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2 TbISWI regulates multiple Pol I transcribed loci and is present at Pol II transcription boundaries

3

in *Trypanosoma brucei*

4

5

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19

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22 RNA polymerase I, chromatin remodeling, transcription control.

23

24 **Abstract**

25           The unicellular eukaryote *Trypanosoma brucei* is unusual in having very little  
26 transcriptional control. The bulk of the *T. brucei* genome is constitutively transcribed by RNA  
27 polymerase II (Pol II) as extensive polycistronic transcription units. Exceptions to this rule  
28 include several RNA polymerase I (Pol I) transcription units such as the *VSG* expression sites  
29 (ESs), which are mono-allelically expressed. TbISWI, a member of the SWI2/ SNF2 related  
30 chromatin remodelling ATPases, plays a role in repression of Pol I-transcribed ESs in both  
31 bloodstream and procyclic form *T. brucei*. Here, we show that TbISWI binds both active and  
32 silent ESs, but is depleted from the ES promoters themselves. TbISWI knock-down results in an  
33 increase in *VSG* transcripts from the silent *VSG* ESs. In addition to its role in repression of the  
34 silent ESs, TbISWI also contributes to the downregulation of the Pol I-transcribed procyclin loci,  
35 as well as nontranscribed *VSG* Basic Copy arrays and mini-chromosomes. We also show that  
36 TbISWI is enriched at a number of strand switch regions which form the boundaries between Pol  
37 II transcription units. These strand switch regions are the presumed sites of Pol II transcription  
38 initiation and termination, and are enriched in modified histones and histone variants. Our  
39 results indicate that TbISWI is a versatile chromatin remodeler that regulates transcription at  
40 multiple Pol I loci, and which is particularly abundant at many Pol II transcription boundaries in  
41 *T. brucei*.

42

43 **Introduction**

44 As an early branching eukaryote *Trypanosoma brucei*, the causative agent of African  
45 Sleeping Sickness, has some unorthodox features. Most of the *T. brucei* genome is constitutively  
46 transcribed by RNA polymerase II (Pol II) as extensive polycistronic arrays, and there is very  
47 little transcriptional control (11). Another unusual feature is that RNA polymerase I (Pol I)  
48 transcribes not only the multi-copy ribosomal DNA (rDNA), but also the genes encoding the  
49 trypanosome major surface proteins Variant Surface Glycoprotein (VSG) and procyclin, which  
50 are controlled in a life-cycle specific fashion (17, 25, 42). Bloodstream form *T. brucei* evades  
51 immune attack by the mammalian host through antigenic variation of its VSG surface coat by  
52 switching between different VSG variants. It is therefore critical for bloodstream form  
53 trypanosomes to tightly control expression of their *VSG* repertoire. Only one of about 1500  
54 different *VSG* genes and pseudogenes is expressed in a mono-allelic fashion from one of about  
55 15 telomeric *VSG* expression site (ES) transcription units (8, 10, 36). Upon differentiation to the  
56 procyclic form insect mid-gut stage, the VSG coat is replaced by an invariant coat of procyclin  
57 (41).

58 Very few regions of the trypanosome genome are non-transcribed. These include the  
59 silent *VSG* genes, which are located in sub-telomeric *VSG* Basic Copy arrays as well as at the  
60 telomeres of large, intermediate and mini-chromosomes (4, 31, 61). Mini-chromosomes are  
61 primarily composed of arrays of 177 bp repeats and are transcriptionally silent (61). In addition,  
62 the rDNA spacer is an 8 kb non-transcribed region in between tandem arrays of rDNA  
63 transcription units (58). Finally, the transcription units containing the *T. brucei* coat protein  
64 genes are turned off in the life-cycle stage where they are not needed. All *VSG* ESs are  
65 downregulated in procyclic form *T. brucei* where VSG is not expressed, and the procyclin

66 transcription units are silenced in bloodstream form *T. brucei* where it would be deleterious for  
67 an invariant surface protein to be expressed on the cell surface (56).

68 It is unclear how *T. brucei* keeps these transcriptionally inactive regions of the genome  
69 silent, as well as the extent to which epigenetic modifications such as chromatin remodeling are  
70 involved in this process. In the case of the ESs, it has recently been shown that the active ES in  
71 bloodstream form *T. brucei* is depleted of nucleosomes (13, 51), indicating that chromatin  
72 remodeling plays a key role in ES activation. This is supported by the discovery that a number  
73 of proteins involved in epigenetic control play a role in ES regulation including the *T. brucei*  
74 ISWI homologue TbISWI (21), the telomere binding protein RAP1 (64) and the histone  
75 methyltransferase DOT1B (14).

76 It has recently become clear that epigenetic marks are also present at the strand switch  
77 regions (SSRs) located between the polycistronic *T. brucei* Pol II transcription units (50, 63).  
78 The divergent SSRs, which contain putative transcription start sites (TSS), are enriched in the  
79 histone variants H2AZ and H2BV as well as histones H3K4me3 and H4K10ac. Convergent  
80 SSRs containing putative transcription termination sites (TTS) are enriched in the histone  
81 variants H3v and H4v (50). The proposed histone labelling of a transcription stop site is another  
82 unique feature of the trypanosome genome.

83 In this study, we investigated the role of TbISWI in regulating gene expression in  
84 different genomic regions of *T. brucei*. We show that TbISWI binds in the *T. brucei* genome  
85 within silent DNA repeats, in silent and active transcription units transcribed by Pol I, as well as  
86 at Pol II-transcribed loci. We also find that TbISWI is particularly enriched at a number of SSRs  
87 between Pol II transcription units containing the presumed Pol II transcription initiation and  
88 termination sites. Using *eGFP* reporter constructs inserted into various genomic loci, we

89 demonstrate that TbISWI is involved in repressing an ES promoter in regions containing silent  
90 *VSGs*, including the *VSG* Basic Copy arrays and the mini-chromosomes, as well as genes  
91 transcribed by Pol I including procyclin. Knock-down of TbISWI resulted in an increase in *VSG*  
92 transcripts from the silent telomeric *VSG* ESs, indicating that it plays a role in keeping the entire  
93 ES silent. Our results indicate that the chromatin remodeler TbISWI is involved in transcription  
94 by both Pol I and Pol II in *T. brucei*.

95

## 96 **Materials and Methods**

### 97 **Trypanosomes.**

98 Bloodstream and procyclic form *T. brucei* 427 cell lines were cultured according to (49),  
99 **except that** the bloodstream form was grown in the presence of 20% foetal calf serum (FCS)  
100 without the addition of SerumPlus. The bloodstream form *T. brucei* 121-SA1 cell has an active  
101 *VSG121* ES, *eGFP* in the silent *VSG221* ES, and a TbISWI RNAi construct (described in (21)).  
102 Additional bloodstream form *T. brucei* lines were made containing a reporter *eGFP* gene  
103 inserted into various loci including: the *VSG* Basic Copy array, the 177 bp repeats of mini-  
104 chromosomes, the procyclin locus, the rDNA spacer, and the tubulin array. All bloodstream  
105 form reporter lines were derived from *T. brucei* S16.221, a derivative of the *T. brucei* S16 ‘single  
106 marker’ cell line (62). S16.221 was obtained by transfecting S16 with the pHNES221Pur1.6  
107 construct (unpublished) that inserts a puromycin resistance gene downstream of the active  
108 *VSG221* ES promoter. Next, the relevant *eGFP* containing reporter construct was transfected  
109 into S16.221, followed by the MC<sup>177</sup> TbISWI-A RNAi construct (21). The lineage of the  
110 different cell lines used for the reporter assays is shown in Fig. 2. *TbISWI* RNAi was induced by  
111 incubating the cells with 750 ng ml<sup>-1</sup> tetracycline. All procyclic form reporter lines were made in

112 a similar fashion, **except that** the parental cell line was *T. brucei* 29-13 (62). Reporter constructs  
113 containing eGFP were first transfected into 29-13 cell lines, followed by the TbISWI RNAi  
114 construct (21).

115 TbISWI was epitope tagged at the C-terminus with a triple myc epitope using the  
116 pMoTAG43M vector (35). Epitope tagging was performed in bloodstream form *T. brucei*  
117 221GPI(VO2+) cells (49) and in wild type procyclic *T. brucei*. In order to ensure functionality  
118 of the tagged TbISWI, cell lines were also made where the second allele of TbISWI was knocked  
119 out using the pSpot5KOPhleo vector.

#### 120 **Constructs.**

121 The sequences of all primers used for cloning are shown in Supplementary Table 1. The  
122 fragments used to target the reporter constructs to different loci are: (i) a mini-chromosomal 177  
123 bp repeat sequence from p2T7<sup>Ti</sup>-177 (59), (ii) a *VSG118* Basic Copy array fragment amplified by  
124 PCR from *T. brucei* genomic DNA (gDNA) using primers BCVSG118-863s and BCVSG118-  
125 2025as, (iii) an rDNA spacer targeting fragment isolated from p2T7<sup>Ti</sup>A/GFP (27), and (iv) a  
126 procyclin targeting fragment amplified by PCR from *T. brucei* gDNA using primers  
127 EP1\_UP\_2480s and EP1\_UP\_3991as. Constructs targeting the first two loci were made with  
128 (+ESp) and without (-ESp) an ES core promoter isolated from rDES1 (43). The reporter gene  
129 cassette consists of an *eGFP* ORF, with a tubulin splice signal at its 5'-end and a tubulin  
130 polyadenylation signal at its 3'-end. The drug resistance cassette consists of a drug resistance  
131 gene flanked by a 5'-tubulin splice signal and a 3'-actin polyadenylation signal and is driven by  
132 an rDNA promoter (48). The reporter constructs **all** contained the blasticidin resistance (BlastR)  
133 gene as the drug marker, **however** the rDNA spacer targeting constructs were **also** made with **the**  
134 phleomycin resistance (PhleoR) gene. The tubulin array targeting reporter construct was derived

135 from pGad8-tubulin (60) by replacing the hygromycin resistance gene (HygroR) by BlastR. All  
136 sequences are available upon request. Verification of the reporter construct integration into the  
137 different genomic loci is described in the Supplementary Material. Integration of reporter  
138 constructs into mini-chromosomes was verified by Pulsed Field Gel (PFGE) analysis and Southern  
139 blot mapping (Sup. Fig. 1C). Reporter construct integration into the *VSG118* Basic Copy array,  
140 the procyclin locus and the tubulin array was verified by PCR (Sup. Fig. 3C, Sup. Fig. 5B and  
141 Sup. Fig. 8B) (primers listed in Sup. Table 2). Integration into the rDNA spacer was confirmed  
142 by Southern blot analysis (Sup. Fig. 7B)(Sup. Table 3).

143 For chromatin immunoprecipitation (ChIP) studies, one of the endogenous TbISWI  
144 alleles was tagged at the C-terminal end with a triple myc epitope as follows: The C-terminal end  
145 of *TbISWI*, excluding the stop codon, was amplified from *T. brucei* 427 genomic DNA using  
146 Spot5CTtag\_s and Spot5tag4934\_as to yield a 701 bp fragment (position 2780- 3480 of the  
147 TbISWI open reading frame). For the downstream region, an 827 bp fragment was produced  
148 using primers Spot5DRtag\_s and Spot5DRtag\_as. Both of these fragments were cloned into the  
149 pMoTAG43M vector, containing HygR for selection (35).

150 The untagged allele of TbISWI was knocked-out through replacement with PhleoR to  
151 ensure functionality of myc-tagged TbISWI. A 499 bp fragment immediately upstream of the  
152 *TbISWI* open reading frame was amplified from *T. brucei* 427 genomic DNA using Spot5-957s-  
153 UPF and Spot5-1455as-US. For the downstream region, a 586 bp fragment was amplified using  
154 primers Spot5-4935s-DSF and Spot5-5520as-DS. These fragments were cloned on either side of  
155 PhleoR in the pBlueScript vector to produce pSpot5KOPhleo. The MC<sup>177</sup> TbISWI-A RNAi  
156 construct used for all reporter lines is described in (21), and the MC<sup>177</sup> Tb11.01.2500 RNAi  
157 construct is described in the Supplementary Material.

158 **Western blotting.**

159 Protein lysates were made from bloodstream and procyclic form *T. brucei* essentially as  
160 described (21) and total lysate from 8-20 x 10<sup>6</sup> cells (equal number of cells per lane in a gel) was  
161 separated by SDS-PAGE. Proteins were transferred to nitrocellulose and probed with either a  
162 monoclonal anti-myc antibody (clone 4A6, 05-724, Upstate) to detect C-terminally tagged  
163 TbISWI or polyclonal anti-BiP (kind gift from Jay Bangs, University of Wisconsin, USA) as a  
164 loading control. Wild type TbISWI was detected using polyclonal anti-TbISWI antibody (21),  
165 and an anti-SMC3 antibody (6) was used as a loading control. Secondary antibodies conjugated  
166 to horseradish peroxidase were used to detect bound antibodies, and signals were visualized  
167 using the Western Lightning Plus ECL system (Perkin Elmer Life Sciences).

168 **Flow cytometry.**

169 Flow cytometry was performed on *T. brucei* cell lines containing an *eGFP* reporter gene  
170 in different genomic locations after induction of RNAi against *TbISWI* for the times indicated. A  
171 Becton Dickinson FACS Calibur (BD Biosciences) was used to monitor the fluorescence of the  
172 cells in the FL-1 channel. The mean of 100,000 events was calculated using CellQuest software  
173 (BD Biosciences). Fold derepression was determined by dividing the mean FL-1 values of  
174 RNAi induced cultures at each time point by the mean FL-1 values of uninduced cultures. For  
175 each cell line, three independent experiments were performed and the standard deviations are  
176 indicated with error bars.

177 **ChIP assays and quantitative PCR.**

178 ChIP was performed in *T. brucei* cells expressing wild type or myc-tagged TbISWI  
179 essentially as described (51). In brief, cultures were fixed in 1% formaldehyde for 1 hour at  
180 room temperature and chromatin from 7 x 10<sup>7</sup> cell equivalents was incubated for 16 hours with

181 an anti-histone H3 antibody (ab1791; AbCam) as a positive control, no antibody as a negative  
182 control, and a monoclonal anti-myc antibody (clone 9E10, M5546; Sigma) to detect TbISWI.  
183 The protein-DNA complexes were incubated with protein A beads (CL4B beads, GE Healthcare)  
184 and eluted in 1% SDS/0.1M NaHCO<sub>3</sub>. DNA was purified using QIAquick PCR purification kit  
185 (QIAGEN). Quantitative PCR (qPCR) was used to amplify regions of interest using the  
186 LightCycler<sup>®</sup> 480 Real-Time PCR System (Roche) (primer details in Sup. Tables 4 and 5). For  
187 the quantitation of TbISWI ChIP experiments, signals are expressed as a percentage of input  
188 immunoprecipitated using an anti-myc antibody after subtraction of signal from the no antibody  
189 control for both the wild type TbISWI and myc-tagged TbISWI cell lines. Quantitation of  
190 histone H3 shows the percentage of input immunoprecipitated after subtraction of signal from  
191 the no antibody control. The data shown is from bloodstream form *T. brucei* and is the average  
192 of three to five independent ChIP experiments, with the standard deviation indicated with error  
193 bars. ChIP-qPCR was also carried out in procyclic form *T. brucei* and produced very similar  
194 results to those found for bloodstream form *T. brucei* in all regions analysed.

195 ChIP material was also analyzed on slot blots using radiolabeled probes as described  
196 previously (51). Hybond-XL membranes (GE Healthcare) were hybridized with probes specific  
197 for the 177 bp repeats (61), the 50 bp repeat regions flanking *VSG* ESs (65) or the telomere  
198 repeats (7). Blots were quantitated using a PhosphorImager (BioRad) and the percentage of  
199 DNA immunoprecipitated was determined using QuantityOne software (BioRad). Signals are  
200 expressed as a percentage of input as described above.

#### 201 **RNA isolation and quantitative reverse-transcriptase PCR.**

202 The bloodstream form *T. brucei* 121-SA1 cell line (21) was used for quantitative reverse-  
203 transcriptase PCR (qRT-PCR). RNAi against *TbISWI* was induced, and total RNA was isolated

204 at various time points using the RNeasy kit (Qiagen). RNA was DNase-treated using the  
205 TURBO DNA-free kit (Ambion) and cDNA was synthesized using the Omniscript RT kit  
206 (Qiagen) with random hexamer primers (Promega). qPCR was performed on a LightCycler<sup>®</sup> 480  
207 Real-Time PCR System using LightCycler<sup>®</sup> 480 SYBR Green I Master Mix (Roche) in  
208 triplicate. The reaction conditions for each primer pair were individually optimized to obtain a  
209 single product of the correct size. The sequences of the primers used are detailed in Sup. Table  
210 4. Control reactions without reverse transcriptase (RT) were made using DNase-treated RNA  
211 from each time point. Levels of transcript were plotted as fold increase relative to the 0 hour  
212 time point after subtraction of the no RT controls. Three independent experiments were  
213 performed, and the standard deviation is indicated with error bars.

214

## 215 **Results**

### 216 **TbISWI binds silent DNA repeats as well as the VSG Basic Copy arrays.**

217 TbISWI was originally identified as binding the 177 bp repeats in DNA affinity  
218 purification experiments, and was subsequently shown to be involved in silencing *VSG* ESs in  
219 both bloodstream and procyclic form *T. brucei* (21). As the 177 bp repeats are primarily located  
220 in the transcriptionally silent mini-chromosomes (61), we investigated whether TbISWI binds to  
221 and represses other silent areas of the *T. brucei* genome. We performed chromatin  
222 immunoprecipitation (ChIP) analysis using an epitope-tagged copy of TbISWI. Bloodstream and  
223 procyclic form *T. brucei* lines expressing a TbISWI-myc fusion protein from one endogenous  
224 *TbISWI* allele were generated. Expression of the myc-tagged TbISWI was verified using  
225 Western blot analysis (Fig. 1A). Functionality of the myc-tagged TbISWI was confirmed by  
226 knocking out the other *TbISWI* allele. The *TbISWI-myc/ TbISWI\_KO* strains grew normally

227 compared to cells expressing wild type TbISWI in both life cycle stages (data not shown). The  
228 myc tag therefore did not detectably interfere with TbISWI function. This cell line was used for  
229 all successive ChIP experiments.

230 ChIP analysis was performed on bloodstream form cell lines containing either wild type  
231 or myc-tagged TbISWI. An  $\alpha$ -myc antibody was used to immunoprecipitate TbISWI, an  $\alpha$ -  
232 histone H3 antibody was used as a positive control, and no antibody was used as a negative  
233 control. Input chromatin and immunoprecipitated material were analyzed using either slot blots  
234 probed for repetitive regions, or by quantitative PCR (qPCR) for individual genes. As expected,  
235 we found that TbISWI binds the 177 bp repeats (Fig. 1B) with 0.05% of the input  
236 immunoprecipitated (Fig. 1C). TbISWI was also found to bind other silent regions including the  
237 50 bp repeat arrays located upstream of *VSG* ESs (about 0.04% immunoprecipitated) (48, 65),  
238 the telomere repeats (0.01% immunoprecipitated) and the subtelomeric *VSG118* Basic Copy  
239 array (0.04% immunoprecipitated). Significant amounts of TbISWI were therefore found  
240 binding all of these nontranscribed regions ( $P < 0.05$ ; see Supplementary Table 6 for statistical  
241 analyses). Similar results were obtained using procyclic form cells (data not shown). The mini-  
242 chromosomes and the *VSG* Basic Copy arrays house the silent *VSG* repertoire used during  
243 antigenic variation. As TbISWI was found binding both of these regions at similar levels, we  
244 next investigated the role of TbISWI in silencing these areas of the genome.

245

246 **TbISWI is involved in down-regulating transcription in *T. brucei* mini-chromosomes and**  
247 ***VSG* Basic Copy arrays.**

248 In order to investigate the role of TbISWI in silencing, we made *T. brucei* reporter cell  
249 lines where the parental bloodstream form *T. brucei* S16.221 line or the procyclic form *T. brucei*

250 29-13 line had an *eGFP* containing construct inserted into the mini-chromosomes, the *VSG118*  
251 Basic Copy Array, the EP procyclin locus, the rDNA spacer or the tubulin transcription unit (Fig.  
252 2). The advantage of using *eGFP* to monitor for transcription is that gene expression at the  
253 population level can be analysed using flow cytometry. This allows one to uncover cell-cycle  
254 specific effects, which is not straightforward with a reporter like luciferase.

255         Constructs integrated into the mini-chromosomes or the *VSG118* Basic Copy array, either  
256 did (+) or did not (-) contain an ES promoter (ESp). Unlike other *T. brucei* Pol I promoters, the  
257 ES promoters are unusually small and lack an upstream control element (39), rendering  
258 transcription highly sensitive to the chromatin state of their genomic context (20, 53). We  
259 therefore used an ES promoter as a “probe” for repressed chromatin. These *T. brucei* reporter  
260 cell lines were subsequently transfected with a TbISWI RNAi construct allowing tetracycline  
261 inducible knock-down of TbISWI. See Materials and Methods for details on the various  
262 constructs and cell lines.

263         To determine whether TbISWI has a role in silencing the *T. brucei* mini-chromosomes,  
264 we integrated an *eGFP* reporter gene into the 177 bp repeat arrays in bloodstream form *T. brucei*  
265 (Fig. 2Ba, BF-MCe in Fig. 3A, Sup. Fig. 1A), and made a similar reporter line with an ES  
266 promoter upstream of *eGFP* (BF-MCESp in Fig. 3B, Sup. Fig. 1B). Correct integration of *eGFP*  
267 was confirmed by Pulsed Field Gel analysis and Southern blot mapping (Sup. Fig. 1C). Both of  
268 these reporter lines were transfected with a TbISWI RNAi construct (21) and two independent  
269 RNAi clones per line were analysed (BF-MCe1, BF-MCe2 and BF-MCESp1, BF-MCESp2).  
270 Western blot analysis confirmed TbISWI protein knock-down after the induction of TbISWI  
271 RNAi (Sup. Fig. 2), and flow cytometry was used to monitor eGFP expression. Four days after  
272 the induction of TbISWI RNAi there was no significant increase of eGFP expression observed in

273 the lines not containing an ES promoter (BF-MCe1 and BF-MCe2 in Fig. 3A) in comparison to  
274 the negative control (BF-MCe). This indicates that blocking TbISWI synthesis **does not lead to a**  
275 significant increase in fortuitous initiation of transcription in the mini-chromosomes. In cell  
276 lines where an ES promoter was present upstream of *eGFP*, knock-down of TbISWI **leads to**  
277 more than six-fold derepression of *eGFP* (BF-MCESp1 and BFMCESp2 in Fig. 3B) when  
278 compared with the negative control (BF-MCESp). This indicates that TbISWI plays a role in  
279 maintaining a repressed chromatin structure around the ES promoter in *T. brucei* mini-  
280 chromosomes.

281 The *VSG* Basic Copy arrays are another transcriptionally silent genomic location in *T.*  
282 *brucei*. Therefore, we also performed similar experiments using cell lines where *eGFP* reporter  
283 constructs (either with or without an ES promoter) were integrated in the *VSG118* Basic Copy  
284 array (Fig. 2Bb, Fig 3C and Fig. 3D, Sup. Fig. 3). Similar to the cell lines where the reporter  
285 construct was integrated in mini-chromosomes, there was **no significant** increase in eGFP  
286 expression **seen** in the absence of an ES promoter (BF-VBe1 and BF-VBe2 in Fig. 3C).  
287 However, if an ES promoter was present (BF-VBESp1 and BF-VBESp2 in Fig. 3D)  
288 approximately six-fold derepression of eGFP was observed after the induction of a block in  
289 TbISWI synthesis.

290 Comparable experiments were also performed in procyclic form *T. brucei*, and similar  
291 results were obtained (Sup. Fig. 4). These data indicate that TbISWI plays a role in maintaining  
292 a repressed chromatin structure in both the *T. brucei* mini-chromosomes and *VSG* Basic Copy  
293 arrays in both bloodstream and procyclic form *T. brucei*. We can not exclude the possibility that  
294 the presence of an rDNA promoter directing transcription of the blasticidin resistance gene  
295 downstream of the reporter cassette could affect the local chromatin state. However, as the

296 results are plotted as fold derepression (ratio of the mean fluorescence of induced cells to  
297 uninduced cells), the observed derepression can be inferred to be a consequence of TbISWI  
298 depletion.

299

300 **TbISWI is present in both silent and active VSG ESs but is depleted from ES promoters.**

301 The active *VSG* is transcribed by Pol I from one of about 15 highly similar polycistronic  
302 *VSG* ES transcription units (depicted schematically in Fig. 4A) (19). We determined the  
303 distribution of TbISWI along the length of these *VSG* ESs using ChIP on wild type or myc-  
304 tagged TbISWI cell lines followed by qPCR as described earlier. Using six different primer  
305 pairs (a-f in Fig. 4A and Fig. 4B), that detect most, if not all *VSG* ESs (i.e. the 14 silent ESs in  
306 addition to the one active ES), we determined that TbISWI is present at significant levels  
307 ( $P<0.05$ ) along the length of the ES, with the exception of the “core” ES promoter (22) where  
308 TbISWI is relatively depleted (primer pair “a” in Fig. 4A and 4B) ( $P<0.05$ ; Sup. Table 7).  
309 Primer pair “a” is located immediately upstream of the putative site of ES transcription initiation.  
310 The relative depletion of TbISWI at ES promoters is particularly striking, considering that most  
311 if not all ESs can be detected with these particular primers. It is thought that the *VSG* ES “core”  
312 promoters of the 14 ‘silent’ ESs contain ‘paused’ Pol I complexes which lead to a low amount of  
313 transcription (Fig. 4B) (2, 43, 55). It is possible that these ‘paused’ Pol I transcription complexes  
314 result in exclusion of TbISWI. This pattern of TbISWI binding is similar to that observed for  
315 histone H3 at this locus (13, 51), and is consistent with TbISWI playing a role in nucleosome  
316 sliding and remodeling (30)(51).

317 Having determined the relative distribution of TbISWI along the *VSG* ESs (Fig. 4A and  
318 4B), we investigated whether there is a correlation between histone occupancy and TbISWI

319 abundance in silent versus active *VSG* ESs. We used the bloodstream form *T. brucei*  
320 221GPI(VO2+) line shown in (Fig. 4C), which has a neomycin resistance gene inserted behind  
321 the promoter of the active *VSGVO2* ES (49). The silent *VSG221* ES contains a puromycin  
322 resistance gene downstream of the ES promoter as well as a *VSG* pseudogene ( $\psi$ ). These are all  
323 single copy genes with the exception of *VSGVO2*, which has an additional copy in a *VSG* Basic  
324 Copy array. To determine the distribution of TbISWI over these silent and active *VSG* ESs,  
325 ChIP and qPCR analysis was performed. Interestingly, significant amounts of TbISWI ( $P < 0.05$ ;  
326 Sup. Table 8) were found binding both active and silent ESs at approximately comparable levels  
327 (Fig. 4D).

328 We had shown earlier that TbISWI is important for silencing genes directly downstream  
329 of a silent ES promoter, although in those experiments we had not found clear evidence for an  
330 increase in transcripts from telomeric *VSGs* present in a number of silent ESs (21). Considering  
331 that TbISWI binds along the length of *VSG* ESs (Fig. 4), we wondered whether it could play a  
332 role in keeping the entire ES silent, including the telomeric *VSG*. We therefore decided to re-  
333 investigate this question using newly available ES sequence data (19).

334

#### 335 **Depleting TbISWI leads to an increase in previously silent telomeric VSG transcripts.**

336 The repertoire of 15 *VSG* ESs from *T. brucei* 427 has recently been sequenced (19). This  
337 new data enabled us to re-investigate whether downregulation of TbISWI results in derepression  
338 of telomeric *VSGs* located in ESs, in addition to upregulation of the ES promoter proximal  
339 regions. The *T. brucei* 121-SA1 *TbISWI* RNAi cell line has an active *VSG121* ES and contains  
340 *eGFP* downstream of the promoter of the silent *VSG221* ES (21). Induction of *TbISWI* RNAi led  
341 to a decrease in TbISWI transcript, and a concurrent 60-fold increase of *eGFP* transcript (Fig.

342 5A). These results agree with previously published data (21). Transcript levels for  $\gamma$ -tubulin  
343 remain relatively unchanged after TbISWI knock-down, which was also observed using a  
344 reporter construct integrated into the  $\alpha$ - $\beta$  tubulin array (Fig. 6E). Transcripts from the active  
345 *VSG121* decrease by about 20%.

346 We next monitored transcripts from eleven silent ES-located *VSGs* as well as *VSG118*,  
347 which is located in a *VSG* Basic Copy array (Fig. 5B). Transcripts from all except for one of the  
348 telomeric ES-located *VSGs* increased significantly ( $P < 0.05$ ) after depletion of TbISWI for up to  
349 72 hours. The observed increase in *VSG* transcripts varied between the different *VSGs* from five-  
350 fold (for *VSG16*) to 70-fold (for *VSG1.8*) (Sup. Table 9). Variability in the degree of  
351 derepression of different ES-located silent *VSGs* was also observed after the knock-down of  
352 RAP1, and presumably reflects a hierarchy of different *VSG* activation frequencies (64).  
353 Interestingly, no statistically significant increase in *VSG14* transcript was observed after TbISWI  
354 knock-down. *VSG14* is located in a drastically truncated ES (BES8), which contains only  
355 *ESAG7* (19). We have never found evidence for activation of BES8 *in vitro* (19), and it is likely  
356 that this tiny ES is dysfunctional.

357 Transcript levels from the *VSG118* Basic Copy also did not increase significantly after  
358 TbISWI knock-down, presumably as there are no promoters (which could be derepressed) in this  
359 area of the genome. In addition, there does not appear to be an increase in levels of fortuitous  
360 initiation of transcription in the *VSG* Basic Copy arrays. A comparable increase in telomeric ES  
361 located *VSG* transcripts was also observed using the *T. brucei* T3-SA1 cell line, where the  
362 *VSGT3* ES is active (data not shown). Although we detected *VSG* transcripts derived from the  
363 silent *VSG* ESs we did not detect VSG protein (result not shown), presumably as Western blot  
364 analysis is much less sensitive than qPCR. These data indicate that TbISWI could play a role in

365 keeping the entire ES silenced. As Pol I transcribes *VSG* ESs, we next asked whether TbISWI is  
366 involved in silencing other Pol I-transcribed loci.

367

368 **TbISWI binds within Pol I and Pol II transcription units but is only involved in silencing**  
369 **Pol I transcribed loci.**

370 Pol I not only transcribes *VSG* genes, but also the EP and GPEET procyclin genes  
371 encoding the invariant surface proteins of insect form *T. brucei* (17, 25, 41-42), as well as the  
372 rDNA. In bloodstream form *T. brucei* the procyclin loci are downregulated, whereas in  
373 procyclic *T. brucei* they are actively transcribed (56). As TbISWI was found to bind along both  
374 silent and active *VSG* ESs, we investigated TbISWI occupancy in one of the procyclin  
375 transcription units (depicted schematically in Fig. 6A) in both life cycle stages. Using ChIP-  
376 qPCR we found that significant amounts of TbISWI ( $P<0.05$ ; Sup. Table 10) bind the EP  
377 procyclin coding region in both bloodstream and procyclic form *T. brucei*. TbISWI is relatively  
378 depleted from the EP promoter (Fig. 6A) in bloodstream form *T. brucei* ( $P<0.05$ ), as was also  
379 observed at the promoters of the many 'silent' ESs which contain paused Pol I complexes (Fig.  
380 4B) (2, 43, 55). It has been proposed that 'paused' Pol I transcription complexes are also present  
381 on 'silent' procyclin promoters in bloodstream form *T. brucei* (54), possibly resulting in the  
382 displacement of TbISWI from this region.

383 We next investigated whether TbISWI is involved in silencing the procyclin locus in  
384 bloodstream form *T. brucei*. To address this, we created a reporter cell line with *eGFP* targeted  
385 downstream of an endogenous procyclin promoter (Fig. 6B, Sup. Fig. 5). This cell line was  
386 transfected with a *TbISWI* RNAi construct and two independent clones were isolated. TbISWI  
387 protein knock-down was confirmed by Western blot analysis (Sup. Fig. 2). Flow cytometry was

388 used to monitor levels of eGFP after TbISWI knock-down. After depleting TbISWI for four  
389 days there was a six-fold increase in eGFP expression (Fig. 6B). These results are similar to the  
390 levels of derepression observed in the mini-chromosomes and *VSG* Basic Copy array (Fig. 3B  
391 and 3D). A similar experiment was carried out in procyclic form *T. brucei* where depleting  
392 TbISWI led to a modest 2.5-fold derepression (Sup. Fig 6A). Therefore, TbISWI appears to play  
393 a role in modulating transcription of the EP procyclin locus in both life cycle stages of *T. brucei*.

394 In mammals, the ISWI containing nucleolar remodelling complex (NoRC) mediates  
395 silencing of the inactive rRNA genes (46). We were therefore interested whether TbISWI binds  
396 in and around *T. brucei* rDNA transcription units (Fig. 6C). ChIP was performed in bloodstream  
397 form *T. brucei* and qPCR was used to detect binding to four regions within the rDNA locus. In  
398 addition, two Pol II transcribed genes were analysed: the gene encoding the large subunit of Pol  
399 I and  $\alpha$ -tubulin. Significant levels of TbISWI were found binding all of these regions ( $P < 0.05$ ;  
400 Sup. Table 11). TbISWI was particularly enriched in the transcriptionally inactive rDNA spacer  
401 (primer pair “a” in Fig. 6C) compared with the 18S rRNA coding region (primer pair “d”, Fig.  
402 6C) ( $P < 0.05$ ). Similar results were found in procyclic *T. brucei* (result not shown). TbISWI  
403 distribution in the rDNA closely resembled that of histone H3 (51), consistent with ISWI-  
404 containing complexes playing a role in nucleosome remodelling.

405 To determine if TbISWI plays a role in silencing the rDNA locus, we created a  
406 bloodstream form reporter line containing a construct with *eGFP* downstream of an ES promoter  
407 located in an rDNA intergenic region between two rDNA transcription units (Fig. 6D, Sup. Fig.  
408 7). After two days induction of *TbISWI* RNAi there was a four-fold increase in levels of eGFP  
409 (Fig. 6D). Comparable derepression was observed in procyclic *T. brucei* (Sup. Fig. 6B). These

410 results indicate that TbISWI has a role in silencing all Pol I transcription units analysed: the  
411 *VSG* ESSs, procyclin, as well as the rDNA spacer region in both life cycle stages.

412 We see that in most cases in these different bloodstream form *T. brucei* reporter lines, *T.*  
413 *brucei* TbISWI protein is greatly depleted 24 hours after induction of TbISWI RNAi (Sup. Fig.  
414 2). An observable growth arrest is typically seen about 48 hours after induction of TbISWI  
415 RNAi (Sup. Fig. 10). The timing of this growth inhibition coincides with the timepoint of  
416 maximal derepression of *eGFP* in most of these reporter cell lines, with the exception of the  
417 minichromosome reporter line, where maximal derepression of *eGFP* was seen a bit later (after  
418 three days). This delay between the reduction of TbISWI protein to very low levels, and the  
419 appearance of a growth or derepression phenotype could indicate that even small amounts of  
420 TbISWI protein are sufficient to maintain normal levels of silencing (21).

421 Most of the *T. brucei* genome is constitutively transcribed by Pol II as extensive  
422 polycistronic units. As TbISWI was found to bind to two Pol II transcribed genes (Fig. 6C), we  
423 investigated if TbISWI could play a role in regulating Pol II transcription. We inserted an *eGFP*  
424 reporter construct into the tubulin array, depleted the cells of TbISWI, and monitored levels of  
425 *eGFP* (Fig. 6E, Sup. Fig. 8). We found no significant change in the levels of *eGFP* after  
426 blocking TbISWI synthesis in both life cycle stages of *T. brucei* (Fig. 6E and Sup. Fig. 6C).

427

#### 428 **TbISWI is enriched in Pol II strand switch regions.**

429 Recent data have identified enrichment of various modified histones and histone variants  
430 in regions between polycistronic Pol II transcription units in *T. brucei* (50, 63). As ISWI  
431 complexes are involved in Pol II transcription initiation and termination in other organisms (30),  
432 this led us to hypothesize that TbISWI may play a role in establishing the epigenetic marks found

433 at strand switch regions (SSR). We analysed three ‘divergent’ and three ‘convergent’ SSRs on  
434 *T. brucei* chromosome 10 using ChIP-qPCR analysis (Fig. 7A). Regions chosen included those  
435 within the SSRs (primers c-e), in the genes directly flanking the SSRs (primers b and f), as well  
436 as in genes at the approximate midpoint of the flanking polycistronic units (primers a and g).  
437 Using anti-histone H3 antibody, histone H3 was found to be relatively uniformly distributed over  
438 divergent and convergent regions (Sup. Fig. 9).

439 The SSRs between two diverging Pol II transcription units are likely to contain Pol II  
440 promoters. Significant levels of TbISWI ( $P<0.05$ ) were found binding three ‘divergent’ SSRs  
441 (D1-D3) and flanking transcription units (Fig. 7B; Sup. Table 12). TbISWI was found to be  
442 significantly enriched in the ‘divergent’ SSRs (primers c-e) compared with genes within the  
443 neighbouring polycistronic transcription units (primers a and g) in divergent regions D1 and D2,  
444 although this was less clear for divergent region D3.

445 The SSRs between two converging Pol II transcription units are likely to contain Pol II  
446 terminators. Lower but significant amounts of TbISWI ( $P<0.05$ ; Sup. Table 13) were found  
447 binding three ‘convergent’ SSRs (C1-C3) and flanking transcription units (Fig. 7C). There was  
448 evidence for enrichment of TbISWI on the SSRs of ‘convergent’ regions C2 and C3, though this  
449 was not found for region C1. TbISWI therefore appears to be enriched at many (particularly  
450 divergent) SSRs, specifically co-localizing with nucleosomes enriched in modified histones and  
451 histone variants known to have roles in regulating transcription by RNA Pol II in other  
452 organisms (3, 47, 50). TbISWI therefore appears to be associated with both Pol I and Pol II  
453 transcription units in *T. brucei*.

454

455 **Discussion**

456 Members of the ISWI family of chromatin remodelers are involved in a wide variety of  
457 processes ranging from transcription activation, repression and termination, as well as the  
458 maintenance of higher order chromatin structure and DNA replication (12). The specialized  
459 function of individual ISWI complexes depends on the protein partners associated with ISWI.  
460 Here, we find that TbISWI is involved in downregulating regions containing silent *VSGs* as well  
461 as other Pol I transcription units. TbISWI was found to be particularly enriched in many Pol II  
462 strand switch regions, which are the proposed sites of Pol II transcription initiation and  
463 termination and are known to contain histone variants and specific histone modifications (50,  
464 63).

465

466 **TbISWI is involved in preventing transcription of the silent VSG repertoire.**

467 For antigenic variation to work, it is critical for bloodstream form *T. brucei* to tightly  
468 control expression of its extensive *VSG* repertoire. *VSGs* are located at most, if not all,  
469 telomeres, as well as in sub-telomeric *VSG* Basic Copy arrays (4, 31, 61). We found that  
470 TbISWI binds mini-chromosomes, the *VSG118* Basic Copy array, as well as *VSG* ESs. Using  
471 *eGFP* reporter constructs inserted into the 177 bp repeats of mini-chromosomes and the *VSG118*  
472 Basic Copy array, we found that TbISWI depletion does not lead to a significant increase in  
473 transcription from these nontranscribed genomic regions. It is possible that even if TbISWI  
474 depletion results in a more “open” chromatin configuration, the absence of promoters in these  
475 regions prevents a more significant increase in transcription.

476 In order to study the role of TbISWI in maintaining a repressed chromatin state at these  
477 regions, constructs containing a small and genome context-sensitive ES promoter were integrated  
478 into these loci. Depletion of TbISWI resulted in 4 to 6-fold derepression of the exogenous ES

479 promoter in both BF and PF *T. brucei*. While it can be argued that this effect of TbISWI  
480 depletion is related to the ES promoter itself, the observation that levels of derepression from an  
481 ES promoter vary from about 6-fold in BF-MCESp1 or BF-VBESp1 to about 60-fold in a silent  
482 ES (21) suggests that TbISWI-mediated repression of the ES promoter is dependent on genomic  
483 context. ES promoter derepression is not simply a consequence of a lethal phenotype, as RNAi  
484 mediated knock-down of another unrelated SNF2 domain containing protein, also led to a  
485 significant growth arrest but no observable ES derepression (Sup. Fig. 10). It is likely that  
486 TbISWI is involved in preventing inappropriate transcription of the various pools of silent *VSG*  
487 genes, presumably by maintaining a repressive chromatin structure which plays a role in  
488 inhibiting fortuitous initiation of transcription. Our results are in agreement with studies from  
489 other eukaryotes, where ISWI chromatin remodelling complexes have been shown to be  
490 involved in transcription repression (12, 16, 30, 45).

491 Previously, we reported that TbISWI is important for silencing the promoters of the silent  
492 *VSG* ESs, although it was unclear whether TbISWI depletion leads to processive transcription  
493 down to the telomeric *VSG* (21). Using new sequence data for *T. brucei* 427 *VSG* ESs (19), we  
494 re-investigated whether TbISWI plays a role in silencing telomeric ES located *VSGs*. After  
495 blocking TbISWI synthesis, we observed significant and reproducible increases in telomeric *VSG*  
496 transcripts. This implies that, contrary to the previous model, TbISWI has a role in keeping the  
497 entire silent ES inactive. The level of derepressed transcription from the silent ESs observed  
498 after TbISWI knock-down, was much lower than levels of transcription from an active ES  
499 (discussed in (21)). The active ES is located in an Expression Site Body (ESB) (34), which is a  
500 subnuclear structure thought to contain a high concentration of Pol I as well as transcription and  
501 RNA processing factors essential for high levels of expression. As the ESB is thought to be **only**

502 able to accommodate a single ES, this restriction could limit the amount of ES derepression  
503 observed after TbISWI depletion.

504

505 **TbISWI is involved in down-regulating Pol I transcription.**

506 Pol I transcribes the multi-copy rDNA transcription units as well as the *VSG* and  
507 procyclin genes. Protein coding genes transcribed by Pol I are tightly regulated, unlike the  
508 constitutively transcribed Pol II transcription units (11). Silent ESs in both bloodstream and  
509 procyclic form *T. brucei* have “poised RNA polymerases” at their promoters, and low levels of  
510 non-processive transcription (2, 43, 55). A similar situation is also seen in the procyclin loci that  
511 are transcribed at a low level in bloodstream form *T. brucei* (54). Using ChIP-qPCR we show  
512 that TbISWI is present on silent *VSG* ESs and on the silent *EPI* procyclin locus in bloodstream  
513 form *T. brucei*, but is relatively depleted from the ES (both silent and active) promoters as well  
514 as procyclin promoters. One possibility is that poised RNA polymerase complexes on these  
515 different promoter regions displace TbISWI from binding. Alternatively, as TbISWI is a  
516 nucleosome remodeler, this relative absence could be a consequence of the relative depletion of  
517 nucleosomes observed on ES and procyclin promoters (51).

518 Both of these Pol I transcribed loci are derepressed when TbISWI synthesis is blocked.  
519 While silent ESs are derepressed 30-60 fold in bloodstream form and 10-17 fold in procyclic  
520 form *T. brucei* (21), a silent procyclin locus in bloodstream form cells is derepressed about 6-  
521 fold. Surprisingly, expression from even the active procyclin locus is upregulated by 2.5 fold in  
522 procyclic *T. brucei* after TbISWI depletion. This suggests that *EPI* procyclin is not transcribed  
523 at its maximal capacity in procyclic form *T. brucei* due to a TbISWI mediated repressive  
524 mechanism. These observations all suggest that TbISWI is involved in regulation of

525 transcription elongation in Pol I transcription units. This function of TbISWI is different to that  
526 seen in the regions containing silent *VSGs* in either the *VSG* Basic Copy arrays or mini-  
527 chromosomes. Here TbISWI presumably modulates chromatin structure, possibly preventing  
528 fortuitous initiation of transcription from cryptic promoters.

529 Our ChIP-qPCR data also shows that TbISWI binds the active ES in bloodstream form *T.*  
530 *brucei* as well as the active *EPI* procyclin locus in procyclic form *T. brucei*. However, the fact  
531 that TbISWI is found binding both active and silent transcription units does not necessarily argue  
532 that it is doing the same thing in these two very different places, as ISWI complexes can have  
533 different functions depending on exactly which proteins ISWI is partnered up with. In  
534 *Saccharomyces cerevisiae* an ISWI complex is present on the active rDNA transcription units  
535 which facilitates transcription (24). In fact the ISWI protein Isw1p is a member of two different  
536 ISWI chromatin remodelling complexes: the Isw1a complex (consisting of Isw1p partnered with  
537 Ioc3p) downregulates transcription initiation (32), while the Isw1b complex (consisting of Isw1p  
538 in association with the Ioc4p and Ioc2p proteins) can facilitate both transcription elongation or  
539 termination (33). Additionally, in other organisms, different ISWI complexes have been shown  
540 to perform distinct functions in Pol I transcriptional control. The ISWI containing B-WICH  
541 complex is involved in activating Pol I transcription by promoting elongation (37-38), whereas  
542 the ISWI containing NoRC complex is involved in repression of Pol I transcription and keeping  
543 the silent rRNA genes inactive (52). It is therefore possible that one TbISWI complex down-  
544 regulates transcription elongation from Pol I transcription units, while another maintains a  
545 chromatin structure at the active loci which is facilitative to transcription.

546

547 **TbISWI is implicated in chromatin remodeling around Pol II transcription units.**

548 Recently in *T. brucei* and the related *T. cruzi*, acetylated and methylated histones were  
549 shown to be enriched in regions where polycistronic transcription units diverge, and which are  
550 proposed to be the sites of transcription initiation (40, 50, 63). The histone variants H2AZ and  
551 H2BV are also enriched in these regions. Convergent Pol II SSRs in *T. brucei* containing  
552 putative transcription termination sites are marked with histone variants H3v and H4v (50).

553 It has been suggested that chromatin remodelling is required to allow for the recognition  
554 of the histone N-terminal tails by histone modifying enzymes (26). Evidence is mounting that  
555 ISWI-containing complexes are necessary for histone methyltransferases and acetyl transferases  
556 to gain access to their histone targets (9, 26, 28). Histone variant H2A.Z-containing  
557 nucleosomes are less stable and help maintain an accessible chromatin structure at transcription  
558 start sites (23). ISWI remodelling complexes have also been found to be involved in histone  
559 variant H2A.Z replacement (18, 57) and have greater activity on H2A.Z-containing nucleosomes  
560 (15). Siegel *et al* have presented a model for the sequence of events leading to Pol II  
561 transcription initiation in *T. brucei* which requires a chromatin remodelling complex to replace  
562 canonical histones with the variants H2AZ and H2BV (50). We find that TbISWI is enriched at  
563 divergent SSR, which are the proposed sites of Pol II transcription initiation. Possibly, TbISWI  
564 has a role in allowing histone chaperones and histone modification enzymes to gain access to  
565 their target substrates in the *T. brucei* SSRs.

566 In summary, our results indicate that TbISWI has at least four different roles in *T. brucei*  
567 transcriptional regulation. First, TbISWI appears to be involved in maintenance of a repressive  
568 chromatin structure in various silent regions of the *T. brucei* genome. Second, it regulates  
569 transcription elongation at the Pol I transcription units encoding the silent *VSG* and *procyclin*  
570 coat protein genes in both bloodstream and procyclic form *T. brucei*. Third, it binds to [multiple](#)

571 regions containing putative Pol II transcription initiation sites, possibly influencing chromatin  
572 remodeling or epigenetic marks in these regions. Fourth, based on the observed ISWI  
573 enrichment at **different** pol II convergent SSRs, it may also play a role in termination of pol II  
574 transcription as has been found for other chromatin remodelling complexes (1, 33). It will now  
575 be crucial to determine the partners of TbISWI. Elucidating the composition and role of the  
576 various TbISWI-containing chromatin remodelling complexes will allow us to dissect the  
577 different functions of TbISWI and unravel the mechanisms that *T. brucei* uses to inherit specific  
578 chromatin states from one generation to the next.

579

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591

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- 790  
791

792 **Figure legends**

793

794 **Fig. 1.**

795 TbISWI binds transcriptionally silent DNA repeats as well as the *VSG* Basic Copy arrays.

796 (A) Bloodstream form (BF) *T. brucei* strains expressing wild-type (wt) or myc-tagged TbISWI  
797 (myc) were analysed with Western blots using an anti-myc antibody (top panel), or an anti-BiP  
798 antibody (bottom panel) as a loading control. The position of TbISWI-myc is indicated with an  
799 arrowhead on the right and size markers in kiloDaltons (kDa) are shown on the left.

800 (B) Chromatin immunoprecipitation (ChIP) was performed on bloodstream form (BF) *T. brucei*  
801 expressing wild-type TbISWI (wt) or myc-tagged TbISWI (myc). The chromatin in the input  
802 material (IN) was immunoprecipitated with an anti-histone H3 antibody ( $\alpha$ -H3) as a positive  
803 control, no antibody (No Ab) as a negative control, and an anti-myc antibody ( $\alpha$ -myc) to  
804 immunoprecipitate TbISWI-myc. The immunoprecipitated DNA was analyzed by slot blots  
805 hybridized with radiolabeled probes specific for the 177 bp, 50 bp, and telomere repeats (Telo).  
806 A representative slot-blot is shown for each probe. While 100% of the DNA  
807 immunoprecipitated using an anti-myc antibody or no antibody was analysed on the slot-blots,  
808 only 0.1% of the input DNA and 1% of the anti-H3 immunoprecipitated DNA was analysed to  
809 avoid saturation and enable accurate quantitation.

810 (C) TbISWI distribution in BF *T. brucei* was analysed by quantitation of slot-blots or by qPCR  
811 for *VSG118* (located in the *VSG* Basic Copy array). Signals are expressed as percentage of input  
812 immunoprecipitated by anti-myc antibody using cell lines expressing TbISWI (wt) or TbISWI-  
813 myc. The data shown is the average of three to five independent ChIP experiments with the

814 standard deviation (SD) indicated with error bars. Significant amounts of TbISWI were found  
815 binding these *T. brucei* genomic regions (P<0.05; Sup. Table 6).

816

817 **Fig. 2.**

818 Schematic of the *T. brucei* reporter cell lines constructed for this study.

819 (A) Parental cell lines for the *T. brucei* reporter lines used. The bloodstream form (BF) *T.*  
820 *brucei* S16.221 cell line has a puromycin resistance gene (PurR) inserted in the active *VSG221*  
821 expression site (transcription indicated with an arrow). Both the *T. brucei* S16.221 and the  
822 procyclic form (PF) *T. brucei* 29-13 lines contain genes encoding the T7 RNA polymerase (T7  
823 RNAP) and the tetracycline repressor (TetR) allowing tetracycline inducible transcription.

824 (B) *T. brucei* reporter lines generated by transfecting different *eGFP* containing reporter  
825 constructs into the parental *T. brucei* S16.221 or 29-13. Experimental detail concerning the  
826 construction and verification of these cell lines is shown in the Supplementary Material. A  
827 *TbISWI* RNAi expressing construct was subsequently introduced into each of these lines to allow  
828 monitoring for derepression of *eGFP* after the induction of *TbISWI* RNAi. a) Reporter cell line  
829 with a construct containing *eGFP* (green box) either with (+) or without (-) an expression site  
830 promoter (ESp, white flag) inserted in a *T. brucei* minichromosome. Telomeric *VSGs* are  
831 indicated with red boxes and the telomere repeats with horizontal arrows. Transcription of the  
832 selectable marker (blue box) is directed by an rDNA promoter (black flag). b) As above, only  
833 the reporter constructs are integrated into the *VSG118* Basic copy array. Flanking *VSGs* are  
834 indicated as VSGX and VSGY. c) The *eGFP* containing reporter construct is integrated behind  
835 an endogenous EPI promoter (EPp, grey flag) in the EP procyclin locus. The EP procyclin  
836 genes and an unknown hypothetical protein (Hyp) are indicated. d) Reporter construct

837 integrated between two ribosomal DNA (rDNA) transcription units (*18S* and *28S* rRNA genes  
838 indicated). An exogenous ES promoter (ESp) is indicated with a white flag. e) The *eGFP*  
839 reporter construct is integrated within the polycistronic tubulin transcription unit. Flanking  $\alpha$ -  
840 and  $\beta$ -tubulin genes are indicated with black boxes.

841

842 **Fig. 3.**

843 TbISWI downregulates an exogenous Expression Site promoter (ESp) located within the silent  
844 mini-chromosomes or *VSG* Basic Copy array by six-fold in bloodstream form *T. brucei*.

845 (A) The schematic shows an *eGFP* reporter construct integrated into the 177 bp repeat arrays in  
846 the mini-chromosomes of BF *T. brucei*. Downstream of the *eGFP* gene is an rDNA promoter  
847 (black flag) and a blasticidin resistance gene (black box). *VSG* genes are indicated with boxes,  
848 and telomere repeats with triangles. A *TbISWI* RNAi construct was transfected into this cell line  
849 and two independent clones were isolated. Cell lines analysed are the parental reporter cell line  
850 (BF-MCe) and two independent *TbISWI* RNAi clones (BF-MCe1 and BF-MCe2). Flow  
851 cytometry was used to monitor eGFP expression after inducing *TbISWI* RNAi with tetracycline  
852 (Tet) for the time indicated in days (d). A representative flow cytometry trace is shown where  
853 the uninduced sample (indicated with a thick black line) is overlaid with a shaded trace  
854 obtained after 4 days induction of *TbISWI* RNAi. The graph shows quantitation of the fold  
855 derepression of *eGFP* after induction of *TbISWI* RNAi. Fold derepression was calculated as the  
856 ratio of mean fluorescence of induced cells to that of the uninduced cells (n=3 +/- SD).

857 (B) As in (A), except the *eGFP* reporter construct integrated in the mini-chromosomal 177 bp  
858 repeats contains an ES promoter (ESp) upstream of *eGFP*. The parental reporter cell line (BF-

859 MCESp) as well as two independent RNAi clones (BF-MCESp1 and BF-MCESp2) were  
860 analysed after the induction of *TbISWI* RNAi.

861 (C) As in (A), however the *eGFP* reporter construct is integrated into the *VSG* Basic Copy array.  
862 The average fold derepression of *eGFP* was quantitated for the parental reporter cell line (BF-  
863 VBe) as well as two independent RNAi clones (BF-VBe1 and BF-VBe2) after the induction of  
864 *TbISWI* RNAi.

865 (D) As in (A), however the reporter construct contains an ES promoter upstream of *eGFP* and is  
866 integrated into the *VSG* Basic Copy array. Quantitation of the average fold derepression of the  
867 *eGFP* gene after tetracycline induction of *TbISWI* RNAi is plotted for the parental reporter cell  
868 line (BF-VBESp) as well as two independent *TbISWI* RNAi clones (BF-VBESp1 and BF-  
869 VBESp2).

870

871 **Fig. 4.**

872 *TbISWI* is distributed along active and silent *VSG* ESs but is depleted from ES promoters.

873 (A) Schematic of a typical *VSG* ES (modified from AnTat1.3A) (5, 29), with the ES promoter  
874 (flag), the expression site associated genes (ESAGs) (numbered boxes), and characteristic ES  
875 repeat arrays (striped boxes) indicated. The region surrounding the ES promoter is expanded  
876 below. Positions of the primer pairs used for qPCR analysis are indicated with lettered brackets.  
877 These primers can be expected to recognize most, if not all ESs.

878 (B) Distribution of *TbISWI* over the length of *VSG* ESs. ChIP was performed on *TbISWI* (wt)  
879 and *TbISWI*-myc expressing BF *T. brucei* cell lines using an anti-myc antibody ( $\alpha$ -myc). qPCR  
880 was used to amplify the regions indicated in (A). Signals are expressed as percentage of input  
881 immunoprecipitated by the  $\alpha$ -myc antibody in the two cell lines (n= 3 to 5 +/- SD). Significant

882 amounts of TbISWI were found binding ESs ( $P < 0.05$ ; Sup. Table 7) with the exception of the ES  
883 promoter region (primer a).

884 (C) Schematic of the BF *T. brucei* 221GPI(VO2+) cell line used for ChIP experiments. Two  
885 *VSG* ESs are shown, with the ES promoters indicated with white flags and transcription of the  
886 active *VSG* ES with an arrow. A neomycin resistance gene (Neo) is located downstream of the  
887 ES promoter in the active *VSGVO2* ES. The silent *VSG221* ES contains a puromycin resistance  
888 gene (Puro) downstream of the ES promoter as well as a single copy *VSG* pseudogene ( $\psi$ ).

889 (D) Distribution of TbISWI over silent and active *VSG* ESs. ChIP analysis was carried out on  
890 BF *T. brucei* cell lines expressing TbISWI (wt) and TbISWI-myc using  $\alpha$ -myc, and chromatin  
891 was immunoprecipitated as in (B). qPCR was used to quantitate amounts of genes shown in (C)  
892 ( $n = 3$  to  $5 \pm$  SD). These genes are all single copy with the exception of *VSGVO2*, which has an  
893 additional copy present in a *VSG* Basic Copy array (BCA). Significant amounts of TbISWI were  
894 found binding both active and silent ESs ( $P < 0.05$ ; Sup. Table 8).

895

896

897 **Fig. 5.**

898 Depleting TbISWI leads to increased transcription of silent telomeric ES-located *VSGs*. RNA  
899 was isolated from the bloodstream form *T. brucei* 121-SA1 cell line (21) after 0, 24, 48 and 72  
900 hours induction of *TbISWI* RNAi. cDNA was synthesized and qPCR was used to quantitate  
901 levels of transcript.

902 (A) The change in transcript levels after the induction of TbISWI RNAi relative to the 0 hour  
903 time point are plotted for *TbISWI*,  $\gamma$ -tubulin ( $\gamma$ Tub), the active *VSG* (*VSG121*), and *eGFP* located  
904 downstream of a silent ES promoter ( $n = 3 \pm$  SD).

905 (B) Changes in transcript levels from silent *VSGs* after the induction of TbISWI RNAi. *VSG*  
906 transcripts from eleven telomeric ES-located *VSGs* as well as a *VSG* located in the *VSG* Basic  
907 Copy array; *VSG118*) were analysed by qPCR (n= 3 +/- SD).

908

909 **Fig. 6.**

910 TbISWI binds within both Pol I and Pol II transcription units, however TbISWI knock-down  
911 leads to increased transcription from Pol I-transcribed genes only.

912 (A) Distribution of TbISWI in the Pol I-transcribed procyclin transcription unit in BF and PF *T.*  
913 *brucei*. At the top is a schematic of the EP procyclin locus (PARP B1) (44). The procyclin  
914 promoter (grey flag), procyclin genes (black boxes), and regions analysed by qPCR (lettered  
915 brackets) are indicated. The graph shows quantitation of ChIP analysis carried out on TbISWI  
916 (wt) and TbISWI-myc expressing BF and PF *T. brucei* cell lines. Signals are expressed as a  
917 percentage of input immunoprecipitated using anti-myc antibody ( $\alpha$ -myc) in the TbISWI (wt)  
918 and TbISWI-myc expressing cell lines (n= 3 to 5 +/- SD). Significant amounts of TbISWI were  
919 found binding the procyclin coding region (primer c) ( $P < 0.05$ ; Sup. Table 10) in both BF and PF  
920 *T. brucei*, and the procyclin promoter (primer a) in BF *T. brucei*.

921 (B) Effect of depleting TbISWI on expression of *eGFP* inserted into the procyclin locus in BF *T.*  
922 *brucei*. A schematic of the cell line containing *eGFP* downstream of the endogenous procyclin  
923 promoter is shown. The procyclin genes *EP1* and *EP2*, as well as a gene encoding a hypothetical  
924 protein (Hyp) are indicated (black boxes). The *eGFP* (green box) containing construct is  
925 integrated behind an endogenous procyclin promoter (grey flag), with the single cross-over  
926 mediated insertion resulting in duplication of the promoter. A downstream rDNA promoter  
927 (black flag) and blasticidin resistance gene (blue box) are also indicated. This cell line (BF-Pro)

928 was transfected with a *TbISWI* RNAi construct and two independent clones were isolated (BF-  
929 Pro1 and BF-Pro2). Flow cytometry was used to monitor eGFP expression after the induction of  
930 *TbISWI* RNAi with tetracycline (Tet) for the time indicated in days (d). A representative FACS  
931 trace is shown with an outline of the trace from the uninduced cell line (black line) overlaid  
932 with the trace showing fluorescence after 4 days induction of *TbISWI* RNAi (green-shaded  
933 trace). The graph shows quantitation of the fold derepression of *eGFP* calculated as the ratio of  
934 mean fluorescence of induced cells to that of uninduced cells (n= 3 +/- SD).

935 (C) Distribution of TbISWI in the Pol I-transcribed rDNA transcription unit compared with Pol  
936 II-transcribed genes in bloodstream form *T. brucei*. A schematic of the region around a  
937 ribosomal DNA (rDNA) transcription unit is shown (58), with the rDNA promoter (black flag),  
938 the rRNA genes (black boxes), and the regions analysed by qPCR indicated with lettered  
939 brackets. The graph shows quantitation of ChIP-ed DNA using qPCR to amplify regions  
940 indicated above, as well as the Pol II-transcribed genes encoding either the large subunit of RNA  
941 polymerase I (Pol I) or  $\alpha$ -tubulin ( $\alpha$ -Tub) which are located within Pol II polycistronic  
942 transcription units. Signals are expressed as a percentage of input immunoprecipitated as in (A)  
943 (n= 3 to 5 +/- SD). Significant amounts of TbISWI were found binding the rDNA region  
944 (primers a-d) and the Pol I and tubulin transcription units in BF *T. brucei* (P<0.05; Sup. Table  
945 11).

946 (D) As in (B), only the BF reporter construct contains an ES promoter (ESp) (white flag)  
947 upstream of *eGFP*. The construct is integrated into the nontranscribed spacer between two  
948 rDNA transcription units (region of primer pair "a" in (C)). The parental reporter line is BF-  
949 rDESp1 and the two *TbISWI* RNAi clones are BF-rDESp1 and BF-rDESp2 (n=3 +/- SD).

950 (E) As in (B), only the *eGFP* reporter construct is integrated into the tubulin array of  
951 bloodstream *T. brucei* and lacks an rDNA promoter upstream of the blasticidin resistance gene  
952 (blue box). The schematic shows the *eGFP* construct integrated between alternating  $\alpha$ -tubulin  
953 and  $\beta$ -tubulin genes (black boxes). Representative flow cytometry traces are shown for a  
954 bloodstream form *TbISWI* RNAi clone. The parental reporter line is BF-tubeG, and the RNAi  
955 clones are BF-tubeG1 and BF-tubeG2 (n=3 +/- SD).

956

957 **Fig. 7.**

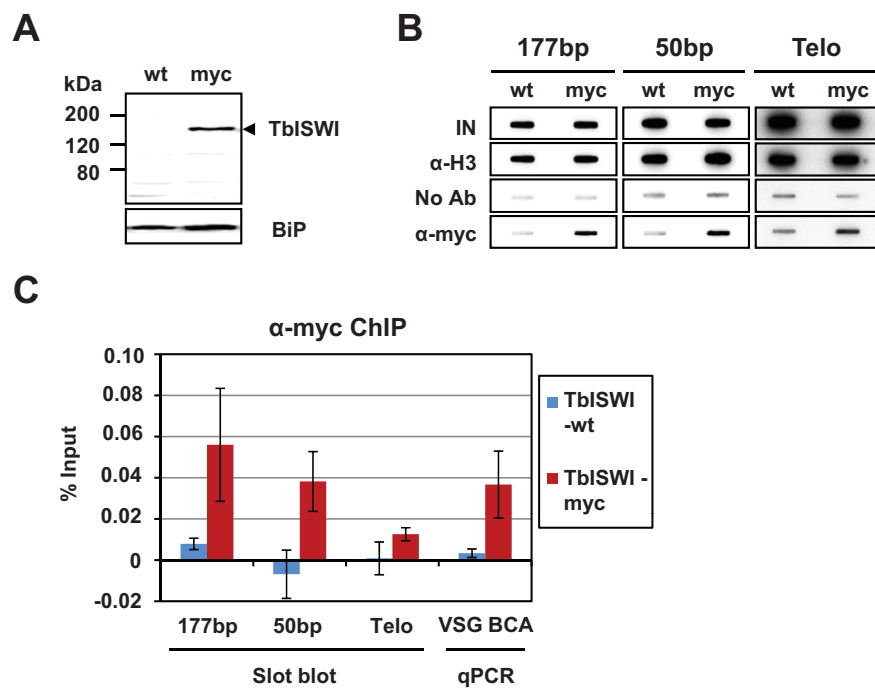
958 *TbISWI* is enriched in the strand switch regions (SSR) between RNA polymerase II-transcribed  
959 polycistronic transcription units.

960 (A) Schematic representation of a region of chromosome 10 according to (50), where grey boxes  
961 represent ORFs and arrows indicate direction of transcription. The positions of three different  
962 divergent SSRs (D1-D3) (containing putative transcription initiation sites) and three different  
963 convergent SSRs (C1-C3) (containing putative transcription termination sites) used for ChIP  
964 experiments are indicated.

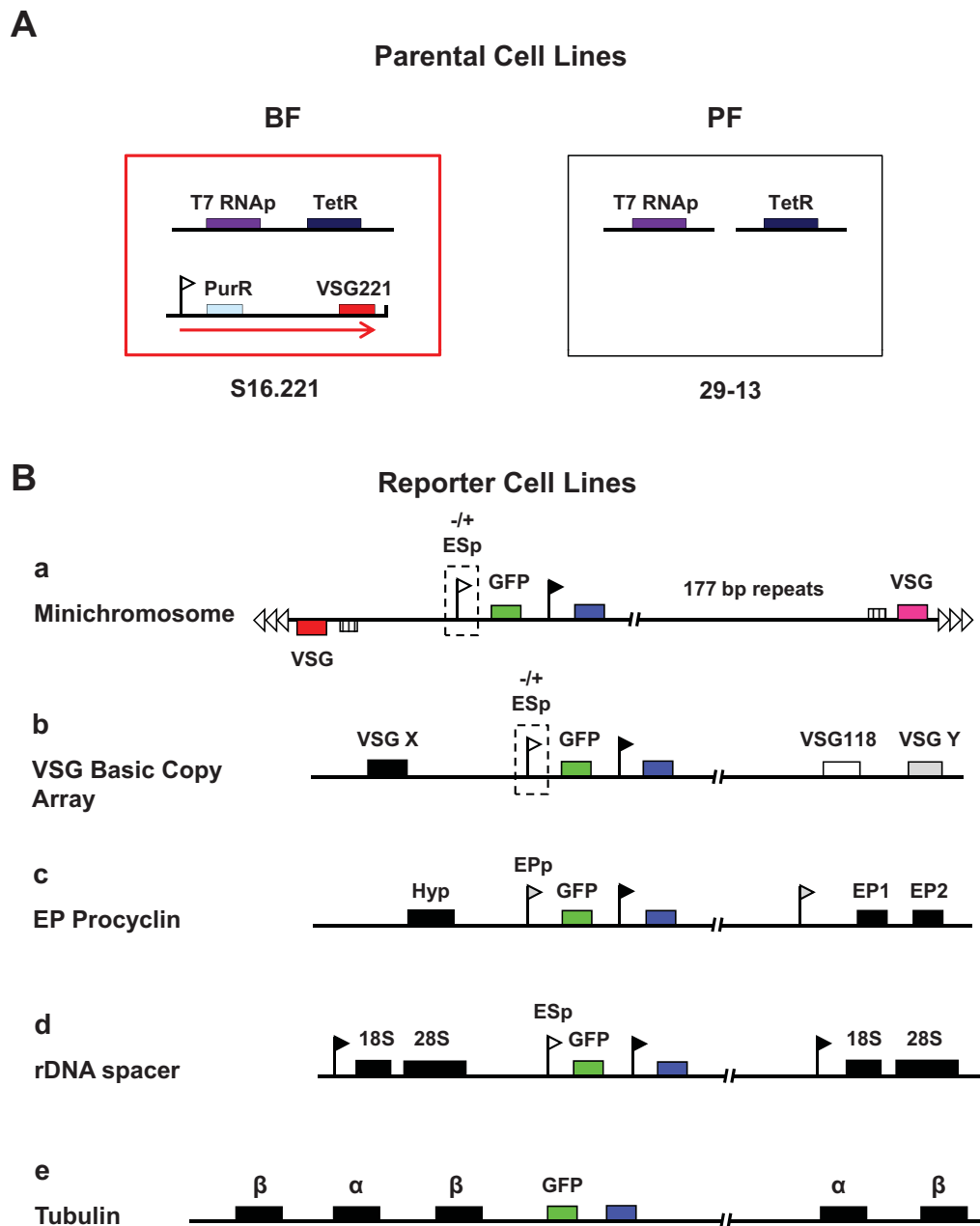
965 (B) The schematic shows a divergent SSR (top panel), with the positions of the primer pairs  
966 used for qPCR analysis indicated with lettered brackets. Primer pairs "a" and "g" are located at  
967 the approximate mid-point of the flanking polycistronic transcription units. The graphs below  
968 show distribution of *TbISWI* over three divergent regions (D1-D3) as indicated in (A). ChIP  
969 analysis was carried out on *TbISWI* (wt) and *TbISWI*-myc expressing BF *T. brucei* cell lines.  
970 qPCR was used to amplify the indicated regions. Signals are expressed as percentage of input  
971 immunoprecipitated by anti-myc antibody in the *TbISWI* (wt) and *TbISWI*-myc expressing cell

972 lines (n=3 +/- SD). Significant amounts of TbISWI were found binding most regions of the D1-  
973 D3 divergent strand switch regions (P<0.05; Sup. Table 12).

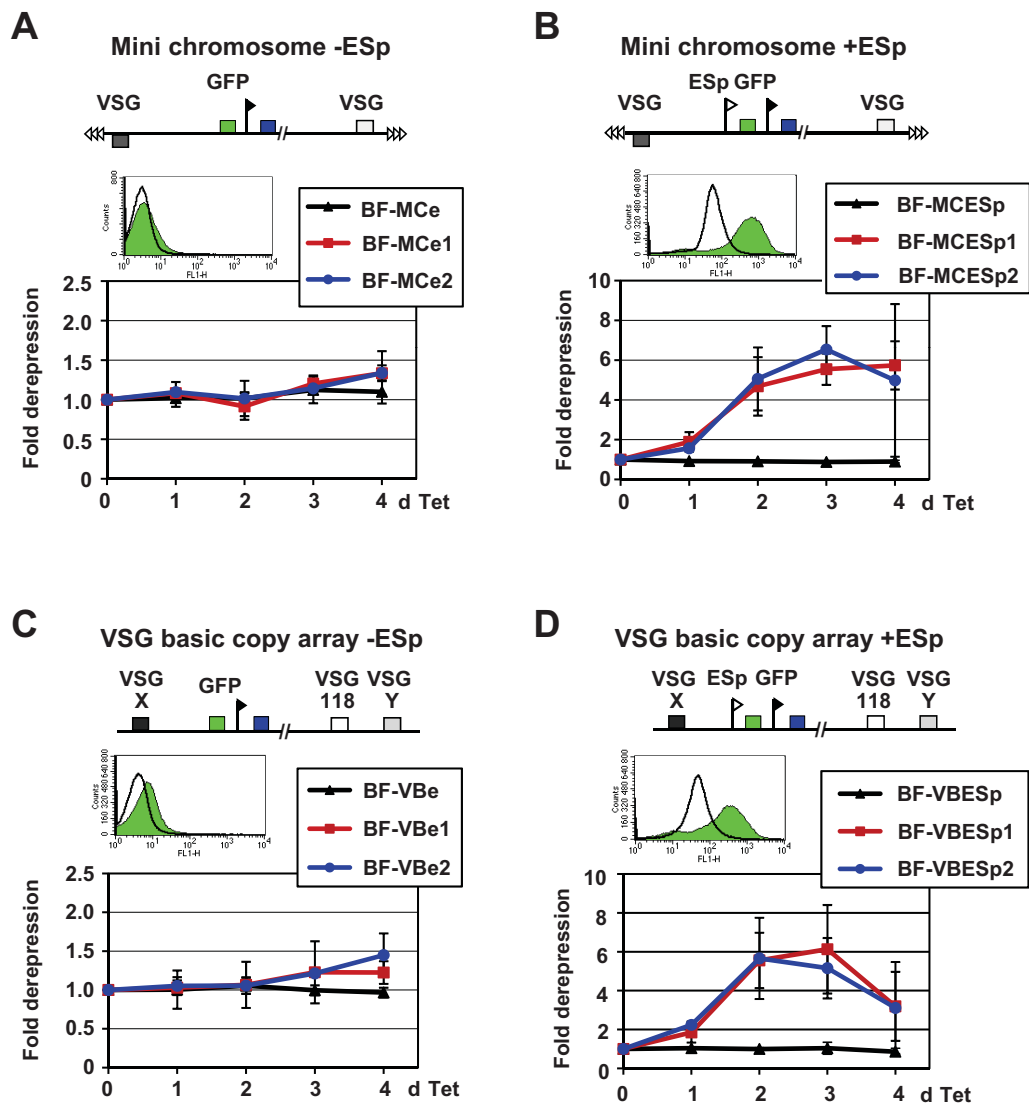
974 (C) Above is a schematic of a convergent SSR, with the positions of the primer pairs used for  
975 qPCR analysis indicated with lettered brackets. The graphs show the distribution of TbISWI  
976 over three convergent regions (C1-C3) as indicated in (A). ChIP was performed, and qPCR was  
977 used to amplify regions shown schematically with signals expressed as percentage of input (n=3  
978 +/- SD). Significant amounts of TbISWI were found binding most regions of the C1-C3  
979 convergent strand switch regions (P<0.05; Sup. Table 13).



**Fig. 1**



**Fig. 2**



**Fig. 3**

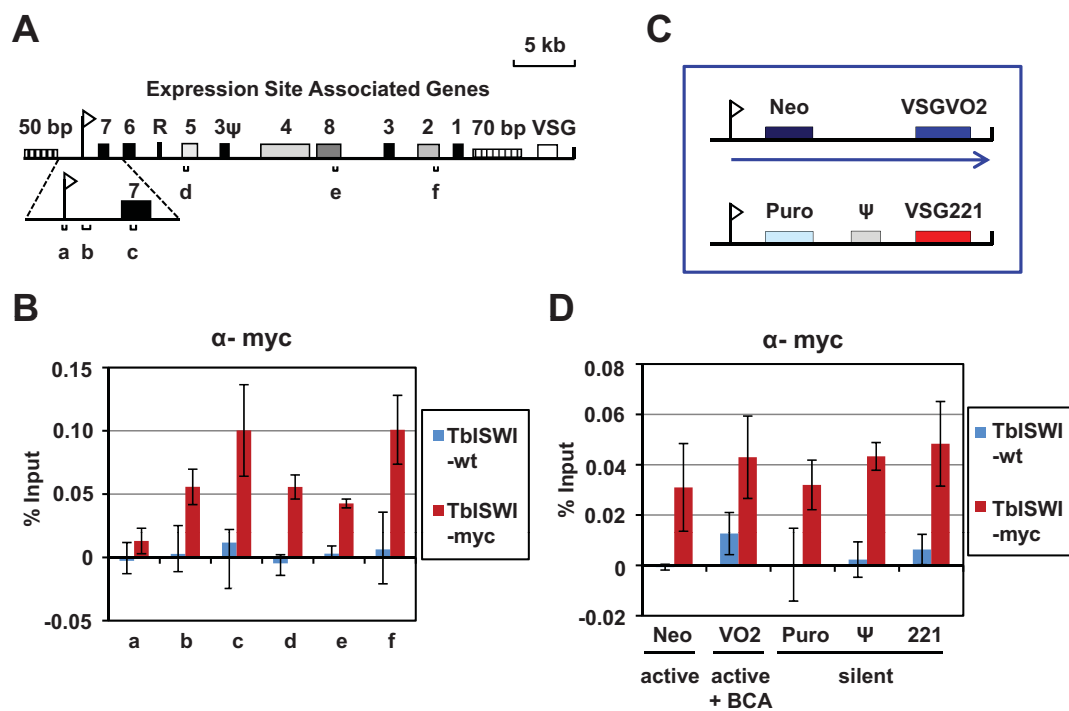


Fig. 4

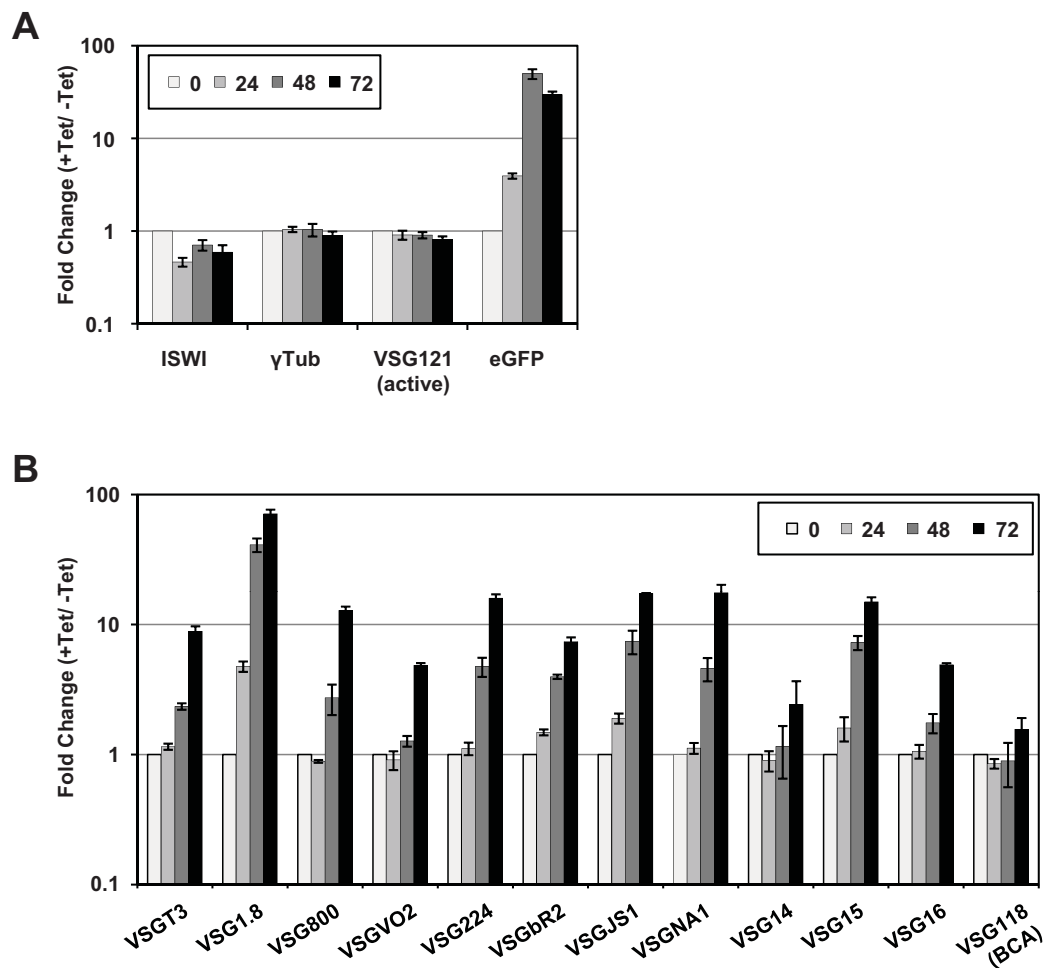


Fig. 5

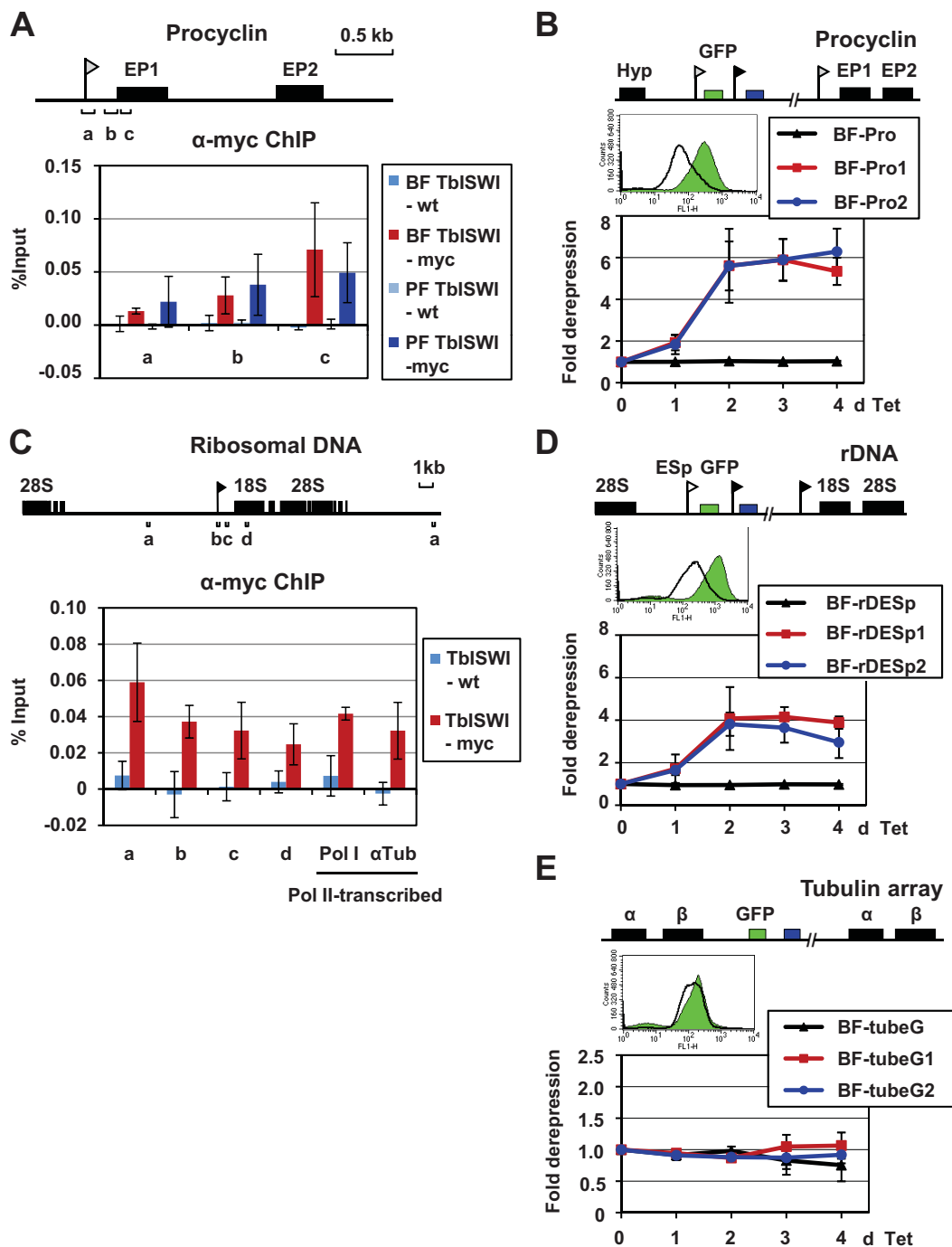
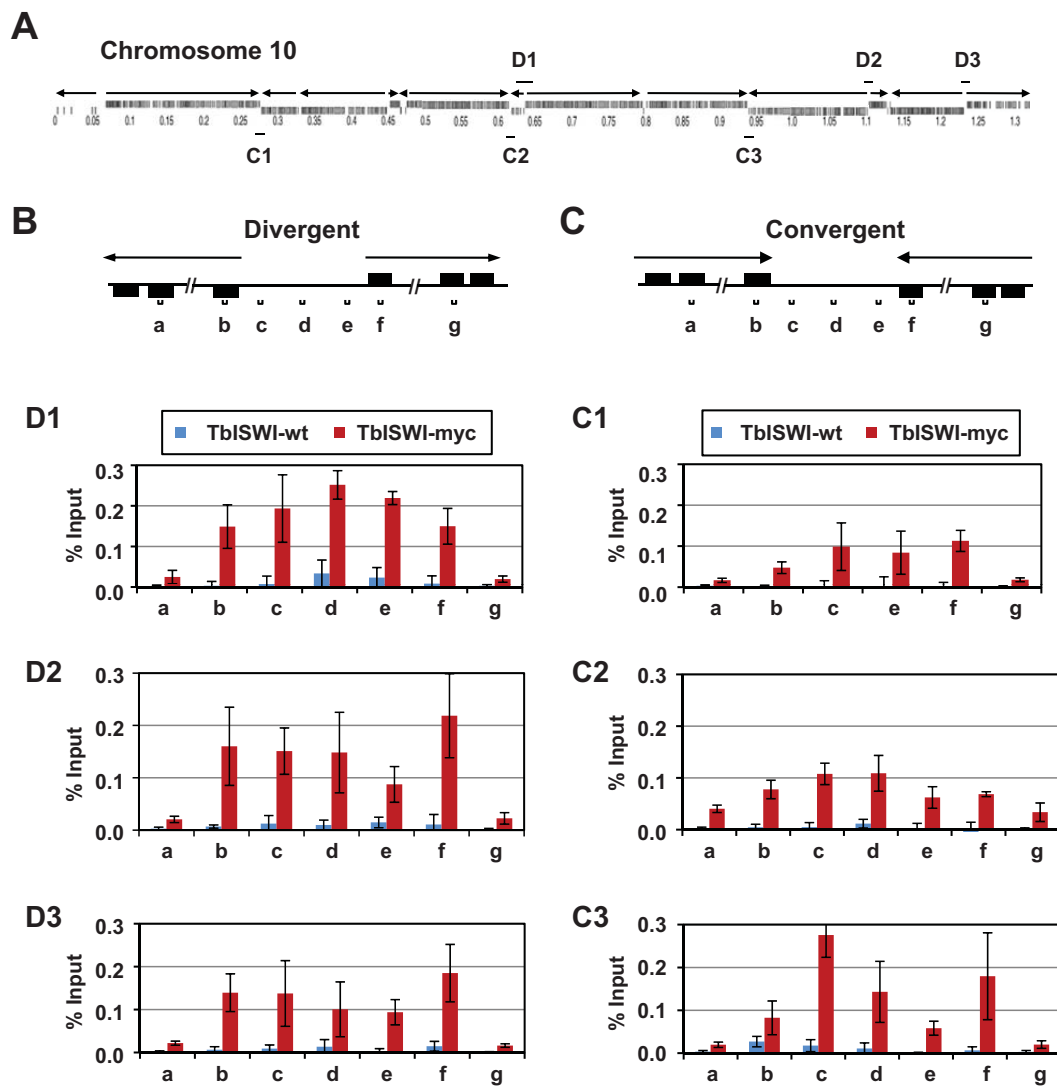


Fig. 6



**Fig. 7**