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# A novel ISWI is involved in *VSG* expression site downregulation in African trypanosomes

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**African trypanosomes show monoallelic expression of one of about 20 telomeric variant surface glycoprotein (*VSG*) gene-expression sites (ESs) while multiplying in the mammalian bloodstream. We screened for genes involved in ES silencing using flow cytometry and RNA interference (RNAi). We show that a novel member of the ISWI family of SWI2/SNF2-related chromatin-remodelling proteins (TbISWI) is involved in ES downregulation in *Trypanosoma brucei*. TbISWI has an atypical protein architecture for an ISWI, as it lacks characteristic SANT domains. Depletion of TbISWI by RNAi leads to 30–60-fold derepression of ESs in bloodstream-form *T. brucei*, and 10–17-fold derepression in insect form *T. brucei*. We show that although blocking synthesis of TbISWI leads to derepression of silent *VSG* ES promoters, this does not lead to fully processive transcription of silent ESs, or an increase in ES-activation rates. *VSG* ES activation in African trypanosomes therefore appears to be a multistep process, whereby an increase in transcription from a silent ES promoter is necessary but not sufficient for full ES activation.**

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## Introduction

Monoallelic transcription of one out of a large family of related genes is a little understood phenomenon in a variety of experimental systems. Examples of this include control of the olfactory receptor genes, whereby only one out of more than 1500 olfactory receptor genes is activated in a mutually exclusive manner in each olfactory neuron (Serizawa *et al*, 2004; Lomvardas *et al*, 2006). Likewise, in the malaria parasite *Plasmodium falciparum*, only one of about 50 *VAR* gene transcription units is activated in stringently monoallelic fashion (Scherf, 2006; Voss *et al*, 2006). Similarly, African trypanosomes show monoallelic expression of one of many highly similar telomeric variant surface glycoprotein (*VSG*) expression sites (ESs) while multiplying in the bloodstream of the mammalian host. Very little is known about how the counting machinery behind this stringent control operates.

The African trypanosome *Trypanosoma brucei* is a protozoan parasite causing African sleeping sickness, transmitted by tsetse flies. Bloodstream-form *T. brucei* is covered with a homogeneous coat of a single VSG. Although trypanosomes have more than 1200 *VSG* genes and pseudogenes (Berriman *et al*, 2005), the active *VSG* is transcribed in a tightly regulated fashion from one of about 20 telomeric *VSG* ES transcription units (Borst and Ulbert, 2001; Becker *et al*, 2004). During a chronic infection, bloodstream-form *T. brucei* successively switches to new VSG types, allowing escape from antibodies against the old VSG. VSG switching can be mediated by activating different ESs or via DNA rearrangements inserting one of many silent *VSG* genes or pseudogenes into an active ES (Barry and McCulloch, 2001). In contrast, in insect-form *T. brucei*, all of the ESs are downregulated, as this life-cycle stage expresses an invariant procyclin coat on its surface rather than VSG (Roditi and Liniger, 2002).

Unusually for a eukaryote, transcription of ESs, as well as of the procyclin transcription units, is mediated by RNA polymerase I (pol I) rather than RNA polymerase II (pol II) (Günzl *et al*, 2003). In bloodstream-form *T. brucei*, the active ES is located in an extranucleolar pol I transcriptional body (expression-site body or ESB), which is hypothesised to contain the transcription and RNA-processing factories necessary for high levels of expression of fully processed transcripts (Vanhamme *et al*, 2000; Navarro and Gull, 2001). Only a single ES can be stably activated at a time, and selection for simultaneous activation of two different ESs gives rise to trypanosomes which appear to be rapidly switching between the two (Chaves *et al*, 1999). ESs are controlled as regulated domains and activation of exogenous promoters, integrated at the chromosome end, cannot be uncoupled from activation of the endogenous ES promoter many tens of kilobases upstream (Horn and Cross, 1995).

Downregulation of ESs differs in a number of characteristics in insect-form compared with bloodstream-form *T. brucei*. In insect-form *T. brucei*, all ESs are downregulated. There are low levels of transcription from silent ESs in both life-cycle stages, but these levels are significantly higher in the insect-form (Rudenko *et al*, 1994). Second, ES downregulation is promoter sequence specific in insect-form *T. brucei*, whereas in bloodstream-form *T. brucei*, both ES and rDNA promoters located within an ES are silenced essentially equally effectively (Rudenko *et al*, 1994, 1995; Horn and Cross, 1995). Last, it has been postulated that there is chromatin-mediated silencing of ESs in insect-form, but not in bloodstream-form *T. brucei*, as assessed using exogenous T7 RNA polymerase to probe for ES chromatin accessibility in both of these life-cycle stages (Navarro *et al*, 1999). It is possible that ES downregulation is mediated primarily at the

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level of transcription elongation rather than transcription initiation (discussed in Pays *et al*, 2004).

We have developed an experimental approach allowing us to screen candidate genes for their role in ES downregulation. Constructs containing fluorescent reporter genes (*DsRed* or *GFP*) were inserted downstream of downregulated ES promoters in both insect and bloodstream-form *T. brucei*. Tetracycline-inducible RNA interference (RNAi) was induced against candidate genes, and ES derepression was monitored by flow cytometry. We identify the first gene shown to play a role in ES control in *T. brucei*, and show that it is a new member of the ISWI family of chromatin-remodelling proteins.

## Results

### *Monitoring expression from downregulated VSG ESs by flow cytometry*

We attempted to identify proteins, which, if depleted by RNAi, cause ES derepression. Our approach was to integrate a construct containing *DsRed* (encoding red fluorescent protein) 216 bp downstream of different endogenous ES promoters into insect-form *T. brucei* 29–13 (Wirtz *et al*, 1999) (construct ESDsB in Figure 1A). This *T. brucei* cell line contains genes encoding T7 RNA polymerase and the tetracycline repressor allowing tetracycline-inducible expression. Clonal *T. brucei* transformants were isolated with the ESDsB construct integrated into chromosomal bands containing either the *VSG121* or *VSG221* ESs (Figure 1A). Using PCR, the constructs were shown to be linked to ES-associated genes (*ESAG*)*7*, the most upstream of the *ESAG*s.

Expression of DsRed in *T. brucei* can be monitored by flow cytometry (Figure 1B). Insect-form *T. brucei* not containing *DsRed* (TBT) did not fluoresce, whereas *T. brucei* with *DsRed* inserted downstream of a highly active rDNA promoter showed high levels of DsRed expression (Dr1 and Dr2 in Figure 1B). This did not lead to a growth arrest, indicating that DsRed is not significantly toxic in insect-form *T. brucei*. In contrast, trypanosomes containing the *DsRed* construct integrated behind inactive ES promoters (D1–D4) only showed levels of fluorescence that were marginally higher than in trypanosomes not containing a *DsRed* gene (Figure 1B).

### *TbISWI is a member of the ISWI family of SWI2/SNF2-related chromatin-remodelling complexes*

We next tested if proteins previously found to bind DNA sequences present in transcriptionally silent regions of the *T. brucei* genome play a role in ES downregulation. TbISWI was originally identified in a screen for DNA-binding proteins interacting with the *T. brucei* 177 bp simple sequence repeats, which constitute the bulk of the transcriptionally inactive

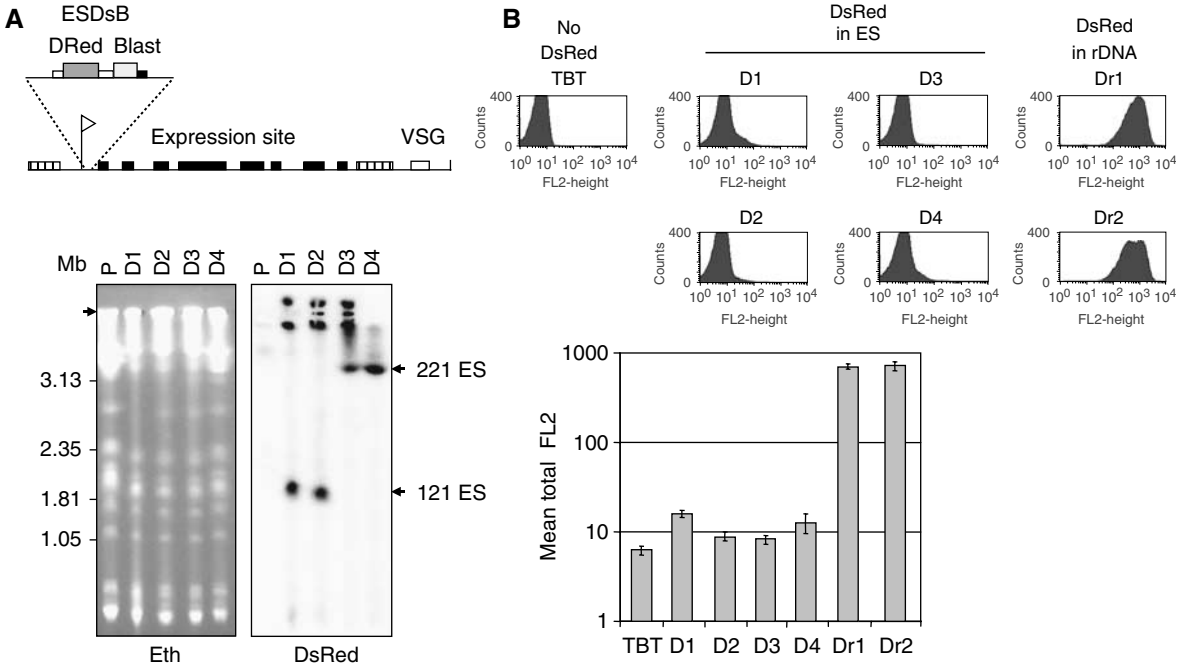


**Figure 1** Monitoring ES derepression using flow cytometry in insect-form *T. brucei*. (**A**) The schematic shows the ESDsB construct integrating downstream of a *VSG* ES promoter. Various genes present within ESs are indicated with black boxes, and characteristic repeat arrays with striped boxes. The construct contains *DsRed* (DRed) and blasticidin resistance (Blast) genes flanked by tubulin (white boxes) or actin (black box) RNA-processing signals. Below: pulsed field gel analysis of insect-form *T. brucei* ESDsB transformants, where DNA from the parental *T. brucei* 29–13 cell line (lane P) is compared with that from *T. brucei* 29–13 transformants D1–D4 (lanes D1–D4). An ethidium stain of the gel (Eth.) is shown with size markers indicated in megabases and the slot indicated with an arrow. The gel was blotted and hybridised with a probe for *DsRed* (panel DsRed). Chromosomal bands containing either the *VSG221* or *VSG121* ESs are indicated with arrows. (**B**) Levels of expression of the *DsRed* gene in inactive ESs in insect-form *T. brucei* as measured by flow cytometry. Above: representative flow cytometry traces are shown, with *DsRed* expression monitored in the FL-2 channel (*x* axis). The *T. brucei* 29–13 TBT cell line (TBT) does not contain *DsRed*. The *T. brucei* 29–13 D1-D4 cell lines contain *DsRed* integrated behind different ES promoters. In the *T. brucei* 29–13 Dr1 and Dr2 cell lines, a *DsRed* containing construct was integrated behind an rDNA promoter in *T. brucei* 29–13. Quantitation of *DsRed* expression from different *DsRed* containing *T. brucei* lines was measured as total mean fluorescence (arbitrary units). Results are the mean of six experiments, with standard deviation indicated by error bars.

*T. brucei* minichromosomes (Wickstead *et al*, 2004). *T. brucei* proteins binding 177 bp repeat containing sequences were isolated and identified by mass spectrometry. These results will be presented elsewhere (Tilston V and K Ersfeld, manuscript in preparation). One of the proteins isolated (provisionally called TbISWI) was found to have a highly conserved SNF2 N-terminal domain (Eisen *et al*, 1995) (e value of $e^{-110}$), a conserved helicase domain ($5e^{-32}$) and a region resembling a myb-like DNA-binding domain ($6e^{-9}$) (Figure 2A). SNF2 domains have DNA-dependent ATPase activity, and are present in the SWI2/SNF2-related class of proteins involved in chromatin remodelling (Mohrmann and Verrijzer, 2005). One of the subclasses of the SWI2/SNF2 family comprises members of the ISWI family (Corona and Tamkun, 2004; Mellor and Morillon, 2004). Sequence database interrogation with the TbISWI sequence preferentially identified ISWI family members from other species. High sequence similarity was found over the SNF2-domain-containing region, particularly over seven regions containing highly conserved ATPase/helicase motifs (Figure 2B).

Members of the ISWI family are recognisable by the presence of both an SNF2 domain and a SANT/SLIDE domain, which is an ISWI-specific subclass of myb domain with DNA-binding activity (Boyer *et al*, 2004). TbISWI has a myb

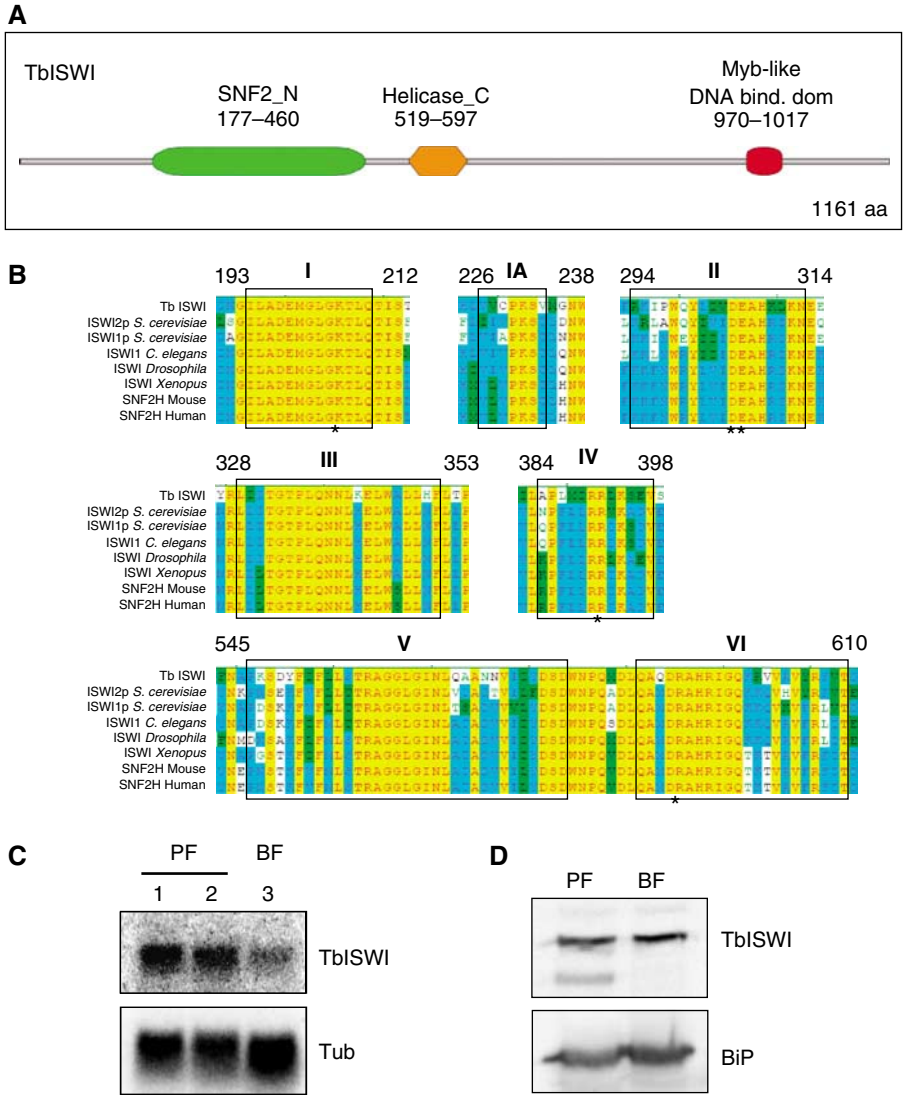


**Figure 2** *T. brucei* TbISWI is a member of the ISWI subclass of SWI2/SNF2-related chromatin-remodelling complexes. (**A**) Schematic of TbISWI with protein domains identified using 3D-JIGSAW (version 2.0). A conserved SNF2 family N-terminal domain is indicated with a green oval, conserved helicase C-terminal domain with an orange hexagon, and a Myb-like DNA-binding domain with a red oval. (**B**) Alignment of TbISWI over the SNF2 and helicase domains with other ISWI family members: *Saccharomyces cerevisiae* ISWI2p, *S. cerevisiae* ISW1p, *Caenorhabditis elegans* ISWI homologue protein 1, *Drosophila melanogaster* ISWI isoform C, *Xenopus laevis* ISWI, *Mus musculus* DNA-dependent ATPase SNF2 H, and *Homo sapiens* SNF2 H (accession numbers in the Materials and methods). Seven conserved regions characteristic of ATPase/helicase motifs are indicated with numbered boxes, with particularly essential amino acids indicated by asterisks (Richmond and Peterson, 1996). Identical amino acids are highlighted in yellow, with conservative or similar amino-acid changes highlighted with blue or green blocks respectively. (**C**) TbISWI transcript is expressed in both insect and bloodstream-form *T. brucei*. Northern blot analysis of RNA from wild-type insect-form *T. brucei* (Lane 1), insect-form *T. brucei* 29–13 (Lane 2), or bloodstream-form *T. brucei* HNIR1(221 +) (Lane 3). The blot was hybridised with a probe for *TbISWI* or tubulin (Tub) as a loading control. (**D**) TbISWI is expressed at comparable levels in both insect-form (PF) or bloodstream-form (BF) *T. brucei*. The Western blot was reacted with an antibody against TbISWI, and as a loading control with an antibody against BiP.

domain, which could play a role in contact of TbISWI with DNA, but does not appear to have a clear SANT/SLIDE domain. We have nonetheless categorised our protein as an ISWI on the basis of the high homology with other ISWIs, and the lack of other ISWI candidates in *T. brucei*. TbISWI is expressed in both insect and bloodstream-form *T. brucei* at comparable levels (Figure 2C and D) as detected using a rabbit polyclonal antibody raised against the C-terminal 207 aa of TbISWI.

### *Inactivation of TbISWI by RNAi in insect-form T. brucei leads to a growth arrest and VSG expression site upregulation*

We first tested the role of TbISWI in the *T. brucei* D1 cell line containing *DsRed* integrated behind an ES promoter located on a chromosomal band containing the *VSG121* ES. Induction of RNAi against TbISWI using a tetracycline-inducible system led to a growth reduction in insect-form *T. brucei* D1-SA1 and D1-SA2 after about 6 days (Figure 3A). This phenotype was also observed using another nonoverlapping *TbISWI* RNAi fragment (result not shown). Using Western blot analysis, there was almost complete depletion of a band, which appeared to correspond to the TbISWI protein after 2 days induction of *TbISWI* RNAi (Figure 3B). This lag between the reduction in TbISWI protein to undetectable levels, and the appearance of the growth arrest could indicate that very low levels of TbISWI can still rescue the cell. Alternatively, the growth arrest could be an indirect consequence of TbISWI knock down.

We next used flow cytometry to monitor DsRed expression from the ES promoter in this cell line after the induction of *TbISWI* RNAi. The *T. brucei* D1-SA1 and D1-SA2 cell lines started to show derepression of DsRed 2–4 days after induction of *TbISWI* RNAi, which reached 10–17-fold background after 6–10 days induction (Figure 3C). Comparable ES derepression was also seen with the *T. brucei* D3-SA1 cell line, where *DsRed* was integrated behind an ES promoter on the *VSG221* ES-containing chromosome (parental *T. brucei* D3 cell line) (Figure 3D). ES derepression was not observed when RNAi was induced against other unrelated essential

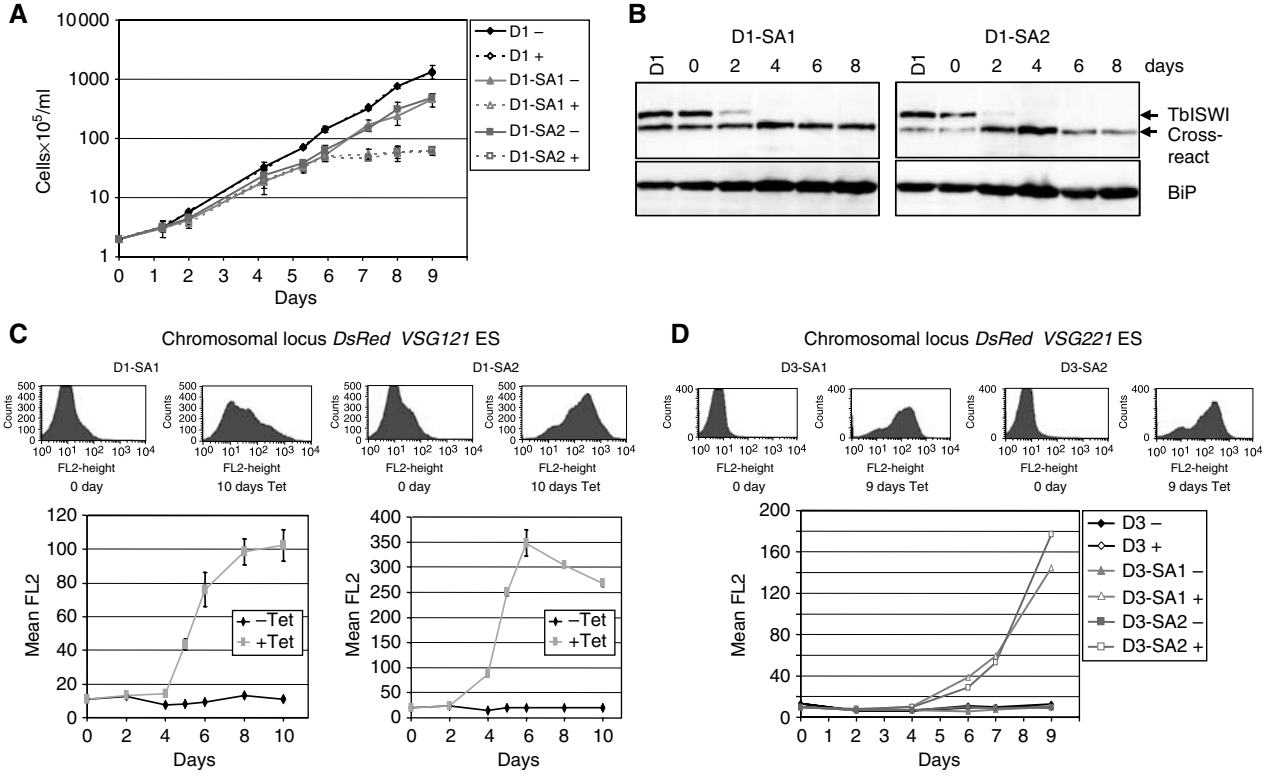


**Figure 3** Blocking synthesis of TbISWI results in ES derepression in insect-form *T. brucei*. (**A**) Growth curves in insect-form *T. brucei* after induction of RNAi against TbISWI with tetracycline. Growth of the parental *T. brucei* 29–13 D1 cell line (D1) is compared with two independent *T. brucei* TbISWI RNAi transformants D1-SA1 and DA-SA2 in the presence (+) or absence (−) of tetracycline. The cumulative cell density is indicated on the *y* axis as cells/ml multiplied by $10^5$. Time is indicated in days. (**B**) Disappearance of TbISWI protein after the induction of TbISWI RNAi in insect-form *T. brucei*. Protein lysates from the parental *T. brucei* 29–13 D1 cell line (D1) were compared with those from *T. brucei* D1-SA1 or D1-SA2. Lysates were isolated from cells after the induction of TbISWI RNAi with tetracycline for 0, 2, 4, 6 or 8 days. TbISWI as well as a cross-reacting band are indicated by arrows. As a loading control the blot was reacted with an antibody against BiP (BiP). (**C**) Derepression of *DsRed* marked ES promoters after induction of RNAi against TbISWI. The *T. brucei* 29–13 D1 cell lines contain a *DsRed* containing construct inserted behind a promoter on the *VSG121* ES containing chromosome. *DsRed* expression was monitored by flow cytometry in the FL-2 channel in the *T. brucei* D1-SA1 or D1-SA2 cell lines grown in the presence (+) or absence (−) of tetracycline (Tet) to induce TbISWI RNAi. The total mean fluorescence is plotted over time. Examples of FACS traces from an uninduced population or one with TbISWI RNAi induced for 10 days are shown above. Results are presented as the mean of three experiments, with standard deviation indicated with error bars. (**D**) Promoters from different ESs are derepressed after the induction of TbISWI RNAi. TbISWI RNAi was induced in the *T. brucei* 29–13 D3 cell line, where *DsRed* is inserted behind an ES promoter on the *VSG221* ES containing chromosome. The parental *T. brucei* 29–13 D3 cell line (D3) does not contain a TbISWI RNAi construct, and is compared with *T. brucei* transformants D3-SA1 and D3-SA2 grown in the presence (+) or absence (−) of tetracycline to induce TbISWI RNAi. Mean fluorescence in the FL2 channel is indicated on the Y-axis. Representative flow cytometry traces are shown for cells grown for 0 or 9 days in the presence of tetracycline.

genes, indicating that derepression of DsRed was not simply a stress response caused by lack of an essential protein. Genes tested included *NUP1* (Rout and Field, 2001), *DAC1* (Ingram and Horn, 2002) and *TDP-1* (Erondu and Donelson 1992), whereby induction of RNAi resulted in a growth reduction within 3 days; however, no significant *VSG* expression-site derepression was observed over a period extending up to 9 days (results not shown).

ES promoters are flanked downstream by different families of *ESAG*s. After induction of *TbISWI* RNAi, transcription from the normally downregulated ES promoters extended through the adjacent *ESAG7*, *ESAG6* and *ESAG5* (Figure 4A and B). These derepressed transcripts were also present as variants with nucleotide lengths longer than expected for mature transcripts, possibly indicating inefficient *trans*-splicing or polyadenylation. Alternatively, these larger *ESAG5* transcripts could be of varying sizes as they are derived from polymorphic *ESAG5* genes present in multiple derepressed ESs. No increase in transcripts was observed from the *ESAG4* or *ESAG8* genes located downstream of *ESAG5* (result not shown). We did not see evidence for upregulated transcripts derived from the 177 bp repeat sequences present on transcriptionally silent minichromosomes after the induction of TbISWI RNAi (result not shown). However, as the 177 bp repeat arrays do not contain RNA-processing signals, fortuitous transcription initiation in these areas of the genome would not necessarily give rise to stable transcripts.

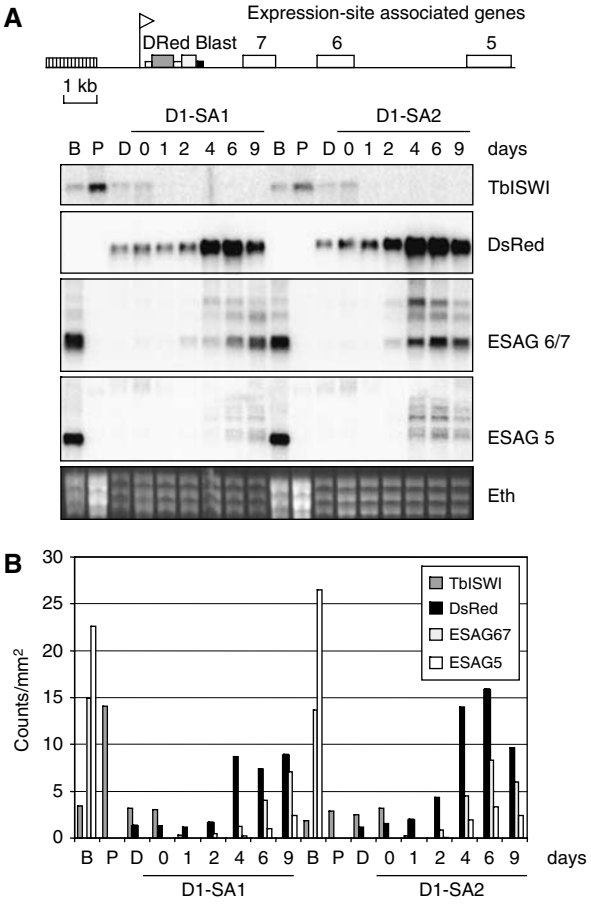


**Figure 4** Transcription of derepressed ESs after the induction of *TbISWI* RNAi in insect-form *T. brucei*. (**A**) A schematic of a typical ES is shown with the promoter indicated by a flag, the integrated ESDsB construct indicated as per Figure 1A, and adjacent ES associated genes (*ESAGs*) with numbered boxes. Northern blot analysis shows RNA from bloodstream-form *T. brucei* 90–13 (B), insect-form *T. brucei* 29–13 (P) and the parental *T. brucei* 29–13 D1 cell line (D), which has a *DsRed* containing construct integrated behind an ES promoter. These lanes are compared with RNA from the *T. brucei* D1-SA1 or D1-SA2 cell lines after the induction of *TbISWI* RNAi for the time in days indicated above. The blot was hybridised with probes for *TbISWI*, *DsRed*, *ESAG6/7*, and *ESAG5*. The ethidium stained gel is shown below (Eth). (**B**) Quantitation of the increase in *DsRed*, *ESAG6/7* and *ESAG5* transcript in the *T. brucei* D1-SA1 and D1-SA2 cell lines after the induction of TbISWI RNAi for the number of days shown below. Radioactive signal is indicated as counts per $mm^2$ in arbitrary units.

### *TbISWI is localised in the nucleus and is present in the chromatin fraction of both insect and bloodstream-form T. brucei*

The cellular localisation of TbISWI was determined by expressing TbISWI-GFP fusion protein from a tetracycline-inducible T7 promoter. TbISWI-GFP protein localised to the nucleus of both insect and bloodstream-form *T. brucei* (Figure 5A). Next, we determined if TbISWI was present in chromatin-enriched cell fractions. Cells can be fractionated into a pellet fraction containing histone H3 as a marker for chromatin, and a supernatant fraction containing nonchromatin-associated proteins, including the nuclear RNA-binding protein La (Arhin *et al*, 2005; DiPaolo *et al*, 2005). At low salt concentrations, TbISWI was present in the pellet fraction together with histone H3, indicating that it is associated with chromatin (Figure 5B). In contrast, the RNA-binding protein La was present in the supernatant. Performing this fractionation procedure in the presence of increasing concentrations of NaCl showed that TbISWI was released into the supernatant at a concentration between 200 and 300 mM NaCl. This indicates that TbISWI binds DNA with a lower affinity than histone H3, which remained associated with the chromatin fraction in up to 500 mM NaCl (Stunnenberg and Birnstiel, 1982). Equivalent results were obtained using fractionated lysates from insect-form *T. brucei* (results not shown). These results are all compatible with TbISWI being a chromatin-associated protein critical for ES downregulation in both insect and bloodstream-form *T. brucei*.

### *TbISWI is essential in bloodstream-form T. brucei and is involved in downregulation of silent VSG ESs*

We next developed an experimental approach allowing us to investigate the role of TbISWI in ES control in bloodstream-form *T. brucei* (Figure 6A). A construct containing *GFP* was integrated downstream of the promoter of the *VSG221* ES in the bloodstream-form *T. brucei* 'single-marker' line containing the T7 RNA polymerase and tetracycline repressor genes allowing tetracycline-inducible expression (Wirtz *et al*, 1999). A construct allowing tetracycline-inducible *VSG221* RNAi was introduced into these VSG221-expressing cells (Sheader *et al*, 2005). Induction of *VSG221* RNAi allows the selection of cells, which have switched to the expression of different VSGs (Aitcheson *et al*, 2005). After screening for cells that had activated the *VSGT3* ES, we integrated a construct containing a blasticidin-resistance gene immediately behind the *VSGT3* ES promoter. This allowed us to maintain cultures of trypanosomes, which were homogeneous for expression of the active *VSGT3* ES. Subsequently, a *TbISWI* RNAi construct ($MC^{177}$ TbISWI-A) was integrated into this cell line, allowing for monitoring for derepression of *GFP* integrated behind

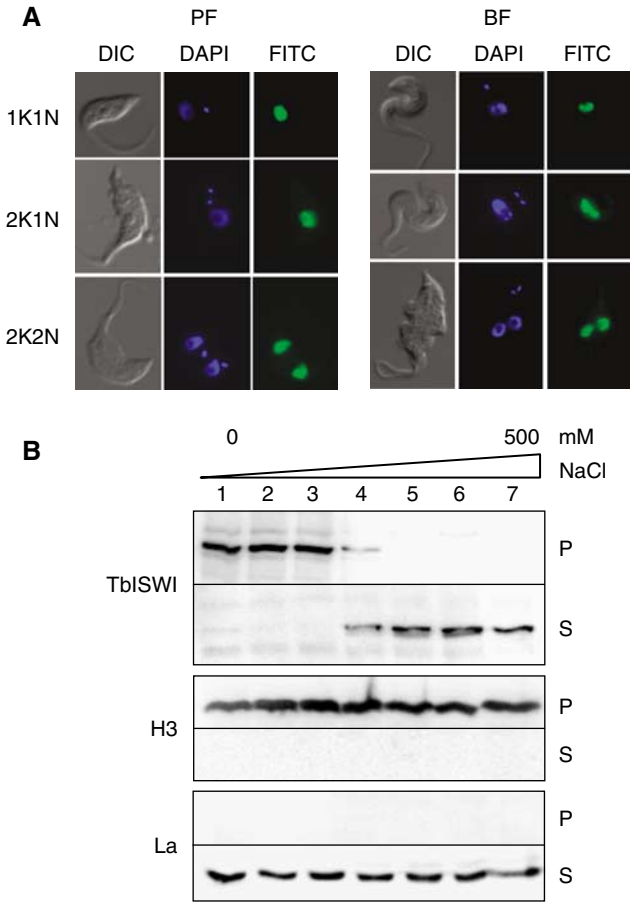


**Figure 5** TbISWI has a nuclear localisation and is present in the chromatin fraction. (**A**) TbISWI-GFP fusion protein localises in the nucleus of both insect-form (PF) and bloodstream-form (BF) *T. brucei*. Results are shown after 8 h induction of TbISWI-GFP in insect-form *T. brucei* (PF) or bloodstream-form *T. brucei* (BF). Panels show trypanosomes imaged using differential interference contrast (DIC). DNA is stained with DAPI, and TbISWI-GFP is visualised in the FITC channel. (**B**) TbISWI colocalises with histone H3 in the chromatin fraction of BF *T. brucei*. Cell lysates were fractionated into a pellet (P) and supernatant (S) fraction in the presence of increasing NaCl (lanes 1–7 respectively 0, 50, 100, 200, 300, 400 and 500 mM NaCl). Panels were reacted with an antibody against TbIWSI (TbISWI), histone H3 (H3) or the RNA-binding protein La (La).

the silent *VSG221* ES promoter after the induction of *TbISWI* RNAi.

Induction of *TbISWI* RNAi in bloodstream-form *T. brucei* resulted in a reduction in growth rate after about 48 h (Figure 6B). The induction of TbISWI RNAi resulted in depletion of TbISWI protein, which was undetectable by 24 h, as monitored by Western blot analysis in *T. brucei* T3-SA1 (Figure 6C). Comparable depletion of TbISWI protein was seen in *T. brucei* T3-SA2 (result not shown). Induction of *TbISWI* RNAi in VSGT3 expressors led to an average 61-fold ($\pm$11) upregulation of the silent *VSG221* ES in two independent *T. brucei* T3 *TbISWI* RNAi transformants (Figure 6D). Levels of GFP expression increased steadily after induction of *TbISWI* RNAi for 24 h, reaching maximal levels at about 80 h after induction.

To determine if derepression is observed in *T. brucei* with different active ESs, we constructed a bloodstream-form *T. brucei* line containing *GFP* in the silent *VSG221* ES, but containing an active *VSG121* ES. This cell line had a construct with the blasticidin-resistance gene inserted within the *VSG121* ES to allow selection of a homogeneous population of VSG121 expressors, in addition to the *TbISWI* RNAi construct ($MC^{177}$ TbISWI-A). Induction of *TbISWI* RNAi led to an average 34-fold ($\pm$12) derepression of the *VSG221* ES in two independent VSG121-expressing *T. brucei* lines (Figure 6E). Western blot analysis showed that depletion of TbISWI was essentially comparable in both the VSGT3- and VSG121-expressing lines (result not shown). Similar to our results with insect-form *T. brucei*, induction of RNAi against a number of unrelated essential genes did not lead to significant ES derepression (result not shown), indicating that we were not observing a nonspecific phenotype caused by stress-related nonspecific RNAi effects.

Using real-time PCR we monitored transcript levels from silent ESs after the induction of TbISWI RNAi. Amounts of *GFP* transcript from the silent *VSG221* ES rose to more than 60-fold background in trypanosomes containing either the *VSGT3* or the *VSG121* ES active (Figure 7). Although transcripts derived from five different telomeric *VSG*s located in silent ESs could sometimes be observed, levels were low and variable (Figure 7). We found no evidence for significant reproduceable increases in *VSG* transcript levels after inducing TbISWI RNAi. This result was the same even if drug selection pressure was removed from the active ES. This indicates that inhibition of the synthesis of TbISWI leads to the derepression of ES promoters, but it does not appear to lead to full activation of the silent ESs, or an increase in rates of ES switching.

It is likely that transcription from the derepressed 'silent' *VSG* expression site promoters does not extend down to the telomeric *VSG*, although we cannot exclude a scenario, whereby transcripts from the derepressed 'silent' ESs are not being processed properly. For technical reasons knock down of TbISWI was performed in cells still containing the *VSG221* RNAi construct. However, as these cells contain a different *VSG* in the active ES, they would not be expected to arrest in the presence of *VSG221* RNAi, as is observed when synthesis of the active VSG is blocked by RNAi (Sheader *et al*, 2005). Lastly, we determined levels of transcript from another downregulated Pol I transcription unit (procyclin). Procyclin transcript did not increase after the induction of TbISWI RNAi for up to 48 h, during which period growth inhibition and *VSG* expression site derepression can be observed (result not shown).

In summary, our results show that TbISWI plays an important role in ES downregulation in both life-cycle stages of *T. brucei*. Inducing ES promoter derepression does not lead to a significant increase in ES activation, indicating that ES activation is a multistep process.

## Discussion

We have identified TbISWI as the first protein shown to play a role in ES downregulation in *T. brucei*. Sequence analysis indicates that TbISWI is a member of the ISWI family of SWI2/SNF2-related chromatin-remodelling proteins. As expected for an ISWI, TbISWI has a nuclear localisation, and is associated with chromatin. TbISWI depletion leads to 30–60-fold derepression of ESs in bloodstream-form *T. brucei*, and 10–17-fold derepression of ESs in the insect-

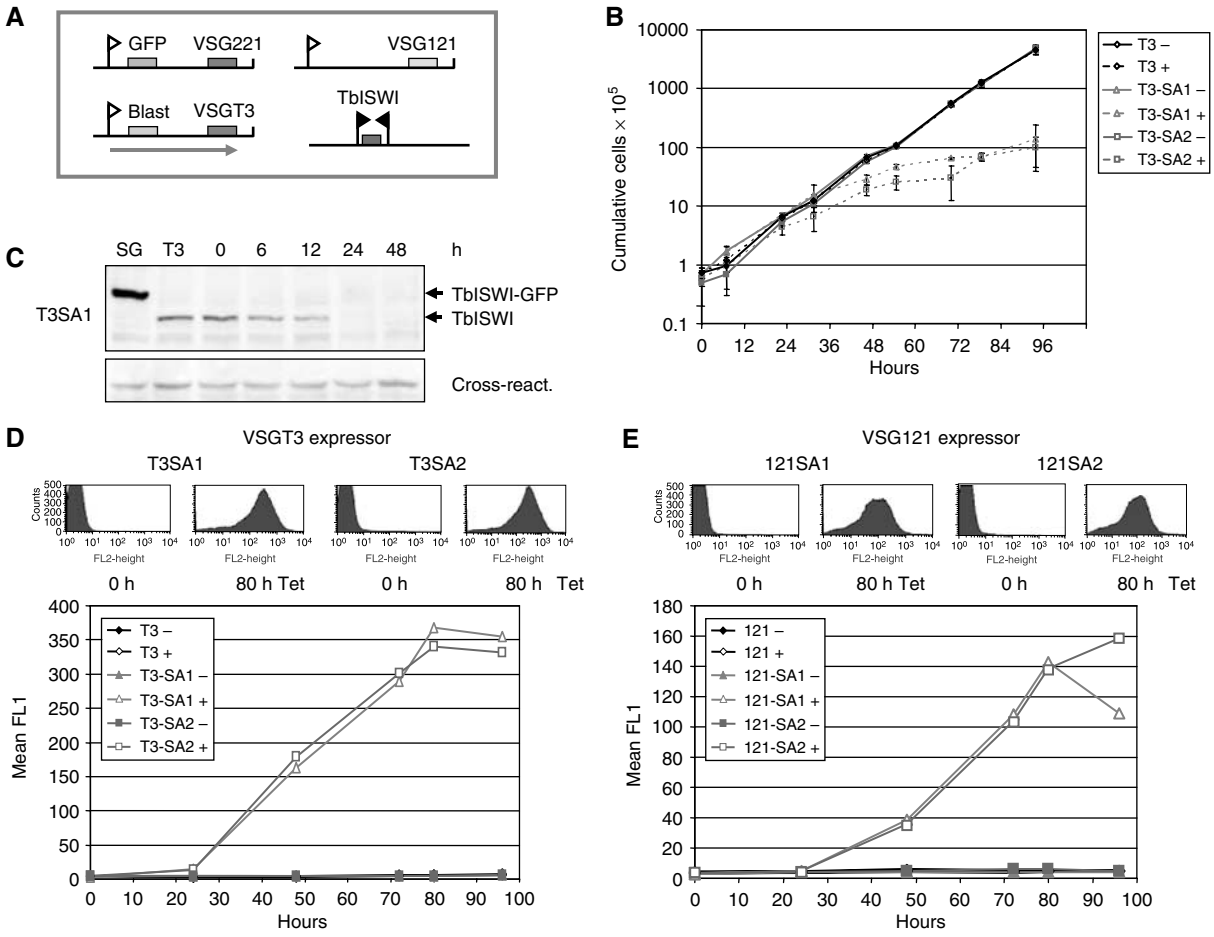


**Figure 6** TbISWI is involved in *VSG* expression site downregulation in bloodstream-form *T. brucei*. (**A**) The bloodstream-form *T. brucei* T3-SA1 cell line used for investigating *VSG* expression site control using RNAi and flow cytometry. The large box indicates a trypanosome expressing VSGT3. ESs are shown with flags for the promoters, and relevant genes including *GFP*, blasticidin resistance (Blast) and *VSG221*, *VSGT3* and *VSG121* with filled boxes. The *T. brucei* T3-SA1 cell line contains genes encoding T7 RNA polymerase and the tetracycline repressor (TetR) allowing tetracycline-inducible transcription from modified T7 promoters (black flags). A TbISWI RNAi construct is integrated into these cells. Derepression of the *GFP* marked *VSG221* ES can be monitored by flow cytometry. (**B**) Growth reduction after the induction of *TbISWI* RNAi in VSGT3 expressing *T. brucei*. *T. brucei* T3SA1 and T3SA2 cell lines were grown in the presence (+) or absence (−) of tetracycline to induce *TbISWI* RNAi. In comparison, the parental VSGT3 expressing cell line *T. brucei* VSGT3-SM (T3) did not contain an RNAi construct. The cell density is indicated as a cumulative amount multiplied by $10^5$, and time is indicated in hours. (**C**) Disappearance of TbISWI protein after induction of *TbISWI* RNAi in bloodstream-form *T. brucei* expressing VSGT3. Protein lysate from bloodstream-form *T. brucei* expressing a TbISWI-GFP fusion protein (SG) was compared with lysate from the parental bloodstream-form *T. brucei* VSGT3-SM cell line (T3) or *T. brucei TbISWI* RNAi transformant T3-SA1, where *TbISWI* RNAi had been induced for 0, 6, 12, 24 or 48 h. The top panels show blots reacted with a polyclonal anti-TbISWI antiserum, with the bands containing TbISWI and the TbISWI-GFP fusion protein indicated with arrows. The bottom panels show an internal cross-reactive band which functions as a loading control. Comparable results were found with the *T. brucei* T3-SA2 cell line. (**D**) Derepression of the *GFP* marked *VSG221* ES after the induction of *TbISWI* RNAi in VSGT3 expressing *T. brucei*. The parental *T. brucei* VSGT3-SM cell line (T3) is compared with the *T. brucei* T3SA1 and T3SA2 cell lines containing the *TbISWI* RNAi construct. The upper panel shows the representative flow cytometry traces with fluorescence in the FL1 channel indicated on the *x* axis. The bottom panel shows the quantitation of a representative experiment through time in the presence (+) or absence (−) of tetracycline to induce *TbISWI* RNAi. (**E**) *GFP* is derepressed after the induction of *TbISWI* RNAi in VSG121 expressing *T. brucei*. The *T. brucei* VSG121-SM cell line (121) is compared with the *T. brucei* 121SA1 and 121SA2 cell lines containing the *TbISWI* RNAi construct. Flow cytometry traces and quantitation of an experiment performed in the presence (+) or absence (−) of tetracycline are as indicated above (C).

form. Despite the striking derepression of ES promoters in the presence of TbISWI RNAi, we have no evidence that this promoter derepression results in an increase in full activation of silent ESs. This indicates that ES activation is multistep pathway, including additional steps in addition to promoter activation.

### *TbISWI as a member of the ISWI family*

Is TbISWI really a member of the ISWI family? ISWI proteins form a subclass of the SWI2/SNF2-related chromatin-remodelling complexes. In addition to the highly conserved SNF2 domain, ISWI proteins have a SANT domain at the C-terminus with strong similarity to the DNA-binding domain of Myb-related proteins (Boyer *et al*, 2002). This domain is separated by a long helical spacer from a SLIDE domain, which is closely related to SANT domains but is unique to ISWI proteins (Grune *et al*, 2003; Mellor and Morillon, 2004). It has been proposed that the SLIDE domain is involved in DNA contact, whereas the SANT domain binds histone tails (Boyer *et al*, 2004).

TbISWI has a highly conserved SNF2 domain, which in database interrogations preferentially identifies ISWI proteins

 

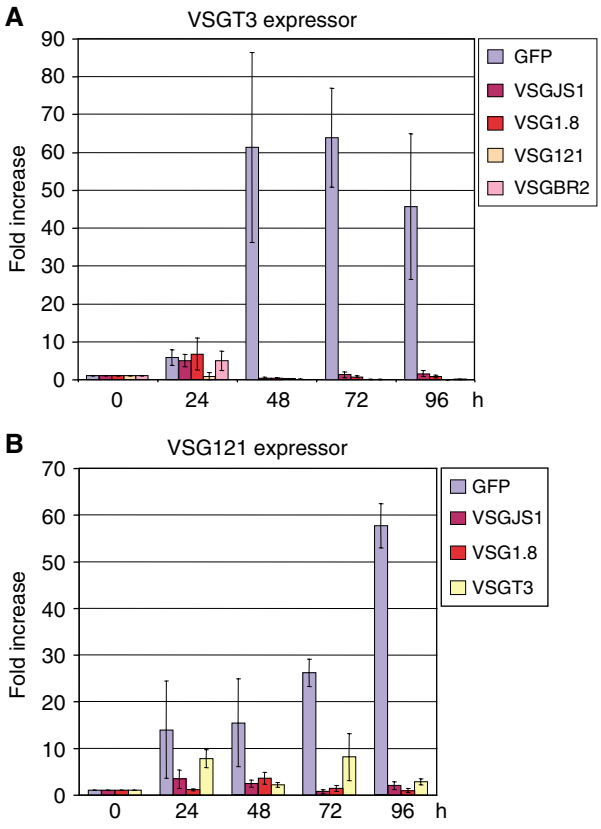


**Figure 7** Derepression of silent ES promoters does not lead to an increase in transcription of telomeric *VSG*s located in silent ESs. (**A**) Transcript analysis in *T. brucei* T3SA1 (*VSGT3* ES active) after the induction of *TbISWI* RNAi for the time indicated in hours. RNA was isolated at various time points, and real-time PCR was used to monitor levels of transcript from *GFP* or various telomeric *VSG*s located in silent ESs. Transcript levels were normalised against γ-tubulin as an internal control, and the fold increase relative to the 0 h time point is shown on the *x* axis. Results are the average of three measurements, with the standard deviation indicated with error bars. (**B**) Analysis of transcription in *T. brucei* 121SA1 (active *VSG121* ES) after the induction of TbISWI RNAi for the times indicated. The experiment was further performed as in (A).

from other species. However, it does not appear to have an obvious C-terminal SANT or SLIDE domain. Instead, it has a region at the C terminus with homology to a myb-like DNA-binding domain. As SANT and SLIDE domains are subclasses of myb domains, we find the similarity to typical ISWI architecture sufficiently close to categorise TbISWI as a member of the ISWI family.

### *The role of TbISWI in VSG expression site regulation*

An unexpected feature of our results is that TbISWI plays a role in ES downregulation in both *T. brucei* life-cycle stages. There are fundamental differences in how ES downregulation is mediated mechanistically in bloodstream versus insect-form *T. brucei*. In the bloodstream-form, ES downregulation is not promoter sequence-specific, and an rDNA promoter inserted into an ES can be turned off and on essentially as well as the endogenous promoter (Horn and Cross, 1995; Rudenko *et al*, 1995). In contrast, in insect-form *T. brucei*, rDNA promoters located within the ES escape downregulation (Rudenko *et al*, 1994; Horn and Cross, 1997).

However, it is unclear how this ES downregulation is mediated. It has been proposed that repressed chromatin does not play a role in ES downregulation in bloodstream-form *T. brucei*. For example, no differences have been found in nuclease sensitivity of silent compared with active ESs (Navarro and Cross, 1998). In their study, a DNase hypersensitive site was detected within the core promoter of both silent and active ESs, arguing that a protein complex is bound irrespective of ES activity. In addition, experiments probing the accessibility of ES chromatin using exogenous T7 RNA polymerase did not find evidence for a repressed chromatin structure in inactive ESs in bloodstream-form *T. brucei* (Navarro *et al*, 1999). In insect-form *T. brucei*, downregulated ESs were not accessible for T7 transcription, arguing that a life-cycle-specific repressed chromatin structure could play a role in ES downregulation in this life-cycle stage (Navarro *et al*, 1999). In contrast, our experiments showing the involvement of a putative chromatin-remodelling protein suggest that chromatin structure does play a role in ES downregulation in both life-cycle stages of *T. brucei*.

ESs are transcribed by pol I rather than pol II (Günzl *et al*, 2003). There is precedent for ISWI family members regulating transcription mediated by pol I as well as pol II. For example, the mammalian ISWI protein SNF2h is present in the multiprotein nucleolar remodelling complex, and plays an active role in establishing the repressed chromatin state, which silences about half of the rDNA transcription units (Strohner *et al*, 2001; 2004).

Although there are arguments for the presence of a repressed chromatin structure in ESs in at least insect-form *T. brucei*, it is still not clear if downregulation of transcription is at the level of transcription initiation or elongation (Vanhamme *et al*, 2000; Pays *et al*, 2004). Arguments for the latter model include the observation that ES shut down as the bloodstream-form trypanosome differentiates to either the insect-form or the nondividing short stumpy form entails progressive stalling of the RNA polymerase on the ES (Pays *et al*, 2004). Our results are compatible with both models for ES control, as ISWI proteins can have multiple roles in transcription regulation.

### *Multiple roles for ISWIs*

ISWI proteins are involved in a variety of processes, and can play a role in transcription elongation and termination as well as transcription initiation (Mellor and Morillon, 2004). These different functional activities are mediated by complexes containing ISWI interacting with different protein partners, allowing the same protein to have different functions within the same organism (Corona and Tamkun, 2004; Mellor and Morillon, 2004). For example, in *Saccharomyces cerevisiae*, Isw1p complexed with the protein partner Ioc3p can prevent transcription initiation at pol II promoters (Moreau *et al*, 2003). However, complexed with the protein partners Ioc2p and Ioc4p, it can prevent transcription elongation of stalled RNA polymerase (Morillon *et al*, 2003). High levels of this latter complex are also thought to allow efficient transcription termination (Morillon *et al*, 2003). A knock down of Isw1p can therefore lead to inappropriate transcription through multiple mechanisms.

Using the data presented here, we cannot distinguish between a scenario, whereby *TbISWI* blocks transcription initiation at silent *VSG* expression site promoters or prevents elongation of already initiated RNA polymerases. The arguments that *VSG* expression site control occurs mainly at the

level of transcription elongation have been presented in Pays *et al* (2004). A plausible explanation for the observed ES derepression in *T. brucei* is that blocking synthesis of TbISWI leads to the release of stalled RNA polymerases. This would be comparable to the role of Isw1p in the Isw1b complex in *S. cerevisiae,* where it is complexed with the proteins Ioc2 and Ioc4. Identification of the TbISWI partners through affinity purification with epitope-tagged TbISWI should give us insight into the number of ISWI-containing complexes present in *T. brucei*. Inducible RNAi-mediated knock downs of these different ISWI-binding partners should allow us to dissect the different roles that TbISWI can play in *T. brucei*. It is possible that ES derepression mediated by TbISWI is operating in a mechanistically different fashion in the two life-cycle stages of *T. brucei*.

### *Other functions for TbISWI*

It is highly unlikely that TbISWI is operating exclusively on ES downregulation. First of all, it was initially identified as a protein binding the 177 bp repeats, which comprise the bulk of the nontranscribed minichromosomes of African trypanosomes. Possibly, it plays a role in preventing fortuitous transcription initiation on these small chromosomes, thereby preventing inappropriate transcription of silent *VSG*s in the bloodstream-form. The role of TbISWI in silencing different transcriptionally inactive regions of the trypanosome genome still needs to be investigated.

TbISWI is the first protein shown to play a role in ES downregulation in *T. brucei*, but there are sure to be more. Our results raise some additional interesting questions. Levels of ES derepression after the induction of TbISWI RNAi in bloodstream-form *T. brucei* are at up to 10% of an active ES. These submaximal levels of transcription could indicate that one or more transcription factors are limiting. As the active ES is transcribed at an extremely high rate, it might therefore be impossible for the cell to maximally transcribe all 20 ESs. Alternatively, location of an ES in the discrete subnuclear location of the ESB (Navarro and Gull, 2001) could provide one or more factors essential for fully competent ES activation.

Our data indicate that ES activation is a multistep process. We did not find evidence that transcription from derepressed ES promoters extends to the telomeric *VSG*, or that there is an increased rate of ES activation after inducing TbISWI RNAi in bloodstream-form *T. brucei.* One explanation is that although silent ES promoters are derepressed in the presence of TbISWI RNAi, they are still excluded from the subnuclear compartment containing the active ES (ESB). This exclusion could prevent full ES activation, as the ESB might be an essential location for fully processive transcription. Nonetheless, we show that TbISWI plays a critical role in at least one of the steps of ES downregulation. In addition, our results show the first indication that chromatin remodelling plays a critical role in ES control in both life-cycle stages of *T. brucei*. Dissecting the different layers of this control will provide a challenge for the future.

## Materials and methods

### *Trypanosome strains*

Insect-form *T. brucei* 29–13 TBT has the TBT construct integrated into *T. brucei* 29–13 (Wirtz *et al*, 1999) (K Hughes and G Rudenko, unpublished results). *T. brucei* 29–13 D1–D4 lines have the ESDsB construct integrated behind ES promoters in *T. brucei* 29–13. *T. brucei* D1-SA1, D1-SA2, D3-SA1 and D3-SA2 lines have an $MC^{177}$ TbISWI-A RNAi construct integrated into either *T. brucei* 29–13 D1 or D3. *T. brucei* 29–13 $MC^{177}$ TbISWI-GFP has the $MC^{177}$ TbISWI-GFP construct integrated into *T. brucei* 29–13.

The bloodstream-form *T. brucei* T3-SM cell line is derived from the 'single marker' cell line (Wirtz *et al*, 1999), and has an active *VSGT3* ES. This cell line was derived from a *VSG221*-expressing line, which had a 221GP1 construct (Sheader *et al*, 2004) integrated into the *VSG221* ES and an $MC^{177}$*VSG221* RNAi construct (Sheader *et al*, 2005) integrated into a minichromosome. *VSG221* RNAi was induced, and a VSG switch variant, which had activated the *VSGT3* ES, was isolated. A construct containing a blasticidin-resistance gene was inserted immediately behind the promoter of the *VSGT3* ES to maintain selection for this active ES. The $MC^{177}$ TbISWI-A construct was integrated into a minichromosome producing the *T. brucei* T3-SA1 and T3-SA2 cell lines. *T. brucei* 121-SA1 and 121-SA2 lines are the same as above, but with the *VSG121* ES active. *T. brucei* 90–13 $MC^{177}$ TbISWI-GFP has the $MC^{177}$ TbISWI-GFP construct integrated into bloodstream-form *T. brucei* 90–13 (Wirtz *et al*, 1999). *T. brucei* HNIR1(221 + ) is described in Rudenko *et al* (1998).

### *Sequence analysis*

Protein domains were determined using programmes including 3D-JIGSAW (version 2.0) and SMART. TbISWI (GeneDB accession No. Tb927.2.1810) was compared with: *S. cerevisiae* ISWI2p (accession no. NP_014948), *S. cerevisiae* ISWI1p (accession no. NP_009804), *Caenorhabditis elegans* ISWI1 (accession no. AAA50636), *Drosophila melanogaster* ISWI isoform C (accession no. NP_725204), *Xenopus laevis* ISWI (accession no. AAH76715), *Mus musculus* SNF2 H (accession no. AAK52454) and *Homo sapiens* SNF2 H (accession no. AAH23144).

### *Constructs*

The target fragments in the ESDsB construct are the same as in the 221GP1 construct (Sheader *et al*, 2004). *DsRed2* (Clontech) is flanked by α-tubulin RNA-processing signals (Rudenko *et al*, 1994). The blasticidin-resistance gene is flanked downstream by an actin intergenic region. The rDDsB construct integrates *DsRed* into an rDNA locus, and has an rDNA promoter on a 520 bp *Alu*I fragment (White *et al*, 1986) cloned in front of the *DsRed* and blasticidin-resistance gene cassette used in the ESDsB construct. An rDNA nontranscribed spacer target fragment from the $p2T7^{Ti}$ A plasmid (LaCount *et al*, 2002) was cloned upstream of the rDNA promoter. The TBT construct has the blasticidin-resistance gene cloned between the intergenic regions of an α-tubulin gene.

The $MC^{177}$ TbISWI-A RNAi construct has the 1190 bp *TbISWI*-A fragment, (positions 4–1194 of the *TbISWI* open reading frame) cloned between the opposing T7 promoters of construct $p2T7^{Ti}$-177 (Wickstead *et al*, 2002). The $MC^{177}$ *TbISWI*-B RNAi target fragment is a 514 bp fragment from the 3′ end of the *TbISWI* gene amplified using 5′-tatctagaACCGGTGCAGTTTTATACGG and 5′-tactcgagGACGCTGCCACTAGTGATGA primers. A blasticidin-resistance gene containing construct was targeted behind the *VSGT3* or *VSG121* ES promoters using target fragments that were PCR amplified from the relevant ES TAR clone (Becker *et al*, 2004). The upstream fragment was amplified using 5′-cctctagaTACGCGTCTACTGAGGTAAGGAATATCGACG-3′ and 5′-ccggatccGTCATGCATGAACCGACAACGGTC-3′ primers. The downstream fragment was amplified using 5′-ccctcgagGGGAGACACTTGCACTTCGAGGTCCG-3′ and either 5′-ccgaagcttGCTTTATCCCGTGCCTACTGCGTC-3′ (*VSG121* ES), or 5′-ccgaagcttGCTTTATCCCGTGGTTCCTTTGTC-3′ for the (*VSGT3 ES*) targeting construct.

The $MC^{177}$ TbISWI-GFP construct allowed inducible expression of TbISWI-GFP fusion protein. The TbISWI open reading frame was amplified from *T. brucei* genomic DNA using primers 5′-tatctagaATGGAGGCACCAGCAGGACG and 5′-taggatccTTATTCCGGAAACTTCCGCT. This was cloned into pDex577 (gift from Keith Gull laboratory) resulting in an in-frame fusion with *GFP*. This construct integrates into *T. brucei* minichromosomes using the 177 bp target sequence (Wickstead *et al*, 2002). TbISWI-His-tagged recombinant fusion protein was made by inserting a TbISWI fragment amplified using primers 5′-taggatccCTTTAACTGCTGTGGAGCGT and 5′-tactcgagTGATTATTCCGGAAACTTCC into pRSetC (Invitrogen).

### *Nucleic acid and protein analysis*

Pulsed field gel analysis was performed using a CHEF-DRIII system (BioRad) (2.5 V/cm for 144 h with 1400–700 s switching time) (Aitcheson *et al*, 2005). Total *T. brucei* RNA was isolated using RNeasy RNA isolation kits (Qiagen). RNA was separated on formaldehyde agarose gels (Sambrook and Russell, 2001), and Northern and Southern blots were hybridised with radiolabelled probes made using a Megaprime kit (Amersham).

Protein lysates were made by centrifuging cells, washing once, and then resuspending in ice cold lysis buffer (50 mM HEPES (pH 7.5), 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA and Roche complete protease inhibitors) at $10^9$ cells/ml. After incubation at 4°C for 15 min with gentle rotation, cells were spun at 14 000 *g* for 15 min at 4°C. After centrifugation, $5 \times 10^6$ cell equivalents of the supernatant were loaded per lane on 6% SDS–polyacrylamide gels. Gels were blotted onto Hybond-P (Amersham) and probed with rabbit polyclonal antibodies against TbISWI-C or BiP (gift from Jay Bangs, University of Wisconsin, USA) (Bangs *et al*, 1993). Detection was carried out using ECL Plus (Amersham). TbISWI-C polyclonal rabbit antiserum was made against His-tagged TbISWI-C fusion protein, which was purified using Ni-NTA agarose (Qiagen). Rabbit polyclonal antisera were produced by Eurogentec (Belgium) using standard immunisation protocols.

The presence of TbISWI in chromatin was determined using a modification of the procedure in DiPaolo *et al* (2005). Protein lysates were prepared as described above in lysis buffer, supplemented with different concentrations of NaCl. Supernatant and pellet fractions were analysed on SDS–PAGE gels ($10^7$ cell equivalents per lane). Anti-histone H3 antibody was a gift from Bob Sabatini and the anti-La antibody was a gift from Elisabetta Ullu.

### *Expression analysis*

Flow cytometry was carried out using a Becton Dickinson FACSCalibur and CellQuest (BD) software. The mean of all events was calculated using Histogram Stats in CellQuest software.

To determine the localisation of the GFP–TbISWI fusion protein, cells expressing the appropriate GFP-TbISWI expression construct were induced with 750 ng $ml^{-1}$ tetracycline for 8 (insect-form *T. brucei*) or 20 h (bloodstream-form). Cells were washed and fixed in 2% paraformaldehyde before further processing for microscopic analysis, which was performed with a Zeiss Axioplan 2 microscope.

Real-time PCR was performed using total RNA isolated from trypanosomes using an RNeasy kit (Qiagen). RNA was treated with DNase I (Roche), and cDNA was made using Omniscript reverse transcriptase (Qiagen) and oligodT(18) primer. Real-time PCR was performed on an ABI 7000 sequencing detection system (SDS) using Brilliant®SYBR®Green PCR master mix (Stratagene). A control without reverse transcriptase was made using DNAse I-treated RNA from each time point. Levels of transcript were normalised using γ-tubulin for each time point and then plotted as fold increase relative to the 0 h time point.

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