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A transcription-independent epigenetic mechanism is associated with antigenic switching in *Trypanosoma brucei*

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ABSTRACT

Antigenic variation in *Trypanosoma brucei* relies on periodic switching of variant surface glycoproteins (VSGs), which are transcribed monoallelically by RNA polymerase I from one of about 15 bloodstream expression sites (BES). Chromatin of the actively transcribed BES is depleted of nucleosomes, but it is unclear if this open conformation is a mere consequence of a high rate of transcription, or whether it is maintained by a transcription-independent mechanism. Using an inducible BES-silencing reporter strain, we observed that chromatin of the active BES remains open for at least 24 hours after blocking transcription. This conformation is independent of the cell-cycle stage, but dependent upon TDP1, a high mobility group box protein. For two days after BES silencing, we detected a transient and reversible derepression of several silent BESs within the population, suggesting that cells probe other BESs before commitment to one, which is complete by 48 hours. FACS sorting and subsequent subcloning confirmed that probing cells are switching intermediates capable of returning to the original BES, switch to the probed BES or to a different BES. We propose that regulation of BES chromatin structure is an epigenetic mechanism important for successful antigenic switching.

INTRODUCTION

RNA Polymerase I (Pol I) transcribes ribosomal DNA genes (rDNA), which account for over 60% of total nuclear transcription (1). Most organisms have large tandem arrays of rDNA genes, but only a fraction is transcriptionally active. Consistent with their transcriptional activity, rDNA genes can be found in one of two chromatin states: a compact nucleosome-rich 'closed state' and an accessible nucleosome-depleted chromatin 'open state' (2). In yeast, replication of DNA converts the chromatin of most rDNA genes into the closed state. When replication is complete and transcription is reinitiated, a stochastic fraction of rDNA genes regains the open state, in a process that is dependent on Pol I. Once chromatin has an open state, its status is maintained by a high-mobility box protein, HMO1, independent from Pol I (3).

Trypanosoma brucei, a unicellular parasite responsible for sleeping sickness, is an unusual eukaryote that also uses Pol I to transcribe genes that encode two classes of abundant surface proteins, the variant surface glycoprotein (VSG) and procyclins (4,5). Periodic exchange of the exposed VSG allows the parasites to evade the host immune system, a process known as antigenic variation. Although the *T. brucei* genome has more than 2000 VSG genes and pseudogenes (6), only one is transcribed at any given time. *VSG*s are transcribed from bloodstream expression sites (BESs), specialized polycistronic units in which a Pol I promoter drives transcription towards the telomere. Between the promoter and the telomere there is a variable number of expression site-associated genes (ESAGs) followed by an array of tandem 70-bp repeats that precede the telomere-proximal *VSG* (7). Among the approximately 15 BESs present in the genome, only one is functionally active, ensuring the monoallelic expression that is the heart of antigenic variation.

VSG switching can happen by two main mechanisms, by recombining a new VSG into the active BES or by switching off a BES and activating another one (*in situ* or transcriptional switch) (8). VSG transcriptional switching involves two BESs, one that is silenced and another that is concomitantly activated. Cross-talk among BESs has been proposed to explain the phenotype observed when two BESs were simultaneously selected with drug selectable markers (9). Davies *et al.* also detected that deletion of a non-coding DNA sequence upstream of the active *VSG*, increased switching frequency to a new BES (10). More recently, Batram *et al.* showed that upon overexpression of an exogenous VSG, the active BES is partially attenuated into an intermediate stage, which may allow probing of silent BESs before commitment to one (11).

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While in the mammalian-infective bloodstream stage, the chromatin of the active and silent BESs is dramatically different. The actively transcribed BES is nucleosome-depleted (open state), while silent BESs are organized in regularly spaced nucleosomes (12,13). The promoters of both active and silent BESs are bound by the multi-subunit class I transcription factor A (CITFA) (14), although to a different extent (15), which is necessary to regulate BES transcription initiation. Several epigenetic factors have been shown to be necessary to prevent transcription from silent BESs (16); others are necessary to ensure fast switching between BESs (17), but only TDP1 has been shown to be a core component of the active BES. TDP1 is a high mobility group box protein that is present in the chromatin of active BES, and at rRNA genes, and it is necessary for their transcription (18,19). The interplay among these factors and the mechanisms by which they affect *VSG* transcription are essentially unknown.

During the life cycle of *T. brucei*, parasites shift between the mammalian host and the Tsetse fly (20). To survive in such different hosts, parasites undergo significant changes in gene expression, which include replacement of the VSG by the similarly abundant procyclins (21). Silencing of the active BES during differentiation is characterized by a progressive downregulation of transcription along the BES (22), together with a re-localization of the BES to the heterochromatic nuclear periphery (23). It has also been shown that the chromatin of the originally active BES becomes less open to T7 polymerase, suggesting that it acquires a more compact structure during differentiation (24).

The goal of this manuscript was to understand the interplay between transcription and chromatin during antigenic variation. We characterized the early events that take place when the active BES is silenced. We found that trypanosomes have a cell-cycle and transcription-independent mechanism to maintain the open chromatin conformation of the active BES, which is dependent upon TDP1. We also observed that, in the first two days after transcriptional silencing is induced, parasites experience an intermediate stage in which several previously silent BESs are temporarily transcribed at higher levels, suggesting that cells probe different BESs before commitment to a new single BES. Our findings provide evidence that regulating chromatin conformation is tightly associated to antigenic switching, an important virulence mechanism of this pathogen.

MATERIALS AND METHODS

Trypanosome cell-lines and plasmid construction

T. brucei bloodstream form (BSF) parasites (strain Lister 427, antigenic type MiTat 1.2, clone 221a) (25) were cultured in HMI-11 as described in (26). Differentiation studies were performed in PL1A, a cell-line described in (27). All transfections were made with an AMAXA nucleofector (Lonza), program X-001, using the previously optimized homemade Tb-BSF buffer (90 mM sodium phosphate, 5 mM potassium chloride, 0.15 mM calcium chloride, 50 mM HEPES, pH 7.3) (28). GLB1 and its derivative cell-lines were modified from the parental 2T1.ESPiGFP:NPT (29), in which the *NPT* gene was replaced by a PCR product containing a stop codon for the *GFP* gene, an *Aldolase* 3′ UTR, *Luciferase* and *BSR* genes. PCR product was obtained using primers with long tails (provided in Supplementary Table S1) that serve as target recombination regions for *GFP* ORF and the BES sequence downstream of *GFP::NPT* 3' UTR.

GLB1-TDP1::TY1 was generated by transfecting pFAB11, which inserts a TY1 tag at the 3' end of one of the *TDP1* endogenous alleles. pFAB11 contains a *Hygromycin resistance* gene, a 5'-truncated *TDP1*, a TY1 tag at the 3'-end of TDP1 and an *Aldolase* 3'UTR. pFAB11 was linearized with *Sma*I (New England Biolabs), which digests in the middle of *TDP1* ORF. GLB1-TDP1::3xcMyc was generated by transfecting pFAB14 which inserts a triple MYC tag in the 3' end of one of the *TDP1* endogenous alleles. pFAB14 contains a *Hygromycin resistance* gene, a 5'-end truncated *TDP1*, a triple MYC tag in the 3'-end of *TDP1 and* the predicted endogenous 3'UTR of *TDP1* (30). pFAB14 was linearized with *Sma*I (New England Biolabs), which digests in the middle of *TDP1* ORF.

GLB1-R15 was obtained by first generating switchers of GLB1 using the BES silencing inducible assay and subsequently transfecting pFAB17 in switchers. pFAB17 contains a 405 nucleotide sequence upstream BES promoter, the BES promoter, an *RFP* ORF lacking NLS, PEST and stop codons, amplified from pCAGGS, a *NPT* ORF lacking the start codon, an *Actin* 3'UTR and a 475 nucleotide sequence downstream of the BES promoter. *RFP* and *NPT* ORFs formed a fused *RFP::NPT* gene. pFAB17 was digested with *Asc*I and *Nde*I (New England Biolabs) prior to transfection. Cloning of VSG transcripts and sequencing allowed identification of the BES in which *RFP::NPT* had integrated. Transfected switchers were reverted to the original BES, essentially as described in (7) by removing G418 and adding 1 μg/ml of tetracycline to the medium. Afterwards, serial dilutions were performed and 10 μg/ml of Blasticidin-S was added to each dilution to select revertants.

All cloning was performed using the In-Fusion® HD Cloning system (Clontech) following to the manufacturer's instructions. The maps of constructs generated in this work are shown in Supplementary Figure S1.

Differentiation assay

Parasites were collected at a density of 1.0–1.5 × 10^6 cells/ml and centrifuged at 650 g for 10 min at room temperature. Cells were resuspended in DTM medium with 6 mM of *cis*-aconitate (Sigma) at a density of 1.5–2 × 10^6 cells/ml and grown at 27°C without CO_2.

Transcript quantification

Parasites were harvested by centrifugation at 650 g for 10 min at 4°C and immediately resuspended in PureZOL (Bio-Rad) or TRIzol (Life Technologies). RNA was isolated following the manufacturer's instructions and RNA quantity and quality was assessed on a NanoDrop 2000 (Thermo Fisher Scientific). cDNA was generated using a Superscript cDNA Synthesis Kit (Life Technologies), according to manufacturer's protocol. Quantitative PCR (qPCR) was performed using 1× SYBR Green PCR Master Mix (Applied Biosystems). Negative controls lacking reverse transcriptase (RT−) were confirmed by qPCR. Amplification

reactions were performed in duplicates. The ΔΔCt method was used to determine transcript levels relative to normalizing gene.

Tetracycline-inducible BES silencing assay

Parasites were centrifuged at 650 g and washed three times with warm HMI-11. Pellets were resuspended in medium with drugs except Blasticidin-S and tetracycline and density was adjusted to 0.5×10^6 cells/ml. Cells were split in two flasks with and without Blasticidin-S and tetracycline (Tet+ and Tet-, respectively).

Survival frequency and commitment assay

To determine the percentage of cells that survive the BES-silencing assay, after washing away drugs, cells were diluted to a density of 10 cells/ml and plated in two 96-well plates with or without tetracycline (Tet+ and Tet-, respectively). Seven days after plating, surviving clones were counted in Tet- and Tet+ plates and its ratio yielded the survival frequency. For commitment assay, cell density of Tet- cultures was determined at 8, 24, 48 and 72 h after BES silencing and subsequently diluted to a density of 10 or 50 cells/ml containing 1 μg/ml of tetracycline. Dilutions were performed with the same drugs and complemented with 1 μg/ml of tetracycline. Diluted cells were then plated in two 96-well plates. All plates containing tetracycline were replenished with fresh tetracycline three days after plating to maintain excess concentration. Six days later, around 20 surviving wells were passaged to new 96-well plates and were analyzed by a FACS High Throughput Sampler (BD Biosciences) to score for expression of GFP.

Cell-cycle profile after BES silencing

At each time point after inducing BES silencing, 2 million cells were centrifuged for 10 min at 1300 g, 4°C and washed once with ice-cold PBS. Cells were resuspended in PBS with 2 mM EDTA and slowly fixed with 2.5 ml of absolute ethanol. After fixing for at least one hour, cells were washed once and resuspended in 1 ml PBS/EDTA. Cells were incubated with 10 μg RNase A and 1 μg of Propidium Iodide for 30 min at 37°C and further analyzed by flow cytometry for DNA content.

Luciferase assay

1.5×10^6 cells were harvested for 5 min at 2800 g, 4°C and washed once with 1 ml cold TDB. Pellets were resuspended in Lysis Buffer (Biotium) and protocol was followed according to the manufacturer's instructions. Luminescence was measured by a microplate reader (Tecan).

FAIRE and Chromatin Immunoprecipitation

Formaldehyde-assisted isolation of regulatory elements (FAIRE) was performed as described previously (31).

H3 and TDP1 ChIPs were carried out in $3–5 \times 10^7$ cells essentially as described elsewhere (12), but with several modifications. Cells fixed for 20 min in a 1:11 dilution of a formaldehyde solution (50 mM HEPES–KOH pH7.5, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA and 11% formaldehyde) in HMI-9 medium. DNA was sonicated in a Bioruptor UCD-200 (Diagenode) for 10 min total (30 seconds, on and off cycles). Lysate was incubated with Dynabeads Protein G (Life Technologies) combined with 10 μg of rabbit anti-H3 antibody (kind gift from Christian Janzen) or mouse anti-TY1 antibody (32) overnight at 4°C. Samples were treated with 80 μg of RNase A for 2 h at 37°C and then with 80 μg of Proteinase K for 2 h at 55°C before being purified using a Gel Extraction Kit (Qiagen). Immunoprecipitated material was quantified by qPCR. Amplification reactions were performed in duplicates.

RPA31 ChIP was carried out in 10^8 cells as described by the Arthur Günzl's laboratory (33), but immunoprecipitation was performed with Protein G Sepharose 4 Fast Flow (GE Healthcare) with a rat anti-RPA31 antibody (kind gift from Arthur Gunzl).

FACS cell sorting

To sort cells based on cell-cycle stage, 10^8 cells were centrifuged for 10 min at 650 g, resuspended in 20 ml of HMI-11 and cross-linked with 1.1% formaldehyde (Sigma) for 10 min. Cross-linking was stopped with 0.125 M of Glycine (Sigma) and cells were washed once with PBS and resuspended in 1 ml PBS/2 mM EDTA. Cells were permeabilized for 5 min with 40 μM digitonin, washed twice with PBS and resuspended in 1 ml PBS/2 mM EDTA. Staining was performed with 1 μl FxCycle Violet Stain (Molecular Probes, Thermo Fisher Scientific) for 30 min protected from light. Cells were sorted according to their DNA content using a FACS Aria sorter (BD Biosciences) and collected in PBS.

To sort cells based on GFP/RFP expression, tetracycline was removed from medium and 24 h later, 2×10^6 cells were collected, washed in TDB and analysed by FACS for GFP and RFP expression. 3000 cells were sorted according to the GFP/RFP expression. The same number of cells was also sorted from a non-induced culture condition (Tet+). 200 (from Tet+ condition) or 1000 cells (from Tet- conditions) were plated in two 96-well plates with or without tetracycline, in the presence of 100 U/ml of PenStrep (Life Technologies). Six days later, surviving wells were passaged to new 96-well plates and were analyzed by a FACS High Throughput Sampler (BD Biosciences) to score for expression of GFP and RFP.

dsRNA production

dsRNA against TDP1 was made using the MEGAscript RNAi Kit (Ambion, Thermo Fisher Scientific) and protocol was followed according to the manufacturer's instructions. The sequence used to deplete TDP1 mRNA is identical to the one published in (18). 20 μg of dsRNA or the same buffer volume (for the mock control) were transfected into 100 million cells, using the X-001 program, and the homemade Tb-BSF buffer in an AMAXA nucleofector. The primers used for amplification of DNA template include a T7 promoter for *in vitro* transcription and are presented in Supplementary Table S1.

Western blotting

2×10^6 cells were lysed using Laemmli sample buffer and treated with 200 U/ml Benzonase (Sigma). After SDS-PAGE separation, proteins were transferred to a nitrocellulose membrane using an iBlot Dry Blotting System (Invitrogen). Primary antibodies used in this work were diluted as follows: 1:1000 for mouse anti-cMyc (clone 9E10, Antibody and Bioresource Core Facility, The Rockefeller University), 1:5000 for rabbit anti-*T. brucei* H2A antibody (custom made), 1:1000 for mouse anti-β-tubulin KMX antibody (34) (gift from Keith Gull). Picture acquisition was made by a Chemidoc XRS+ (Bio-Rad). Quantification was performed using the Image Lab software (Bio-Rad).

VSG staining of live cells

0.5×10^6 cells were harvested for 5 min at 2800 g, 4°C. Cells were resuspended in 50 μl of cold HMI-11 in which Alexa Fluor 647 anti-VSG13 conjugated antibody had been previously diluted (1:5000). After 15 min of incubation at 4°C with gentle shaking, cells were washed three times in cold TDB, resuspended in cold TDB, and immediately analyzed on a FACS Fortessa (Becton Dickinson Biosciences). Data were processed with FlowJo software (FlowJo, LLC).

RESULTS

Transcription and chromatin dynamics during differentiation

When a bloodstream parasite undergoes transcriptional VSG switching or when it differentiates into procyclic forms, the active BES must be silenced and its chromatin closed (12,13,24). In both processes, the interplay between transcription and chromatin is unknown. What is the order of these events: does chromatin condensation lead to a transcriptional silencing, or does transcription stop first and chromatin is condensed later? We began to tackle this problem by characterizing what happens during differentiation (Figure 1), and then in BES switching (Figure 2).

To study the interplay between transcriptional silencing and chromatin condensation in the active BES during differentiation, we induced cell differentiation *in vitro* by adding the chemical trigger *cis*-aconitate and lowering the temperature from 37°C to 27°C. We measured levels of mRNA at different time points, using a cell-line in which BES1 is actively transcribed (Figure 1A). As expected, *VSG2* transcript levels rapidly decreased, reaching 2% of the bloodstream form levels, 24 h after inducing differentiation (Figure 1B). Ribosomal DNA (rDNA) *18S* transcripts remained unchanged, as expected (22), and transcript levels of silent VSG9 were slightly reduced. As previously observed, transcript levels of RNA Polymerase II (Pol II)-transcribed genes, *β-tubulin* and *GAPDH*, also decreased (22,35,36), the second reflecting the reduced metabolic dependence of glycolysis in procyclic forms.

To test if this transcriptional silencing of the active BES was a consequence of chromatin conformational changes, we assessed nucleosome occupancy during differentiation by FAIRE (37) (Figure 1C). This technique allows the purification and quantification of DNA with low protein content from what we call a more 'open' chromatin conformation (12,31). We observed that, throughout the first 24 h of differentiation, chromatin of silent *VSG9*, *β-tubulin* and *GAPDH* kept the same FAIRE-enrichment, suggesting that the chromatin remained equally condensed. Importantly, at BES1, although chromatin condensed slightly (2.5-fold at *VSG2*), it remained 12-fold more open than a silent *VSG*.

These results show that, during differentiation, the drop in mRNA levels of the active BES starts earlier and it is more pronounced than the condensation of chromatin (Figure 1D), suggesting that chromatin does not close immediately after transcription is halted.

Transcription and chromatin dynamics during BES switching

Next we hypothesized that, during BES switching, chromatin condensation may also be delayed relative to transcription silencing. We postulated that, if open chromatin of the active BES was exclusively the consequence of high transcription, induction of transcription silencing should lead to an immediate condensation of chromatin. To test this hypothesis, we used a BES-inducible silencing system to block the transcription at the active BES and we followed transcription and chromatin dynamics. Because BES switching happens at very low frequency, we adapted a previously established reporter strain in which BES1 has a tetracycline operator sequence at the promoter followed by a GFP reporter (29). When tetracycline is removed from the culture medium, the heterologous tetracycline repressor is free and binds to the tetracycline operator, thus sterically blocking Pol I transcription. We introduced a *luciferase* gene downstream of the *GFP* gene since this is a more sensitive transcriptional reporter (Figure 2A). This cell-line was named GLB1, for *GFP*, *luciferase* and *BSR* in BES1.

During the first 8 h after tetracycline was removed from the medium (Tet-), GLB1 cells grew at the same rate as the control (Tet+), after which cell growth lagged until 48–72 h (Figure 2B). This lag phase was characterized by abnormal cell morphology and motility (data not shown) and cell-cycle arrest in G_2/M, with a considerable accumulation of cells with polyploidy or abnormal DNA content (Figure 2C). Quantification of cell death using propidium iodide, which only stains dead cells or in late apoptosis (38) (Supplementary Figure S2), showed a significant number of stained cells from 24 to 72 h after tetracycline removal, reaching a peak of 7.5% at 48 h. Consistent with this significant number of dead cells for several days, clonogenic assay confirmed that only 8% of the initial population of cells survive the silencing assay (Figure 2D). After 96 h, surviving cells took over the culture and grew at normal rate of around 8 h per population doubling. As expected, all surviving clones no longer expressed GFP, indicating they had successfully switched to a new BES (Figure 2E).

To test the efficiency of the steric blockade of Pol I upon tetracycline removal, we checked whether Pol I was evicted from BES1 chromatin fiber by conducting ChIP against the RPA31 subunit of Pol I (Supplementary Figure S3). As expected, before BES silencing, Pol I was present in the *18S* rDNA gene and in the active BES genes (*Luciferase* and *VSG2*) and essentially absent from Pol II loci (*β-tubulin* and *GAPDH*). 8 hr after BES silencing (tetracycline removal), the levels of Pol I in the active BES decreased to similar background levels detected in the silent *VSG9, β-tubulin*

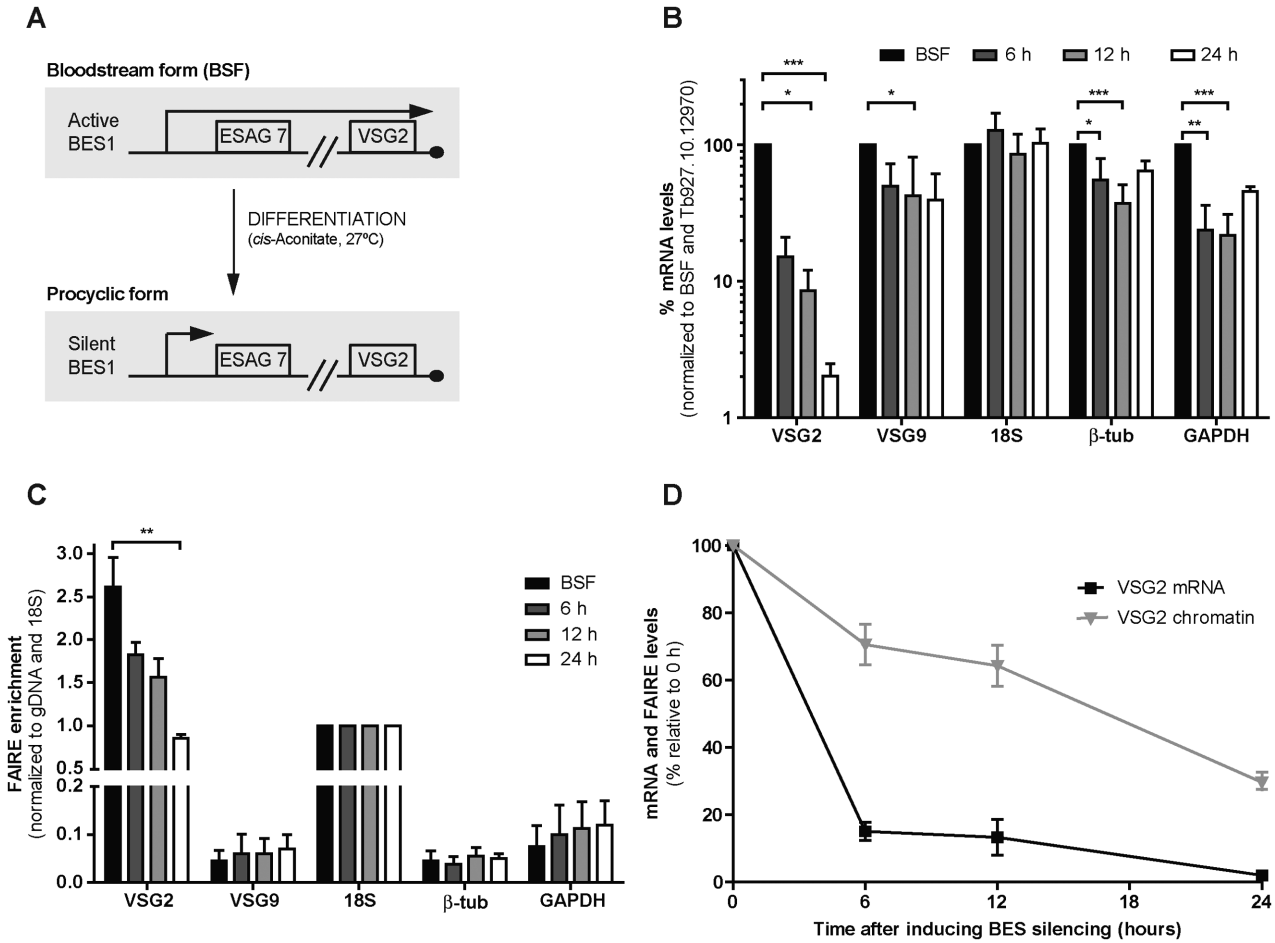

Figure 1. BES transcriptional silencing precedes chromatin condensation during differentiation. (**A**) A bloodstream form cell-line that expressed *VSG2* (BES1) was differentiated to procyclic forms by adding *cis*-aconitate to the medium and changing temperature from 37°C to 27°C. During differentiation, BES1 is silenced. Procyclic forms do not express VSG at the surface. Quantification of mRNA levels and (**B**) chromatin conformation (**C**) 6, 12 and 24 h after induction of differentiation. (**B**) Transcript levels were measured by qPCR and normalized to bloodstream form (BSF) levels and *Tb927.10.12970* (56), a gene previously shown to maintain constant transcript levels during differentiation. Four to six independent experiments were analyzed. (**C**) DNA purified from FAIRE was quantified by qPCR and normalized to *18S* as its transcript levels also remained constant throughout differentiation (23) and FAIRE signal was more intense than *Tb927.10.12970*. Three to five independent experiments were analyzed. (**D**) Comparison between transcript levels and FAIRE enrichment for *VSG2* gene. Values were extracted from analysis in (**B**) and (**C**). Statistical significance was determined by one-way ANOVA with Bonferroni post-test comparison.. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

and *GAPDH*. Hence, Pol I is indeed efficiently removed from chromatin fiber upon BES silencing.

To characterize the dynamic of chromatin structure once transcription is halted, we focused on the first 8 h because this is the period in which cells grow well and present a normal morphology (Figure 2B, Supplementary Figure S2). As a proxy of transcription, we followed luciferase activity 2, 5 and 8 h after removing tetracycline (Figure 3A). Luciferase activity showed an exponential decrease to ∼20% of the initial activity at 5 hr and to only 5% 8 h after tetracycline removal. Luciferase activity was confirmed by quantifying mRNA transcript levels 8 hr after inducing BES silencing (Figure 3B). *18S* rDNA and *β-tubulin* did not suffer transcriptional changes, confirming that BES silencing only affected expression sites and it did not cause any other major indirect changes in the rest of the genome. *Luciferase* and *BSR* transcripts decreased 90% of the initial levels, while active *VSG2* decreased less (32%) probably due to its stability (half-life around 4.5 h) (35). Concomitantly, we observed a 4–8-fold transcriptional up-regulation of several silent VSGs, which is likely a result from either newly activated BESs or derepressed silent BESs.

To determine the chromatin structure of the inducibly silenced BES, we performed FAIRE (Figure 3C) and histone H3 chromatin immunoprecipitation (ChIP) (Figure 3D). By FAIRE, we observed that, both at 5 and 8 h after BES silencing, the chromatin at the active BES remained highly enriched in the aqueous phase, indicating an open conformation. During this period, FAIRE-enrichment of silent BESs (*VSG9* and *VSG13* genes) remained unchanged. Although at 8 h the chromatin of the active *VSG2* presented a 2-fold decrease in FAIRE-enrichment, it was still 123-fold higher than FAIRE-enrichment of the same gene six days post-silencing, when this expression site was completely silenced. Six days post-silencing, genes from previously silent BESs (*VSG9* and *VSG13*) showed an increase of three-fold

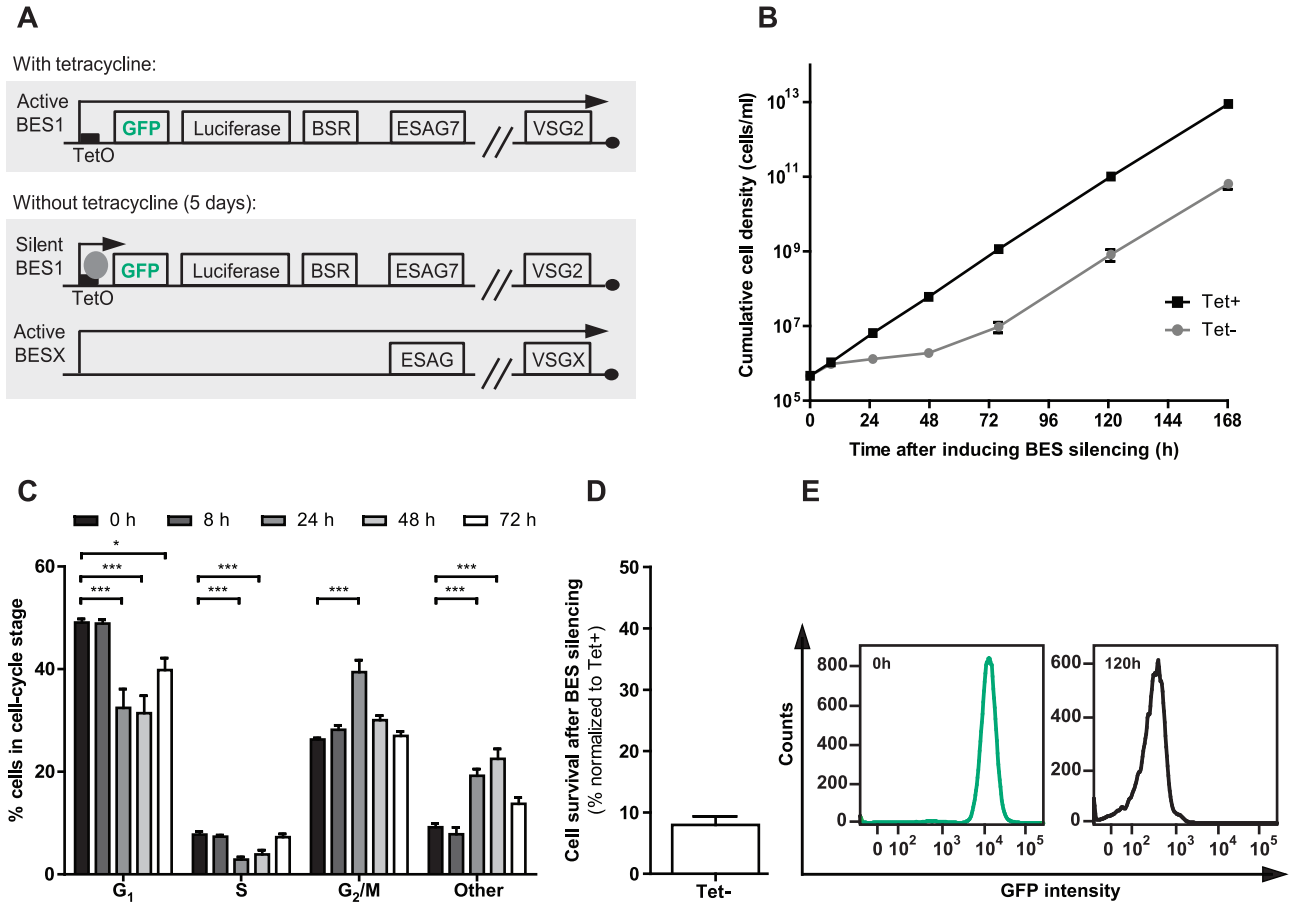

Figure 2. BES silencing causes growth delay and G_2/M cell-cycle arrest but only after more than 8 h of induction. (**A**) In the bloodstream reporter cell-line, GLB1, removing tetracycline from the medium induces BES1 silencing and subsequent activation of another BES. **Upper panel** shows that in the presence of tetracycline, BES1 is actively transcribed. BES1 contains a tet operator sequence (TetO, black rectangle), *GFP*, *luciferase* and *BSR* genes downstream of the promoter. **Lower panel** shows the outcome of tetracycline removal: BES1 becomes silent (because tetracycline repressor (grey circle) binds TetO, sterically blocking Pol I transcription) and a new BES is activated. *BSR*, Blasticidin-S Resistance. (**B**) Growth curves of cells in the presence (Tet+, black curve) or after removal (Tet-, grey curve) of tetracycline. Four independent experiments were analyzed. (**C**) Cell-cycle profile of GLB1 at different time-points after removal of tetracycline. 'Other' represents cells with abnormal DNA content. Four independent experiments were analyzed. Statistical significance was determined by a two-way ANOVA with Bonferroni post-test comparison. $*P < 0.05$; $***P < 0.001$. (**D**) Percentage of GLB1 cells that survive the BES silencing induction was determined by a clonogenic assay and normalized to Tet+ cells. Four independent experiments were analyzed. (**E**) Flow cytometry analysis of GFP expression of cells at 0 h (**left panel**) and 120 h (**right panel**) after tetracycline removal.

in FAIRE enrichment. This increase was expected not to be maximal (up to around 100) because FAIRE was performed on a mixed population of switchers. As expected, *18S* rDNA and *β-tubulin* did not show major chromatin alterations at any time. These results were mirrored by histone H3 ChIP: at 8 h, chromatin of originally active BES1 (*BSR* and *VSG2*) was still heavily depleted of histone H3, which is consistent with a high FAIRE-enrichment. *VSG2* had slightly more histone H3, but the levels were still much lower than those detected at silent *VSG9* or *VSG13*. As expected for a healthy parasite population, ChIP of other control genes was not affected during the first 8 h post-silencing induction, including at the *18S* rDNA.

Overall, our results show that, during BES switching, chromatin condensation lags significantly behind transcriptional silencing, suggesting that *T. brucei* has a mechanism of maintaining chromatin open when transcription has been halted.

Chromatin conformation is cell-cycle independent

In yeast, the ratio between open and closed rDNA genes changes throughout the cell-cycle: entrance into S phase leads to repression of most rDNA genes and their chromatin becomes more compact, while transcription re-initiation in G_2 re-opens chromatin (3). As BESs are transcribed by Pol I, we hypothesized that chromatin of active BES may also close as a function of the cell-cycle. To test this hypothesis, we stained GLB1 fixed cells with FxCycle Violet DNA stain and we FACS-sorted them, according to the DNA content, into G_1, S and G_2/M subpopulations (Figure 4A). The chromatin conformation of these subpopulations was subsequently assessed by FAIRE (Figure 4B). The first observation was that the FAIRE-enrichment of sorted subpopulations revealed patterns very similar to unsorted cells (*BSR*, *luciferase* and *18S* rDNA 50–100; *VSG9*, *β-tubulin* and *GAPDH* around 1) (Figure 3C), suggesting that the sorting procedure did not affect chromatin confor-

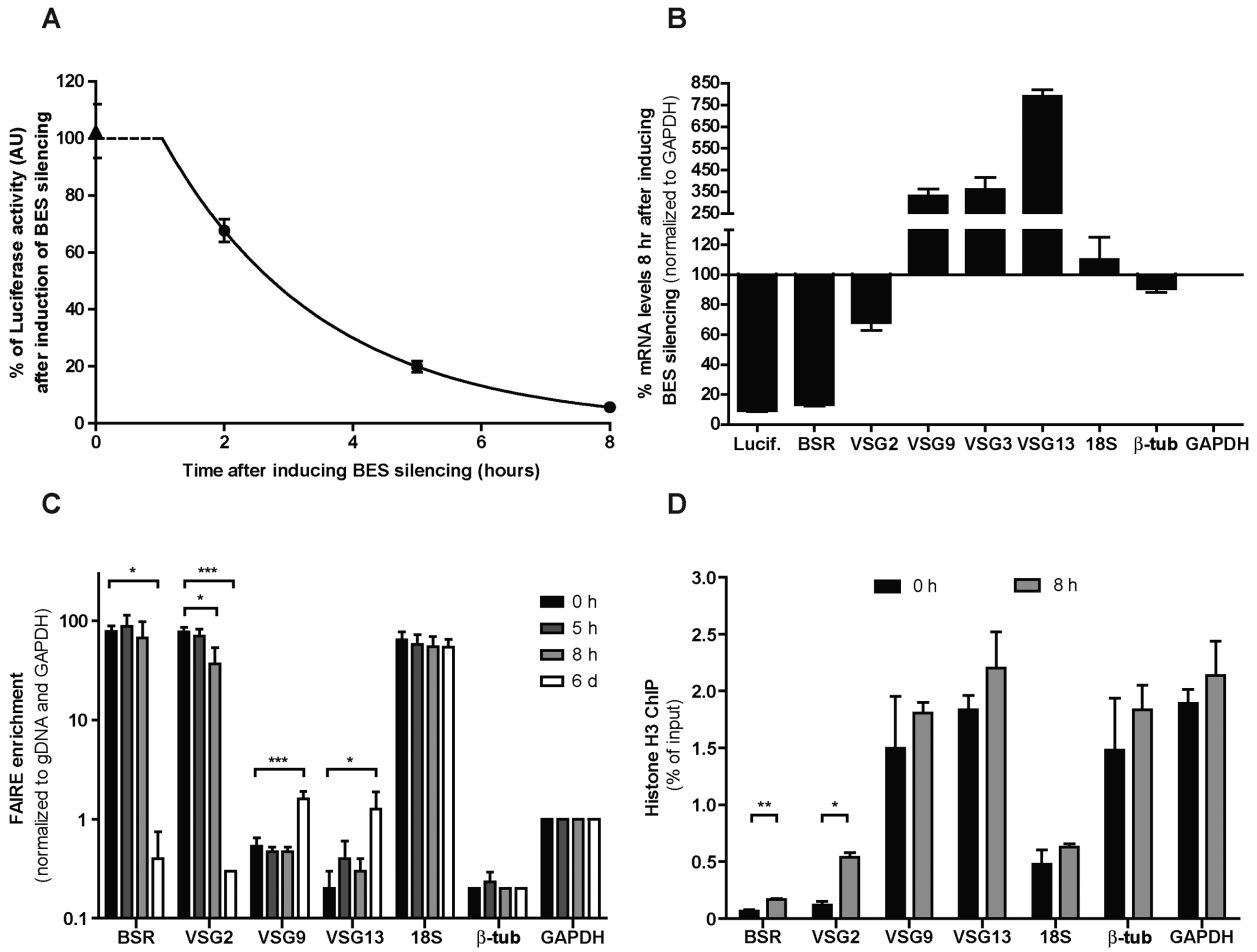

Figure 3. BES chromatin retains an open conformation despite its transcription being reduced 90%. (**A**) % of luciferase activity of Tet- relative to Tet+ cells after 0, 2, 5 and 8 h of tetracycline removal. Curve represents the best decay fit for time-points 2, 5 and 8 h. Five to seven independent experiments were analyzed for each time-point. (**B**) % of mRNA levels after 8 h of tetracycline removal relative to Tet+ cells, measured by qPCR and normalized to *GAPDH* transcripts. Five independent experiments were analyzed. (**C**) Chromatin conformation was measured by FAIRE at 0, 5, 8 h and 6 days after tetracycline removal. DNA isolated by FAIRE was quantified by qPCR and normalized to gDNA copy number and *GAPDH*. Three independent experiments were analyzed. Statistical significance was determined by one-way ANOVA with Bonferroni post-test comparison. (**D**) Nucleosome occupancy was determined by histone H3 ChIP at 0 and 8 h after tetracycline removal. Immunoprecipitated DNA was compared to the total input material. Three independent experiments were analyzed. Statistical significance was determined by a paired *t*-test against time-point 0 h. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

mation of these genomic loci. Second, for each gene, the FAIRE-enrichment was constant overall for the three stages of the cell-cycle, suggesting that chromatin conformation is essentially insensitive to cell-cycle. We cannot exclude the possibility that very rapid and transient chromatin changes in conformation take place, which could not be captured at the time-resolution used here.

Unlike rDNA genes in yeast, we observed no major changes in the chromatin conformation of the active BES throughout the cell-cycle, which suggests that the mechanism that keeps BES chromatin open in the absence of transcription open is very likely cell-cycle independent.

TDP1 maintains chromatin open in the absence of transcription

In budding yeast, a Pol I transcriptional restriction for four hours under G_1 arrest does not affect the conformation of rDNA chromatin, which is maintained by HMO1, a high mobility group box (HMGB) protein (3). In. *T. brucei*, TDP1, a high-mobility group box protein, facilitates transcription of Pol I transcribed genes (18). Here, we tested if TDP1 was necessary to maintain chromatin open in the absence of Pol I transcription. For this, we tagged an endogenous allele of *TDP1* with a TY1 epitope and we performed ChIP. As previously reported (18), we found TDP1 highly enriched in the active BES (*Luciferase* and *VSG2* genes) and *18S* rDNA (0 h) (Figure 5A). After 8 h of transcriptional silencing, the chromatin of the promoter-proximal region of the BES contained the same amount of TDP1, whereas the telomeric region had two-fold less TDP1. Nonetheless, these levels were still higher (7–9-fold) than in silent *VSG*s. As TDP1 inversely correlates with histone H3 (18), this result is in accordance with the H3 distribution after 8 h of BES silencing (Figure 3C and D), and it shows that TDP1 is still largely present in the active BES when its transcription is reduced by at least 90% (Figure 3A). The surviving switchers (six days post-silencing induction) presented, as

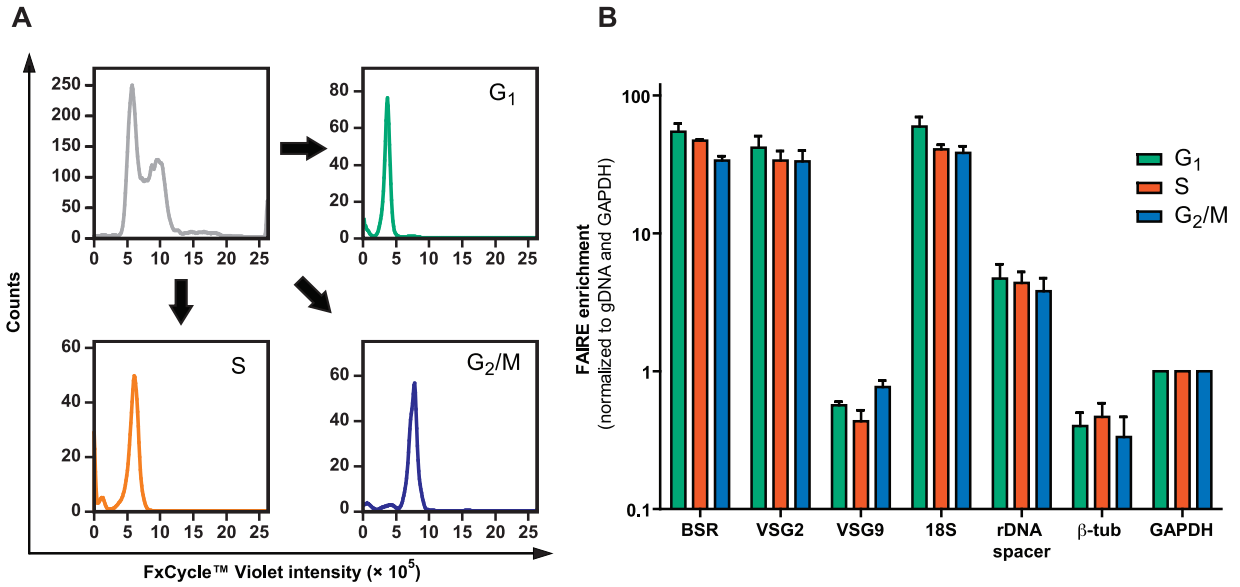

Figure 4. Chromatin conformation of BESs is cell-cycle independent. (**A**) Nuclear DNA of GLB1 cells was stained by FxCycle Violet and sorted by flow cytometry in G_1 (red), S (orange) and G_2/M (blue) cell-cycle stages. Panel in grey represents original population, colored panels represent analysis of post-sorted populations. (**B**) Chromatin conformation of the three cell-cycle populations was measured by FAIRE. DNA isolated by FAIRE was quantified by qPCR and normalized to gDNA copy number and *GAPDH*. Three independent experiments were analyzed. Statistical significance was determined by a 1-way ANOVA with Bonferroni post-test comparison.

expected, very low levels of TDP1 in the previously active BES.

To test if TDP1 is necessary to maintain chromatin open when the active BES is silenced, we depleted TDP1 by transiently transfecting an anti-TDP1 dsRNA (18). This method has been previously used to knock-down α-tubulin transcripts (39). Transfection with buffer (mock control), showed no changes in TDP1 levels over time (Figure 5B, Supplementary Figure S4A). However, transfection of anti-TDP1 dsRNA lead to a reduction of TDP1 protein levels to ∼18% after 24 h and remained low until 48 h, after which TDP1 levels increased (Figure 5B). Consistent with a previous report (18), we observed that 24 h of TDP1 depletion resulted in a ∼43% reduction of luciferase activity, confirming the role of TDP1 as a transcriptional facilitator of the active BES (Supplementary Figure S4B). To test if TDP1 is necessary to maintain open chromatin status in the absence of transcription, we transfected TDP1 dsRNA or buffer into the BES inducible reporter strain and, after 19 h, we transcriptionally silenced the active BES by removing tetracycline from the medium. FAIRE was used to characterize chromatin status 5 h post-silencing (which corresponds to 24 h post TDP1 depletion) in four conditions: when BES silencing was induced or not, and in the presence or absence of TDP1 (Figure 5C). As expected, chromatin changes were not detected for control genes: Pol II-transcribed genes, nor in silent *VSG9*. Chromatin of *18S* rDNA, although transcribed by Pol I, was not affected either, which is consistent with observations in yeast, in which HMO1-null mutants do not lead to condensation of rDNA chromatin (3). Relative to non-transfected control, mock control cells did not display changes in chromatin in any loci, including BES1, indicating that transfection *per se* did not significantly affect chromatin conformation (Figure 5C).

When TDP1 was depleted but BES1 remained active (TDP1 KD, Tet+), chromatin remained open in the promoter region and began to close at the telomere (*VSG2* is 2.6-fold more closed than mock transfection), which is consistent with the observations of Narayanan *et al.* upon depletion of TDP1 by RNA interference (18). However, when TDP1 was depleted and BES1 was silenced (TDP1 KD, Tet-), chromatin of *VSG2* closed even further and chromatin of *luciferase* also closed slightly (Figure 5C). Next we compared the change of FAIRE-enrichment after and before silencing was induced (Tet- versus Tet+), in both conditions: mock transfection (presence of TDP1) and upon TDP1 knock-down (Figure 5D). For most genes in both conditions, this fold-change of FAIRE-enrichment is around 1, indicating that chromatin after 5 h of silencing is not dramatically different and absence of TDP1 does not cause global changes in chromatin during this period. However, in the *VSG2* gene we detected a significant reduction in the fold-change of FAIRE-enrichment between mock and TDP1 depleted conditions. These results indicate that TDP1 is necessary to keep an open chromatin conformation when transcription of BES is halted.

We conclude that TDP1 is a key player of the chromatin of active BES, especially at the telomeric end. Narayanan *et al.* has previously shown that TDP1 maintains chromatin nucleosome-depleted and facilitates transcription. Here we show that TDP1 is crucial to keep an open chromatin in a BES that has been recently silenced (especially at the telomeric end of BES).

BES probing precedes commitment to switching

Next we investigated the advantages of having a mechanism that keeps chromatin open when transcription has been silenced. We hypothesized that this may be a mechanism that

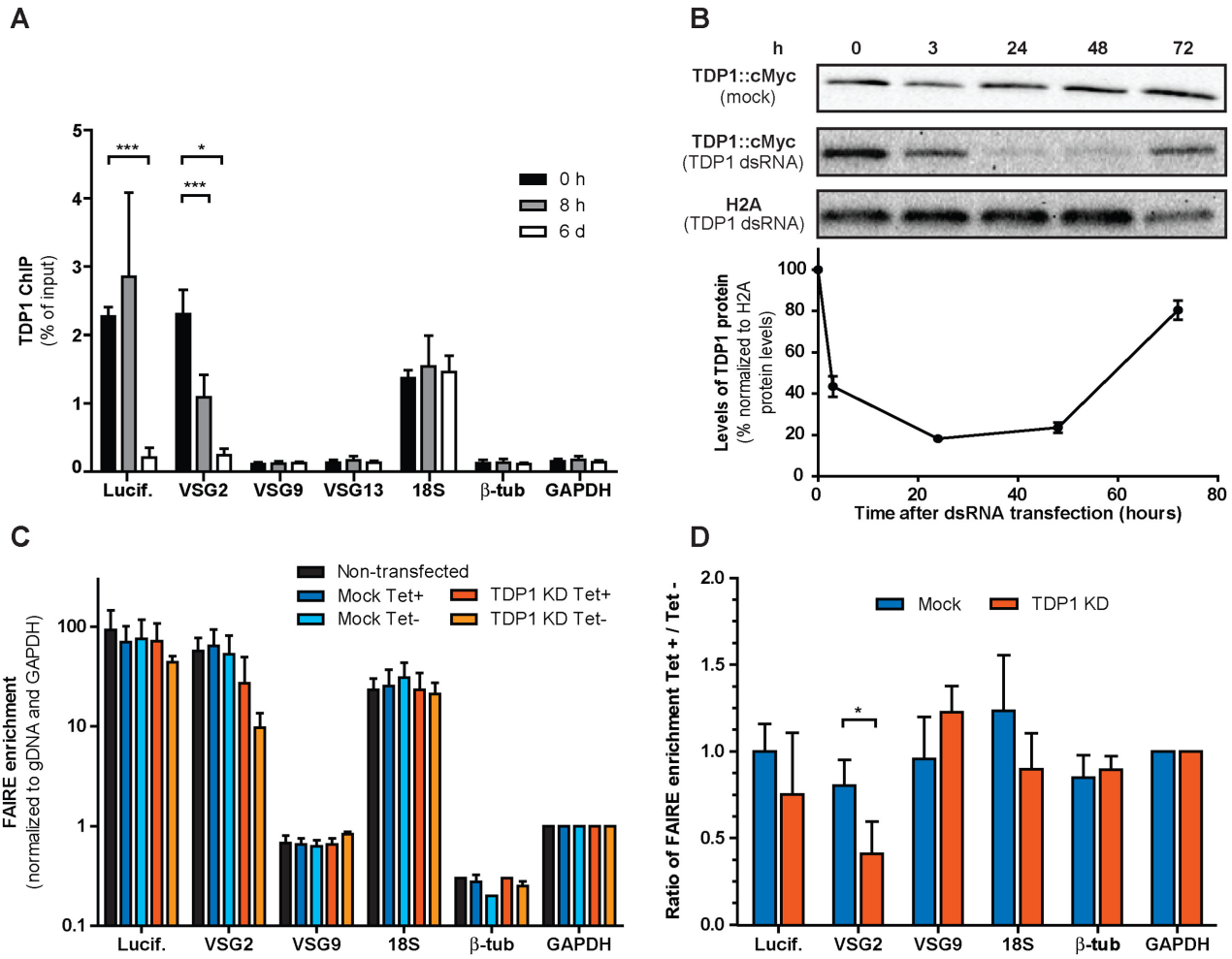

Figure 5. When active BES is silenced, TDP1 keeps its chromatin open. (**A**) TDP1 ChIP at 0, 8 h and 6 days after tetracycline removal in GLB1-TDP1::TY1, a cell-line in which one endogenous allele of TDP1 is fused with a TY1 tag. Immunoprecipitated DNA was compared to the total input material and normalized to *18S* DNA. Statistical significance was determined by a one-way ANOVA with Bonferroni post-test comparison. Three independent experiments were analyzed. (**B**) Western blotting analysis of TDP1 protein after 3, 24, 48 and 72 h of transfection with buffer (mock) or anti-TDP1 dsRNA in GLB1-TDP1::3xcMyc, a cell-line in which one endogenous allele of TDP1 is fused with a triple c-MYC tag that is more sensitive for western blot. Time-point 0 h indicates mock control cells transfected only with buffer. Each lane corresponds to lysates from 2×10^6 cells. Quantification of TDP1 signal is indicated in the **lower panel**. TDP1 protein levels were normalized for H2A protein levels and mock control. Four independent experiments were analyzed. (**C**) Chromatin conformation of GLB1-TDP1::3xcMyc cells after 5 h of BES silencing (24 h of TDP1 depletion) was measured by FAIRE. DNA isolated by FAIRE was quantified by qPCR and normalized to gDNA copy number and to *GAPDH*. Four independent experiments were analyzed. Statistical significance was determined by an unpaired t-test comparing Tet- to Tet+ in each condition (Mock or TDP1 KD). (**D**) Ratio of FAIRE enrichment (calculated from data in panel C) between Tet- and Tet+ for Mock and TDP1 KD conditions. Statistical significance was determined by an unpaired *t*-test. $*P < 0.05$; $***P < 0.001$.

allows parasites to probe different silent BESs before committing to a new BES. To test this hypothesis, first, we determined how long cells take to commit to a second BES. For that, we repeated the BES silencing assay but we re-added tetracycline to the cells 8, 24 or 48 h post-silencing. Tetracycline relieves the transcriptional block of Tet repressor protein and thus allows cells that re-activate BES1 to survive (Figure 6A). Cells were cloned by limiting dilution and GFP-intensity of each clone was measured by FACS. GFP-positive clones indicate that cells re-activated BES1, while GFP-negative clones indicate cells that switched to a new BES. We observed that, none of the wells were GFP-negative at 8 h, suggesting that all surviving cells could potentially reactivate BES1. Instead, adding tetracycline at 24 h resulted in 49% of clones no longer expressing GFP, while at 48 h this number was 96%. These results show that most surviving cells are already committed to a new BES two days after silencing was induced. These results also show that during the first 8 h of switching, most cells are not committed to a VSG switch and can revert to transcribe the original BES.

We postulated that if commitment of most cells happens between 8 and 48 h, during this period we may be able to detect cells transiently probing new BESs at intermediate levels. Such cells were detected by Chaves *et al.* (9). To test if transcription of silent BESs increases before commitment, we constructed another reporter strain, GLB1-R15, in which an *RFP::NPT* fusion gene was inserted downstream of the silent BES15 in GLB1 cell-line (Figure 6B). This reporter allowed us to test at single-cell level whether

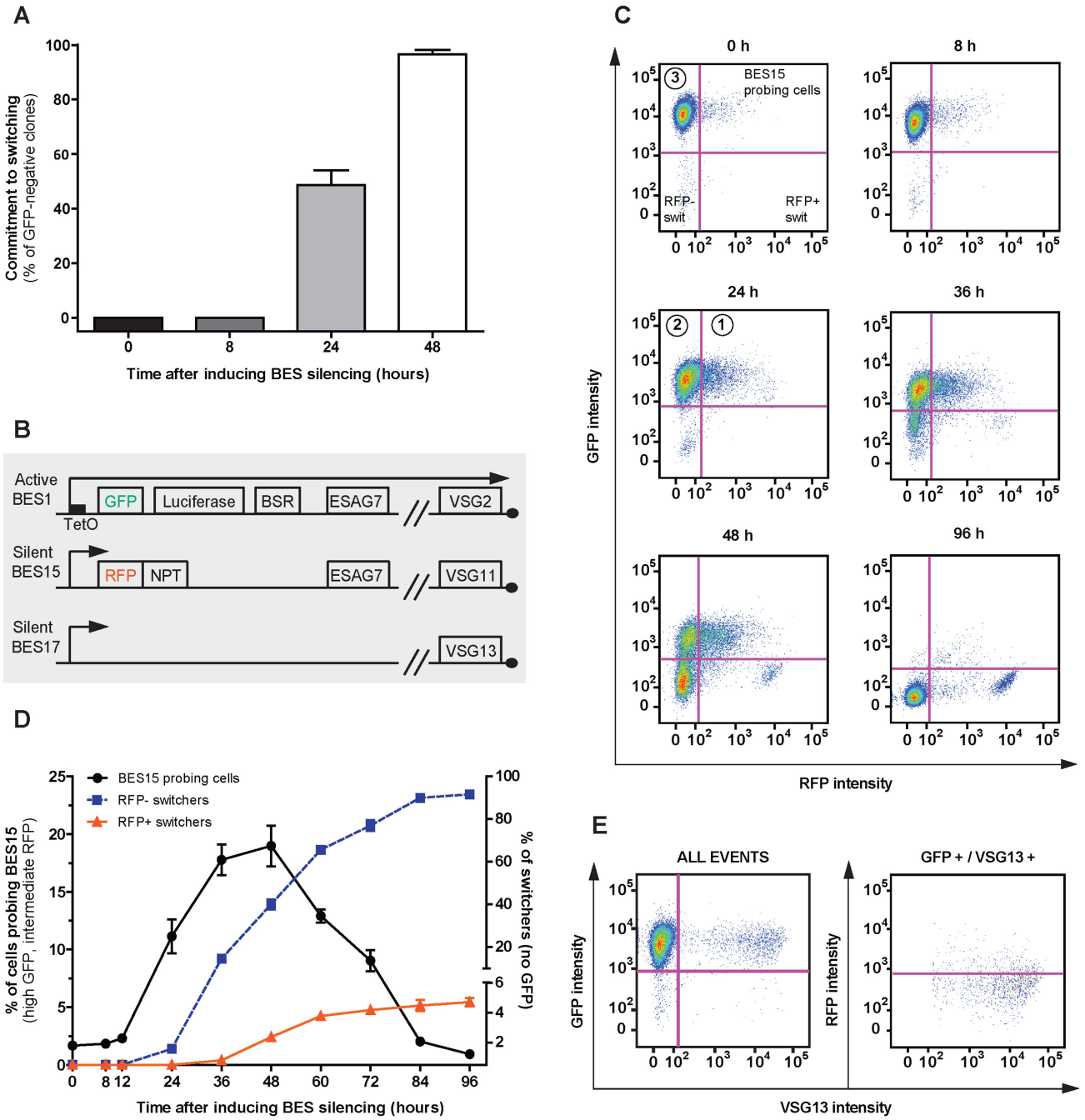

Figure 6. Cells transcriptionally probe silent BESs for up to two days, when most cells are committed to switching. (**A**) Commitment assay. 8, 24 or 48 h after inducing BES silencing, tetracycline was added back to the medium and cells were cloned. Six days later FACS was used to assess if clones were GFP-positive, indicating re-expression of original BES1. A minimum of 95 total individual wells was analyzed for each time-point between five individual experiments. (**B**) The cell-line GLB1-R15 is a derivative of GLB1, in which the fused gene *RFP::NPT* was introduced downstream the promoter of a silent BES. *NPT*, Neomycin Phosphotransferase. (**C**) Representative examples of FACS plots at several time-points post-BES silencing showing GFP and RFP expression (see Supplementary Figure S5 for complete set of time points). (**D**) Proportion of probing cells was assessed by measuring number of cells expressing RFP at intermediate levels and still present high levels of GFP (black line, left Y axis); switchers were defined as GFP-negative cells and either RFP- or RFP+ (blue and orange lines, respectively, right Y axis) after tetracycline removal. Three independent experiments were analyzed. Circled 1,2 and 3 labels indicate the sorted populations described in Figure 7. (**E**) **Left panel** - Representative example of a FACS plot at 24 h post-BES silencing showing VSG13 and GFP expression. **Right panel** - Representative example of a FACS plot showing VSG13 and RFP expression in the cells present on the top right gate in **left panel**. Four independent experiments were analyzed.

silent BES15 was being transiently more transcribed during switching (Figure 6C and D, Supplementary Figure S5). Before silencing was induced, a small number of cells (1–2%) expressed low levels of RFP (11–14-fold higher intensity than background levels), consistent with previous studies showing that silent BES are transcribed at low rate (40,41). 12 h after silencing was induced, we observed an increase in the proportion of total cells expressing RFP (2–3%), suggesting that more cells are transcribing BES15. The number of cells probing BES15 increased with time up until 36–48 h, in which around 20% of the cells showed elevated levels of RFP (Figure 6D).

At 36 h, two new and distinct GFP-negative populations were detected: one expressed high levels of RFP (0.7%) (∼200-fold higher intensity than RFP background levels) and the other was RFP-negative (14.5%), suggesting that these cells are switchers that silenced BES1 and activated BES15 (orange curve) or another BES (blue curve), respectively (Figure 6D). In this mixed population of cells, the switcher subpopulations became more predominant with time, while the number of cells probing silent BES15 gradually decreased. At 96 h, switchers were almost the sole populations in culture (around 5% expressed RFP and 92% did not).

Is probing restricted to the promoter region or does it span an entire BES? Because there is no antibody against the VSG11 of BES15, we used an anti-VSG13 antibody to test if we could detect VSG13 (from BES17) at the cell surface of silencing-induced cells. Silencing was induced in GLB1-R15 and, at 24 h, cells were stained with anti-VSG13 (BES17) and analyzed by FACS (Figure 6E). We observed that ∼5% of cells that expressed GFP also expressed heterogeneous levels of VSG13. Of these, ∼20% simultaneously expressed intermediate levels of RFP and VSG13, indicating that cells can simultaneously probe two BESs, BES15 (RFP) and BES17 (VSG13). These results also show that probing is not restricted to the promoter region and, at least in some cells, the entire BES is upregulated all the way until the telomeric end of BES17.

Taken together, we conclude that parasites transiently increase the transcription levels of silent BESs for around 2 days, when commitment to a new BES is almost complete; probing spans the entire BES, until the VSG gene and, albeit at a low frequency, two BESs can be simultaneously probed in individual cells.

BES probing is a reversible intermediate switching step

Next we investigated whether cells that partially upregulated RFP (probing BES15) are true switching intermediates. We chose to characterize the 24 h time-point because its FACS profile is less heterogeneous (Figure 6C and Supplementary Figure S5) and there are fewer dead cells (Supplementary Figure S2). We induced BES silencing for 24 h, FACS-sorted GFP+/RFP+ cells, and placed them in culture in limiting dilutions in the presence or absence of tetracycline (Population 1, indicated in 24 hr FACS plot Figure 6C). We also FACS-sorted and plated GFP+/RFP- cells (Population 2) because, although BES15 was not upregulated, other BESs may be upregulated. As a control, a population of cells in which silencing was not induced (Population 3) was also sorted (Figure 7A). Cells that attempt to reactivate BES1 die in the absence of tetracycline and survive in the presence of this drug. Therefore, the number of surviving clones six days after sorting reflects the efficiency of BES switching of each sorted population at 24 h post-silencing. Overall, in the absence of tetracycline, 3–5% of the clones survived the silencing assay, which is consistent with 8% of surviving clones measured in the clonogenic assay of the whole population (Figure 2D) and confirms that, with this inducible system, although we can dramatically increase the switching frequency from around 1 in a million cells to 1 in 10 cells, 9 in 10 cells still fail to switch to a new BES, even in the populations 1 and 2. When tetracycline was added to the culture medium after sorting, the number of surviving clones doubled (7–9%), which was expected because at 24 hr half of the population is still capable of returning to the originally active BES1 (Figure 6A).

Seven days post BES silencing, surviving clones were characterized by FACS for their expression of GFP and RFP, which we used as reporter of BES activity: GFP+/RFP- profile indicates clones that probably did not switch and reactivated BES1; GFP-/RFP+ indicates switchers to BES15; and GFP-/RFP- indicates switchers to another BES. No clones were obtained that simultaneously expressed GFP and RFP. For both populations 1 and 2, all post-sorting clones obtained in the absence of tetracycline no longer expressed GFP and thus represent switchers. Interestingly, from population 1, 57% (12 clones on average of three experiments) of switchers expressed RFP, while 43% (nine clones) did not, indicating that cells can probe one BES at 24 h post-silencing and eventually switch to another BES (Figure 7B). From population 2, all switcher clones were RFP-negative (13 clones), indicating that switchers activated a BES other than BES15 (Figure 7B). These results suggest that if a BES is not probed at 24 h, apparently it is not fully activated later.

When sorted populations were plated in the presence of tetracycline we obtained some clones that expressed GFP and not RFP, as expected and consistent with the fact that at 24 h there are still many cells that are not committed for switching and reactivate BES1 (Figure 7A). Post-sorting clones from population 1 showed similar proportions of the three different types of GFP/RFP expression profiles, indicating a large plasticity of this population before commitment (Figure 7B). In contrast, most post-sorting clones from population 2 reactivated BES1 (77%, 24 clones) and none activated BES15 (Figure 7B), suggesting once again that if a BES is not probed at 24 h, it seems not to be activated later.

Clones obtained after sorting population 3, in which silencing was never induced, resulted as expected in 100% of clones expressing GFP and not RFP (Figure 7B), consistent with BES1 remaining active. This shows that the stress associated with sorting did not induce any unexpected changes in BES expression.

Taken together, our results indicate that cells in a probing state are not a dead end product resulting from the silencing inducible system used in this work. Importantly, these data show that probing cells can revert to the original BES1, or switch and commit to the probed BES, or switch and commit to another BES. Probing is therefore a reversible step

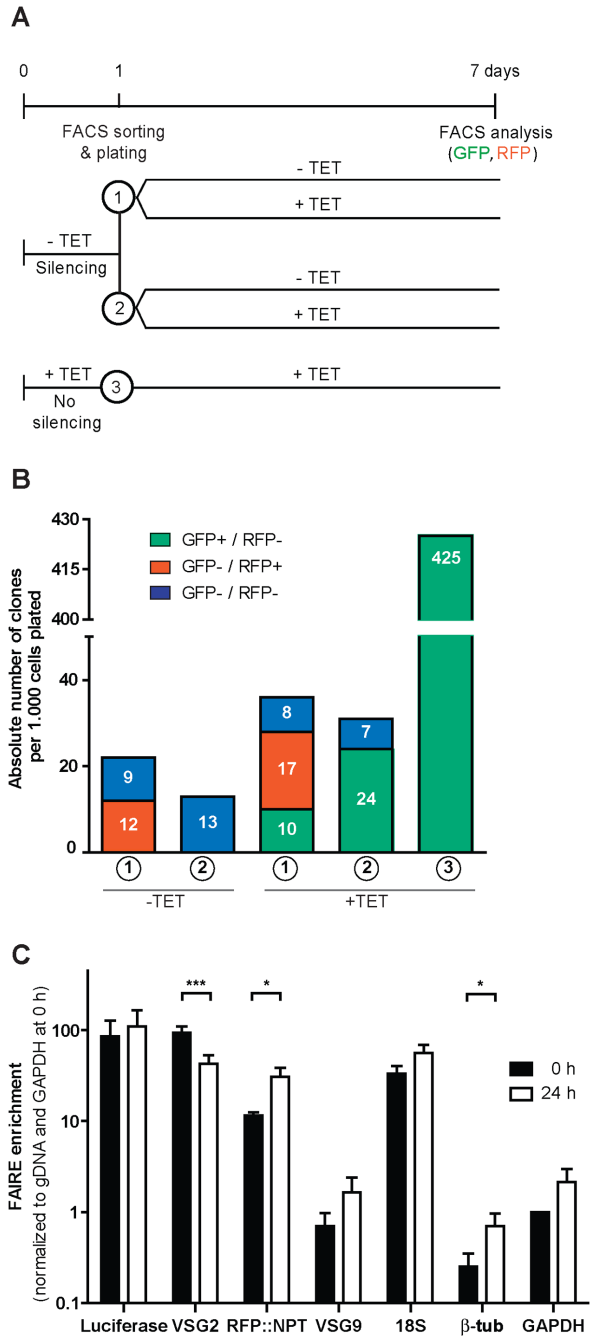

Figure 7. Probing cells are switching intermediates that can choose different fates. (**A**) Experimental design of FACS sorting and subsequent phenotype characterization. Tetracycline was removed from the medium of GLB1-R15 cell-line. One day later, two populations 1 and 2 (indicated in Figure 6C) were sorted and plated by limiting dilution. Population 1 consists of cells that express GFP and RFP, while population 2 consists of cells that express GFP, but not RFP. As control, a third population of GFP+/RFP- cells was sorted from a culture kept under tetracycline pressure. Clones obtained after six days were characterized in terms of expression of GFP and RFP by FACS. (**B**) GFP and RFP expression of surviving clones was assessed by FACS. Numbers in white show mean number of clones with each specific phenotype from three individual experiments. **(C)** Chromatin conformation of GLB1-R15 cells after 24 h of BES silencing was measured by FAIRE. DNA was quantified by qPCR and normalized to gDNA copy number and to *GAPDH* at 0 h. Statistical significance was determined by a paired *t*-test against time-point 0 h. $*P < 0.05$; $***P < 0.001$.

during BES switching, allowing cells to reactivate the originally active BES or activate previously silent BESs.

Given that at 24 h, the population consists mainly of cells capable of switching or reverting to original BES (with only around 3% of dead cells, Supplementary Figure S2), we decided to check whether chromatin of the originally active BES1 remains accessible during this period in this cell-line (GLB1-R15). For this, silencing was induced and cells were collected for FAIRE at 0 and 24 h post-silencing (Figure 6E). At 24 h, chromatin in *VSG2* became significantly more compact than at 0 h, which is consistent with the trend previously observed by FAIRE in GLB1 cell-line at 8 h (Figure 3C), but the conformation is still 25-fold more open than that of a silent *VSG9*. Consistent with probing phenotype, we detected a significant increase in chromatin accessibility of the *RFP::NPT* gene. All other tested genes showed a slight increase in accessibility, which may be due to the fact that, at 24 h, the culture has some cells arrested in G_2/M or with an abnormal DNA content (Figure 2C), or already dead (Supplementary Figure S2).

Taken together, our data reveal a novel association between the alterations of chromatin structure at the active BES and BES switching. Chromatin is kept essentially open for at least 24 h, while silent BESs are reversibly probed before committing to a new BES.

DISCUSSION

In this study, we showed that the chromatin structure of the active BES is cell-cycle independent and TDP1 keeps its open conformation when transcription is halted, especially in the telomeric region. We propose that these properties are critical for the dynamics of switching between BESs because it gives time for cells to reversibly probe multiple silent BESs before committing to a new BES.

Transcription and chromatin dynamics in the active BES

The initial trigger that leads to VSG switching remains a mystery. However, several studies in which transcription of the active BES was somewhat interrupted or diminished, either due to loss of the VSG upstream sequence (CTR) (10) or replacement of a BES promoter by the T7 promoter (24), resulted in more frequent *in situ* switching, suggesting one of the earliest events during BES switching is the silencing of the active BES. In this work, we first checked if halting transcription is also one of the earliest steps during differentiation from bloodstream to procyclic forms, a process in which the active BES needs to be silenced while procyclin genes are upregulated. We show that, 24 h after inducing differentiation, transcription of the active BES is highly reduced to 2% (Figure 1B), while the chromatin of this locus remains essentially open (Figure 1C). We conclude that during differentiation, transcription silencing precedes chromatin changes, and thus it is likely that a similar mechanism happens during BES switching in bloodstream forms.

In the tetracycline-inducible system developed by the Horn lab, when the tetracycline repressor binds the tetracycline operator, it sterically blocks Pol I transcription, which results in silencing of the initially active BES and activation of a new one (Figure 2). In this work, we confirmed

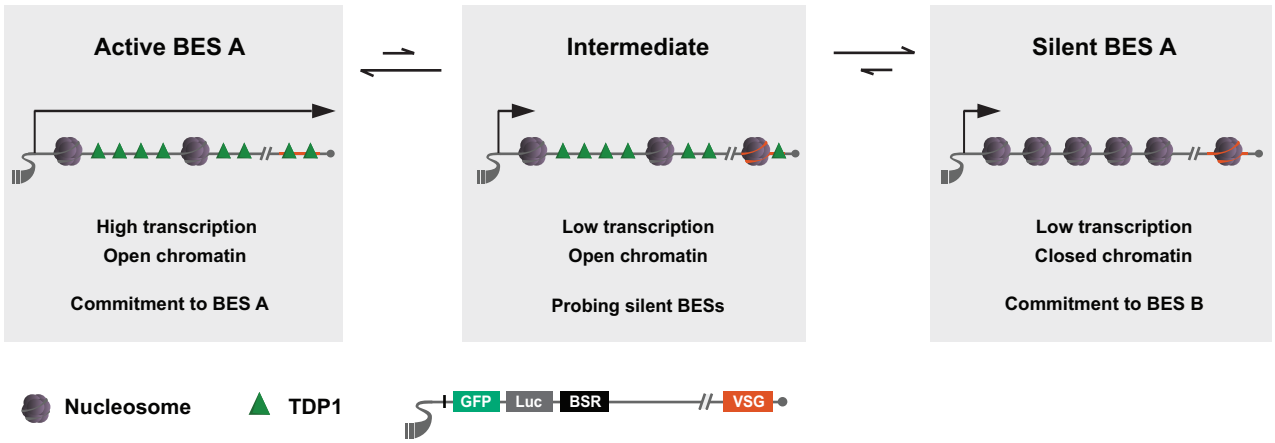

Figure 8. Model for VSG expression site switching: transcriptional probing silent BESs before commitment is associated to a temporary maintenance of open chromatin by TDP1. Chromatin of the actively transcribed BES possesses an open chromatin enriched in TDP1. Upon transcriptional silencing of the active BES, cells undergo an intermediate state characterized by stabilization of the active BES open chromatin by TDP1 while probing silent BESs. Commitment to switch or not switch seems to take around two days. This decision can either be returning to the initial active BES (which, in our reporter cell-line, because BES1 is blocked by tetracycline repressor, it would result in cell death) or switching to a new BES. In this later case, the chromatin of the originally active BES loses TDP1 and becomes nucleosome-enriched.

that inducible BES silencing promotes a rapid decrease in transcript levels of genes from initially active BES (at least 90% drop in the first 8 h, as accessed by transcript levels of the unstable *luciferase* reporter) and an eviction of Pol I from chromatin fiber, which confirms that transcription of active BES is efficiently and rapidly stopped (Figure 3A, B and Supplementary Figure S3). During this period, chromatin remained in an open state despite transcription being halted (Figure 3C and D). By ChIP, we observed a slight enrichment of histone H3 in the *VSG2* gene and a decrease in TDP1, suggesting a more compact chromatin mainly at the telomeric end of BES.

Our study also shows that chromatin conformation of the active BES is cell-cycle independent. Using FAIRE, we detected no significant differences in chromatin conformation of active BES in cells in G_1, S and G_2/M. Given that, when parasites divide, most daughter cells use the 'mother's' BES and only very few switch to a new BES, it is tempting to speculate that keeping an open chromatin structure serves as an epigenetic marker that, after cell division, signals which BES should be used by the daughter cells. Perhaps TDP1 interacts with the cohesion complex, which is necessary after S phase to maintain the two sister chromatids of the active BES associated to the single Expression Site Body while waiting for chromosome segregation during mitosis (42). This hypothesis deserves further investigation in the future.

TDP1 maintains open chromatin when BES has been silenced

High-mobility group box (HMGB) proteins are essential nuclear components in chromatin structure, transcriptional activity and DNA damage repair (43–45). In yeast, HMO1 is a HMGB protein that is associated with Pol I transcription machinery (46), which can competitively displace histone H1 (47) and also maintain chromatin in an open conformation (3). In *T. brucei*, TDP1 is an essential HMGB protein that is highly enriched in Pol I loci (18,19). Like yeast HMO1, TDP1 is a facilitator of Pol I transcription and it is necessary to keep chromatin nucleosome-depleted in a steady-state situation. Here, we show that when BES silencing was induced in the absence of TDP1, chromatin became significantly more compact, indicating that TDP1 is necessary to maintain an open chromatin structure in the absence of transcription. This role is consistent with what has been observed in yeast, in which HMO1 maintains Pol I chromatin open when transcription is halted (3).

In eukaryotes, deposition of canonical histones is normally replication-dependent (48). Although our data shows that maintenance of BES chromatin structure is cell-cycle independent (Figure 4), we predict that during the rare event of BES switching, chromatin remodelling occurs in S phase of the cell-cycle. Thus, during replication, eviction of TDP1 from a silencing BES may be an important step to complete a BES switching. Regulating the timing of TDP1 eviction may be a means to determine for how long a cell stays in a 'probing' stage, before commitment.

It is interesting to observe that, in two different circumstances (BES transcriptional silencing in wild-type or TDP1 depleted conditions), the chromatin of the BES telomeric end is more rapidly closed than chromatin close to the BES promoter (which can be up to 50 kb upstream from telomere). Telomeres possess a highly controlled chromatin structure and, in trypanosomes, they are very important for antigenic variation (49). It is possible that, upon transcriptional silencing, loading of nucleosomes onto chromatin happens faster at the telomeric region. Tiengwe *et al.* found that although most origins of replication are found in core regions of the chromosome, VSG genes also harbor origins of replication, which could drive inward replication and deposition of nucleosomes (50). Spreading of such chromatin condensation may happen evenly towards the BES promoter or it may happen in steps if the BES has insulator elements, such as the 70-bp repeat array for example, which could prevent spreading of nucleosome loading into the upstream ESAGs.

Probing and commitment to a new BES

Chaves *et al.* proposed that, during a switching event, a cell pre-activates a silent BES before a natural intermediate rapidly and transiently express two VSGs and ultimately commits for switching or reverts to the original BES (9). A more recent study, in which BES transcriptional attenuation was observed as a consequence of ectopically overexpressing a silent VSG, also proposed that this attenuation could give time for parasites to probe silent BES before switching to a fully competent BES (11). The nature of what cells are probing is unknown. It is possible that cells are testing the order of VSG switching, or if ESAGs are functional, or if previously untested or mosaic VSGs are functional.

Our results are in agreement with these models of 'probing before decision' and we propose that regulation of chromatin structure may be the underlying epigenetic mechanism. We show here that trypanosomes test silent BES before a choice is made and this is associated to keeping chromatin of the active BES open (Figure 8). Our commitment assay showed that, 8 hr after silencing, no cells are committed to switching although silent VSG transcripts are already detected (Figure 3B). Half of the cells are committed 24 h post-silencing, while most of them are committed at 48 h. From 12 to 48 h, more cells probe silent BESs, but only a fraction of the cells expresses higher levels of RFP, which indicates that not all BESs are being probed at the same time in each cell. Nevertheless, we found that at least two BESs can be probed simultaneously by the same cell (Figure 6E). At 24 h, a small fraction of cells becomes GFP-negative, consistent with committed switching and this population becomes more predominant with time. These results show that probing of one or more BESs is a reversible process that lasts around two days, during which cells either switch to the probed BES, switch to a different BES or revert to BES1. Reverting to original BES1 is probably facilitated by the fact that BES1 retains an open chromatin structure for at least 24 h (Figure 7C).

In some aspects, our model resembles the formation of poised chromatin. Poised chromatin at Pol II transcribed genes is characterized by the presence of bivalent marks: the co-existence of activating and repressive histone marks within the same domains (51). This epigenetic status has been associated to genes that are transcribed at low levels but need to be in a prepared state for developmental fates of rapid activation or repression, such as differentiation of embryonic stem cells or activation of T-cells (52,53). A poised chromatin state has also been found at *var* genes in *Plasmodium falciparum*, the causative agent of malaria. Switching between *var* genes is essential for antigenic variation (54). It is possible that *T. brucei* uses a TDP1-dependent mechanism as a means of temporarily keeping the active BES in a poised state, ready for being activated or repressed. In the future, it will be interesting to test whether this mechanism of keeping chromatin temporarily open also facilitates VSG switching by recombination. In lymphocytes, for example, it has been already shown that the chromatin that is accessible for V(D)J recombination typically displays elevated acetylation of histones H3 and H4, which is a hallmark of a more accessible chromatin (55).

In this study, we showed that the chromatin structure is kept open by TDP1 probably to facilitate probing, before cells commit to a new BES. It is intriguing that, during differentiation, chromatin of the originally active BES is also hold open for some time while transcription has already been reduced. This suggests that differentiating cells may also undergo an intermediate stage, which may be reversible. This is consistent with findings by Batram *et al.*, in which attenuation of the active BES results in an intermediate stage that can reversibly progress in two directions: differentiation to procyclic or returning to proliferation in the mammalian form. The implications of chromatin dynamics in differentiation should be further investigated, and preferably in a pleomorphic strain, whose differentiation process is more similar to what happens *in vivo*.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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