A Histone Methyltransferase Modulates Antigenic Variation in African Trypanosomes

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To evade the host immune system, several pathogens periodically change their cell-surface epitopes. In the African trypanosomes, antigenic variation is achieved by tightly regulating the expression of a multigene family encoding a large repertoire of variant surface glycoproteins (VSGs). Immune evasion relies on two important features: exposing a single type of VSG at the cell surface and periodically and very rapidly switching the expressed VSG. Transcriptional switching between resident telomeric VSG genes does not involve DNA rearrangements, and regulation is probably epigenetic. The histone methyltransferase DOT1B is a nonessential protein that trimethylates lysine 76 of histone H3 in Trypanosoma brucei. Here we report that transcriptionally silent telomeric VSGs become partially derepressed when DOT1B is deleted, whereas nontelomeric loci are unaffected. DOT1B also is involved in the kinetics of VSG switching: in ΔDOT1B cells, the transcriptional switch is so slow that cells expressing two VSGs persist for several weeks, indicating that monoallelic transcription is compromised. We conclude that DOT1B is required to maintain strict VSG silencing and to ensure rapid transcriptional VSG switching, demonstrating that epigenetics plays an important role in regulating antigenic variation in T. brucei.

Introduction

Post-transcriptional histone modifications play important roles in the regulation of chromatin structure and gene expression. Unlike acetylation, which is in general associated with transcription activation, histone methylation can activate or repress transcription depending upon the genomic location and the position of the modified amino acid in the histone chain [1]. Histone methylation mainly occurs on lysine or arginine residues that are located in the N-terminal tails of histones H3 and H4. One exception is lysine 79 of histone H3 (H3K79), which is located in the globular domain of H3 and is methylated by Dot1 in yeast [2,3] and hDOT1L in humans [4]. Very little is known about the function of H3K79 methylation. In yeast, it has a role in maintaining heterochromatin, probably indirectly, by limiting the spreading of Sir2 and Sir3 proteins into euchromatin [2]. In yeast and mammalian cells, H3K79 methylation appears to be involved in the detection of DNA damage [5,6] and in the development of leukemia as a result of Hox gene activation [7].

Antigenic variation is one of the most elegant systems that have evolved to evade host immune defenses. Trypanosoma brucei, the unicellular parasite that causes African sleeping sickness, expresses a single type of variant surface glycoprotein (VSG) at the cell surface and escapes the host immune response by periodically exchanging it with a different VSG (reviewed in [8]). The expressed VSG is always transcribed from one of the ~15 bloodstream expression sites (BESs), which are always located at telomeres [9]. To ensure monoallelic VSG expression, only one BES is transcribed by RNA polymerase I at any time. This active BES localizes to a specialized extranucleolar compartment, the expression site body (ESB), which is proposed to contain the transcription machinery and regulatory factors that are required for complete processing of BES transcripts [10,11]. One of the mechanisms used to change the transcribed VSG is coordinated silencing and activation of different BESs. This stochastic process occurs at a low frequency but is very rapid. Attempts to select cells with two simultaneously active BESs revealed that switching intermediates are very unstable and short-lived [12]. BES switching does not seem to require DNA rearrangements [13], which suggests that it is mediated by epigenetic mechanisms. Although an ISWI homologue was shown recently to be involved in silencing BES promoter-proximal regions [14], no well-characterized chromatin remodeling factors are known to participate in VSG gene regulation.

The function and structure of chromatin in T. brucei is very poorly understood. DNA is not methylated, but it contains an unusual modified base β-glucosylhydroxymethyluracil (J) [15], which is mainly present in telomeric repeats and silent BESs [16] but whose function remains unknown. The histone tails of T. brucei are highly diverged from other well-studied eukaryotes. Nevertheless all core histones are subject to a few post-transcriptional modifications [17,18], including some unusual ones such as methylation of the N-terminal alanine

References

1. M. Figueiredo, C. J. Janzen, G. A. M. Cross (2008) A histone methyltransferase modulates antigenic variation in African trypanosomes. PLoS Biol 6(7): e161. doi:10.1371/journal.pbio.0060161

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Abbreviations: BES, bloodstream expression site; CRD, cross-reacting-determinant; DOT, disruptor-of-telomeric silencing; DP, downstream promoter region; ESB, expression site body; FACS, fluorescent-activated cell sorting; HA, hemagglutinin; IFA, immunofluorescence analysis; NEO, neomycin resistance gene; PAG3, procyclin-associated gene 3; PUR, purumycin resistance gene; RT-PCR, reverse-transcriptase PCR; UP, upstream promoter region; VSG, variant surface glycoprotein

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**Author Summary**

The surface of *Trypanosoma brucei*, a unicellular parasite that lives in the bloodstream of its mammalian host, is coated with glycoprotein (VSG) molecules. To evade elimination by the immune system, this parasite replaces its coat with one tailored from another glycoprotein variant. Even though there are hundreds of VSG genes in the genome, this process, called antigenic variation, works because all are silenced except for the one that encodes the current coat. In this work, we show that the chromatin-modifying enzyme DOT1B helps to epigenetically regulate the number of VSGs each parasite can have at a time and how fast each parasite can switch from one coat to another. In parasites lacking DOT1B, silent VSG genes become partially active and the switch from one VSG to another slows down, allowing two different VSGs to appear on the surface of an individual parasite at the same time. Our studies reveal the importance of epigenetics in regulating VSG genes and provide new insights toward the understanding of this unique survival device.

**Results**

**DOT1B Is Required for Complete BES Silencing**

To test whether DOT1B is involved in transcriptional regulation of BESs, we generated a cell line in which genes conferring resistance to puromycin or G418 were introduced immediately downstream of the promoters of two BESs (Figure 1A), an approach first used in [21]. In a first step, the active BES1 was tagged with a puromycin resistance gene (PUR) downstream of the promoter. In silent BESs, transcription initiates at the BES promoter but is rapidly attenuated. This low level of transcriptional activity allowed the random integration of the neomycin resistance gene (NEO) immediately downstream of any “silent” BES promoter. PCR and pulsed-field gel electrophoresis genotyping showed that NEO had integrated at BES17. Because this double-tagged cell line actively expresses VSG221, we named it PN221. This reporter strain served two purposes: to eliminate cells that spontaneously silence either BES17 or BES1 and to place unique molecular markers into the residues of H2A, H2B, and H4. Accordingly, the genome of *T. brucei* contains candidates for multiple histone-modifying enzymes (reviewed in [19]), including two disruptor-of-telomeric silencing (DOT) methyltransferases, DOT1A and DOT1B, that are responsible for the methylation of H3K76 (corresponding to H3K79 in yeast and mammals). DOT1A is essential for viability and is responsible for dimethylation of H3K76, which is important for cell cycle regulation. DOT1B is not essential and exclusively trimethylates H3K76, a modification apparently required for complete differentiation into the insect stage of the life cycle in culture [20]. In this study, we examined the role of DOT1B in VSG transcriptional regulation. We found that DOT1B is required for complete BES silencing but not for regulating the expression of nontelomeric genes. When we selected for activation of a second BES in ΔDOT1B cells, we observed that many cells expressed two VSGs at their surface. With continuous selection, switching was eventually completed. We conclude that DOT1B is necessary for monoallelic VSG expression and rapid switching kinetics, which demonstrates the importance of a histone-modifying enzyme for VSG regulation.

**Figure 1.** Disruption of DOT1B Results in Derepression of Silent VSGs

(A) Schematic representation of PN221 genetic background, in which the actively transcribed BES1 is tagged with a PUR and the silent BES17 is tagged with NEO. VSG221 (MITat1.2) and VSG13 (MITat1.13) genes are located ~50 kb downstream of the promoter (arrow) and ~1 kb upstream of the telomere (circle).

(B) Transcript quantification by quantitative RT-PCR of WT PN221 and two ΔDOT1B PN221 bloodstream clones, grown in the presence of 1 μg/ml puromycin. VSG224 (MITat1.3), VSG800 (MITat1.18), VSGVO2 (MITat1.9), VSG13, and NEO are genes present in silent BESs, whereas VSG221 is actively transcribed in BES1. PAG3 is present in one procyclin locus, which is transcriptionally downregulated in bloodstream forms. β-Tubulin genes are present in a tandem array that is transcribed by RNA polymerase II. Actin was used as the reference gene. Error bars indicate the standard deviation of sextuplicates of the same cDNA sample.

(C) Relative enrichment of ΔDOT1B transcripts relative to the wild-type parental reference (average of four independent measurements). When column height is >1, ΔDOT1B transcript levels are higher than those in parental PN221.

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otherwise highly conserved DNA sequences that flank BES promoters.

PN221 and two clones of ΔDOT1B PN221 were grown for at least 5 d in the presence of a high concentration of puromycin. This treatment killed any cells that may have undergone a BES switch, ensuring that the active BES in the entire population was BES1. Real-time quantitative reverse-transcriptase PCR (RT-PCR) was used to measure the transcript levels of several genes. In the absence of DOT1B, the transcripts of four silent VSGs and NEO were approximately 10-fold higher compared to those of the parental cell line,
indicating that the absence of DOT1B reduces BES silencing (Figure 1B and 1C). The fact that both the promoter-proximal NEO and the telomeric VSG13 of BES17 showed a similar increase in transcript levels suggests that the entire BES17 is partially derepressed. No significant differences were found for the highly expressed VSG221, whose transcripts are 10^4–10^5 more abundant than silent VSG mRNAs. No changes were seen in mRNA levels of non-BES genes, such as actin and β-tubulin, which are transcribed by RNA polymerase II, or for procyclin-associated gene 3 (PAG3), whose transcription by RNA polymerase I is largely repressed at this stage of the life cycle [22].

Taken together, these results show that DOT1B is necessary for complete BES silencing. Derepression due to loss of DOT1B was not observed in a non-BES gene, which suggests that the absence of this methyltransferase affects only a subset of genes, including the VSGs.

**DOT1B Is Required for Monoallelic VSG Expression**

One of the mechanisms to change the VSG coat relies on transcriptional silencing of one BES and activating another. As the deletion of DOT1B affected VSG silencing, we hypothesized that BES switching might be affected by the absence of DOT1B. In vitro VSG switching occurs at a very low frequency, which means that the proportion of switchers of a population is so small that most methods cannot detect them. To overcome this problem, positive drug selection was used to select for cells that stochastically activated BES17. In the original PN221 reporter strain, the vast majority of cells are puromycin-resistant and G418-sensitive (unpublished data). When high concentrations of G418 (75 µg/ml) were added to 2 × 10^6 cells of 5 to 12 PN221 parental or ΔDOT1B subclones, most cells died. Approximately 8 G418-resistant clones were obtained per subclone after 1–2 wk of incubation, which suggests that parental and ΔDOT1B clones switch at a similar frequency. Nine days after adding G418, protein dot-blot analysis showed that all G418-resistant clones from parental cells, but only a fraction of ΔDOT1B clones, expressed VSG13 (Figure 2A). Three classes of ΔDOT1B clones were detected (Figure 2A): those that exclusively expressed VSG221 (Class I), those that expressed VSG221 and VSG13 (Class II), and those that exclusively expressed VSG13 (Class III). With continuous G418 selection, VSG221-expressing clones became VSG221/VSG13 double-expressing clones, and these eventually expressed only VSG13 (Figure 2B). These results indicated that ΔDOT1B cells are capable of switching from BES1 (VSG221) to BES17 (VSG13) at a similar frequency as wild-type cells, but the switchover proceeds more slowly.

To study VSG expression in individual cells, indirect immunofluorescence analysis (IFA) was performed using purified anti-VSG221 and anti-VSG13. IFA on a mixture of wild-type PN221 and PN13 cells confirmed that both anti-VSG sera are specific (Figure 2C, upper panel). All cells in ΔDOT1B Class II expressed VSG221, but some also expressed VSG13 (Figure 2C, middle and lower panels), indicating that the VSG coat is composed of more than one type of VSG. Both VSGs seem to be uniformly distributed over the cell surface. The number of detectable double-expressers varied with time and between clones, ranging from 10–60% of the total number of cells. From these results we conclude that DOT1B is required for monoallelic VSG expression.

The presence of two VSGs at the surface of a cell suggested that two BESs might be simultaneously active. Because both BESs were tagged with selectable markers, we could use high-level resistance to puromycin or G418 as a measure of the
transcriptional status of BES1 or BES17, respectively. Before selection with G418, VSG221-expressing parental and ΔDOT1B cells were puromycin-resistant and G418-sensitive, which indicated that BES1 was active and BES17 silent. After G418 selection, switched parental cells were sensitive to puromycin, but ΔDOT1B Class I and ΔDOT1B Class II cells were resistant. In fact, both classes of ΔDOT1B cells were able to grow in the presence of both puromycin and G418 with a doubling time (~7 h) only slightly longer than that of wild-type cells (Figure S1). In contrast, Class III cells were puromycin-sensitive. Pulsed-field gel electrophoresis and PCR analysis showed that no DNA rearrangements had occurred (unpublished data).

Taken together, these results (summarized in Table 1) show that DOT1B is necessary to maintain monoallelic expression in the cell population. In the absence of DOT1B, BES switching can still occur, but more slowly. A stable intermediate stage can be detected in which BES1 and BES17 are simultaneously active, conferring resistance to both drugs that DOT1B is necessary to maintain monoallelic expression in the cell population. In the absence of DOT1B, BES switching can still occur, but more slowly. A stable intermediate stage can be detected in which BES1 and BES17 are simultaneously active, conferring resistance to both drugs that DOT1B is necessary to maintain monoallelic expression in the cell population. In the absence of DOT1B, BES switching can still occur, but more slowly. A stable intermediate stage can be detected in which BES1 and BES17 are simultaneously active, conferring resistance to both drugs.

**Table 1. Phenotype of Wild-Type and ΔDOT1B Double-Tagged Clones**

| Cell Line          | VSG Expressed | Drug Resistance | Expression Status |
|--------------------|---------------|-----------------|-------------------|
| PN221              | VSG221        | +++             | Active            |
| PN13               | VSG13         | +               | Active            |
| ΔDOT1B PN221       | VSG221        | +               | Active            |
| ΔDOT1B Double-Expresser (Class I and II) | VSG221 and VSG13 | +++             | Active Range of activity |
| ΔDOT1B PN13 (Class II and III) | VSG13       | –               | Derepressed       |

To confirm that the levels of surface VSG and mRNA are correlated, RNA and protein levels in independent ΔDOT1B clones with variable levels of VSG13 expression were quantified by northern blotting and FACS analysis (Figure S3B). The correlation between VSG13 mRNA and surface protein levels was very high (+0.99), indicating that surface VSG staining reflects steady-state mRNA levels and suggests that the ΔDOT1B switching intermediates simultaneously transcribe two BESs.

**Partial BES Activation Is Stably Inherited**

After prolonged G418 selection, a population of cells exclusively expressing VSG13 appears among ΔDOT1B Class II clones, and this population eventually outgrows the double-expressing cells. To confirm that these pure VSG13 expressers originate from a double-expressing cell, FACS was used to sort only those cells that were brightly stained with both anti-VSG221 and anti-VSG13 (marked with a square in Figure 4A). These cells were subcloned and cultivated for 60 d in a high concentration of G418. FACs analysis showed that, in all subclones, a population of pure VSG13-expressers emerged between 8 and 24 d and gradually became more abundant, eventually leading to a homogeneous population of VSG13 expressers (Figure 4B). These results show that a ΔDOT1B double-expressing cell is capable of completing the switching process between BES1 and BES17, which confirms that a double-expresser is a switching intermediate.

To test whether partial activation of BES17 was dependent on drug selection, double-expressers were sorted, subcloned, and cultured without antibiotics for 30 d. Except for a larger
Pearson correlation between RNA and protein series is

VSG13 were selected based on the quantification by FACS analysis.

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independent

D

percentage of the levels in wild-type PN13 (sample #10). Five
measured by northern and FACS analysis and are indicated as a
(B) VSG

(D) DOT1B Class II clones (panels d and e), and one DOT1B Class III clone (panel f).

(B) VSG RNA and protein levels in multiple DOT1B clones were
measured by northern and FACS analysis and are indicated as a
percentage of the levels in wild-type PN13 (sample #10). Five
independent DOT1B Class II clones expressing different levels of
VSG13 were selected based on the quantification by FACS analysis.
Pearson correlation between RNA and protein series is +0.99.
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DOT1B Regulates Antigenic Variation in T. brucei

Other VSG Genes Remain 10-Fold Derepressed in Double
Expressers

If DOT1B only affects the kinetics of specific drug-selected
BES switching, then we expected VSG genes in other BES to
remain repressed in ADOT1B double-expressers. Transcript
levels of silent telomeric VSGs 224, 800, and VO2 were measured by quantitative RT-PCR. Three ADOT1B double-
expressing clones expressing different levels of VSG13 were
compared to ADOT1B single-expressers (one ADOT1B
PN221 and two ADOT1B PN13 clones). Figure 5 shows that
transcript levels of silent VSGs in the three ADOT1B double-
expressing clones were not significantly different from the
levels in the single-VSG-expressing clones ADOT1B PN221
and ADOT1B PN13. The control gene, PAG3, also remained
essentially unchanged. VSG221 transcript levels in ADOT1B
double-expressers were identical to those of ADOT1B PN221,
in agreement with the FACS data, which showed that BES1
remained fully active in these two types of clones (Figure 3A).
In contrast, in ADOT1B PN13, VSG221 transcripts were, on
average, $3 \times 10^4$-fold lower, which reflects the rapid silencing
of BES1. These results confirm that silent VSGs remain largely
repressed in ADOT1B double-expressers, displaying only the
basal 10-fold derepression described previously for ADOT1B
PN221 expressers (Figure 1).

Partially Active BES17 Is Not Found in a New
Extranucleolar Body

The active BES localizes in the extranucleolar ESB [10]. It
has been proposed that limited access to the ESB and its
singularity could be the reason why only one BES is fully
active. As ADOT1B double-expressers have two active BESs,
we asked whether a second ESB was present in these cells. To
count ESBS, we epitope-tagged RPB6z, a well-characterized
subunit of the RNA polymerase I complex that localizes in the
nucleolus and the ESB [23]. An N-terminally hemagglutinin
(HA)-tagged RPB6z has been shown to be fully functional in
the T. brucei procyclic form and to not interfere with Pol I
activity in vitro [24]. HA-RPB6z-tagged cell lines were
generated in parental and ADOT1B cells. PCR and western
blotting were used to confirm correct genomic integration
and expression levels (unpublished data). ADOT1B double-
expressers were subsequently selected with G418 and char-
acterized by FACS (unpublished data). We chose ADOT1B
double-expressing clones in which at least 40% of the cells
showed 40–50% of maximum VSG13 expression. IFA with an
anti-HA antibody revealed one nucleolus in practically all
double-expressers. The nature of a second extranucleolar
body was confirmed by FACS (unpublished data). We chose
ADOT1B double-expressers displaying only the
basal 10-fold derepression described previously for ADOT1B
PN221 expressers (Figure 1).

Figure 3. Two BESs Are Simultaneously Active in ADOT1B Double-

Expressers

(A) Living cells were stained with Alexa Fluor 488 anti-VSG221 and Alexa
Fluor 647 anti-VSG13 and analyzed by FACS. Intensities of VSG221 and
VSG13 staining are shown on the x and y axes, respectively. Percentages
refer to the proportion of the total cells in a given quadrant. Each panel
represents an independent clone: parental PN221 and PN13 clones
(pannels a and b), one ADOT1B Class I clone (panel c), two ADOT1B Class II
clones (panels d and e), and one ADOT1B Class III clone (panel f).

(B) VSG RNA and protein levels in multiple ADOT1B clones were
measured by northern and FACS analysis and are indicated as a
percentage of the levels in wild-type PN13 (sample #10). Five
independent ADOT1B Class II clones expressing different levels of
VSG13 were selected based on the quantification by FACS analysis.
Pearson correlation between RNA and protein series is +0.99.
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proportion of cells with undetectable levels of VSG13
between 5 and 30 d postsorting, the FACS profile of the
sorted cells (Figure 4C) was very similar to the profile of
the starting population (Figure 4A). When a high concentration
of G418 was added back to the medium, no cell death or
growth lag was observed, and cells continued to grow
exponentially, only slightly slower than in the absence of
drug (Figure 4D). These results showed that partial activation
of BES17 persisted and was stably inherited in the absence of
drug pressure.
that the partially active BES17 is located in an existing RNA Pol I site, such as the nucleolus or the ESB.

**Discussion**

Monoallelic transcription of a single member of a multigene family is a phenomenon seen in several eukaryotes. In mammals, for example, a single olfactory receptor gene is selected from a family of ~1,000 [25]. For several pathogenic protozoa, this mechanism is the basis for antigenic variation (reviewed in [26]). For example, the human malaria parasite *Plasmodium falciparum* transcribes only one of ~60 *var* genes on the surface of infected red blood cells [27]. To switch from one gene to another, most organisms use epigenetic transcriptional control mechanisms that are poorly characterized. In this study, we provide the first evidence that a well-characterized histone methyltransferase, DOT1B, affects antigenic variation in *T. brucei* on three levels: maintenance of complete BES silencing, rapid transcriptional switching between BESs, and, as a consequence, VSG monoallelic expression.

We previously reported that RT-PCR detected no VSG derepression in ΔDOT1B cells [20]. Here, we used a more sensitive approach, quantitative RT-PCR, to quantify the transcript levels of silent VSGs. In the absence of DOT1B, silent BESs were 10-fold derepressed in single- and double-expressers, indicating that DOT1B is required for maximum repression of silent BESs. Importantly, disruption of DOT1B does not seem to affect the expression of all genes: no changes were detected in the transcript levels of non-BES genes β-tubulin or actin, which are transcribed by RNA polymerase II, or PAG3, whose transcription by RNA polymerase I is partially repressed at this stage of the life cycle [22]. This selectivity is consistent with what has been described in *Saccharomyces cerevisiae*, in which loss of Dot1 only derepresses telomeric marker genes and—to a lesser extent—the HM loci [28]. In yeast, this derepression is most likely an indirect consequence of the redistribution of Sir proteins throughout the genome [2]. Although telomeric silencing has
been described in *T. brucei* [29], no causal proteins have been identified. Nevertheless, it is tempting to speculate that disruption of DOT1B causes a similar cascade of events in *T. brucei*, which results in an indirect derepression of telomeric BESs but not of other loci.

Although silent BESs are partially derepressed in ΔDOT1B cells, the number of G418-resistant clones arising is similar to that of wild-type cells, indicating that partial derepression of a silent BES does not affect the BES switching frequency. This is consistent with previous observations showing that the rate-limiting step of a BES switch is silencing of the previously active BES rather than activation of a new one [30,31]. After 1 wk of selection, all parental G418-resistant clones were puromycin-sensitive and exclusively expressed VSG13, indicating that BES1 was completely and rapidly silenced and BES17 was fully activated. In contrast, after the same time, mostΔDOT1B G418-resistant clones were resistant to both puromycin and G418 and expressed maximum levels of VSG221 and variable levels of VSG13, indicating that two BESs were simultaneously transcribed. To our knowledge, so far, this is the only gene whose disruption leads to the loss of monoallelic VSG expression.

A previous study showed, by using a double-BES-tagged cell line similar to ours, that double-resistant clones could be obtained in which ~65% of the cells simultaneously expressed two VSGs at the surface [12]. There are two important features that distinguish these cells from ΔDOT1B double-resistant clones. First, ΔDOT1B double-resistant clones are obtained by selecting for resistance to only one antibiotic (G418), whereas double-resistant wild-type clones could only be obtained by simultaneously selecting with two antibiotics. Second, ΔDOT1B double-resistant cells are stable and inheritable for at least 30 d in the absence of drug selection, whereas wild-type double-resistant clones rapidly lose double resistance and revert to a 50:50 mixture of trypanosomes with only one of the two marked BESs active. We propose, however, that both studies isolated a comparable switching intermediate, but whereas this is a transient state in wild-type cells, it is stable and inheritable in the absence of DOT1B.

On the basis of FACS and quantitative RT-PCR, we were able to quantify the activities of BES1 and BES17 in switching intermediates. Whereas BES1 remained 100% active, BES17 showed variable expression levels. Interestingly, we never found ΔDOT1B double-expressers that expressed wild-type levels of VSG13, indicating that, although the entire BES17 is transcribed, the level of transcription is not maximal. These results suggest an important VSG regulation mechanism that strictly prevents two BESs from being fully transcribed but that is permissive to one BES being fully active and another BES being partially active. We do not know how such a limitation is imposed, but it is possible that it relies on the competition for a limiting transcription factor present in the ESB [10]. This nuclear compartmentalization hypothesis is consistent with our RNA polymerase I localization studies. We observed no difference in the number of RNA Pol I sites between wild-type and ΔDOT1B cells, suggesting that there is a spatial restriction of Pol I transcription sites in the nucleus.

We speculate that BES17 is expressed either in the nucleolus or in the unique ESB, close to BES1. This model is consistent with what was observed in double-resistant wild-type clones, in which the two rapidly switching active BESs often were found in close proximity in the nucleus [12], and with the localization of a partially active BES at the periphery of the nucleolus [32]. Despite the localization in a Pol I competent compartment, BES17 is only partially activated, which suggests that another limiting factor is missing. We cannot exclude the possibility that the levels of expression of BES17 are too low to allow the detection of an additional RPB6z site, but this seems unlikely because, for these experiments, we chose ΔDOT1B double-expressers where at least 40% of the cells were displaying 40–50% of maximal VSG13 expression. If BES17 is transcribed by half the amount of Pol I complexes than BES1, then we should still be able to detect a bright spot.
After 8–24 d of continuous G418 selection, switching intermediates eventually express only VSG13, suggesting that the competition for a limiting factor is resolved in some double-resistant cells. Consequently, this leads to the rapid silencing of BES1 and full activation of BES17. BES switching is most likely a complex mechanism that involves several events in a precise order. Nothing is known about the intermediates of a BES switch, which means that we cannot exclude the possibility that double-resistant cells are not a switching intermediate but simply a fraction of the population selected by drug pressure. If the latter was true, then one would predict that the number of G418-resistant clones would be higher in ADOT1B because the drug would select not only for VSG13 expressers but also for the fraction of cells with higher transcription levels in BES17. Also, if drug double-expressers were a product of drug selection, then, when pressure is removed, one would predict that the double-resistance phenotype should be lost, as demonstrated by [12] and discussed above. None of these predictions is consistent with our data, suggesting that double-expressers are more likely switching intermediates and, consequently, DOT1B is involved in the kinetics of switching.

The VSG profile detected by FACS indicates that ADOT1B double-expressers have the same amount of VSG221 molecules at the surface as a VSG221 expresser and an extra 5–90% of the total VSG13 usually present in a VSG13 expresser. As VSG molecules form a tightly packed surface coat, we speculate that the volume of the cells might be slightly larger to accommodate the excess of VSG molecules. A similar observation was made previously, when two VSGs were simultaneously expressed at the same levels as one normally is [39]. By means that we can only speculate upon, the cells seem able to accommodate up to twice as much VSG expression.

Several studies suggest that silencing of a previously active BES and activation of a new BES are tightly coupled (reviewed in [34]), which predicts the existence of a sensing mechanism that facilitates cross-talk between BESs. It remains unclear what step of switching is affected in ADOT1B: activation of BES17 or silencing of BES1. If DOT1B is a transcription activator, as shown in cancer cells [7], then its absence may interfere with BES17 activation. In this scenario, double-resistant cells are a consequence of a defective BES17 activation that leads, due to incomplete cross-talk between BESs, to BES1 remaining fully active. Alternatively, the effects of DOT1B depletion may be an indirect consequence of a disruption of telomeric silencing. In this case, the absence of DOT1B may interfere with silencing of BES1, and by cross-talk, a second BES is temporarily prevented from being fully activated. Interestingly, no population of ADOT1B double-expressers ever was found with intermediate levels of VSG221, indicating that, even in the absence of DOT1B, BES1 either remains fully active or becomes fully silenced. These results indicate that DOT1B is not implicated in the all-or-nothing property of the machinery that silences a previously active BES.

BES switching probably requires the ordered recruitment of complex machinery that recognizes a specific chromatin configuration and remodels it to establish a new chromatin structure in the two involved BESs. In this study, we showed that a histone-modifying enzyme is part of this machinery, which unveils the importance of chromatin modifications in antigenic variation of African trypanosomes.

Materials and Methods

Strains and media. T. brucei bloodstream-form parasites (strain Lister 427, antigenic type MiTat1.2, clone 221a) [35] were cultured at 37 °C in HMI-9 medium [36]. The “single maker” cell line expresses PCNA and the tet repressor and expresses detectable epitopes under control of the T7 promoter and tet operator [37]. Stable transfections were performed as described using either a BTX electroporator [38] or an AMAXA Biosystems nucleofector. In the latter, 40 million cells were resuspended in T-cell solution and transfected using program X-001. DOT1B alleles (Accession Number: XM_001218759) were deleted using a PCR-based approach that conferred resistance to hygromycin and phleomycin as described previously [39]. Loss of both alleles was confirmed by PCR and western blotting with an anti-H3K79me3 antibody [20]. In drug sensitivity assays, 104 cells were diluted in 10 ml of culture medium containing 40 μg/ml of G418 for 48 h. Cell growth was determined 48 h later. To measure population doubling time, cell growth was monitored for at least 8 d under similar conditions.

Generation of a switching-reporter strain and tagged proteins. An in situ switching reporter strain was derived from wild-type clone 221a, in which PUR and NEO were integrated downstream of different BES promoters. Integration of PUR in BES1 was obtained by transfecting 221a cells with a construct (pLF12) consisting of three fragments cloned into pBluescriptSK+ in the following 5’ to 3’ order: the upstream promoter region (UP), PUR box, and the Aladlospe acceptor site at 3’ UTR, and the downstream promoter region (DP) (Figure S3). UP is the 1590-bp fragment spanning from the 50-bp repeat array to 240 bp downstream of the ES transcription start site. DP is the 1150-bp region spanning from 287 bp downstream of the ES transcription start site to ESAG7. The BES1/PUR cell line was subsequently transfected with pLF13, which introduced NEO in an identical position of a second random BES. After multiple clones were generated, the cell line chosen as in situ switch reporter integrated PUR at the actively transcribed BES1 and NEO at the silent BES17. Because it expresses VSG221, this cell line was named PN221.

An influenza HA tag was introduced at the 5’ end of one of the RPB6z alleles by transfection with pLP96, derived from a pPURO-HA-RPB6z construct [24] in which the PURO cassette was replaced by a HindIII to SacI fragment encoding blasticidin resistance, flanked upstream by the hsp70 intergenic region and downstream by the β-tubulin intergenic region. HA-RPB6z-tagged cell lines were generated in single marker and ADOT1B backgrounds. Single marker was preferred over wild-type 221 for future uses of the tagged cell line.

Real-time quantitative RT-PCR and northern blot analysis. Total RNA was extracted from ~109 bloodstream-form cells with RNA STAT-60 (TEL-TEST, Inc.) following the manufacturer’s instructions. Approximately 20 μg of RNA was DNAse-treated, phenol-chloroform-extracted, and ethanol-precipitated. cDNA was synthesized with oligo(dT)18 primers using the Stratascript First Strand Synthesis System (Stratagene). Approximately 5 μl of three serial dilutions of cDNA from 1:100, and 1:1000 was used for quantitative RT-PCR with SYBR green (Applied Biosystems). Amplification reactions were performed in duplicates in an Applied Biosystems 7900HT. Primer sequences are listed in Table S1. Relative levels of gene expression were measured using the comparative cycle threshold Ct method (User Bulletin 2, ABI, http://www.appliedbiosystems.com), using β-tubulin or actin as reference genes. For northern analysis, 3 μg of total RNA was resolved on a 1.5% formaldehyde agarose gel, transferred to a nylon membrane, and detected with a random primer-labelled specific probe. Sequences are available upon request.

Anti-VSG antibodies. Anti-VSG13 (also known as MITat1.13) serum was obtained by immunizing a rabbit with purified native VSG13, as described previously [40]. Rabbit anti-VSG13 and rabbit and chicken anti-VSG221 (MITat1.2) were depleted of glycosylphosphatidylinositol-related VSG cross-reacting-determinant (CRD) antibodies by passing through an affinity column of a different VSG [41]. For FACS, live cells were stained with affinity-purified antibodies directly conjugated to Alexa Fluor 488 (anti-VSG221) or Alexa Fluor 647 (anti-VSG13).

VSG immunodetection. VSG expression was assayed by dot blotting. Briefly, 2–3 × 105 trypanosomes were concentrated into 10 μl of HMI-9 medium by centrifugation at 2,500g for 4 min. Approximately 0.2 μl of cell suspension was dropped onto a 22 mm ECL membrane (Amersham), previously incubated in PBS pH 7.2 for 5 min. After being air-dried for 5 min, the membrane was processed as for western blotting. Primary anti-VSG antibodies were detected

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Amanitin inhibits transcription by RNA Pol II and III, allowing the exclusive detection of RNA polymerase I transcripts. DNA was detected with DAPI (blue). Scale bar: 2 μm.

(B) Quantification of Pol I transcript foci in parental and ADOT1B double-expressor clones, in different stages of the cell cycle. An average of 40 cells/clone was counted.

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Figure S3. Constructs to Generate Reporter Strain pLF12 and pLF13 constructs used to introduce drug-selectable markers into BESs. Dashed lines represent the targeting sequences UP and DP. Gray boxes represent the aldolase splice-acceptor site and 3' UTR. pLF12 and pLF13 differ only in the drug-selectable marker. Genotyping of clones obtained after transfection of both plasmids into wild-type 221 cells led to the identification of the PN221 cell line in which pLF12 had targeted the actively transcribed BES1 and pLF13 the silent BES17.

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Table S1. Primer Sequences Used for Quantitative RT-PCR

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Author contributions. LMF conceived and designed the experiments. LMF, CJJ, and GAMC performed the experiments. LMF, CJJ, and GAMC wrote the paper.

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