recombination with a puromycin acetyl transferase (*PAC*) selectable marker to obtain a bloodstream form *TbSTT3A,B,C*<sup>+/-</sup> mutant (Supplementary Figure S1C). This deletion did not affect the glycosylation profile of



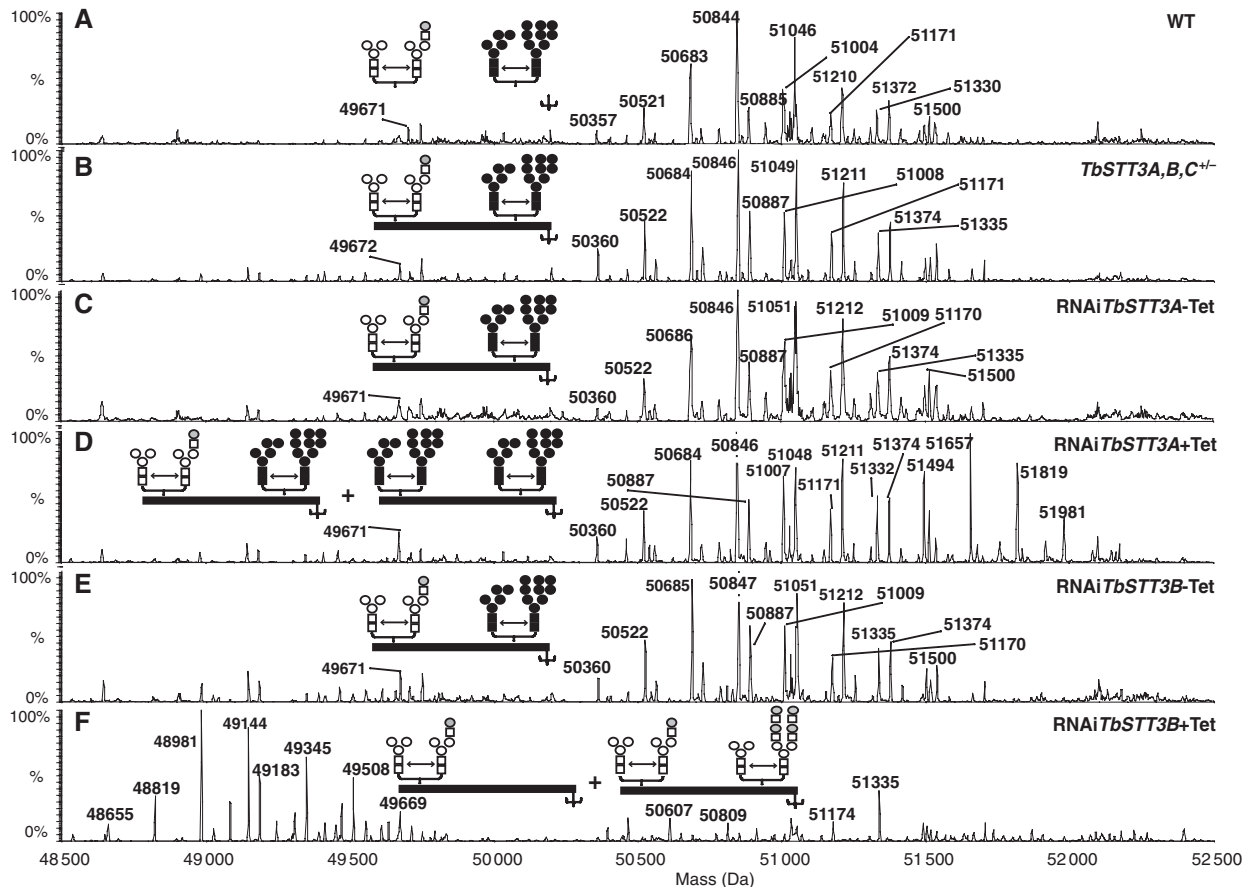
**Figure 1** Complementation of yeast  $\Delta$ *stt3* by *T. brucei* OTases. (A) Yeast cells with (*STT3*) or without ( $\Delta$ *stt3*) genomic *STT3*, with the yeast *STT3* gene encoded on a URA3 plasmid (p*STT3*), and with various Tb*STT3* genes (pTb*STT3A*; pTb*STT3B*; pTb*STT3C*) or with empty vector (p) on a LEU2 plasmid were grown in serial dilution on minimal media (MM) or media containing 5-FOA for selection of *ura*-cells and (B) after selection were grown at different temperatures in serial dilution on MM. (C) Glycosylation occupancy of all sites measured for each strain, mapped from 100% occupancy, black, to 0% occupancy, white. Values are averages of three independent measurements. Data are shown in Tables S1 and S2. (D) Proportion of threonine in the +2 position of efficiently and poorly glycosylated sequons. (E) Cross correlation of site-specific glycosylation efficiency by Tb*STT3B* and Tb*STT3C*. (F) Glycosylation of TOS1\_417 and CCW14\_87 sites by *T. brucei* *STT3B* and *STT3C* that are not used by yeast OTase. Error bars show range.

the VSG221 reporter glycoprotein (Figure 2A and B) but it provided us with a heterozygote cell line in which subsequent selective RNAi knockdowns of Tb*STT3*s would be maximised.

A 498 nt internal coding sequence was used to generate a bloodstream form cell line containing an inducible double-stranded RNA (dsRNA) construct targeting Tb*STT3A* in the Tb*STT3A,B,C*<sup>+/-</sup> heterozygote (Supplementary Figure S1E). Tetracycline induction substantially reduced Tb*STT3A* mRNA without affecting Tb*STT3B* mRNA (Figure 3A). The Tb*STT3A* knockdown did not have a major effect in the growth of the parasite in culture (Figure 3B) but it had a clear effect on the glycosylation of newly synthesised (ER-associated) VSG221. Thus, the majority of the ER-associated VSG221 in the Tb*STT3A* knockdown contained two EndoH-sensitive N-glycans, instead of one EndoH-sensitive and one EndoH-resistant N-glycan (Figure 3C). Analysis of cell surface-derived sVSG221 after 72 h of RNAi induction showed the presence of two distinct bands after EndoH digestion, suggesting this preparation contained two forms of sVSG221 with one and two EndoH-sensitive N-glycans, respectively, (Figure 3D). Further analysis of the cell surface-derived sVSG221 by

ES-MS confirmed that two sets of overlapping glycoforms were present: one set identical to wild-type sVSG221 and another set with masses compatible with a modified sVSG221 with oligomannose structures at both N-glycosylation sites (Figure 2D; Supplementary Table SIII). This assignment was supported by subsequent Pronase digestion and ES-MS and ES-MS/MS identification of the sVSG221 glycopeptides (Supplementary Table SIV; Supplementary Figure S3A). The apparently greater proportion of modified versus wild-type VSG221 in the newly synthesised VSG221 versus mature cell surface-derived sVSG221 (compare Figure 3C and D) suggests that the small proportion of wild-type VSG221 present in the ER is enriched during ER exit and/or transit to the cell surface, which is consistent with the apparent importance of the correct glycosylation of the Asn263 site for VSG221 structure and folding (Blum *et al*, 1993; Izquierdo *et al*, 2009).

These data suggest that Tb*STT3A* is responsible for the transfer of Man<sub>5</sub>GlcNAc<sub>2</sub> to Asn263 of VSG221 and that, in its absence, Man<sub>9</sub>GlcNAc<sub>2</sub> is transferred to that site instead via Tb*STT3B*. The model described in Manthri *et al* (2008) predicts that only sites that receive Man<sub>5</sub>GlcNAc<sub>2</sub> can be processed to complex structures, because of the absence of



**Figure 2** Mass spectrometric analyses of intact mature sVSG221 glycoforms before and after selective knockdown of TbSTT3A and TbSTT3B expression. Samples of sVSG221 from (A) wild type cells, (B) *TbSTT3A,B,C<sup>+/-</sup>*, (C) *TbSTT3A,B,C<sup>+/-</sup>-STT3ARNai* minus Tet, (D) *TbSTT3A,B,C<sup>+/-</sup>-STT3ARNai* plus Tet, (E) *TbSTT3A,B,C<sup>+/-</sup>-STT3BRNai* minus Tet and (F) *TbSTT3A,B,C<sup>+/-</sup>-STT3BRNai* plus Tet were analysed by ES-MS. The spectra show the masses of the various glycoforms of the intact mature sVSG221 molecules. The inset cartoons represent our interpretation of those glycoform masses in terms of the ranges of N-glycans present at each of the two N-glycosylation sites. These assignments combine additional data from the ES-MS and ES-MS/MS analyses of Pronase glycopeptides from the same sVSG221 preparations (Supplementary Figure S3; Table SIV). In the inset cartoons, endo-H-resistant N-glycans are in white and endo-H-sensitive N-glycans are in black. Circles and squares (filled and open) represent mannose and N-acetylglucosamine residues, respectively, and grey circles represent galactose residues.

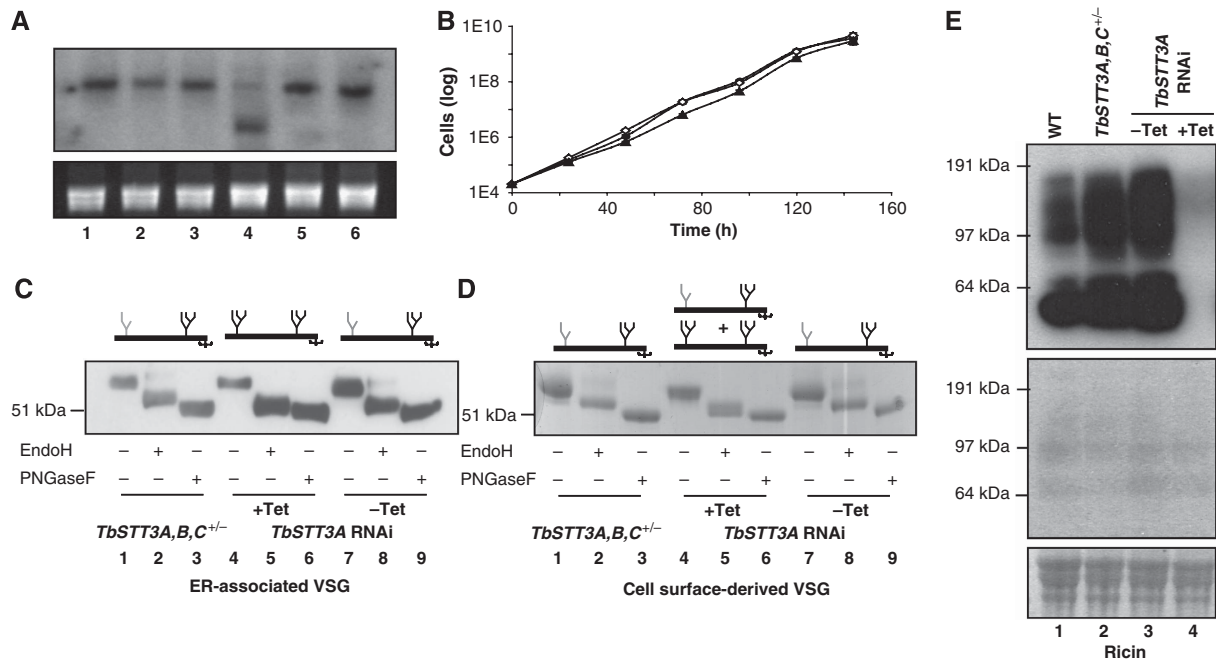
Golgi  $\alpha$ -mannosidase II gene in the *T. brucei* genome. Therefore, we also looked at the non-VSG glycoproteins of the parasite by solubilising parasite ghosts (after osmotic shock-mediated release of sVSG) and performing SDS-PAGE and ricin lectin blotting. The terminal galactose-specific lectin ricin detects *T. brucei* glycoproteins expressing complex N-linked oligosaccharides, including the giant poly-N-acetylglucosamine-containing N-glycans unique to this organism (Atrih *et al*, 2005). The striking reduction in ricin binding to the glycoproteins of the TbSTT3A knockdown cells (Figure 3E) is in complete agreement with the aforementioned model and suggests that it is general, rather than restricted to VSG N-glycosylation.

**The *TbSTT3B* gene encodes a *Man<sub>9</sub>GlcNAc<sub>2</sub>*-specific OTase responsible for most or all oligomannose N-glycans in *T. brucei* glycoproteins**

The same approach was taken to assess the function of *TbSTT3B*. A 467 nt internal coding sequence was used to create a bloodstream form *TbSTT3A,B,C<sup>+/-</sup>* heterozygote containing an inducible dsRNA construct targeting both *TbSTT3B* and *TbSTT3C* (Supplementary Figure S1E). As *TbSTT3C* mRNA is not present in bloodstream or procyclic

form cells (Supplementary Figure S1B), tetracycline induction for 72 h leads to the selective knockdown of *TbSTT3B* mRNA (Figure 4A). As for *TbSTT3A*, the *TbSTT3B* knockdown did not substantially affect the growth of the parasite in culture (Figure 4B). Analysis of both newly synthesised, ER-associated, VSG221 and cell surface-derived sVSG221 after 72 h of *TbSTT3B* silencing showed the presence of two sets of VSG221 glycoforms (Figure 4C and D). The lower molecular weight band co-migrates with wild-type VSG221 treated with EndoH, suggesting it has only one occupied N-glycosylation site, whereas the higher molecular weight band has two EndoH-resistant N-glycans. Analysis of intact and Pronase digested cell surface-derived sVSG221 after 72 h of *TbSTT3B* knockdown by mass spectrometry (Figure 2F; Supplementary Tables SIII, SIV; Supplementary Figure S3B) confirmed the presence of a lower molecular weight group of sVSG221 glycoforms lacking glycans at Asn428 and a higher molecular weight group with Asn263 and Asn428 sites occupied by paucimannose and/or complex glycans, respectively.

These results are also consistent with our model for VSG221 N-glycosylation (Manthri *et al*, 2008), such that removal of *TbSTT3B* has no effect on the glycosylation of Asn263 but greatly reduces the efficiency of the glycosylation



**Figure 3** The effects of RNAi silencing of *STT3A* on protein glycosylation in *T. brucei*. (A) Northern blot of total RNA extracted from the following cell lines: wild type (lane 1), *TbSTT3A, B, C<sup>+/-</sup>* (lane 2), *TbSTT3A, B, C<sup>+/-</sup>-STT3ARNAi* minus Tet (lane 3), *TbSTT3A, B, C<sup>+/-</sup>-STT3ARNAi* plus Tet (lane 4), *TbSTT3A, B, C<sup>+/-</sup>-STT3BRNAi* minus Tet (lane 5) and *TbSTT3A, B, C<sup>+/-</sup>-STT3BRNAi* plus Tet (lane 6) were probed with the *STT3A* probe (upper panel) and stained with ethidium bromide for rRNA as loading control (lower panel). (B) Growth of the following *T. brucei* cells at 37°C: wild type (closed circles), *TbSTT3A, B, C<sup>+/-</sup>-STT3ARNAi* minus Tet (open squares) and *TbSTT3A, B, C<sup>+/-</sup>-STT3ARNAi* plus Tet (closed triangles). (C) Cell ghosts containing ER-associated VSG221 and (D) cell surface-derived sVSG221 from the following cells: *TbSTT3A, B, C<sup>+/-</sup>* (lanes 1–3), *TbSTT3A, B, C<sup>+/-</sup>-STT3ARNAi* plus Tet (lanes 4–6) and *TbSTT3A, B, C<sup>+/-</sup>-STT3ARNAi* minus Tet (lanes 7–9) were digested or not, as indicated, with EndoH or PNGaseF were subjected SDS-PAGE and either western blot with anti-VSG221 antibody (C) or staining with Coomassie blue (D). The cartoons above the gels in panels (C) and (D) summarise the types of VSG221 present with respect to EndoH-resistant biantennary paucimannose and complex N-glycans (grey) and EndoH-sensitive triantennary oligomannose N-glycans (black). (E) Blots of total glycoprotein extracts of the following cells: wild type (lane 1), *TbSTT3A, B, C<sup>+/-</sup>* (lane 2), *TbSTT3A, B, C<sup>+/-</sup>-STT3ARNAi* minus Tet (lane 3) and *TbSTT3A, B, C<sup>+/-</sup>-STT3ARNAi* plus Tet (lane 4) were incubated with ricin without (upper panel) and with carbohydrate inhibitors (specificity control, middle panel) or stained with Ponceau red (loading control, lower panel).

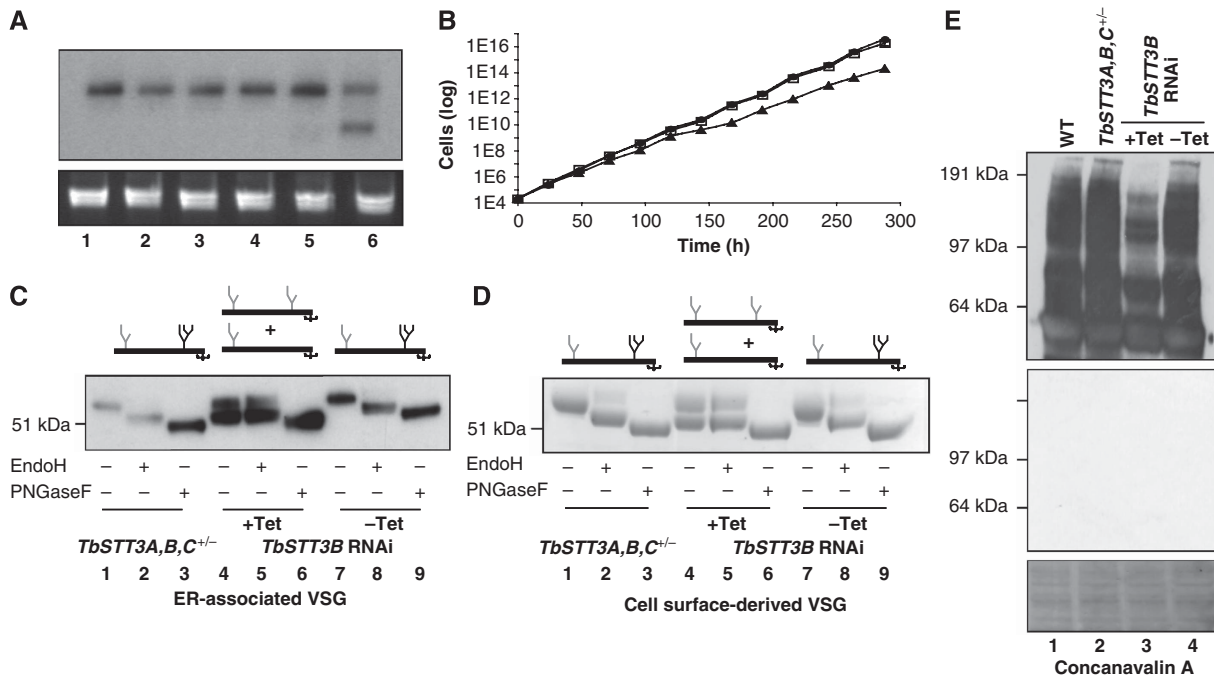
of the Asn428 site (which is normally served by the transfer of  $\text{Man}_9\text{GlcNAc}_2$  via *TbSTT3B*). We confirmed the generality of the results seen with the VSG221 reporter by analysing the aforementioned non-VSG glycoprotein preparation by SDS-PAGE and concanavalin A (ConA) blotting (Figure 4E). ConA binds with highest affinity to oligomannose and hybrid glycans containing a  $\text{Man}\alpha 1-3(\text{Man}\alpha 1-6)\text{Man}\alpha 1$ -motif and less well to complex biantennary-type structures (Brewer and Bhattacharyya, 1986). Thus, the significant reduction in ConA binding to the glycoproteins from the *TbSTT3B* knock-down cells suggests that most or all of the conventional triantennary oligomannose N-linked glycans have disappeared in parallel with *TbSTT3B* knockdown.

#### ***TbSTT3A* and *TbSTT3B* have distinct acceptor sequon specificities as well as oligosaccharide donor specificities**

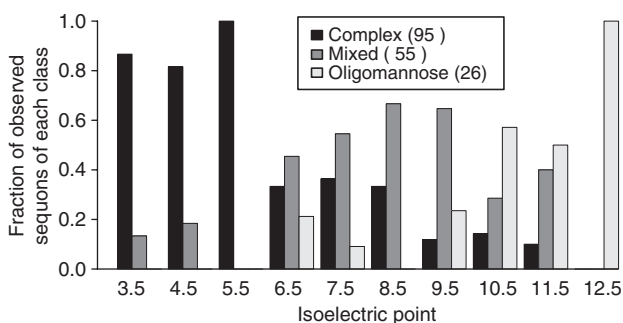
To establish if there are consensus sequences or patterns that control site-specific N-glycosylation by *TbSTT3A* or *TbSTT3B*, we developed a mass spectrometry approach to analyse wild-type bloodstream form *T. brucei* glycoproteins, summarised in 'Materials and methods' and in Supplementary Figure S4. Briefly, the glycoproteins were captured from VSG-depleted trypanosome lysates by sequential affinity chromatography on immobilised ricin followed by immobilised ConA. The ricin- and ConA-binding fractions were eluted with appropriate sugars and individually processed

by digestion with endoH followed by PNGaseF. Thus, endoH-sensitive glycopeptides appear 203 Da heavier by mass spectrometry because of the GlcNAc residue left attached to the relevant Asn residues by endoH whereas the remaining endoH-resistant, but PNGaseF-sensitive, sites appear 1 Da heavier by mass spectrometry because of the conversion of the relevant Asn residues to Asp by PNGaseF. Using this method, we noticed that many of the amino-acid sequences immediately adjacent to the sequons presenting complex or paucimannose N-glycans contained aspartic and/or glutamic acid residues, suggesting that charge might have a function in sequon recognition by *TbSTT3A*. To test this hypothesis, the isoelectric points (pIs) of the sequons themselves, plus five residues N- and C-terminal to them, were calculated. The exclusively EndoH-resistant sites, that is those modified by *TbSTT3A*, tend to be found within more acidic regions of protein sequence (mean  $\pm$  s.d. pI of  $4.8 \pm 1.7$ ) whereas the exclusively EndoH-sensitive sites, that is those modified by *TbSTT3B*, tend to be within neutral to basic regions (mean  $\pm$  s.d. pI of  $9.5 \pm 2.2$ ). The mixed sites, that is those that have been partly modified by *TbSTT3A* and partly by *TbSTT3B*, are relatively evenly spread between these pI extremes (Figure 5; Supplementary Tables SV and SVI). Therefore, the different polypeptide acceptor substrate specificities of *TbSTT3A* and *TbSTT3B* seem to be, at least in part, related to the pIs of the peptide sequences immediately around the N-glycosylation sites.





**Figure 4** The effects of RNAi silencing of *STT3B* on protein glycosylation in *T. brucei*. (A) Northern blot of total RNA extracted from the following cell lines: wild type (lane 1), *TbSTT3A,B,C<sup>+/-</sup>* (lane 2), *TbSTT3A,B,C<sup>+/-</sup>-STT3ARNAi* minus Tet (lane 3), *TbSTT3A,B,C<sup>+/-</sup>-STT3ARNAi* plus Tet (lane 4), *TbSTT3A,B,C<sup>+/-</sup>-STT3BRNAi* minus Tet (lane 5) and *TbSTT3A,B,C<sup>+/-</sup>-STT3BRNAi* plus Tet (lane 6) were probed with the *STT3B* probe (upper panel) and stained with ethidium bromide for rRNA as loading control (lower panel). (B) Growth of the following *T. brucei* cells at 37°C: wild type (closed circles), *TbSTT3A,B,C<sup>+/-</sup>-STT3BRNAi* minus Tet (open squares) and *TbSTT3A,B,C<sup>+/-</sup>-STT3BRNAi* plus Tet (closed triangles). (C) Cell ghosts containing ER-associated VSG221 and (D) cell surface-derived sVSG221 from the following cells: *TbSTT3A,B,C<sup>+/-</sup>* (lanes 1–3), *TbSTT3A,B,C<sup>+/-</sup>-STT3BRNAi* plus Tet (lanes 4–6) and *TbSTT3A,B,C<sup>+/-</sup>-STT3BRNAi* minus Tet (lanes 7–9) were digested or not, as indicated, with EndoH or PNGaseF were subjected SDS-PAGE and either western blot with anti-VSG221 antibody (C) or staining with Coomassie blue (D). The cartoons above the gels in panels (C) and (D) summarise the types of VSG221 present with respect to EndoH-resistant biantennary paucimannose and complex N-glycans (grey) and EndoH-sensitive triantennary oligomannose N-glycans (black). (E) Blots of total glycoprotein extracts of the following cells: wild type (lane 1), *TbSTT3A,B,C<sup>+/-</sup>* (lane 2), *TbSTT3A,B,C<sup>+/-</sup>-STT3BRNAi* plus Tet (lane 3) and *TbSTT3A,B,C<sup>+/-</sup>-STT3BRNAi* minus Tet (lane 4) were incubated with ConA without (upper panel) and with carbohydrate inhibitors (specificity control, middle panel) or stained with Ponceau red (loading control, lower panel).



**Figure 5** Distribution of the type of N-glycan occupancy related to the isoelectric point (pI) of the acceptor site peptide. The predicted pI values for each sequon  $\pm 5$  amino acids were binned in unit intervals from 3 to 13. For each interval, bars are shown to indicate the observed proportions of sites modified with exclusively EndoH-resistant glycans (black), EndoH-sensitive glycans (white), both EndoH-resistant and EndoH-sensitive glycans (grey).

***TbSTT3C* expressed in yeast seems to have a similar preference for acidic acceptor sequences to *TbSTT3A* expressed in *T. brucei***

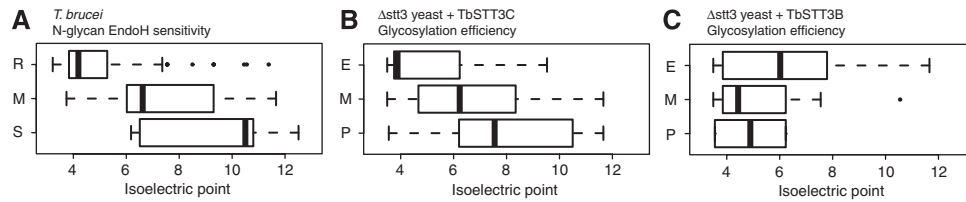
As mentioned earlier, we cannot make a direct comparison between the specificities of *TbSTT3A* and *TbSTT3C* because *TbSTT3A* does not complement *Astt3* yeast and *TbSTT3C* is not expressed in trypanosomes. However, in *T. brucei*, sites receiving endoH-resistant paucimannose/complex glycans through

*TbSTT3A* are relatively acidic and the same is true of sites exhibiting high glycosylation efficiency in *Astt3* + *TbSTT3C* yeast cells (Supplementary Table S1). This is apparent from the data plotted in (Figure 6). Figure 6A shows the distributions of pIs of the acceptor sequons ( $\pm 5$  amino acids) for sites recognised principally by *TbSTT3A* (endoH-resistant sites), principally by *TbSTT3B* (endoH-sensitive sites) and by both (mixed sensitivity sites) in *T. brucei*. Figure 6B and C show the comparable distributions of pIs for sites that are efficiently (>80%), poorly (<20%) and moderately (20–80%) glycosylated in *Astt3* + *TbSTT3C* and *Astt3* + *TbSTT3B* yeast cells, respectively. These data illustrate the similar preferences of *TbSTT3C* and *TbSTT3A* for acidic sites, and the lack of any obvious preference of *TbSTT3B* when it is expressed in either *Astt3* yeast or in *T. brucei*.

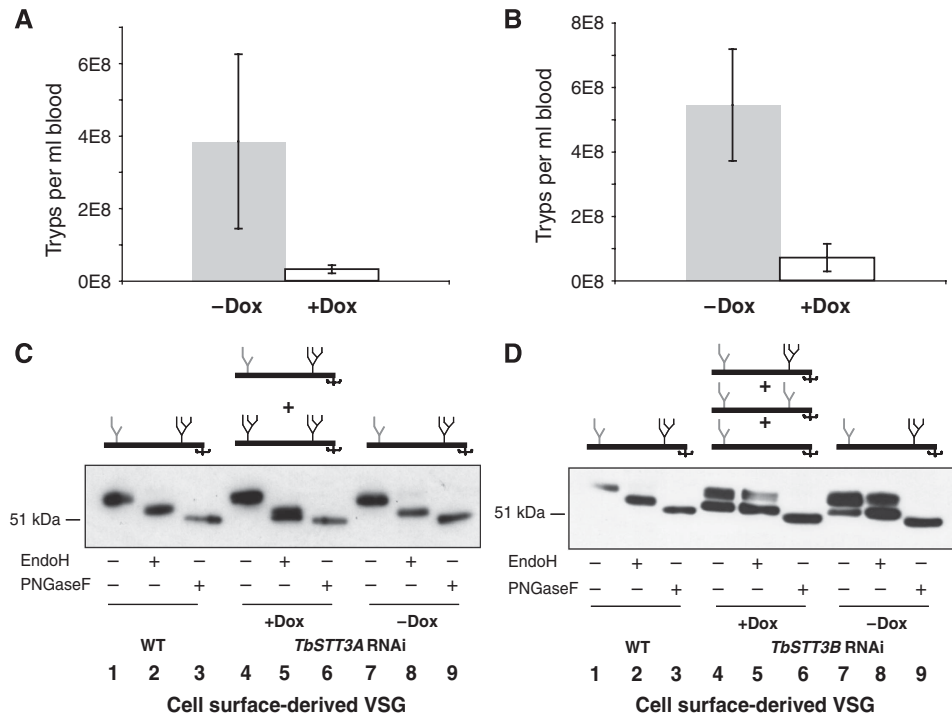
**Both *TbSTT3A* and *TbSTT3B* catalysed N-glycosylation mechanisms are essential for infectivity in mice**

Both N-glycosylation mechanisms are, individually, non-essential for parasite growth in culture (Figures 3B and 4B). However, we found that simultaneous knockdown of *TbSTT3A* and *TbSTT3B* halted the growth of bloodstream form trypanosomes in culture (Supplementary Figure S1F), showing that protein N-glycosylation *per se* is essential in culture.

We next investigated whether the *TbSTT3A/TbSTT3B* redundancy seen in culture was also evident in animal



**Figure 6** Correlations of N-glycan type in *T. brucei* and N-glycosylation efficiency in  $\Delta$ stt3 yeast with acceptor site pI. (A) Box plot (Tukey, 1977) of predicted pI distributions for the sequons  $\pm$  5 amino acids occupied by endoH-resistant (R), mixed (M) or endoH-sensitive (S) N-glycans. (B, C) Box plots of predicted pI distributions for the sequons  $\pm$  5 amino acids exhibiting efficient (E), moderate (M) and poor (P) N-glycosylation in  $\Delta$ stt3 + TbSTT3C and TbSTT3B yeast cells, respectively. Note: Box plots are concise ways of illustrating a distribution of values. The black line shows the median, the box encompasses 50% of the data, the whiskers, 75% of the data and the outliers are shown as discrete points.



**Figure 7** Animal infectivity studies with *STT3ARNAi* and *STT3BRNAi* cell lines. (A) *T. brucei* *TbSTT3A,B,C<sup>+/-</sup>-STT3ARNAi* cells or (B) *TbSTT3A,B,C<sup>+/-</sup>-STT3BRNAi* cells present in the blood of mice dosed with (white bar) and without doxycycline (grey bar) for 2 days before and throughout the 3 day infection. (C) Purified sVSG221 of wild-type cells grown in culture (lanes 1–3, as a control), and *TbSTT3A,B,C<sup>+/-</sup>-STT3ARNAi* plus doxycycline (lanes 4–6) and *TbSTT3A,B,C<sup>+/-</sup>-STT3ARNAi* minus doxycycline (lanes 7–9) cells isolated from mouse blood, digested or not, as indicated, with EndoH or PNGaseF were subjected SDS-PAGE and western blot with anti-VSG221 antibody. (D) Purified sVSG221 of wild-type cells grown in culture (lanes 1–3, as a control), and *TbSTT3A,B,C<sup>+/-</sup>-STT3BRNAi* plus doxycycline (lanes 4–6), and *TbSTT3A,B,C<sup>+/-</sup>-STT3BRNAi* minus doxycycline (lanes 7–9) cells isolated from mouse blood, digested or not, as indicated, with EndoH or PNGaseF were subjected SDS-PAGE and western blot with anti-VSG221 antibody.

infections. Although both RNAi cell lines grew well in doxycycline-free mice, the infections in the presence of RNAi-inducing doxycycline were very low in both cases (Figure 7A and B). Western blot analysis of the VSG221 from the few surviving cells showed that they were expressing significant amounts of wild-type N-glycosylated VSG221 (Figure 7C and D). Most likely these cells proliferated because they had lost some of the effects of the doxycycline-induced *TbSTT3* knockdown, a well-known phenomenon in *T. brucei* RNAi experiments. These data suggest that both mechanisms of protein N-glycosylation are independently required for parasite growth in mice.

## Discussion

In common with the related kinetoplastids *T. cruzi* and *L. major* (Ivens *et al*, 2005), and with the unrelated protist

*Giardia lamblia* (Morrison *et al*, 2007), the genome of *T. brucei* lacks identifiable homologues of all known OTase subunits except for the catalytic subunit, STT3, for which it has three intact genes, *TbSTT3A,B* and *C* (Berriman *et al*, 2005; Kelleher and Gilmore 2006). The goal of this work was to elucidate the specificities and roles of the three *TbSTT3* proteins in *T. brucei*.

Although we cannot formally exclude the possibility that heterologous expression of *TbSTT3s* in yeast might affect their specificities, or that STT3 protein levels necessarily correlate directly with enzymatic activity, the heterologous complementation of *Stt3p* mutant yeast strains with STT3 proteins from protozoan organisms seems, thus far, to be a good experimental system to study STT3 function and specificity (Shams-Eldin *et al*, 2005; Castro *et al*, 2006; Nasab *et al*, 2008; Hese *et al*, 2009). Studies on the four STT3 genes of *L. major* showed that three of them complement the yeast

*Astt3* mutant with different efficiencies, that they can function without association with other yeast OTase subunits (Nasab *et al*, 2008; Hese *et al*, 2009) and that they have overlapping but distinct peptide substrate specificities (Nasab *et al*, 2008; Hese *et al*, 2009). Similar *ex vivo* studies carried out here showed that TbSTT3B and TbSTT3C are functional single-subunit OTases able to rescue the yeast  $\Delta$ *Stt3* mutant. Furthermore, the glycosylation site occupancy data highlighted the different polypeptide acceptor specificities of TbSTT3B and TbSTT3C and, in particular, a preference for TbSTT3C (which rescued the yeast *Astt3* mutant at the expense of a severe growth phenotype) for acceptor sequons in relatively acidic local environments. Despite these successes, the *ex vivo* approach provided no information on TbSTT3A because its acceptor and/or donor specificity is/are incompatible with the growth of *Saccharomyces cerevisiae*. To address this issue, we studied the expression of the *TbSTT3* genes in the bloodstream and procyclic form *T. brucei* and the functions of the two expressed genes (*TbSTT3A* and *TbSTT3B*) in bloodstream form trypanosomes using inducible RNAi knockdown in bloodstream form parasites.

*TbSTT3A* is more highly expressed in bloodstream form than procyclic form *T. brucei* and its selective knockdown produced a profound glycosylation phenotype in bloodstream form parasites that show that TbSTT3A is largely or solely responsible for the transfer of the biantennary  $\text{Man}_5\text{GlcNAc}_2$  precursor during the biosynthesis of glycoproteins in the ER of *T. brucei*. The data also confirmed that the processing of biantennary  $\text{Man}_5\text{GlcNAc}_2$  attached to protein is the only route to complex N-glycans in this organism (Manthri *et al*, 2008). In contrast, TbSTT3B is highly expressed in both life cycle stages and its selective knockdown in bloodstream form parasites showed that TbSTT3B is largely or solely responsible for the transfer of the triantennary  $\text{Man}_9\text{GlcNAc}_2$  precursor during the biosynthesis of glycoproteins in the ER of *T. brucei* and confirmed that the processing of triantennary  $\text{Man}_9\text{GlcNAc}_2$  attached to protein is the only route to oligomannose N-glycans in this organism (Manthri *et al*, 2008). As noted earlier (Manthri *et al*, 2008), the strict demarcation between these different routes to complex and oligomannose N-glycans seems to be due to the absence of the Golgi mannosidase II activity that permits the conversion of triantennary  $\text{Man}_9\text{GlcNAc}_2$  to  $\text{Man}_3\text{GlcNAc}_2$  in other eukaryotes.

This clear selectivity for different LLO donors by TbSTT3A and TbSTT3B is novel. Mammalian and yeast OTase complexes, which operate in organisms with full repertoires of the ALG genes responsible for LLO biosynthesis, are selective for  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2\text{-PP-Dol}$  whereas protists and fungi that lack groups of ALG genes (Samuelson *et al*, 2005) display either little specificity for donor LLO structure or are tuned to selectively transfer the end LLO product of their modified dolichol cycles (Kelleher *et al*, 2007). In the case of *T. brucei*, the end product of LLO synthesis is  $\text{Man}_9\text{GlcNAc}_2\text{-PP-Dol}$  and TbSTT3B does indeed have a preference for this donor whereas TbSTT3A has specificity for the first topologically competent LLO intermediate  $\text{Man}_5\text{GlcNAc}_2\text{-PP-Dol}$ .

The two OTases also differ substantially in apparent peptide acceptor substrate specificity, with TbSTT3A showing unusual selectivity for glycosylation sequons flanked by acidic residues. TbSTT3C also showed a similar preference for acidic sites; however, it is difficult to compare TbSTT3A

and TbSTT3C because the two could not be studied in the same organism. Nevertheless, it is tempting to speculate that TbSTT3C (which is more closely related to TbSTT3B) might have hybrid properties, perhaps sharing the features of the acidic peptide acceptor specificity of TbSTT3A with the more conventional  $\text{Man}_9\text{GlcNAc}_2\text{-PP-Dol}$  LLO donor specificity of TbSTT3B. It may be that TbSTT3C is expressed at specific parasite life cycle stage(s) when the organism might require oligomannose structures attached to relatively acidic glycosylation sites. In this respect, it is worth noting that the cell-surface BARP glycoproteins, which are specifically expressed in the epimastigote form of the parasite in the tsetse salivary glands (Urwyler *et al*, 2007), present one or two relatively acidic N-glycosylation sites per molecule.

Analysis of VSG glycosylation in the TbSTT3A RNAi knockdown shows that, in the absence of TbSTT3A, TbSTT3B is able to transfer  $\text{Man}_9\text{GlcNAc}_2$  to the acidic Asn263 site rather efficiently, such that most of the VSG in the ER contains  $\text{Man}_9\text{GlcNAc}_2$  at both Asn263 and Asn428 (Figure 4C). This is consistent with our model that TbSTT3A rapidly modifies acidic sites, possibly in a co-translational fashion, whereas TbSTT3B subsequently glycosylates any remaining sites, regardless of pI. In other words, TbSTT3A has distinct acceptor peptide substrate specificity, whereas TbSTT3B has very broad specificity. The latter is also borne out by the yeast data in Figure 1. This helps to explain why in the procyclic form of the parasite, which expresses predominantly TbSTT3B, the relatively acidic N-glycosylation sites of the procyclins are occupied by triantennary oligomannose  $\text{Man}_5\text{GlcNAc}_2$  glycans (Treumann *et al*, 1997) and why the lysosomal glycoprotein p67 contains complex N-glycans in the bloodstream form of the parasite but exclusively oligomannose structures in the procyclic form (Kelley *et al*, 1995).

It is interesting to note that, though in the TbSTT3A knockdown the ER resident VSG is predominantly modified with oligomannose glycans at both Asn263 and Asn428, the mature cell-surface VSG is made up of these mutant VSG glycoforms plus wild-type VSG glycoforms (Figures 3D and 4D). Presumably, the incomplete knockdown of TbSTT3A mRNA allows some wild-type VSG to be synthesised and this is enriched during the process of ER exit and/or transit to the cell surface. It is possible that incorrect glycosylation of the Asn263 site might destabilise VSG221 and lead to its degradation. This site seems to be particularly important in VSG221 because the wild-type paucimannose and small complex N-glycans at Asn263 occupy the same space as a short  $\alpha$ -helix of peptide in other VSG variants (Blum *et al*, 1993) and because this site seems to interact selectively with the calreticulin/UDP-glucose:glycoprotein glucosyltransferase protein folding/quality control system (Izquierdo *et al*, 2009).

Analysis of the VSG molecules in the TbSTT3B RNAi knockdown shows that, in the absence of TbSTT3B, TbSTT3A barely modifies Asn428 at all (Figures 3F, 4C and D) and, when it does, it transfers exclusively  $\text{Man}_5\text{GlcNAc}_2$ , leading to the synthesis of complex glycans at this site. The underglycosylation of Asn428 in the TbSTT3B knockdown is more severe than that seen in the *TbALG3* null mutant, which has normal levels of TbSTT3A and TbSTT3B but can only make  $\text{Man}_5\text{GlcNAc}_2\text{-PP-Dol}$  (Manthri *et al*, 2008). This indicates that TbSTT3B that normally uses  $\text{Man}_9\text{GlcNAc}_2\text{-PP-Dol}$  can in its absence use  $\text{Man}_5\text{GlcNAc}_2\text{-PP-Dol}$ , albeit relatively inefficiently, to glycosylate Asn428.



In summary, the available data show that (i) TbSTT3A has strict specificity for the Man<sub>5</sub>GlcNAc<sub>2</sub>-PP-Dol LLO donor and is highly selective for acidic peptide acceptor sites. (ii) TbSTT3B is selective, but not specific, for the Man<sub>9</sub>GlcNAc<sub>2</sub>-PP-Dol LLO donor and has broad specificity for peptide acceptor sites. (iii) TbSTT3C can use Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub>-PP-Dol donor and has more selectivity for acidic peptide acceptors than TbSTT3B. Although we conclude that local pI around the glycosylation sequon correlates with sequon usage by the different TbSTT3s, we acknowledge that other factors, such as position in the polypeptide and, thus, local secondary and tertiary structure are also likely to have significant functions in differential N-glycosylation in *T. brucei*. These aspects are currently under investigation.

Some subunits of the multiprotein complex OTase of higher eukaryotes tune protein- or site-specific N-glycosylation activity (Wilson and High, 2007; Ruiz-Canada *et al*, 2009; Schulz *et al*, 2009). This additional control or regulation of OTase activity possibly arises from an evolutionary advantage in some organisms for increased diversity of glycosylation sites. Although higher eukaryotes may have achieved this by the addition of proteins to the OTase complex, the data presented here support the hypothesis that gene duplication and diversification of STT3 activity and specificity have occurred in *T. brucei*. Regardless of the reason, the dual N-glycosylation mechanism used by *T. brucei* produces an extraordinarily high N-glycosylation efficiency, with 96% of all sites occupied as compared to ~50–60% for other eukaryotes (Petrescu *et al*, 2004). It is remarkable that minor differences in amino-acid sequence, especially between TbSTT3B and TbSTT3C, lead to substantial alterations in their polypeptide acceptor substrate specificities (Supplementary Figure S2). The WWDYG motif and the surrounding amino-acid sequences are totally conserved in the three proteins, whereas the residues in and near the DxxK motif (Igura *et al*, 2008) present more variability. The novel LLO donor and acceptor peptide specificities of the *T. brucei* STT3 proteins certainly raise possibilities for their usage in glycoprotein engineering in heterologous systems.

The exact reason(s) why *T. brucei* has evolved parallel and selective routes to oligomannose and complex glycan synthesis is not clear. However, it could have occurred to compensate for *T. brucei*'s apparent loss of Golgi mannosidase II genes (which disallows complex glycan formation) and/or to provide a 'fast-track' to paucimannose/complex glycan synthesis from Man<sub>5</sub>GlcNAc<sub>2</sub>-PP-Dol. The latter might provide a selective advantage when one considers that about 50% of the N-glycosylation sites across all of its highly abundant VSG molecules are paucimannose or complex (Mehlert *et al*, 1998). Thus, fast tracking would minimise the drain on Dol-P-Man donors (necessary for converting Man<sub>5</sub>GlcNAc<sub>2</sub>-PP-Dol to Man<sub>9</sub>GlcNAc<sub>2</sub>-Dol) that are also required for GPI anchor biosynthesis, which is prodigious in this organism because it makes and turns over a 10-fold excess of GPI precursors (Ralton *et al*, 1993). Thus, overall, one might speculate that the unusual physiology of this protozoan parasite, which must synthesise an entire contiguous coat of protective surface glycoprotein to survive, may have provided the evolutionary pressure for N-glycosylation acceptor sites and OTase specificities to co-evolve.

Finally, it is surprising that the separate knockdown of TbSTT3A and TbSTT3B, with consequent disappearance of most paucimannose/complex or oligomannose N-glycans, respectively, from all glycoproteins in the parasite can be tolerated in culture, with only a marginal growth phenotype in both cases. On the other hand, both STT3 activities are independently essential in a mouse infection model. This result emphasises that gene functionality should be tested *in vivo*, that is in an animal model of infection, before making final conclusions about gene essentiality. The essentiality of both TbSTT3A and TbSTT3B *in vivo* and their novel specificities (particularly that of TbSTT3A) may offer potential therapeutic targets for future exploitation.

## Materials and methods

### TbSTT3 plasmid constructs

Each of the three *TbSTT3* ORFs were amplified from genomic DNA by PCR and cloned into a pGEM vector; and into a pBAD *Escherichia coli* expression vector, introducing a C-terminal myc-His6 tag; and then into a pRS425\_GPD\_CYC1 yeast expression vector (pTbSTT3A, pTbSTT3B and pTbSTT3C).

### Yeast manipulation

Yeast strains used were Y24390 (EUROSCARF, Frankfurt, Germany), YBS1 (Mat@ *his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 Δstt3::kanMX4 pSTT3*), YBS2 (Mat@ *his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 Δstt3::kanMX4 pTbSTT3B*) and YBS3 (Mat@ *his3Δ1 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 Δstt3::kanMX4 pTbSTT3C*) (Nasab *et al*, 2008). Cells were grown at permissive temperature in YPD; for maintaining plasmids, in minimal medium; for selection of kanMX4 carrying strains with 100 μg/ml G418; or for selection of *ura*-cells with 5-fluoroorotic acid (5-FOA) (Boeke *et al*, 1987) and 1 M sorbitol. Haploid cells with genomic deletion of *STT3* and expressing a TbSTT3 were created essentially as described (Castro *et al*, 2006), but using pSTT3 (Zufferey *et al*, 1995) containing the yeast *STT3* locus in a URA3 YEp352 vector. Addition of 5-FOA to the media allowed for selection of cells that had lost the URA plasmid encoding the wild-type yeast STT3. These cells could only survive if expression of a plasmid-borne *T. brucei* STT3 paralogue complemented the lack of yeast STT3.

### Site-specific glycosylation occupancy determination

Proteins covalently linked to the cell wall polysaccharide matrix were prepared from yeast cells, detected with liquid chromatography–electrospray ionisation–tandem mass spectrometry (LC-ESI-MS/MS), and analysed as described earlier (Schulz and Aebi, 2009). Glycosylation sites with more than 95% occupancy were defined as being 'efficiently' glycosylated.

### Cultivation of trypanosomes

Bloodstream form *T. brucei* genetically modified to express T7 polymerase and the tetracycline repressor protein were cultivated in HMI-9 medium as described in Wirtz *et al* (1999).

### Generation of constructs and transformation of bloodstream form *T. brucei*

530-bp 5'- and 406-bp 3'-UTR sequences were PCR amplified from genomic DNA using HiFi *Taq* Platinum with 5'-atgCGGCCGcgaacggatggcagtcatttgc-3' and 5'-gtttaaactcggaccgtcaagctgtacgtataa ggagggg-3' and 5'-gacggtccgtaagttaaacggatcggatgctgtgttaggg-3' and 5'-ataagtaaCGGCCGCaatcgcgaacctccacaagc-3' as forward and reverse primers, respectively. The two PCR products were used together in a further PCR reaction to yield a product containing the 5'-UTR linked to the 3'-UTR by a short *Hind*III and *Bam*HI cloning site (underlined) and *Sall* and *Not*I restriction sites at each end (capital letters). The PCR product was cloned into the *Sall* and *Not*I sites of the pGEM-5Zf(+) vector (Promega) and the *PAC* drug resistance gene, with exogenous T7 promoter and terminator sequences, was introduced into the targeting vector through the *Hind*III and *Bam*HI sites. For RNAi internal coding sequences were PCR amplified from genomic DNA using HiFi *Taq* Platinum with 5'-cgcGGATCCcctcttgggggtgc-3' and 5'-ccgCTCGAG

attggtagatcagtcacg -3' (*Bam*HI and *Xho*I sites, respectively, in capital letters) for Tb927.5.890 (*TbSTT3A*), and 5'-cgcGGATCCgattcttttggttacc -3' and 5'-ccgCTCGAGctcagaatataatccgaa -3' (*Bam*HI and *Xho*I sites, respectively, in capital letters) for Tb927.5.900 (*TbSTT3B*). The 498 nt (*TbSTT3A*) and 467 nt (*TbSTT3A*) internal coding sequences were cloned into p2T7TA (Alibu *et al*, 2005), cut with *Bam*HI and *Xho*I, to generate p2T7-STT3A and p2T7-STT3B. These plasmids were then digested with *Not*I, and purified for transfection. Finally, for RNAi of *TbSTT3A/B/C* a 493 nt internal coding sequence was PCR amplified from genomic DNA using HIFI *Taq* Platinum with 5'-cgcGGATCCtagccattcatcgtgtgc -3' and 5'-ccgCTCGAGggaacgcaaatcgcaagg -3' (*Bam*HI and *Xho*I sites, respectively, in capital letters) and cloned into p2T7TA, to generate p2T7-TbSTT3All. These plasmids were then digested with *Not*I, precipitated and washed in 70% ethanol, dissolved in sterile distilled water, and used for *T. brucei* transformation (Wirtz *et al*, 1999).

### Northern blotting

Total RNA for Northern blots was prepared using Qiagen RNeasy Midi kits. Samples of RNA (5 µg) were run on formaldehyde-agarose gels and transferred to Hybond N nylon membrane (Amersham Biosciences) for hybridisation with [ $\alpha$ -<sup>32</sup>P] dCTP-labeled *T. brucei* Tb927.5.890 or Tb927.5.900 probe (Amersham Rediprime II).

### Small-scale soluble-form VSG isolation

Soluble-form VSG (sVSG) was isolated from 100 ml cultures containing  $\sim 2 \times 10^8$  bloodstream form *T. brucei* as described in Cross (1984).

### ES-MS analysis of intact and Pronase digested VSG

Samples of the sVSG preparations were diluted to  $\sim 0.07$  µg/µl in 50% acetonitrile, 1% formic acid, loaded into nanotips (Micromass type F), and analysed by positive ion ES-MS on a Q-Star XL instrument (Applied Biosystems). Data were collected and processed using the Bayesian protein reconstruction algorithm of Analyst software. Samples of sVSG were digested with Pronase and the resulting glycopeptides enriched and analysed by ES-MS and ES-MS/MS as described in Manthri *et al* (2008).

### ER-VSG SDS-PAGE and western blotting

sVSG-depleted *T. brucei* ghosts (Bohme and Cross, 2002), equivalent to  $\sim 2 \times 10^5$  cells were subjected to SDS-PAGE and transferred to polyvinylidene difluoride Hybond-P membranes (Amersham Biosciences). The membranes were incubated for 1 h with anti-VSG221 rabbit polyclonal antibody diluted 1:4000 in PBS and then 1 h with anti-rabbit conjugated to horseradish peroxidase diluted 1:10000 with the same buffer. Membranes were developed with ECL (Amersham Biosciences) according to the manufacturer's instructions.

### Lectin blotting of *T. brucei* cell extracts

Bloodstream form *T. brucei* cells were washed twice in trypanosome dilution buffer, solubilised with 2% SDS and 4 M urea, subjected to SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences). The membranes were incubated for 1 h with biotin-conjugated ricin (Vector Labs) diluted 1/3000, with or without inhibitors (10 mg/ml galactose and 10 mg/ml lactose) or 1 h with ConA (Sigma) diluted 1/3000, with or without inhibitor (0.5 M  $\alpha$ -methyl mannose). Then, membranes were incubated for 1 h with extravidin-HRP diluted 1/10000, and developed with ECL (Amersham Biosciences) according to the manufacturer's instructions.

### Mouse infection studies

The RNAi inducible cells were subcultured and grown without selection drugs for 48 h with and without 1 µg/ml tetracycline. The parasites were then introduced into groups of five mice (dosed with and without doxycycline, respectively) by intraperitoneal injection of  $3 \times 10^5$  parasites. The plus doxycycline group of animals were dosed with doxycycline in the drinking water (0.2 mg/ml in a 5% sucrose

solution) for 1 week before infection and until the experiment was terminated. Infections were assessed by tail bleeding and cell counting.

### Extraction of ricin and ConA-binding glycoproteins

Bloodstream form *T. brucei*, were isolated from infected rats, purified over DEAE-cellulose and subjected to hypotonic lysis to release cytosolic components as well as the majority of the VSG coat as sVSG (Cross, 1984). The cell ghost pellets ( $10^{11}$  cell equivalents) were solubilised in 50 ml of 8 M urea, 3% SDS, 50 mM Tris-HCl, pH 6.8. The SDS/urea extract was diluted 50 times in buffer A (50 mM Tris-HCl, pH 6.8, 400 mM NaCl, and 0.8% Triton X-100, 0.1 mM TLCK, 1 µg/ml leupeptin and 0.1% sodium azide). Ricin-coupled agarose (Vector Laboratories, 4-ml packed volume) was added to the suspension, rotated gently overnight at 4°C, recovered by gentle centrifugation and packed into a 5 cm  $\times$  1 cm column. The column was washed with 5 volumes of buffer A and bound material was eluted with 30 mg/ml lactose and 30 mg/ml galactose in 4-fold diluted buffer A. ConA-coupled Agarose (Vector Laboratories) was added to the recovered supernatant from the earlier pull-down with Ricin-Agarose adjusted earlier to 150 mM NaCl, 5 mM CaCl<sub>2</sub>, 5 mM MnCl<sub>2</sub> and 1 mM MgCl<sub>2</sub>, and recovered by gentle centrifugation and packed into a 5 cm  $\times$  1 cm column. Bound material was eluted with 0.5 M methyl- $\alpha$ -D-mannopyranoside in 4-fold diluted Buffer A.

### Sequential endoglycosidase digestion of eluted glycoproteins and mass spectrometry analysis

Aliquots of the eluted Ricin-bound and ConA-bound fractions were independently subjected to EndoH followed by PNGaseF digestion (Roche Applied Science), subjected to SDS-PAGE and the gel lanes were then divided into 10 independent slices. Proteins in each slice were excised, reduced and alkylated, by the addition of 10 mM DTT and then 50 mM iodoacetamide. The samples were digested with 25 µl of 12.5 µg/ml modified trypsin (Roche Applied Science) in 20 mM ammonium bicarbonate. An aliquot of the tryptic digest (recovered in 1% formic acid in water) was analysed by LC-MS on an LTQ-Orbitrap mass spectrometer (ThermoElectron) coupled to a Dionex 3000 nano-LC system. The Orbitrap was set to analyse the survey scans at 60 000 resolution and the top five ions in each duty cycle selected for MS/MS in the LTQ linear ion trap. The raw files were processed to generate a Mascot generic file using the program Raw2msm and searched against an in-house curated *T. brucei* database using the Mascot search engine v.2.2 (Matrix Science) run on an in-house server using the following criteria: peptide tolerance = 6 p.p.m., trypsin as the enzyme and carboxyamidomethylation of cysteine as a fixed modification. Variable modifications were oxidation of methionine, N-acetylglucosamine modification of Asn and deamidation of Asn to Asp.

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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## Conflict of interest

The authors declare that they have no conflict of interest.

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