Molecular Cell *23*, 497–507, August 18, 2006 ©2006 Elsevier Inc. DOI 10.1016/j.molcel.2006.06.027

Selective Di- or Trimethylation of Histone H3 Lysine 76 by Two DOT1 Homologs Is Important for Cell Cycle Regulation in *Trypanosoma brucei*

Christian J. Janzen,[1] Sandra B. Hake,[2] Joanna E. Lowell,[1] and George A.M. Cross[1,*]
[1] Laboratory of Molecular Parasitology
[2] Laboratory of Chromatin Biology
The Rockefeller University
New York, New York 10021

Summary

DOT1 is an evolutionarily conserved histone H3 lysine 79 (H3K79) methyltransferase. K79 methylation is associated with transcriptional activation, meiotic checkpoint control, and DNA double-strand break (DSB) responses. *Trypanosoma brucei* has two homologs, DOT1A and DOT1B, which are responsible for dimethylation and trimethylation of H3K76, respectively (K76 in *T. brucei* is synonymous to K79 in other organisms). K76 dimethylation is only detectable during mitosis, whereas trimethylation occurs throughout the cell cycle. Deletion of DOT1B resulted in dimethylation of K76 throughout the cell cycle and caused subtle defects in cell cycle regulation and impaired differentiation. RNAi-mediated depletion of DOT1A appears to disrupt a mitotic checkpoint, resulting in premature progression through mitosis without DNA replication, generating a high proportion of cells with a haploid DNA content, an unprecedented state for trypanosomes. We propose that DOT1A and DOT1B influence the trypanosome cell cycle by regulating the degree of H3K76 methylation.

Introduction

A mounting number of histone posttranslational modifications (PTMs) has been described in recent years. The functions of many of these PTMs, and the enzymes that are responsible for them, are being intensively studied (reviewed in Fischle et al. [2003] and Lachner et al. [2003]). Analysis of the impact of histone PTMs on chromatin structure and gene regulation led to the "histone code" hypothesis, in which histone PTMs establish epigenetic information that can be propagated from one generation to the next (Strahl and Allis, 2000).

DOT1 was discovered in a screen to identify genes that interfere with transcriptional silencing at telomeres in *Saccharomyces cerevisiae* (Singer et al., 1998). Deletion or overexpression of *DOT1* disrupts silencing at telomeres and HM loci, and overexpression interferes with silencing at the rDNA array (Singer et al., 1998; van Leeuwen et al., 2002). Dot1p exhibits methyltransferase activity toward histone H3 lysine 79 (H3K79), a residue located in a loop within the histone-fold domain (Feng et al., 2002; Lacoste et al., 2002; Ng et al., 2002; van Leeuwen et al., 2002). H3K79 methylation is thought to prevent the formation of heterochromatin, as disruption of *DOT1* or mutation of H3K79 increases the interaction of silencing proteins Sir2 and Sir3 with chromatin in vivo (van Leeuwen et al., 2002). Methylation of K79 is also enriched on H3.3, a histone variant found at transcriptionally active loci in *Drosophila* (McKittrick et al., 2004).

Dot1p is also important for meiotic checkpoint control in yeast, ensuring proper chromosome segregation. In the absence of *DOT1*, *zip1* or *dmc1* mutants, which show chromosome segregation defects, inappropriately progress through meiosis and generate inviable meiotic products (San-Segundo and Roeder, 2000). Recent studies have also suggested that H3K79 methylation is directly involved in the detection of DSBs (Game et al., 2005; Giannattasio et al., 2005; Huyen et al., 2004; Wysocki et al., 2005). Binding of the conserved checkpoint protein 53BP1 to methylated H3K79 seems to be an early event by which eukaryotic cells sense and respond to DSBs (Huyen et al., 2004). In yeast, the loss of H3K79 methylation prevents phosphorylation of the 53BP1 homolog Rad9, which is required to recruit and activate the Rad53 kinase (Giannattasio et al., 2005; Wysocki et al., 2005). Activation of Rad53 is a key step in the signal transduction cascade that is routinely used as a marker for DSB checkpoint activation. Although the role of Dot1p in cell cycle checkpoints after DNA damage has been investigated thoroughly, Dot1p has not been implicated in normal cell cycle progression. However, Dot1p homologs appear to be essential for normal growth in mammalian cells and in *Drosophila* (Okada et al., 2005; Shanower et al., 2005).

Little is known about chromatin structure, epigenetic regulation, and cell cycle control in *Trypanosoma brucei*, the unicellular parasitic protozoan that is responsible for African Sleeping Sickness. Studies in our laboratory suggest that epigenetic mechanisms regulate expression of variant surface glycoproteins (VSGs), the major surface proteins of African trypanosomes (reviewed in Cross et al. [1998]). By regularly switching the VSG, the trypanosome population escapes the host's immune system. The active VSG gene is transcribed from 1 of ~20 essentially identical telomere-proximal expression sites (ESs) (reviewed in Barry and McCulloch [2001]). Trypanosome telomeres repress the expression of reporter genes, and this position effect might contribute to the regulation of telomeric VSG genes (Horn and Cross, 1995). After parasite ingestion by *Glossina*, the tsetse, the bloodstream form differentiates to the procyclic stage, in which all ESs are transcriptionally repressed. Although some studies have hinted that chromatin remodeling may be involved in developmental ES silencing (Navarro et al., 1999), only few functional studies of chromatin remodeling or histone-modifying enzymes have been reported (Garcia-Salcedo et al., 2003; Ingram and Horn, 2002), and histone PTMs have only recently been identified in trypanosomes (Janzen et al., 2006).

Four potential histone deacetylases (DAC1–4) have been characterized in *T. brucei* (Ingram and Horn, 2002). Neither enzymatic activity nor specificity was addressed, but DAC4-negative parasites displayed a delay in G2/M phase of the cell cycle, suggesting that histone

*Correspondence: george.cross@rockefeller.edu

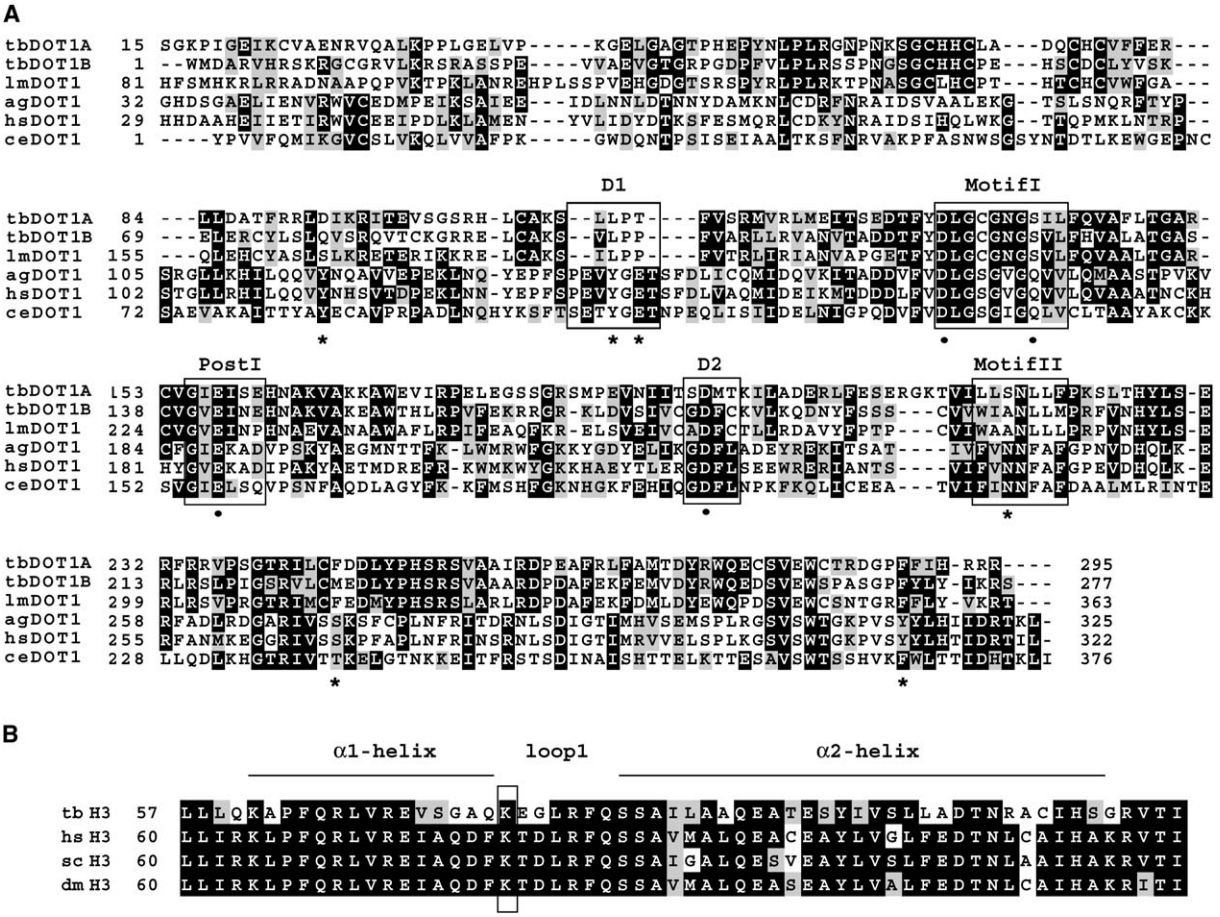

Figure 1. Amino Acid Alignment of the Conserved DOT1 Core Region

(A) The alignment includes selected members of the DOT family: *tb*DOT1A (*T. brucei*, Tb08.26N11.380), *tb*DOT1B (*T. brucei*, Tb927.1.570), *ag*DOT1 (*Anopheles gambiae*, EAA03558), *hs*DOT1 (*Homo sapiens*, NP_115871), and *ce*DOT1 (*Caenhorabditis elegans*, NM_058569). Secondary structural elements (motif D1, Motif I, Post I, D2, and Motif II) are indicated above the aligned sequences and marked by boxes. Black background indicates sequence identity; gray background indicates sequence similarity. Dots below the sequence indicate amino acids involved in SAM binding; asterisks indicate amino acids that might interact with the lysine target.

(B) Alignment of the H3 core region from various species: *tb*H3 (*T. brucei*, Q810K5), *hs*H3 (*H. sapiens*, P16106), *sc*H3 (*Saccharomyces cerevisiae*, P02303), and *dm*H3 (*Drosophila melanogaster*, P16106). α1 helix, loop1, and α2 helix are indicated above the sequence. Backgrounds indicate sequence identity and similarity as above. Lysine K79 (K76 in *T. brucei*) is boxed. Alignments and shadings were performed with ClustalW 1.74 and Boxshade 3.2, respectively (http://www.ch.embnet.org).

acetylation is important for efficient cell cycle progression. The presence of a potential *DOT1* homolog in trypanosomes was recognized when *DOT1* was characterized in yeast (van Leeuwen et al., 2002). We have identified two homologs, which we refer to as DOT1A and DOT1B. Using gene disruption, RNA interference (RNAi), antibodies, and mass spectrometry, we demonstrate that DOT1A is essential for viability and is responsible for the dimethylation of H3K76, which is synonymous to H3K79 in other organisms. In contrast, DOT1B is not essential for viability and exclusively trimethylates H3K76. The possible functions of DOT1A and DOT1B in cell cycle control and developmental differentiation will be discussed.

Results

DOT1A and DOT1B Are Homologs of DOT1

A BLASTp search of the *T. brucei* genome revealed two genes encoding proteins with predicted molecular masses of 33 and 31 kDa, with significant similarity to the catalytic domain of human DOT1 (Figure 1A). We named them DOT1A and DOT1B (for the 33 kDa and the 31 kDa polypeptide, respectively). *Trypanosoma cruzi* and *Leishmania major* also contain both homologs. Alignment of DOT1A and DOT1B with other members of the DOT1 family revealed conserved motifs that are important for substrate binding and for interaction with H3K79 (Min et al., 2003). One of the most conserved sequences is the DxGxGxG motif I, which is found in S-adenosyl-L-methionine (SAM) binding proteins (Figure 1A, Motif I). Motif I forms the SAM binding pocket together with other conserved motifs called post-I, II, D1, and D2 (Min et al., 2003). D1 and D2 seem to be characteristic for DOT1 proteins, and D1 is also thought to form part of the lysine binding channel. I, post-I, and II motifs are also found in other SAM binding methyltransferases. Most of the amino acids that bind directly to SAM are conserved in both trypanosome homologs (Figure 1A, dots), but the conserved glutamine in motif I has been replaced by

serine, and D1 appears to be absent from DOT1A and DOT1B. Based on the crystal structure of the yeast Dot1p (Sawada et al., 2004), other conserved residues have been proposed to be involved in H3K79 interaction (Figure 1A, asterisks), but these residues are not conserved in the trypanosome DOT1 homologs. This is not surprising, because the structure of the binding site is most likely dependent on the residues adjacent to K79. The putative target for the trypanosome DOT1 homologs (K76) lies at a similar position in a short loop between the α1 and α2 helices (Figure 1B), based on the nucleosome structure (Luger et al., 1997), but the flanking amino acids are not conserved.

DOT1B Is Essential for Developmental Transitioning to Procyclic Forms

To examine the function of DOT1A and DOT1B, we tried to generate DOT1A- or DOT1B-deficient bloodstream trypanosomes by replacing both alleles of each gene with selectable markers. We could not delete both DOT1A alleles, suggesting that DOT1A is essential, which was confirmed by RNAi experiments (see below). In contrast, ΔDOT1B cells were viable and morphologically indistinguishable from wild-type cells, and growth rates of two independent ΔDOT1B clones were identical to wild-type cells. Although DOT1 is involved in telomeric silencing in yeast (Singer et al., 1998), we could not detect transcripts from inactive telomeric VSG genes in ΔDOT1B cells by RT-PCR (data not shown), suggesting that DOT1B is not involved in VSG repression in bloodstream-form trypanosomes.

Bloodstream-form trypanosomes can be differentiated to procyclic (tsetse midgut) forms in vitro by dropping the temperature from 37°C to 20°C–27°C and adding *cis*-aconitate (Brun and Schonenberger, 1981; Engstler and Boshart, 2004). When wild-type or DOT1B single-allele knockout cells (DOT1B$^{+/-}$) were exposed to the differentiation signals, they grew normally after a 24 hr lag that is typical for this strain (Figure 2A). In contrast, two ΔDOT1B cell lines suffered growth arrest and died after several days. Cell cycle profiles of arrested ΔDOT1B cells, determined by flow cytometry, were indistinguishable from those of growing wild-type cells (data not shown), suggesting that the growth was not arrested at a specific phase of the cell cycle. One of the early events of differentiation is the downregulation of VSG and upregulation of procyclic-specific surface proteins (Roditi et al., 1989). To test if DOT1B plays a role in the initiation of differentiation, we analyzed mRNA levels of the active VSG and the Procyclin EP1-2 gene (Figure 2B). In both wild-type and ΔDOT1B cells, the active VSG was downregulated rapidly and EP1-2 mRNA was readily detectable after 24 hr (Figure 2B), suggesting that the initiation of differentiation was not affected in ΔDOT1B cells. We analyzed mRNAs of other genes that are known to be induced upon differentiation (ZFP1, COXVI, COXII, and CAP5.5) by quantitative real-time RT-PCR (data not shown). These mRNAs were equally abundant in wild-type and ΔDOT1B cells, suggesting that developmental regulation of stage-specific gene expression is not impaired in ΔDOT1B trypanosomes.

A possible explanation for the growth arrest might be that DOT1B is essential in procyclic forms, but this is not the case because it was possible to delete both DOT1B

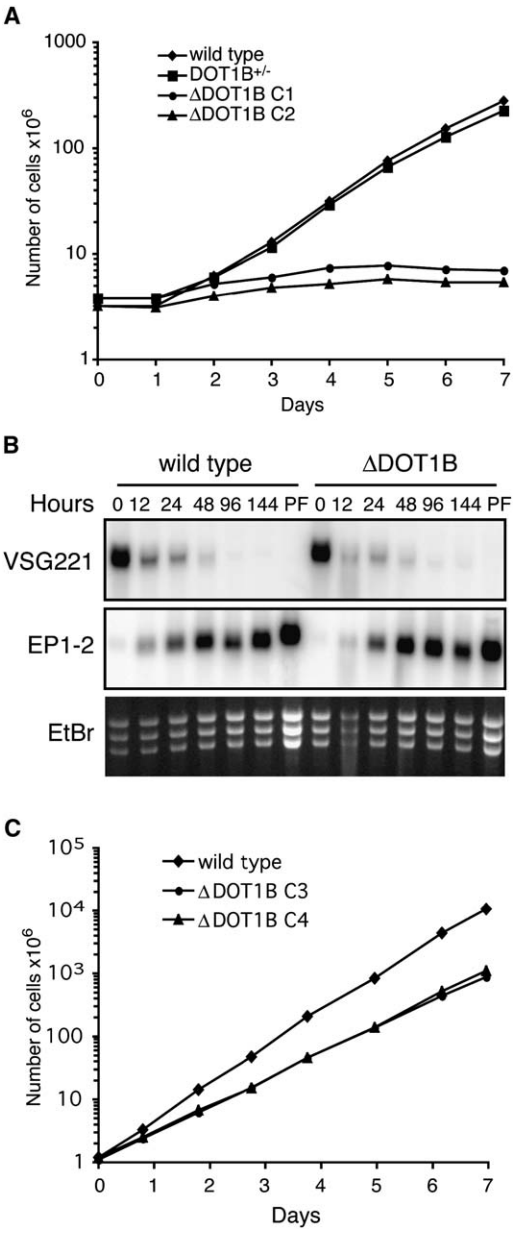

Figure 2. DOT1B Is Essential for Differentiation

(A) Cumulative growth after inducing differentiation of wild-type (diamonds), DOT1B single-allele knockouts (squares), and two ΔDOT1B cell lines (circles and triangles) of bloodstream to procyclic forms in vitro.

(B) Changes in stage-specific mRNAs during differentiation. VSG (VSG221) and Procyclin (EP1-2) mRNAs were monitored by Northern blot in wild-type and ΔDOT1B cells. Ethidium bromide staining (EtBr) of the agarose gel served as loading control.

(C) Cumulative growth of wild-type (diamonds) and two ΔDOT1B procyclic cell lines (circles and triangles).

alleles in an established procyclic cell line (Figure 2C). However, the population doubling time of the ΔDOT1B procyclic cells was ~4 hr longer than that of wild-type cells (Figure 2C, ΔDOT1B C3 and C4).

DOT1B Directs the Trimethylation of H3K76

To test the hypothesis that DOT1B is an H3K76-specific methyltransferase, we purified histones from wild-type

and ΔDOT1B procyclic forms, to compare the degree of H3K76 methylation by mass spectrometry. Wild-type H3 revealed two major ions that matched the predicted mass of peptide 70-VSGAQKE-78 (718 Da) plus an additional 28 or 42 Da, indicating the presence of two or three methyl groups, respectively (Figure 3A, top left). Sequencing of the two peptides by tandem mass spectrometry (MS/MS) confirmed their identity and mapped the modification to K76 (Figure 3A, top right). The peak corresponding to H3K76me3 was undetectable in H3 from ΔDOT1B trypanosomes (Figure 3A, bottom left). The identity of the 746.4 Da peptide in the ΔDOT1B trypanosomes was confirmed by MS/MS (Figure 3A, bottom right). These data suggest that DOT1B is exclusively responsible for the trimethylation of H3K76. Unmodified or monomethylated peptides were not detected, which suggests that these species are either absent or minor. To prove that these marks would be detectable, we performed MS analysis on synthetic tri-, di-, mono-, and nonmethylated peptides (Figure S1 in the Supplemental Data available with this article online). Although the signals from di-, mono-, and nonmethylated peptides were weaker, they were clearly detectable.

To confirm the different methylation states of H3K76 and to explore the function of the different methyl marks, we generated polyclonal antibodies against di- and trimethylated peptides. After affinity purification, both antibodies detected one major band of 15 kDa on Western blots of whole-cell lysates and reacted with HPLC-purified trypanosome H3 (data not shown). Antibody specificity was confirmed by peptide competition assays (Figure 3B). Only the dimethylated peptide could eliminate the signal of the dimethyl-specific antibody, and only the trimethylated peptide could compete with the trimethyl-specific antibody. The specificity was also confirmed by ELISA (data not shown).

Western blot analysis of lysates from wild-type cells, single-allele knockouts (DOT1B$^{+/-}$), and two clones of ΔDOT1B trypanosomes were performed with both antibodies. H3K76me3 could not be detected in the ΔDOT1B cell lines, confirming the MS results (Figure 3C, middle). In wild-type cells, only a faint band was visible with the dimethyl antibody (Figure 3C, top). This signal increased slightly in DOT1B$^{+/-}$ cells and strongly in two independent ΔDOT1B clones. These data demonstrate that K76 is di- and trimethylated in *T. brucei* and confirm that DOT1B exclusively directs tri-methylation.

To further confirm that DOT1A and DOT1B are directly responsible for K76 methylation, we tested the activity of recombinant DOT1A and DOT1B in vitro. Mononucleosomes purified from ΔDOT1B trypanosomes, and therefore dimethylated or RP-HPLC-purified histone H3, were incubated with recombinant GST, GST-DOT1A, or GST-DOT1B fusion proteins (Figure 4). DOT1B completely converted dimethylated mononucleosomes to the trimethylated state. As with DOT1 in other organisms (Feng et al., 2002; Lacoste et al., 2002; Ng et al., 2002; van Leeuwen et al., 2002), purified H3 was not a substrate for DOT1A or DOT1B. Dimethylation of nucleosomes by DOT1A could not be demonstrated in vitro because unmodified nucleosomes do not appear to exist in wild-type cells, nucleosomes from ΔDOT1B are dimethylated, and ΔDOT1A cells are not viable.

Dimethylated H3K76 Is a Marker for Mitosis

Two interesting questions arose from the Western blot analysis (Figure 3C): what is the source of the faint dimethyl signal in wild-type cells, and why does the intensity of the dimethyl mark increase in ΔDOT1B trypanosomes? To address these questions, we first examined whether the intensity or nuclear distribution of H3K76me2 might be cell cycle regulated. *T. brucei* cannot be synchronized, but the cell cycle position of individual cells is easily determined by structural markers, including the number and position of the kinetoplast, a discrete physical structure that represents the mitochondrial genome, the number and shape of the nuclei, and the shape of the parasite itself (Robinson et al., 1995; Sherwin and Gull, 1989; Woodward and Gull, 1990). The divisions of the kinetoplast and the nucleus are tightly coordinated. In procyclic-form trypanosomes, the kinetoplast replicates first and starts to divide while the nucleus is still in G2, and kinetoplast segregation is completed before the nucleus enters mitosis. In early mitosis, the kinetoplasts are clearly separated from each other and the nucleus is elongated. During mitosis, one kinetoplast and the daughter nucleus migrate to the posterior of the cell.

We monitored the H3K76me2 mark during cell cycle progression. Dimethylated K76 could not be detected during G1 or G2 (Figure 5A, G1 and G2) but was first observed at the beginning of mitosis (Figure 5A, early mitosis). The dimethylation signal increased during progression through mitosis and remained detectable after karyokinesis (Figure 5A, mitosis and postmitosis). Because H3K76me2 was only detectable in mitotic cells, we concluded that this modification plays a specific role during mitosis. In contrast, the H3K76me3 mark was present throughout the cell cycle (Figure 5B), with no obvious variation in signal intensity (Figure 5B).

As expected, H3K76me3 was undetectable in ΔDOT1B cells (Figure 5D): faint signals in some G1-phase cells were attributed to nonspecific crossreactivity. In contrast to wild-type cells, a strong cell cycle-independent dimethylation signal was detectable in every ΔDOT1B trypanosome (Figure 5C). In wild-type cells, about 20% of the population is in mitosis/cytokinesis (reviewed in McKean [2003]). Because H3K76 is dimethylated in all of the ΔDOT1B mutants, they should have at least 5-fold more dimethylated H3K76 compared to wild-type cells, which explains the increase of the dimethyl signal intensity in the Western blot analysis (Figure 3C). The ΔDOT1B population contained aberrant cells, including ~5% of anucleated parasites called "zoids" (Figure 5C, top, asterisk), which also occur in trypanosomes compromised in mitosis after rhizoxin treatment (Robinson et al., 1995), or under adverse growth conditions (Cross and Manning 1973), but never in healthy wild-type cells. About 1% of the cells had three or more nuclei (Figure 5C, middle). Both observations suggest that a proportion of the cells failed to coordinate mitosis and cytokinesis, which is consistent with the reduced growth rate of the ΔDOT1B population. This defect is apparent in another example of a cell that has two nuclei (Figure 5C, bottom, asterisk), most likely from the previous cell cycle, although one dividing kinetoplast would place it in G2. These data suggest that ΔDOT1B trypanosomes can be impaired in cell cycle

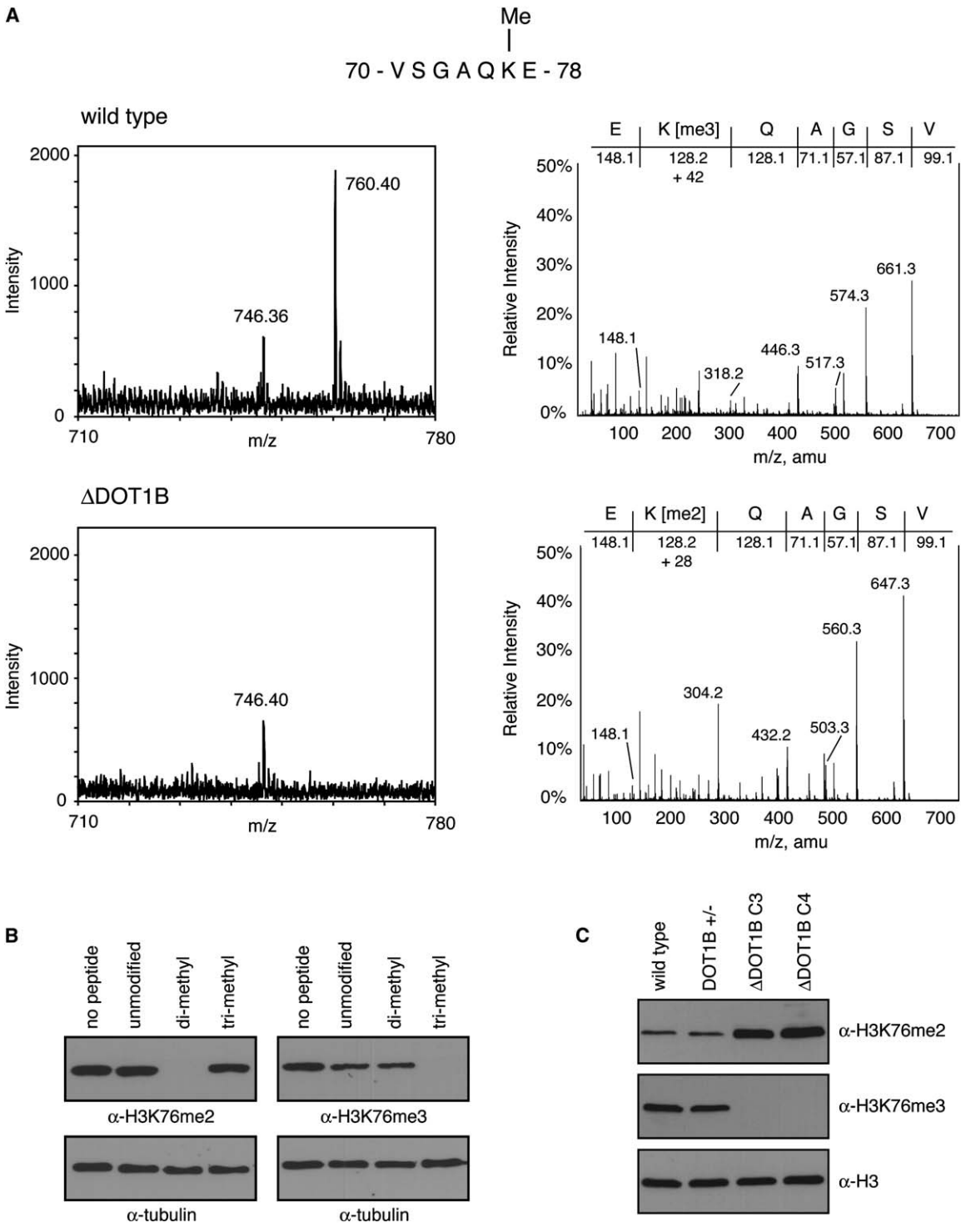

Figure 3. DOT1B Directs Trimethylation of H3K76

(A) Mass spectrometry. Purified H3 from wild-type (top panels) and ΔDOT1B procyclic cells (bottom panels) was digested with Endoproteinase Glu-C and peptide 71–77 subjected to MALDI-TOF spectrometry (left panels). The 760.4 Da and 746.4 Da peptides of wild-type and ΔDOT1B cells, respectively, were sequenced by tandem mass spectrometry (right panels).

(B) Western blot analysis on whole trypanosome extracts using the di- or trimethyl-specific antibodies and peptide competitors. An anti-tubulin antibody was used to monitor loading.

(C) Western blot analysis of the methylation state of K76 in wild-type, DOT1B single knockout, and two ΔDOT1B cell lines. An α-H3 peptide-specific antibody (AbCam 1791) was used to monitor loading.

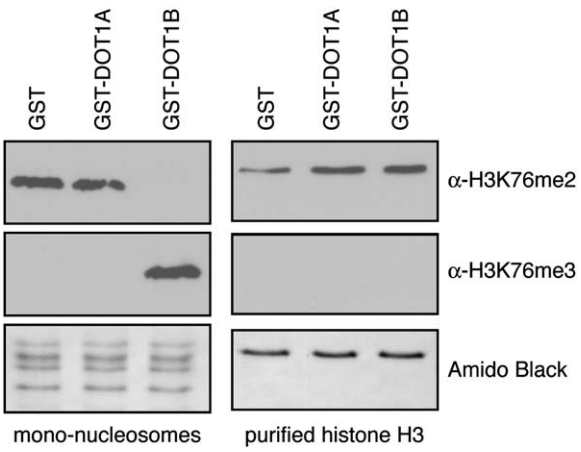

Figure 4. Recombinant DOT1B Methylates Mononucleosomes In Vitro

About 0.5 μg of recombinant GST, GST-DOT1A, or GST-DOT1B was incubated with mononucleosomes (left panels) or RP-HPLC-purified histone H3 (right panels). Methylation was evaluated by Western blot analysis using dimethyl (top panels) or trimethyl antibodies (middle panels). Equal input was monitored by Amido Black staining (bottom panels).

regulation, even though the majority of the population survives without DOT1B. The impairment could be due to lack of H3K76me3 or the consequent constitutive dimethylation.

DOT1B Expression Is Controlled by Conserved Sequences in the 5′ UTR

We failed to rescue the ΔDOT1B phenotypes using a standard inducible expression vector (pLew100), in which *DOT1B* was flanked by UTRs that confer high constitutive expression levels (Wirtz et al., 1999). Induction was lethal in procyclic forms (data not shown), suggesting that DOT1B expression has to be regulated carefully. Little is known about mRNA regulation in *T. brucei,* but an octamer consensus sequence ([C/A]ATAGAA[G/A]) that is responsible for cell cycle regulation of several mRNAs has been described in the related organism *Crithidia fasciculata* (Brown and Ray, 1997; Pasion et al., 1996). We identified two similar motifs in the 5′ UTR of *DOT1B* (Figure 6A), prompting us to generate an expression construct encoding an epitope-tagged DOT1B flanked by the entire native *DOT1B* 5′ and 3′ UTRs. After transfection of a ΔDOT1B cell line, H3K76me3 was restored to a level comparable to wild-type cells in two independent clones (Figure 6B, middle). We also observed a decrease in dimethylation in the same cell lines (Figure 6B, top), suggesting that the conserved sequences in the 5′ UTRs are important for correct DOT1B expression. An enzymatically inactive DOT1B failed to complement the ΔDOT1B phenotype. We could not confirm that the epitope-tagged DOT1B was expressed in a cell cycle-dependent manner, because the expression levels were too low for immunofluorescence detection.

Dimethylation of H3K76 by DOT1A Is Important for Cell Cycle Control in Bloodstream Forms

We wanted to verify that DOT1A is responsible for K76 dimethylation. As discussed earlier, this could not be addressed by biochemical methods. Because DOT1A is essential, we used an inducible RNAi system to knock down DOT1A mRNA. After 24 hr of RNAi induction, the trypanosomes stopped growing and subsequently died (Figure 7A). After 4 days, a subpopulation of RNAi-resistant cells emerged, as frequently happens with the inducible RNAi system. We analyzed cells 12 and 24 hr after induction, to avoid secondary effects in dying cells. RNA analysis confirmed that the growth phenotype coincided with DOT1A mRNA depletion (Figure 7B). A minor decrease of DOT1A mRNA was already detectable in uninduced cells, which is probably attributable to leaky transcription from the RNAi construct (Figure 7B, top). However, dsRNA accumulated markedly after 12 hr, and DOT1A mRNA was barely detectable after 24 hr. Induction of DOT1A dsRNA had no detectable effect on DOT1B mRNA levels, after adjustment for the slight variation in RNA loading demonstrated by the tubulin-specific signal and the ethidium bromide staining of ribosomal RNA (Figure 7B, bottom two panels). In parallel, we harvested cells for Western blot analysis to reveal the methylation status of H3K76. Twenty-four hours postinduction, H3K76me2 decreased drastically (Figure 7C, top). In contrast, the trimethylation signal did not decrease, supporting our previous findings that trimethylation of H3K76 only requires DOT1B. Antibodies specific for *T. brucei* histone H2AZ (Lowell et al., 2005) and tubulin served as loading controls. RNAi with an unrelated protein demonstrated that the observed effects were specific for DOT1A depletion (Figure S2B).

As the H3K76me2 mark is mitosis specific, we wanted to determine whether DOT1A depletion interfered with mitotic cell cycle control in bloodstream forms. DNA in DOT1A RNAi cells was stained with propidium iodide to analyze the cell cycle profile by flow cytometry (Figure 7D). Without RNAi induction, cell cycle profiles were indistinguishable from wild-type cells (Figure 7D, top panels). However, a population of cells with a DNA content of 1C appeared 12 hr postinduction (Figure 7D, C1). After 24 hr, these unprecedented and apparently haploid cells accounted for more than 20% of the population. These data suggest that H3K76me2 plays an important role in mitotic cell cycle control and that depletion of DOT1A might force the cells into an additional round of nuclear division and cytokinesis without preceding DNA synthesis. Depletion of H3K76me3 did not result in cell cycle defects in the bloodstream form (Figure S2A).

Discussion

T. brucei diverged early in the eukaryotic lineage (Stevens et al., 2001). Studying histone modifications and histone-modifying enzymes in trypanosomes gives us the opportunity to understand conserved and fundamentally different pathways at the same time. DOT1A and DOT1B exemplify this opportunity. DOT1A is responsible for the dimethylation of histone H3K76, whereas DOT1B trimethylates the same residue. Relatively few studies have addressed the consequences of different methylation states of the same histone lysine residue. Set1 is responsible for both di- and trimethylation of H3K4 in yeast (Santos-Rosa et al., 2002).

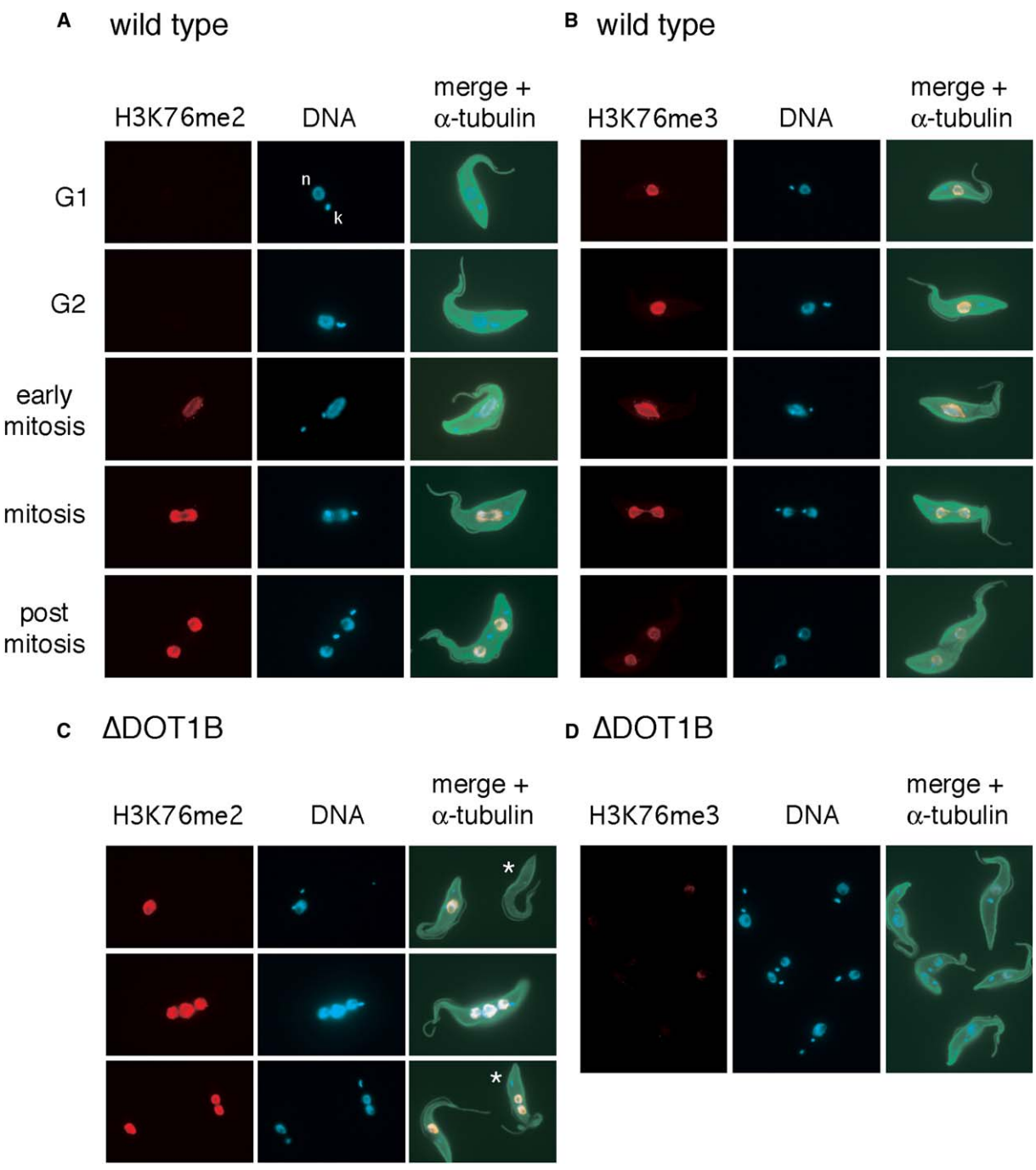

Figure 5. H3K76me2 Is a Marker for Mitosis

(A) Cell cycle-dependent dimethylation of K76 (red) in wild-type procyclic cells. Cell cycle stages of the parasite, defined by the number and position of the DAPI-stained kinetoplasts (k) and the nuclei (n), are indicated on the left. Parasite outlines were visualized by staining with α-tubulin antibody (green).

(B) Trimethylation of K76. Staining and labeling as in (A).

(C) K76 dimethylation and cell cycle regulation defects in ΔDOT1B cells. Staining is as described in (A). An anucleate cell and a G2 cell with two nuclei are marked by asterisks in the top and bottom panels, respectively. The middle panel shows a polynucleated parasite.

(D) K76 trimethylation is not detectable in cells. Staining is as described in (A).

Trimethylation was only found at active genes, whereas dimethylation occurs in both active and inactive euchromatin. In mice, dimethylation of H3K9 is dependent on the methyltransferase G9a, whereas trimethylation is directed by Suv39h1 and Suv39h2 (Peters et al., 2003; Rice et al., 2003). The different methylation states of H3K9 are associated with different domains of silent chromatin. In contrast, di- and trimethylation of H3K76 in *T. brucei* appear to affect fundamentally different processes. H3K76me2 by DOT1A is essential for viability, but DOT1B is not, although its absence causes subtle defects in mitosis in procyclic forms.

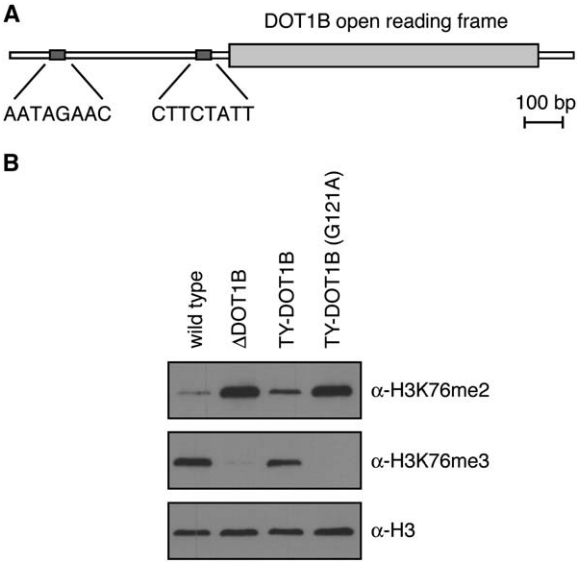

Figure 6. DOT1B Expression Is Controlled by Conserved Sequences in the 5′ UTRs

(A) Schematic representation of the (C/A)ATAGAA(G/A) consensus sequences in the 5′ UTR of *DOT1B* (the second motif is inverted).
(B) Western blot analysis of the methylation state of K76 in wild-type, ΔDOT1B, and two TY-DOT1B cell lines using the dimethyl (top) and trimethyl (middle) antibodies. An H3 peptide-specific antibody (Ab-Cam 1791) was used to monitor loading (bottom).

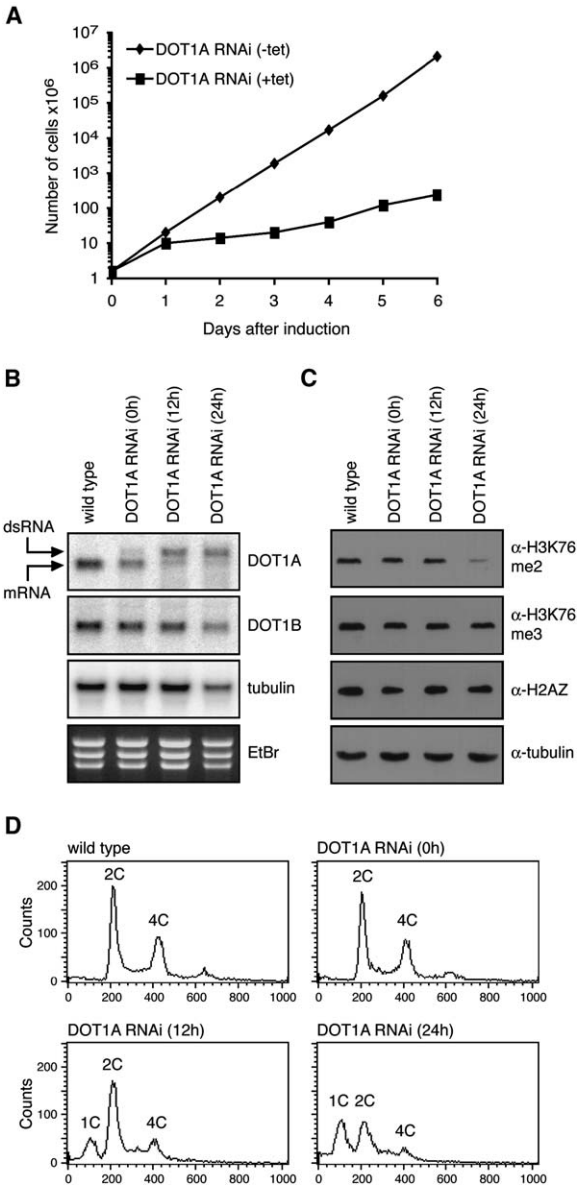

Figure 7. DOT1A Is Essential in Bloodstream Forms and Directs Dimethylation of H3K76

(A) Cumulative growth of parasites after induction of DOT1A RNAi.
(B) Northern blot analysis during DOT1A RNAi induction for 12 and 24 hr, with probes specific for DOT1A, DOT1B, and tubulin . Ethidium bromide staining (EtBr) served as loading control.
(C) Western blot analysis of the K76 methylation state in cell lysates 12 and 24 hr after RNAi induction. α-tubulin and α-H2AZ antibodies served as loading controls (bottom panels).
(D) Cell cycle analysis after RNAi induction. Cells were fixed at 0, 12, and 24 hr after RNAi induction, stained with propidium iodine, and analyzed by FACS. Wild-type cells served as control. DNA content is indicated as 1C, 2C, and 4C.

The differentiation of trypanosomes from bloodstream to procyclic forms has been quite well studied, and several proteins that are required for differentiation have been identified. Like DOT1B, a mitogen-activated protein kinase 2 (MAP2) can be deleted in bloodstream forms without apparent consequences (Muller et al., 2002). MAP2 deletion mutants initiate differentiation but subsequently arrest at random phases of the cell cycle. Because the DOT1B and MAP2 deletion phenotypes are so similar, DOT1B might be involved in a differentiation checkpoint that requires K76 methylation to activate genes that regulate cell cycle progression. Recent studies have shown that dot1p is involved in cell cycle checkpoint control in *S. cerevisiae* and in the activation of kinases after DNA damage (Game et al., 2005; Giannattasio et al., 2005; Huyen et al., 2004; San-Segundo and Roeder, 2000; Wysocki et al., 2005). Alternatively, widespread H3K76me3 may be required for the efficient regulation of many genes that are required to respond to a sudden environmental change.

One of our most striking findings is the mitosis-specific dimethylation of K76 and its apparent role in mitotic cell cycle control. Little is known about cell cycle-dependent regulation of DOT1 or the different K79 methylation states in other organisms. In humans, where K79 is mono- and dimethylated, the dimethyl mark appears to be cell cycle regulated (Feng et al., 2002). Although H3K79me2 is detectable throughout the cell cycle, it decreases during S phase, reaches the lowest levels in G2 phase, and increases again during mitosis. In yeast, H3K79me2 seems to increase slightly in late S phase and remains constant during G2 phase and mitosis (Zhou et al., 2006). This differs from *T. brucei*, where dimethylated K76 is only present during mitosis. Dimethylation increased dramatically in ΔDOT1B trypanosomes and was no longer cell cycle dependent, suggesting that mitosis-specific dimethylation of K76 is determined by cell cycle regulation of DOT1B expression or activity, which normally converts all dimethylated K76 to trimethylated K76 in interphase. If DOT1B is absent during S phase, G2 phase, and mitosis, freshly synthesized histones can only be dimethylated by DOT1A after

incorporation into chromatin. The fact that K76me2 is first detectable in early mitosis might suggest that dimethylation is a slow or carefully regulated process. Alternatively, both DOT1A and DOT1B might be cell cycle regulated. Sequential upregulation of DOT1A before mitosis (to generate K76me2) and DOT1B after mitosis (to convert all K76me2 to K76me3) would restrict K76me2 to mitosis. Consistent with these models, we were only able to reintroduce an ectopic copy of DOT1B by using its natural 5′ UTR, which contains two copies of a conserved sequence that has been shown to confer cell cycle-regulated expression of several mRNAs in *Crithidia fasciculata* (Brown and Ray, 1997; Pasion et al., 1996). Because these regulatory elements appear to mediate extremely low expression levels, it was impossible to confirm cell cycle-dependent expression of DOT1B by IF or Western blot. Alternatively, there might be cell cycle-regulated proteins that specifically bind to H3K76me2 to protect this mark from trimethylation by DOT1B. We also cannot exclude the possibility that a demethylase might remove one methyl group from trimethylated K76 during mitosis, although such an activity has not been described in any system. Histone demethylases have been described that target specific methylated arginines (Cuthbert et al., 2004; Wang et al., 2004) or dimethylated, but not trimethylated, lysine (Shi et al., 2004). The recently discovered family of Jumonji C domain histone demethylases, however, seems to include enzymes that can also target trimethylated histones (reviewed in Trojer and Reinberg [2006]).

How can we explain the cell cycle regulation defects in ΔDOT1B that occur in procyclic, but not in bloodstream, trypanosomes? Important functional differences exist between the trypanosome and mammalian cell cycle control machinery (reviewed in Hammarton et al. [2003b]). Early studies suggested that bloodstream forms, but not procyclic forms, have a mitosis to cytokinesis checkpoint (Ploubidou et al., 1999). The lack of this checkpoint allows procyclic forms to enter cytokinesis in the absence of mitosis, causing the formation of anucleate zoids. Procyclic forms lacking the mitotic cyclin CYC6 also undergo cytokinesis without the completion of mitosis, whereas a mitotic checkpoint in bloodstream forms appears to inhibit cytokinesis in the absence of CYC6 (Hammarton et al., 2003a). As the H3K76me2 mark emerges early in mitosis and is strongest during and after karyokinesis, dimethylation might function in the regulation of the mitosis to cytokinesis transition. In ΔDOT1B procyclic forms, K76 is dimethylated throughout the cell cycle, which might entice some of the cells into premature cytokinesis, resulting in zoids and polynucleated trypanosomes and the observed growth phenotype. A mitotic checkpoint might prevent this in bloodstream forms. In contrast, depletion of DOT1A in bloodstream forms is lethal and results in the accumulation of apparently haploid cells, pointing to a severe deregulation of mitosis and/or cytokinesis that allows another round of karyokinesis without DNA replication. Haploid trypanosomes have never been observed before, despite genetic experiments that argue for their existence at some point in the life cycle (reviewed in Gibson [2001]). Because Dot1p is involved in a meiotic checkpoint in yeast (San-Segundo and Roeder, 2000), it is possible that modulation of K76 methylation mediates meiosis in trypanosomes, but the details will be a challenge for future studies.

Experimental Procedures

Trypanosome Cell Lines and Plasmid Construction

T. brucei bloodstream forms (strain Lister 427, antigenic type MITat 1.2, and clone 221a [Doyle et al., 1980]) were cultured in HMI-9 medium at 37°C (Hirumi and Hirumi, 1989). Bloodstream and procyclic derivatives named "single marker" and "29-13," respectively, constitutively express T7 RNA polymerase and the Tet repressor (Wirtz et al., 1999). Single-marker bloodstream forms were cultured in HMI-9 containing 2.5 μg/ml G418 (Sigma) and 29-13 procyclics in SDM-79 (Brun and Schonenberger, 1979) containing 15 μ/ml G418 and 25 μg/ml hygromycin (Sigma). Bloodstream forms were differentiated to procyclics as described previously (Overath et al., 1986). A PCR-based gene disruption method was used to generate DOT1B-deficient cell lines (Baudin et al., 1993). The loss of DOT1B genes was confirmed by Southern and Northern blot analysis. To generate pHD309-TY-DOT1B, the entire 5′ UTR (1027 bp) and the entire 3′ UTR (555 bp) of DOT1B were amplified by PCR and cloned into pHD-309-BSR (Wirtz et al., 1994). The DOT1B ORF was amplified with a primer pair that introduced the sequence of a TY-epitope (MEVHTNQDPLDA) at the 5′ end and cloned between the UTRs. To generate DOT1A RNAi cell lines, the entire ORF of DOT1A was amplified by using an AmpliTaq kit (Roche). The PCR product was cloned into AhdI-digested p2T7TA-blue plasmid (Alibu et al., 2005). Constructs were linearized with NotI prior to transfection (Wirtz et al., 1994).

Northern Blot Analysis

Total RNA was prepared with RNA STAT60 (Tel TestTM). Three micrograms of RNA was separated on a 1.5% formaldehyde agarose gel and transferred to a nitrocellulose membrane. After UV crosslinking, the membrane was incubated with a DNA probe specific for VSG 221 or Procyclin EP1-1 (Roditi and Clayton, 1999). The probes were radioactively labeled by using a Prime-It random primer labeling kit (Stratagene).

Histone Purification and Mass Spectrometry

Nuclei from 2×10^{10} procyclic forms were isolated (Rout and Field, 2001), washed in 10 mM Tris (pH 8.0), 0.05% NP-40, and 75 mM NaCl, and resuspended in the same buffer containing 400 mM NaCl. After sonication, histones were extracted by adding 0.4 M H_2SO_4 and incubating overnight at 4°C and precipitated with trichloroacetic acid. The precipitates were fractionated by RP-HPLC between 0% and 60% acetonitrile (v/v) in 0.1% trifluoroacetic acid (v/v), dried, and redissolved in water. For matrix-assisted laser-desorption/ionization time-of-flight spectrometry (MALDI-TOF), histone H3 was digested with endoproteinase Glu-C (Roche). MALDI-TOF data were obtained with a DE-STR mass spectrometer (Applied Biosystems) operating in the delayed extraction and reflector modes. Tandem mass spectrometry of the precursor ions (m/z 760.4 for wild-type cells and m/z 746.4 for ΔDOT1B cells) was performed on a QSTAR XL instrument (Applied Biosystems). Mass spectrometry was performed at The Rockefeller University Proteomics Resource Center.

Western Blot Analysis and Antibodies

1×10^6 procyclic forms were washed in PBS and lysed in RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS). Lysates were separated on a 15% SDS-PAGE gel and transferred onto a nitrocellulose membrane. Polyclonal antibodies specific for dimethylated or trimethylated histone H3K76 were raised by immunizing rabbits with KLH-conjugated peptides VSGAQK[Me_2]EGLRFC or VSGAQK[Me_3]EGLRFC, respectively. Antisera were affinity purified with the corresponding peptides immobilized to SulfoLink coupling gel (Pierce). Antibody specificity was evaluated by ELISA and peptide competition assays. Monoclonal α-tubulin antibody TAT (gift of Keith Gull), polyclonal anti-H2AZ (Lowell et al., 2005), and α-H3 (ab1791, Abcam) were used as controls. Primary antibodies were

detected with horseradish-peroxidase-conjugated sheep anti-rabbit and sheep anti-mouse antibodies (Amersham-Pharmacia).

In Vitro Histone Methyltransferase Assay

Mononucleosomes were prepared as previously described (Lowell et al., 2005). Equal amounts of mononucleosomes or RP-HPLC-purified histone H3 from ΔDOT1B cells were incubated with ~500 ng recombinant GST, GST-DOT1A, or GST-DOT1B in 200 μl assay buffer (50 mM Tris [pH 8.0], 100 mM NaCl, 50 mM KCl, 5 mM CaCl2, 2.5% glycerol, 0.025% NP-40, and 0.5 mM S-adenosylmethionine) for 10 min at RT. Aliquots of the reaction were separated on a 15% SDS-PAGE gel and transferred onto a nitrocellulose membrane. Input controls were stained with amidoblack solution. Histone methyltransferase activity was detected by Western blot.

Flow Cytometry Analysis

2×10^7 bloodstream forms were washed with trypanosome dilution buffer (Cross, 1975), resuspended in 1 ml cold PBS, and fixed by adding 2.5 ml cold 95% ethanol while vortexing. Cells were incubated at 4°C for 1 hr, washed with PBS, and resuspended in 1 ml PBS plus 10 μg RNaseA. Incubation was continued at 37°C for 30 min. DNA was stained by adding 10 μg propidium iodide for 30 min at room temperature. The stained cells were analyzed on a FACSCalibur flow cytometer for relative DNA content based on red fluorescence.

Immunofluorescence

This protocol was carried out as previously described (Munoz-Jordan and Cross, 2001) with minor modifications. 1×10^6 procyclic forms were washed with PBS before fixing in 2% formaldehyde in PBS for 10 min at 4°C. The fixed cells were attached to glass cover slips by centrifugation at 2000 × g for 5 min at 4°C and permeabilized with 0.2% NP-40 in PBS for 10 min at room temperature. Polyclonal or monoclonal primary antibodies were visualized with tetramethyl rhodamine isothiocyanate (TRIC)-conjugated donkey anti-rabbit or fluorescein isothyocyanate (FITC)-conjugated goat anti-mouse antibodies (Jackson Laboratories), respectively. Cells were mounted in antifade mounting solution (Vectashield, Vecta Laboratories Inc.) and analyzed with a Nikon Optiphot microscope.

Supplemental Data

Supplemental Data include two figures and can be found with this article online at http://www.molecule.org/cgi/content/full/23/4/497/DC1/.

Acknowledgments

We thank Keith Gull and colleagues for gifts of antibodies, all members of the Cross lab for useful suggestions, Luisa Figueiredo and Veena Mandava for carefully reading the manuscript, and David Allis for valuable discussions and for comments on the manuscript. We are grateful to the staff of the Proteomics and Flow Cytometry Resource Centers of The Rockefeller University for advice and analytical support. This work was supported by the National Institutes of Health (AI50614 and AI21729) and by a Rockefeller University Women and Science Fellowship to S.B.H.

Received: February 16, 2006
Revised: May 16, 2006
Accepted: June 27, 2006
Published: August 17, 2006

References

Alibu, V.P., Storm, L., Haile, S., Clayton, C., and Horn, D. (2005). A doubly inducible system for RNA interference and rapid RNAi plasmid construction in *Trypanosoma brucei*. Mol. Biochem. Parasitol. *139*, 75–82.

Barry, J.D., and McCulloch, R. (2001). Antigenic variation in trypanosomes: enhanced phenotypic variation in a eukaryotic parasite. Adv. Parasitol. *49*, 1–70.

Baudin, A., Ozier-Kalogeropoulos, O., Denouel, A., Lacroute, F., and Cullin, C. (1993). A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. Nucleic Acids Res. *21*, 3329–3330.

Brown, L.M., and Ray, D.S. (1997). Cell cycle regulation of RPA1 transcript levels in the trypanosomatid *Crithidia fasciculata*. Nucleic Acids Res. *25*, 3281–3289.

Brun, R., and Schonenberger, M. (1979). Cultivation and in vitro cloning of procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium. Acta Trop. *36*, 289–292.

Brun, R., and Schonenberger, M. (1981). Stimulating effect of citrate and cis-aconitate on the transformation of *Trypanosoma brucei* bloodstream forms to procyclic forms *in vitro*. Z. Parasitenkd. *66*, 17–24.

Cross, G.A.M. (1975). Identification, purification and properties of clone-specific glycoprotein antigens constituting the surface coat of *Trypanosoma brucei*. Parasitology *71*, 393–417.

Cross, G.A.M., and Manning, J.C. (1973). Cultivation of *Trypanosoma brucei* sspp. in semi-defined and defined media. Parasitology *67*, 315–331.

Cross, G.A.M., Wirtz, L.E., and Navarro, M. (1998). Regulation of *VSG* expression site transcription and switching in *Trypanosoma brucei*. Mol. Biochem. Parasitol. *91*, 77–91.

Cuthbert, G.L., Daujat, S., Snowden, A.W., Erdjument-Bromage, H., Hagiwara, T., Yamada, M., Schneider, R., Gregory, P.D., Tempst, P., Bannister, A.J., et al. (2004). Histone deimination antagonizes arginine methylation. Cell *118*, 545–553.

Doyle, J.J., Hirumi, H., Hirumi, K., Lupton, E.N., and Cross, G.A.M. (1980). Antigenic variation in clones of animal-infective *Trypanosoma brucei* derived and maintained in vitro. Parasitology *80*, 359–369.

Engstler, M., and Boshart, M. (2004). Cold shock and regulation of surface protein trafficking convey sensitization to inducers of stage differentiation in *Trypanosoma brucei*. Genes Dev. *18*, 2798–2811.

Feng, Q., Wang, H., Ng, H.H., Erdjument-Bromage, H., Tempst, P., Struhl, K., and Zhang, Y. (2002). Methylation of H3-lysine 79 is mediated by a new family of HMTases without a SET domain. Curr. Biol. *12*, 1052–1058.

Fischle, W., Wang, Y., and Allis, C.D. (2003). Histone and chromatin cross-talk. Curr. Opin. Cell Biol. *15*, 172–183.

Game, J.C., Williamson, M.S., and Baccari, C. (2005). X-ray survival characteristics and genetic analysis for nine *Saccharomyces* deletion mutants that show altered radiation sensitivity. Genetics *169*, 51–63.

Garcia-Salcedo, J.A., Gijon, P., Nolan, D.P., Tebabi, P., and Pays, E. (2003). A chromosomal SIR2 homologue with both histone NAD-dependent ADP-ribosyltransferase and deacetylase activities is involved in DNA repair in *Trypanosoma brucei*. EMBO J. *22*, 5851–5862.

Giannattasio, M., Lazzaro, F., Plevani, P., and Muzi-Falconi, M. (2005). The DNA damage checkpoint response requires histone H2B ubiquitination by Rad6-Bre1 and H3 methylation by Dot1. J. Biol. Chem. *280*, 9879–9886.

Gibson, W. (2001). Sex and evolution in trypanosomes. Int. J. Parasitol. *31*, 643–647.

Hammarton, T.C., Clark, J., Douglas, F., Boshart, M., and Mottram, J.C. (2003a). Stage-specific differences in cell cycle control in *Trypanosoma brucei* revealed by RNA interference of a mitotic cyclin. J. Biol. Chem. *278*, 22877–22886.

Hammarton, T.C., Mottram, J.C., and Doerig, C. (2003b). The cell cycle of parasitic protozoa: potential for chemotherapeutic exploitation. Prog. Cell Cycle Res. *5*, 91–101.

Hirumi, H., and Hirumi, K. (1989). Continuous cultivation of *Trypanosoma brucei* bloodstream forms in a medium containing a low concentration of serum protein without feeder cell layers. J. Parasitol. *75*, 985–989.

Horn, D., and Cross, G.A.M. (1995). A developmentally regulated position effect at a telomeric locus in *Trypanosoma brucei*. Cell *83*, 555–561.

Huyen, Y., Zgheib, O., Ditullio, R.A., Jr., Gorgoulis, V.G., Zacharatos, P., Petty, T.J., Sheston, E.A., Mellert, H.S., Stavridi, E.S., and

Halazonetis, T.D. (2004). Methylated lysine 79 of histone H3 targets 53BP1 to DNA double-strand breaks. Nature *432*, 406–411.

Ingram, A.K., and Horn, D. (2002). Histone deacetylases in *Trypanosoma brucei*: two are essential and another is required for normal cell cycle progression. Mol. Microbiol. *45*, 89–97.

Janzen, C.J., Fernandez, J.P., Deng, H., Diaz, R., Hake, S.B., and Cross, G.A. (2006). Unusual histone modifications in *Trypanosoma brucei*. FEBS Lett. *580*, 2306–2310.

Lachner, M., O'Sullivan, R.J., and Jenuwein, T. (2003). An epigenetic road map for histone lysine methylation. J. Cell Sci. *116*, 2117–2124.

Lacoste, N., Utley, R.T., Hunter, J.M., Poirier, G.G., and Cote, J. (2002). Disruptor of telomeric silencing-1 is a chromatin-specific histone H3 methyltransferase. J. Biol. Chem. *277*, 30421–30424.

Lowell, J.E., Kaiser, F., Janzen, C.J., and Cross, G.A.M. (2005). Histone H2AZ dimerizes with a novel variant H2B and is enriched at repetitive DNA in *Trypanosoma brucei*. J. Cell Sci. *118*, 5721–5730.

Luger, K., Mader, A.W., Richmond, R.K., Sargent, D.F., and Richmond, T.J. (1997). Crystal structure of the nucleosome core particle at 2.8-angstrom resolution. Nature *389*, 251–260.

McKean, P.G. (2003). Coordination of cell cycle and cytokinesis in *Trypanosoma brucei*. Curr. Opin. Microbiol. *6*, 600–607.

McKittrick, E., Gafken, P.R., Ahmad, K., and Henikoff, S. (2004). Histone H3.3 is enriched in covalent modifications associated with active chromatin. Proc. Natl. Acad. Sci. USA *101*, 1525–1530.

Min, J., Feng, Q., Li, Z., Zhang, Y., and Xu, R.M. (2003). Structure of the catalytic domain of human DOT1L, a non-SET domain nucleosomal histone methyltransferase. Cell *112*, 711–723.

Muller, I.B., Domenicali-Pfister, D., Roditi, I., and Vassella, E. (2002). Stage-specific requirement of a mitogen-activated protein kinase by *Trypanosoma brucei*. Mol. Biol. Cell *13*, 3787–3799.

Munoz-Jordan, J.L., and Cross, G.A.M. (2001). Telomere shortening and cell cycle arrest in *Trypanosoma brucei* expressing human telomeric repeat factor TRF1. Mol. Biochem. Parasitol. *114*, 169–181.

Navarro, M., Cross, G.A.M., and Wirtz, E. (1999). *Trypanosoma brucei* variant surface glycoprotein regulation involves coupled activation/inactivation and chromatin remodeling of expression sites. EMBO J. *18*, 2265–2272.

Ng, H.H., Feng, Q., Wang, H., Erdjument-Bromage, H., Tempst, P., Zhang, Y., and Struhl, K. (2002). Lysine methylation within the globular domain of histone H3 by Dot1 is important for telomeric silencing and Sir protein association. Genes Dev. *16*, 1518–1527.

Okada, Y., Feng, Q., Lin, Y., Jiang, Q., Li, Y., Coffield, V.M., Su, L., Xu, G., and Zhang, Y. (2005). hDOT1L links histone methylation to leukemogenesis. Cell *121*, 167–178.

Overath, P., Czichos, J., and Haas, C. (1986). The effect of citrate/cis-aconitate on oxidative metabolism during transformation of *Trypanosoma brucei*. Eur. J. Biochem. *160*, 175–182.

Pasion, S.G., Hines, J.C., Ou, X., Mahmood, R., and Ray, D.S. (1996). Sequences within the 5′ untranslated region regulate the levels of a kinetoplast DNA topoisomerase mRNA during the cell cycle. Mol. Cell. Biol. *16*, 6724–6735.

Peters, A.H., Kubicek, S., Mechtler, K., O'Sullivan, R.J., Derijck, A.A., Perez-Burgos, L., Kohlmaier, A., Opravil, S., Tachibana, M., Shinkai, Y., et al. (2003). Partitioning and plasticity of repressive histone methylation states in mammalian chromatin. Mol. Cell *12*, 1577–1589.

Ploubidou, A., Robinson, D.R., Docherty, R.C., Ogbadoyi, E.O., and Gull, K. (1999). Evidence for novel cell cycle checkpoints in trypanosomes: kinetoplast segregation and cytokinesis in the absence of mitosis. J. Cell Sci. *112*, 4641–4650.

Rice, J.C., Briggs, S.D., Ueberheide, B., Barber, C.M., Shabanowitz, J., Hunt, D.F., Shinkai, Y., and Allis, C.D. (2003). Histone methyltransferases direct different degrees of methylation to define distinct chromatin domains. Mol. Cell *12*, 1591–1598.

Robinson, D.R., Sherwin, T., Ploubidou, A., Byard, E.H., and Gull, K. (1995). Microtubule polarity and dynamics in the control of organelle positioning, segregation, and cytokinesis in the trypanosome cell cycle. J. Cell Biol. *128*, 1163–1172.

Roditi, I., and Clayton, C. (1999). An unambiguous nomenclature for the major surface glycoproteins of the procyclic form of *Trypanosoma brucei*. Mol. Biochem. Parasitol. *103*, 99–100.

Roditi, I., Schwarz, H., Pearson, T.W., Beecroft, R.P., Liu, M.K., Williams, R.O., and Overath, P. (1989). Procyclin gene expression and loss of the variant surface glycoprotein during differentiation of *Trypanosoma brucei*. J. Cell Biol. *108*, 737–746.

Rout, M.P., and Field, M.C. (2001). Isolation and characterization of subnuclear compartments from *Trypanosoma brucei*. identification of a major repetitive nuclear lamina component. J. Biol. Chem. *276*, 38261–38271.

San-Segundo, P.A., and Roeder, G.S. (2000). Role for the silencing protein Dot1 in meiotic checkpoint control. Mol. Biol. Cell *11*, 3601–3615.

Santos-Rosa, H., Schneider, R., Bannister, A.J., Sherriff, J., Bernstein, B.E., Emre, N.C., Schreiber, S.L., Mellor, J., and Kouzarides, T. (2002). Active genes are tri-methylated at K4 of histone H3. Nature *419*, 407–411.

Sawada, K., Yang, Z., Horton, J.R., Collins, R.E., Zhang, X., and Cheng, X. (2004). Structure of the conserved core of the yeast Dot1p, a nucleosomal histone H3 lysine 79 methyltransferase. J. Biol. Chem. *279*, 43296–43306.

Shanower, G.A., Muller, M., Blanton, J.L., Honti, V., Gyurkovics, H., and Schedl, P. (2005). Characterization of the *grappa* gene, the *Drosophila* histone H3 lysine 79 methyltransferase. Genetics *169*, 173–184.

Sherwin, T., and Gull, K. (1989). The cell division cycle of *Trypanosoma brucei brucei*: timing of event markers and cytoskeletal modulations. Philos. Trans. R. Soc. Lond. B Biol. Sci. *323*, 573–588.

Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J.R., Cole, P.A., and Casero, R.A. (2004). Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell *119*, 941–953.

Singer, M.S., Kahana, A., Wolf, A.J., Meisinger, L.L., Peterson, S.E., Goggin, C., Mahowald, M., and Gottschling, D.E. (1998). Identification of high-copy disrupters of telomeric silencing in *Saccharomyces cerevisiae*. Genetics *150*, 613–632.

Stevens, J.R., Noyes, H.A., Schofield, C.J., and Gibson, W. (2001). The molecular evolution of Trypanosomatidae. Adv. Parasitol. *48*, 1–56.

Strahl, B.D., and Allis, C.D. (2000). The language of covalent histone modifications. Nature *403*, 41–45.

Trojer, P., and Reinberg, D. (2006). Histone lysine demethylases and their impact on epigenetics. Cell *125*, 213–217.

van Leeuwen, F., Gafken, P.R., and Gottschling, D.E. (2002). Dot1p modulates silencing in yeast by methylation of the nucleosome core. Cell *109*, 745–756.

Wang, Y., Wysocka, J., Sayegh, J., Lee, Y.H., Perlin, J.R., Leonelli, L., Sonbuchner, L.S., McDonald, C.H., Cook, R.G., Dou, Y., et al. (2004). Human PAD4 regulates histone arginine methylation levels via demethylimination. Science *306*, 279–283.

Wirtz, E., Hartmann, C., and Clayton, C. (1994). Gene expression mediated by bacteriophage T3 and T7 RNA polymerases in transgenic trypanosomes. Nucleic Acids Res. *22*, 3887–3894.

Wirtz, E., Leal, S., Ochatt, C., and Cross, G.A.M. (1999). A tightly regulated inducible expression system for dominant negative approaches in *Trypanosoma brucei*. Mol. Biochem. Parasitol. *99*, 89–101.

Woodward, R., and Gull, K. (1990). Timing of nuclear and kinetoplast DNA replication and early morphological events in the cell cycle of *Trypanosoma brucei*. J. Cell Sci. *95*, 49–57.

Wysocki, R., Javaheri, A., Allard, S., Sha, F., Cote, J., and Kron, S.J. (2005). Role of Dot1-dependent histone H3 methylation in G1 and S phase DNA damage checkpoint functions of Rad9. Mol. Cell. Biol. *25*, 8430–8443.

Zhou, H., Madden, B.J., Muddiman, D.C., and Zhang, Z. (2006). Chromatin assembly factor 1 interacts with histone H3 methylated at lysine 79 in the processes of epigenetic silencing and DNA repair. Biochemistry *45*, 2852–2861.