Histone H4 lysine 14 acetylation in *Leishmania donovani* is mediated by the MYST-family protein HAT4

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Post-translational modifications (PTMs) of histones regulate almost all facets of DNA metabolism in eukaryotes, such as replication, repair, transcription and chromatin condensation. While histone PTMs have been exhaustively examined in yeast and higher eukaryotes, less is known of their functional consequences in trypanosomatids. Trypanosome histones are highly divergent from those of other eukaryotes, and specific PTMs have been identified in histones of *Trypanosoma* species. The characterization of three MYST-family histone acetyltransferases (HATs) in *Trypanosoma brucei* had earlier identified the HATs responsible for acetylation of two lysine residues, K4 and K10, in the N-terminal tail of histone H4. This report presents the results of what we believe to be the first study of a HAT in a *Leishmania* species. The HAT4 gene of *Leishmania donovani*, the causative pathogen of visceral leishmaniasis, was cloned and expressed in fusion with GFP in *Leishmania* promastigotes. We found that HAT4–GFP behaves differently from typical eukaryotic MYST-family HATs, which are usually constitutively nuclear, in that it is cytosolic throughout the cell cycle, although the protein is also present in the nucleus in post-mitotic cells. Substrate-specificity analyses revealed that LdHAT4 acetylates the N terminus of histone H4, but not those of H2A, H2B or H3. Nor does it acetylate the C terminus of H2A. The primary target of HAT4-mediated acetylation is the K14 residue of H4, although K2 may be a minor site as well. H4K14 acetylation in *Leishmania* may occur either in the cytoplasm prior to histone deposition, or soon after mitosis in the nucleus.

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INTRODUCTION

Histones are a part of the nucleosomal structure of eukaryotic chromatin, and they carry a host of various post-translational modifications (PTMs), especially on their tail domains, that act in consortium or sequentially to regulate several activities such as chromatin condensation, transcriptional activation, replication timing, and DNA repair. Over 60 different residues on histones have been detected to have PTMs, and the roles of specific modifications in various biological processes have been determined. While the best-characterized histone modifications are acetylations, methylations and phosphorylations, other modifications such as ubiquitylations and sumoylations of lysine residues have also been reported

(reviewed by Kouzarides, 2007; Bannister & Kouzarides, 2011). These PTMs on specific residues form the histone code, and a multitude of histone-modifying enzymes are responsible for the formation of this code. The histone code hypothesis, first proposed by Strahl and Allis over a decade ago (Strahl & Allis, 2000), postulates that histone PTMs modulate cellular processes through effector proteins that recognize specific histone modifications, via specific domains present in them. The interaction of effector protein with chromatin may lead to one or more useful outcomes, such as the recruitment of essential transcription factors to promoters, or the further modification of other histone residues, which in turn influence downstream processes (Yun *et al.*, 2011). Histone modifications are not uniformly distributed throughout the genome. Microarray analysis in *Saccharomyces cerevisiae* has revealed that histones are hyperacetylated in promoters of genes that are actively expressed (Millar & Grunstein, 2006), and lysine trimethylation is enriched in coding regions of genes. In general, acetylation, methylation, phosphorylation and ubiquitination are implicated in transcriptional activation, while methylation, ubiquitination,

Abbreviations: HAT, histone acetylase; PTM, post-translational modification.

The GenBank/EMBL/DDBJ accession numbers for the *Leishmania donovani* sequences are JF742059, JF742060 and JF742061.

Three supplementary figures are available with the online version of this paper.

sumoylation and deimination are implicated in transcriptional repression.

The sequences of trypanosome histones are highly divergent from those of higher eukaryotes. Analysis of PTMs on the N-terminal tails of histones in *Trypanosoma brucei* and *Trypanosoma cruzi* (da Cunha *et al.*, 2006; Janzen *et al.*, 2006; Mandava *et al.*, 2007) has revealed that H4 is heavily acetylated and methylated in its N-terminal tail, and that the H3 tail carries several modifications as well. In contrast to other eukaryotes, the H2A and H2B N-terminal tails have very few modifications, while the H2A C-terminal tail is heavily modified (Janzen *et al.*, 2006; Mandava *et al.*, 2007). A comparison of the sequences of histones H2A, H2B, H3 and H4 of *Leishmania donovani* (Tritryp GeneDB; GenBank accession nos JF742060 and JF742061) with those of other *Leishmania* species as well as *Trypanosoma* species (Berriman *et al.*, 2005; El-Sayed *et al.*, 2005; Ivens *et al.*, 2005; Peacock *et al.*, 2007) reveals that the tails of H2A, H2B, H3 and H4 are highly conserved among trypanosomatids (Fig. 1a). This suggests that the PTMs found on these tails may also be conserved across these species. The presence of relatively fewer histone-modifying enzymes in these protozoans suggests that the functional redundancy of these enzymes may be lower than that seen in other eukaryotes. Determining the cellular functions of these enzymes may therefore be comparatively simpler, and deciphering the roles of individual histone modifications may be more directly possible.

There are three main families of histone acetylases (HATs): the GNAT, MYST and CBP/p300 families (Stern & Berger, 2000), of which the CBP/p300 family is specific to metazoans. The preferred sites of acetylation are lysine residues in the N-terminal tails of histones, especially histones H3 and H4, with the sites of acetylation being largely conserved from *S. cerevisiae* to metazoans. Analysis of whole-genome sequences of *Trypanosoma* and *Leishmania* species (Berriman *et al.*, 2005; El-Sayed *et al.*, 2005; Ivens *et al.*, 2005; Peacock *et al.*, 2007) reveals the presence of three MYST-family HATs in *T. brucei* and four MYST-family HATs in *T. cruzi* and *Leishmania* species. HATs 1–3 of *T. brucei* have been investigated in some depth, and HAT1 and HAT2 found to be essential for cell survival (Kawahara *et al.*, 2008; Siegel *et al.*, 2008). The TbHAT2 and TbHAT3 proteins acetylate histone H4 at the K10 and K4 positions, respectively.

Leishmania species are the causative agents of leishmaniasis, a group of diseases that occurs in three forms: cutaneous, subcutaneous and visceral. Visceral leishmaniasis (VL) is fatal if not treated in a timely manner. Every year, approximately 500 000 new cases of VL are reported, of which 90% are from the Indian subcontinent and Sudan. In these regions, *L. donovani* is the causative agent of VL, and therefore understanding the cellular biology and physiology of this pathogen is important for the development of new measures for therapeutic intervention. This study examines the fourth HAT of the MYST family that is

absent in *T. brucei*, HAT4. We find that HAT4, unlike the other three HATs of *L. donovani* (HATs 1–3), is cytosolic. Biochemical analysis reveals that *L. donovani* HAT4 is responsible for acetylation of the lysine residue at the 14th position of H4 (H4K14), a residue that is believed to be analogous to the H4K16 residue of other eukaryotes.

METHODS

***Leishmania* cultures.** *L. donovani* 1S promastigotes were cultured in M199 medium, as described previously (Kumar *et al.*, 2008).

Cloning of HAT4 gene. *L. donovani* genomic DNA was isolated as described previously (Kumar *et al.*, 2009), and the HAT4 gene was amplified using genomic DNA as template, and primers HAT4-F and HAT4-R (5'-CACCGGATCCGAATTCATGGAGGTCGGCCAG-3' and 5'-TCCTGCAGTCCGTGGTGGTAGTACGGCGTGT-3', respectively), with the highly proof-reading enzyme *Phu* DNA polymerase (Finnzymes). The amplicon was cloned into pENTR/D-TOPO (Invitrogen) for sequencing. For expression in *Leishmania* promastigotes, the HAT4 gene was amplified using primers HAT4-GFP-F and HAT4-GFP-R (5'-CACCGGATCCACCATGGAGGTCGGCCAG-3' and 5'-TCGATATCTCCGTGGTGGTAGTACGGCGTGT-3', respectively), and cloned into the *Bam*HI and *Eco*RV sites of pXG-/GFP+ (a kind gift from Professor Stephen Beverley, Washington University School of Medicine, St. Louis, MO) and pXG-/GFP+/FLAG (a derivative of pXG-/GFP+ that expresses the gene in fusion with a FLAG tag at the C-terminal end; Minocha *et al.*, 2011b), creating plasmids pXG/HAT4-GFP and pXG/HAT4-FLAG.

Site-directed mutagenesis. The HAT4-C381A mutant was created by overlap PCR using primers HAT4-GFP-F and HAT4-C381A-R (5'-GGCAGCGTGACAATGGCCGAGAGCGTGTGAT-3') to amplify the N-terminal part, and HAT4-GFP-R and HAT4-C381A-F (5'-ATCACACGCTCTCGGCCATTGTACACGCTGCC-3') to amplify the C-terminal part of the mutant. The mixture of the amplicons obtained in these two PCRs was used as template to amplify the full-length mutant gene HAT4-C381A, using HAT4-GFP-F and HAT4-GFP-R primers. The amplicon was cloned into pXG-/GFP+/FLAG to create the plasmid pXG/HAT4-C381A-FLAG for expression in *Leishmania*.

***Leishmania* transfections.** *Leishmania* promastigotes were transfected as described by Robinson & Beverley (2003). G418 (100 µg ml⁻¹) was added 42 h after transfection. HAT4-GFP expression was analysed 6–8 days after induction of selection pressure. HAT4-FLAG expression (wild-type and C381A mutant) was analysed 8–10 days after drug-induced selection pressure. Pulldowns of HAT4-FLAG (wild-type and mutant) for HAT assays were performed 2 weeks (or later) after transfections.

Synchronization and flow cytometry analysis. Synchronization of *Leishmania* promastigotes and flow cytometry analyses were carried out as described previously (Minocha *et al.*, 2011a). Briefly, cells were blocked with 5 mM hydroxyurea and released into drug-free M199 medium. Cells were harvested at different intervals for flow cytometry analysis. G1, S and G2/M phases are indicated on the flow cytometry histograms by the gates M1, M2 and M3, respectively, which were derived from the data using CellQuest Pro software.

Preparation of *Leishmania* extracts. *Leishmania* whole-cell extracts were prepared using the M-PER kit (Pierce Biotechnologies).

Pulldowns of FLAG-tagged proteins. For pulldowns of FLAG-tagged proteins, whole-cell lysates were made from promastigotes that

Fig. 1. (a) Histones are highly conserved across trypanosomatids. CLUSTAL W analysis of the N-terminal tails of H2A, H2B, H3 and H4, with the corresponding regions of histones from other trypanosomatids, and of the C-terminal tail of H2A with the corresponding region of H2A from other trypanosomatids; viewed using Jalview multiple alignment editor (Waterhouse *et al.*, 2009). Mature histones lack the initial methionine. The coloured residues represent amino acids grouped according to their physicochemical properties. Pink, aliphatic/hydrophobic; orange, aromatic; purple, glycine/proline; blue, basic; green, hydrophilic; red, acidic. (b) The HAT4 amplicon. A band of the expected size of 1.8 kb was obtained after PCR using 25 ng genomic DNA as template. (c) Schematic representation of the conserved domains in LdHAT4. Comparison of domain structure of LdHAT4 with the MYST-family acetyltransferases Esa1, Sas2 and Hbo1. ZF, zinc finger motif; A, motif A; C and E, conserved cysteine and glutamate residues involved in catalysis; shaded grey boxes, core acetyltransferase domain.

were transfected with HAT4-FLAG or HAT4-C381A-FLAG proteins, and the lysates were incubated for 2 h at 4 °C with FLAG M2 agarose beads (Sigma Aldrich) equilibrated with 1 × PBS, using a nutator mixer. After washing the beads extensively with 1 × PBS containing 0.2 % Triton X-100 to remove unbound and non-specifically bound proteins, the beads were boiled in 3 × SDS sample-loading buffer, and analysed by SDS-PAGE followed by Western blotting. To perform

HAT assays, the bead-bound FLAG-tagged proteins were directly used in HAT assays after extensive washing.

Direct fluorescence analysis. To examine the subcellular localization of HAT4, promastigotes transfected with pXG/HAT4-GFP were harvested, washed with PBS, and fixed with 2 % paraformaldehyde for 20 min. Cells were spread on poly-lysine-coated coverslips,

and the coverslips were mounted in anti-fade solution containing DAPI (Vectashield, Vecta Laboratories). Cells were viewed and images of cells were acquired using a confocal microscope (LSM 510; Carl Zeiss MicroImaging) and a 100 $\times$ objective.

HAT assay. HAT assays were performed using HAT4-FLAG and HAT4-C381A-FLAG, pulled down from transfectant *Leishmania* promastigotes as described above. The assays were performed using HAT Assay kit (Active Motif) according to the manufacturer's instructions. The substrates we used in these assays were histone peptides derived from the tails of *L. donovani* histones (peptides were manufactured by either Peptron Inc., South Korea, or Abgent, USA).

HAT4-FLAG and HAT4-C381A-FLAG pulldowns were equally divided into five or six parts, each part corresponding to pulldown from 8×10^8 cells. One part was used in HAT assay reactions performed in the absence of histone peptide substrate. This gave background acetylation values in the case of HAT4-C381A-FLAG (negative control), and background acetylation plus autoacetylation values in the case of HAT4-FLAG. HAT4-FLAG autoacetylation levels were determined by subtracting background acetylation values obtained in the HAT4-C381A-FLAG reaction from values obtained in the HAT4-FLAG reaction. The other parts of each pulldown were used to perform the HAT assay in the presence of histone peptide substrate. HAT4-mediated acetylations (autoacetylation plus peptide acetylation) were determined by subtracting the value obtained in the HAT4-C381A-FLAG reaction with peptide from the value obtained in the HAT4-FLAG reaction with peptide. The values are represented as bar charts. Each experiment was performed twice, and similar results were obtained in each case. For each experiment, one dataset is shown here, while the second dataset is presented in the supplementary data.

RESULTS

Cloning of HAT4 from *L. donovani*

Four HATs of the MYST family have been annotated in all of the *Leishmania* whole-genome sequences (Ivens *et al.*, 2005; Peacock *et al.*, 2007), and three of these (HATs 1–3) are conserved across all *Leishmania* species as well as *T. brucei* and *T. cruzi*. The fourth member, HAT4, is absent in *T. brucei*. The HAT4 gene of *L. donovani* (strain 1S) was cloned by amplification of the gene using genomic DNA as template, and primers designed based on the published *Leishmania major* sequence (TriTrypDB ID: LmjF.13.0170), as no sequence of *L. donovani* was available at the time. The amplicon obtained was of the expected size of ~1.8 kb (Fig. 1b), and its authenticity was confirmed by cloning followed by sequencing (GenBank accession no. JF742059). A comparison of the sequence of LdHAT4 with that of other trypanosomatids revealed ~37% identity and ~50% similarity with HAT4 of *T. cruzi*, and 81–98% identity and 86–99% similarity with HAT4 of other *Leishmania* species. The sequence showed 98.5% identity and 99% similarity with the sequence of HAT4 from *L. donovani* strain BPK282A1 (TriTryp GeneDB ID: LdBPK_130170.1). Analysis of the LdHAT4 sequence indicated that two motifs present in the MYST family of HATs, motif A (Q/RXXGXG/A) and a zinc finger motif (CXXCX₁₂HX_{3–5}C), are present in this protein (molecular mass ~ 68 kDa) (Fig. 1c). While motif A (amino acids 410–415) plays a role in the binding of

coenzyme A, the zinc finger motif (amino acids 229–251) is important for substrate recognition, as this domain interacts with the globular region of the nucleosomal core. Like Sas2, Esa1 and Hbo1, LdHAT4 also carries a core acetyltransferase domain (between residues 186 and 501). MYST-family HATs mediate histone acetylation through the formation of an acetyl-cysteine enzyme intermediate, the formation of which is arbitrated by a glutamate residue. It is postulated that the glutamate residue may aid cysteine deprotonation so that acetyl transfer can follow (Yan *et al.*, 2000, 2002). The Cys and Glu residues that are crucial for HAT catalytic activity are usually conserved in all members of this family. Although we found HAT4 to contain the conserved Cys residue at position 381 (Fig. 1c), we were unable to detect the conserved Glu residue. In the absence of any apparent candidate glutamate in LdHAT4, it is possible that the role of glutamate has been taken on by another acidic residue such as aspartate. Further detailed experimentation will be needed to ascertain this. *Leishmania* HATs 1–3 are more typical as they contain both the conserved Cys and Glu residues.

HAT4 is constitutively cytosolic

To examine the subcellular localization of HAT4 in *Leishmania*, we raised antibodies to purified recombinant LdHAT4 expressed in *Escherichia coli*. However, although we raised antibodies in both mice and rabbits, and immunized multiple animals, we were unable to obtain antibodies of high enough sensitivity. Therefore, we expressed HAT4 in fusion with GFP at its C-terminal end in *Leishmania* promastigotes, using vector pXG-/GFP+ (see above; Ha *et al.*, 1996). *Leishmania* promastigotes transfected with pXG/HAT4-GFP were first analysed to ascertain expression of full-length HAT4-GFP by Western blot analysis, using anti-GFP antibodies (Fig. 2a). To examine subcellular localization at different stages of the cell cycle, we adopted the approach of using kinetoplast morphology and segregation pattern as a marker for specific stages of the cell cycle, in immunofluorescence studies (Minocha *et al.*, 2011a; Siegel *et al.*, 2008; Wheeler *et al.*, 2011). The subcellular localization of HAT4-GFP was analysed by examining direct fluorescence of HAT4-GFP (Fig. 2b). In *L. donovani*, G1 and early S phase cells have one roundish or short rod-like kinetoplast (1N1K); late S phase and early G2/M cells have an elongated kinetoplast (1N1K); and late G2/M cells have either one nucleus and two kinetoplasts (1N2K) or two nuclei and one kinetoplast (2N1K). Cells with two nuclei and two kinetoplasts (2N2K) are designated post-mitotic (Minocha *et al.*, 2011a). Our studies revealed that HAT4-GFP was cytoplasmic at all stages (Fig. 2b). In post-mitotic cells, however, it was present throughout the cell (Fig. 2b and Supplementary Fig. S1). This contrasts with what we have observed with HATs 1–3, which have been found to be nuclear using a similar approach.

Fig. 2. Analysis of HAT4-GFP expression. (a) Western blot analysis of 50 µg whole-cell extracts made from untransfected *L. donovani* (Ld1S) and HAT4-GFP transfectant promastigotes. Analysis was done using anti-GFP antibody (1:2000 dilution, Invitrogen). (b) Direct fluorescence analysis of HAT4-GFP expression. G1/early S phase cells: one nucleus, one short round kinetoplast (1N1K). Late S phase/early G2/M cells: one nucleus, one elongated kinetoplast (1N1K). Late G2/M phase cells: two nuclei, one kinetoplast (2N1K). Post-mitosis cells: two nuclei, two kinetoplasts (2N2K). Images were collected using a confocal microscope. A Z series of the post-mitotic stage is provided in Supplementary Fig. S1. Bars, 2 µm.

Characterization of HAT4 activity

While no direct information is available about the acetylation status of specific residues of histones in *Leishmania* species, work from the Myler laboratory suggests that the N-terminal tail of histone H3 is acetylated at regions that initiate divergent polycistronic transcription (Thomas *et al.*, 2009). Detailed analyses of *T. brucei* and *T. cruzi* histones (da Cunha *et al.*, 2006; Mandava *et al.*, 2007) have identified specific residues that are acetylated and methylated in H2A, H2B, H3 and H4. Histone H3 has been found to be acetylated at its N-terminal end, but the specific residues modified have not yet been identified. To determine the substrate specificity of LdHAT4 we used a fluorescence-based assay that uses histone peptide as substrate, as described in Methods. In this assay, the HAT protein (HAT4-FLAG in this case) causes the transfer of an acetyl group from acetyl-CoA to the histone substrate, resulting in an acetylated histone substrate and

free CoA. The free thiol groups on the CoA react with the developer provided in the kit, and the resultant fluorescence is detected with excitation at 360–390 nm and emission at 450–470 nm. Attempts to characterize histone acetylation activity with recombinant HAT4 expressed in *E. coli* did not succeed, possibly because the recombinant protein did not have the correct conformation or because of the absence of the PTMs that are essential to the protein's activity. Therefore, HAT4 was expressed in fusion with a FLAG tag at its C-terminal end in *Leishmania* promastigotes. For this, plasmid pXG/HAT4-FLAG was transfected into *Leishmania* cells, and HAT4-FLAG expression in transfectants was examined by Western blot analysis of whole-cell extracts using anti-FLAG antibodies (Sigma Aldrich). A HAT4 mutant, HAT4-C381A-FLAG (HAT4 with the conserved Cys residue that is predicted to be essential for catalysis mutated to alanine), was also likewise expressed and analysed. As is evident from Fig.

3(a), both proteins were robustly expressed in transfectants. To perform HAT activity assays, HAT4-FLAG was pulled down from exponentially growing transfectant cells using FLAG M2 agarose beads (Sigma Aldrich). As a negative control, equivalent amounts of HAT4-C381A-FLAG that had been similarly pulled down were used in parallel reactions. HAT4-C381A-FLAG was found to be devoid of HAT activity. [Acetylation levels using this mutant protein were similar to those that we obtained in HAT assays using ORC-FLAG (replication initiator protein), and were only a little over reactions containing buffer plus substrate only.] Background, HAT4-FLAG autoacetylation levels, and HAT4-FLAG-mediated peptide acetylation plus autoacetylation levels were determined as described in Methods, using HAT4-C381A-FLAG reaction values as a control.

Five peptide substrates derived from the N or C termini of histones H2A, H2B, H3 and H4 (Fig. 3b), whose sequences were obtained either from the N termini of LdH3 and LdH4 cloned in the laboratory (GenBank accession nos JF742060 and JF742061, respectively), or from H2A and H2B sequences of *L. donovani* strain BPK282A1 (Tritryp GeneDB IDs: LdBPK_211160.1.1 and LdBPK_190040.1.1,

respectively), were used as substrates. Pulled down wild-type and C381A mutant HAT4-FLAG proteins (Fig. 3c, left panel) were used in assays to test the possibility of the five peptides serving as substrates. In the absence of peptide substrate, HAT4-FLAG displayed autoacetylation. When the N-terminal peptides of H2A, H2B and H3, and the C-terminal peptide of H2A, were added as substrates to the reactions, HAT4-mediated acetylation levels were near to autoacetylation levels (Fig. 3c, right panel, and Supplementary Fig. S2a). In contrast, when H4 N terminus peptide substrate was added to the reaction, HAT4-mediated acetylation levels were 5-6 fold higher than autoacetylation levels (Fig. 3c and Supplementary Fig. S2a). All five peptides were acetylated using purified p300, which served as a positive control (Supplementary Fig. S2b). Thus, HAT4 clearly utilized H4 as substrate.

Five main sites of acetylation have been identified in the N terminus of H4 in *Trypanosoma* species: K4 (in 73 % of H4), K2, K5, K10 and K14 (each in less than 10 % of H4). In *T. brucei*, H4K4 acetylation is brought about by TbHAT3, and H4K10 acetylation by TbHAT2 (Kawahara *et al.*, 2008; Siegel *et al.*, 2008). As *L. donovani* H4 does not have a lysine residue at the fifth position, there were four

Fig. 3. (a) Analysis of HAT4-FLAG expression. Western blot analysis of 50 μ g whole-cell extracts made from untransfected *L. donovani* (Ld1S), and from HAT4-FLAG and HAT4-C381A-FLAG transfectant promastigotes, using anti-FLAG antibody (1 : 7500 dilution, Sigma Aldrich). (b) Histone peptide substrates. Sequences of peptides used in HAT assays, corresponding to the N terminus of H2A, H2B, H3 and H4, and the C terminus of H2A. (c) HAT assay using H2A, H2B, H3 and H4 substrates. HAT4-FLAG proteins (wild-type and mutant) were pulled down from 4.8×10^9 promastigotes. Left panel: Western blot analysis of one-tenth of input bead-bound proteins. Anti-FLAG antibody was used at a 1 : 7500 dilution. Right panel: HAT assay products were estimated after removing the bead-bound proteins by centrifugation. Products were estimated by excitation at 390 nm and detecting emission at 490 nm. A second dataset is provided in Supplementary Fig. S2(a).

likely residues in the H4 peptide that were potential sites of HAT4-mediated acetylation: K2, K4, K10 and K14. To address the possibility of K4, K10 and K14 being HAT4 acetylation sites, we performed HAT assays using H4 peptide that was already acetylated at the K4 position [H4K4(Ac)], K10 position [H4K10(Ac)] or K14 position [H4K14(Ac)] (Fig. 4a). The results of the experiments demonstrated that prior blocking of the K4 and K10 sites by acetylation did not diminish the ability of HAT4 to utilize the H4 substrate. However, when the K14 site was blocked by prior acetylation, HAT4-mediated acetylation was only slightly above autoacetylation levels (Fig. 4b and Supplementary Fig. S3a). Thus, while the K4 and K10 residues do not appear to be sites of HAT4-mediated

acetylation, K14 seems to be the major site of acetylation by HAT4. Substrate specificity was further investigated using three H4 mutant peptides (Fig. 4a). The H4K4A-K10A-K14A peptide retains only one lysine, at the second position. The H4K2A-K4A-K10A peptide retains only the lysine at the 14th position, and the H4K14A peptide carries a single (lysine to alanine) mutation at the 14th position. We found that HAT4-mediated acetylation of H4K4A-K10A-K14A was only 1.5-fold greater than autoacetylation levels, and acetylation of H4K14A was only slightly greater. In contrast, acetylation of the H4K2A-K4A-K10A peptide was about fourfold greater than autoacetylation levels (Fig. 4c and Supplementary Fig. S3b). Reactions with H4 (wild-type) peptide carried out simultaneously revealed that it

(a) H4K4 (Ac) : A K G K(Ac) R S A D A K S S Q K R
 H4K10 (Ac) : A K G K R S A D A K(Ac) S S Q K R
 H4K14 (Ac) : A K G K R S A D A K S S Q K(Ac) R
 H4K4A-K10A-K14A : A K G A R S A D A A S S Q A R
 H4K2A-K4A-K10A : A A G A R S A D A A S S Q K R
 H4K14A : A K G K R S A D A K S S Q A R

Fig. 4. (a) Modified H4 peptide substrates. Sequences of H4 peptide derivatives used in the HAT assay. (b) HAT assay using acetylated H4 substrates. HAT4-FLAG proteins were pulled down from 4×10^9 promastigotes. Left panel: Western blot analysis of one-twelfth of input bead-bound proteins using anti-FLAG antibody. Right panel: measurement of HAT assay products by excitation at 390 nm and detecting emission at 490 nm. A second dataset is provided in Supplementary Fig. S3(a). (c) HAT assay using mutated H4 substrates. HAT4-FLAG proteins were pulled down from 4×10^9 promastigotes. Left panel: Western blot analysis of one-sixth of input bead-bound proteins. Right panel: HAT assay products were analysed by excitation at 390 nm and detection of emission at 490 nm. A second dataset is provided in Supplementary Fig. S3(b).

Fig. 5. (a) Overexpression of HAT4 does not affect the growth of *Leishmania* promastigotes. The growth patterns of Ld1S promastigotes transfected with empty FLAG vector and HAT4-FLAG transfectants were analysed by counting cells every 24 h. Closed circles, HAT4-FLAG; closed squares, Ld1S transfected with empty FLAG vector; error bars, SD. (b) Overexpression of HAT4 does not appear to affect cell cycle progression in *Leishmania* promastigotes. Flow cytometry analysis of Ld1S transfected with FLAG vector and Ld1S expressing HAT4-FLAG. Data from 25 000 events were collected for each time point. In the overlay histogram panels, light-grey-filled histograms represent Ld1S/FLAG and mid-grey lines represent HAT4-FLAG transfectants. G1, S and G2/M phases are indicated on the histograms by the gates M1, M2 and M3, respectively. Log and HU refer to logarithmically growing and hydroxyurea-blocked, respectively.

catalytic activity. These data indicate that the K14 residue of LdH4 is the major site of HAT4 acetylation.

We examined the growth pattern of promastigotes overexpressing HAT4-FLAG, in comparison with *Leishmania* promastigotes harbouring an empty FLAG vector. As seen in Fig. 5(a), no apparent difference in growth or cell viability was detected. Comparative analysis of flow cytometry profiles of promastigotes overexpressing HAT4-FLAG with promastigotes harbouring the empty FLAG vector revealed that they moved through the cell cycle at comparable rates (Fig. 5b).

DISCUSSION

In eukaryotes, histone modifications, especially acetylation, play an important role in transcriptional regulation as well as in other processes such as DNA replication and repair. In the absence of direct experimental data assigning modifications to specific histone residues in *Leishmania* species, we exploited the fact that histone sequences, especially the N-terminal tails, are highly conserved among trypanosomatids (Fig. 1a), and therefore the histone PTMs are likely to be conserved as well. PTMs in *T. brucei* and *T. cruzi* histones have mainly been identified by MS analysis (da Cunha *et al.*, 2006; Janzen *et al.*, 2006; Mandava *et al.*, 2007). Mature histones lack the first methionine residue. The N termini of the core histones have relatively fewer PTMs than do those of higher eukaryotes, while the C terminus of H2A is unusually hyperacetylated. Of the five main sites of acetylation in the N terminus of H4, H4K4 acetylation is the most abundant. Acetylations of K4, K10 and K14 of histone H4 in *T. cruzi* have been demonstrated to increase after S phase, indicating rapid acetylation of newly synthesized H4. H4K4 acetylation is more prominent in replicating forms of the parasite and colocalizes with densely packed chromatin, while H4K10 and H4K14 acetylations are distributed at the interface of dense and nondense chromatin (Nardelli *et al.*, 2009). In *T. brucei*, H4K4 acetylation is predominantly detected in G2/M (Siegel *et al.*, 2008). Some *Trypanosoma* PTMs may be synonymous

was a more efficient substrate than the H4K2A-K4A-K10A peptide. It is possible that the alteration of the three lysines to alanine residues in the H4K14 peptide affects the interaction of HAT4 with the substrate and thereby decreases

with the PTMs seen in other eukaryotes. H4K4 and H4K10 acetylation events are believed to be synonymous with H4K5 and H4K12 acetylation, both PTMs that play a role in appropriate histone deposition. H4K14 acetylation is believed to be synonymous with H4K16 acetylation, which affects higher-order chromatin compaction in higher eukaryotes and also regulates transcription.

This study reports what are believed to be the first findings about HAT4 in any of the trypanosomatids, and as far as we are aware is the first report of a HAT in any *Leishmania* species. Analysis of the LdHAT4 sequence revealed the presence of three domains typically found in MYST-family HATs: the core acetyltransferase domain, motif A and a zinc finger motif, as well as the Cys residue that is conserved among all members of this family. We found the Cys residue (Cys 381) to be essential for catalytic activity, as mutating it to alanine made the protein inactive (Figs 3 and 4). HAT4 was found to be cytosolic throughout the cell cycle (Fig. 2b), though it was present in the nucleus as well in post-mitotic cells (Fig. 2b and Supplementary Fig. S1). While this analysis was carried out using HAT4–GFP and does not examine endogenous HAT4 due to non-availability of sensitive antibodies, using a similar approach we have found HAT1–GFP, HAT2–GFP and HAT3–GFP to be constitutively nuclear (our unpublished results). This behaviour of HAT4, though deviant from the usual behaviour of MYST-family HATs in higher eukaryotes, which usually localize to the nucleus, is similar to what is seen in apicomplexan protozoans. MYST-family HATs of *Toxoplasma gondii* and *Plasmodium falciparum* have been found to localize to both the nucleus and the cytosol, and MYST-B of *Toxoplasma gondii* has been found to be predominantly cytosolic (Smith *et al.*, 2005; Miao *et al.*, 2010; Vonlaufen *et al.*, 2010). The mammalian MYST-family HAT Tip60 has also been found to be distributed between the nucleus and the cytoplasm (Zhang *et al.*, 2007).

To determine the substrate specificity of LdHAT4, a series of peptides derived from *Leishmania* histone tail sequences were synthesized and tested as potential substrates in HAT assays. The results obtained suggest that HAT4 mediates acetylation of the 14th lysine of histone H4 (H4K14) in *Leishmania*. Considering the subcellular localization of LdHAT4, it is possible that H4K14 acetylation occurs in the cytoplasm prior to histone deposition on chromatin. Alternatively, H4K14 acetylation may occur after histone deposition in the nucleus, soon after mitosis is complete. To analyse these aspects, antibodies specific to H4acetylK14 will need to be produced. This modification-specific antibody will also be a useful tool in genetic studies aimed at deciphering the role of HAT4 *in vivo*. Though conserved in almost all the trypanosomatids, the H4K14 residue is absent in *L. major* (Fig. 1a). This points to HAT4 having other substrates in *L. major*. These might be other residues in the core histones, or might be non-histone protein substrates. The yeast histone acetyltransferase Hbo1 has been demonstrated to acetylate several component proteins of pre-replication complexes (Iizuka *et al.*, 2006).

H4K14 acetylation is believed to be analogous to that of the H4K16 residue of other eukaryotes, a site that is marked by acetylation to varying degrees in different species. The role of H4K16 acetylation is best understood in budding yeast, where it disrupts higher-order chromatin structure (Shogren-Knaak *et al.*, 2006; Shogren-Knaak & Peterson, 2006). Brought about mainly by the MYST-family HAT Sas2 as well as by Esa1, over 80 % of the H4 in *S. cerevisiae* carries this acetylation (Kimura *et al.*, 2002; Smith *et al.*, 2003), which is more dominant in euchromatic regions. This acetylation mark is not as widely prevalent in higher eukaryotes, a fact that is reflected in the comparatively more decondensed state of chromatin in yeast than in other eukaryotes. H4K16 acetylation status regulates the extent of silent heterochromatin in yeast, favouring the maintenance of euchromatin domains (Kimura *et al.*, 2002; Suka *et al.*, 2002). This study lays the foundation for future studies aimed at determining the role of the H4K14 acetylation event in *Leishmania* species. Genetic approaches targeting the HAT4 gene will enable detailed investigation of the role of H4K14 acetylation in the organism's cellular processes, and functional dissection may shed light on the role of HAT4 in growth and pathogenesis.

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REFERENCES

- Bannister, A. J. & Kouzarides, T. (2011). Regulation of chromatin by histone modifications. *Cell Res* 21, 381–395.
- Berriman, M., Ghedin, E., Hertz-Fowler, C., Blandin, G., Renauld, H., Bartholomeu, D. C., Lennard, N. J., Caler, E., Hamlin, N. E. & other authors (2005). The genome of the African trypanosome *Trypanosoma brucei*. *Science* 309, 416–422.
- da Cunha, J. P., Nakayasu, E. S., de Almeida, I. C. & Schenkman, S. (2006). Post-translational modifications of *Trypanosoma cruzi* histone H4. *Mol Biochem Parasitol* 150, 268–277.
- El-Sayed, N. M., Myler, P. J., Bartholomeu, D. C., Nilsson, D., Aggarwal, G., Tran, A. N., Ghedin, E., Worthey, E. A., Delcher, A. L. & other authors (2005). The genome sequence of *Trypanosoma cruzi*, etiologic agent of Chagas disease. *Science* 309, 409–415.
- Ha, D. S., Schwarz, J. K., Turco, S. J. & Beverley, S. M. (1996). Use of the green fluorescent protein as a marker in transfected *Leishmania*. *Mol Biochem Parasitol* 77, 57–64.
- Iizuka, M., Matsui, T., Takisawa, H. & Smith, M. M. (2006). Regulation of replication licensing by acetyltransferase Hbo1. *Mol Cell Biol* 26, 1098–1108.

- Ivens, A. C., Peacock, C. S., Worthey, E. A., Murphy, L., Aggarwal, G., Berriman, M., Sisk, E., Rajandream, M. A., Adlem, E. & other authors (2005). The genome of the kinetoplastid parasite, *Leishmania major*. *Science* 309, 436–442.
- Janzen, C. J., Fernandez, J. P., Deng, H., Diaz, R., Hake, S. B. & Cross, G. A. (2006). Unusual histone modifications in *Trypanosoma brucei*. *FEBS Lett* 580, 2306–2310.
- Kawahara, T., Siegel, T. N., Ingram, A. K., Alsford, S., Cross, G. A. & Horn, D. (2008). Two essential MYST-family proteins display distinct roles in histone H4K10 acetylation and telomeric silencing in trypanosomes. *Mol Microbiol* 69, 1054–1068.
- Kimura, A., Umehara, T. & Horikoshi, M. (2002). Chromosomal gradient of histone acetylation established by Sas2p and Sir2p functions as a shield against gene silencing. *Nat Genet* 32, 370–377.
- Kouzarides, T. (2007). Chromatin modifications and their function. *Cell* 128, 693–705.
- Kumar, D., Mukherji, A. & Saha, S. (2008). Expression and subcellular localization of ORC1 in *Leishmania major*. *Biochem Biophys Res Commun* 375, 74–79.
- Kumar, D., Minocha, N., Rajanala, K. & Saha, S. (2009). The distribution pattern of proliferating cell nuclear antigen in the nuclei of *Leishmania donovani*. *Microbiology* 155, 3748–3757.
- Mandava, V., Fernandez, J. P., Deng, H., Janzen, C. J., Hake, S. B. & Cross, G. A. (2007). Histone modifications in *Trypanosoma brucei*. *Mol Biochem Parasitol* 156, 41–50.
- Miao, J., Fan, Q., Cui, L., Li, X., Wang, H., Ning, G., Reese, J. C. & Cui, L. (2010). The MYST family histone acetyltransferase regulates gene expression and cell cycle in malaria parasite *Plasmodium falciparum*. *Mol Microbiol* 78, 883–902.
- Millar, C. B. & Grunstein, M. (2006). Genome-wide patterns of histone modifications in yeast. *Nat Rev Mol Cell Biol* 7, 657–666.
- Minocha, N., Kumar, D., Rajanala, K. & Saha, S. (2011a). Kinetoplast morphology and segregation pattern as a marker for cell cycle progression in *Leishmania donovani*. *J Eukaryot Microbiol* 58, 249–253.
- Minocha, N., Kumar, D., Rajanala, K. & Saha, S. (2011b). Characterization of *Leishmania donovani* MCM4: expression patterns and interaction with PCNA. *PLoS ONE* 6, e23107.
- Nardelli, S. C., da Cunha, J. P., Motta, M. C. & Schenkman, S. (2009). Distinct acetylation of *Trypanosoma cruzi* histone H4 during cell cycle, parasite differentiation, and after DNA damage. *Chromosoma* 118, 487–499.
- Peacock, C. S., Seeger, K., Harris, D., Murphy, L., Ruiz, J. C., Quail, M. A., Peters, N., Adlem, E., Tivey, A. & other authors (2007). Comparative genomic analysis of three *Leishmania* species that cause diverse human disease. *Nat Genet* 39, 839–847.
- Robinson, K. A. & Beverley, S. M. (2003). Improvements in transfection efficiency and tests of RNA interference (RNAi) approaches in the protozoan parasite *Leishmania*. *Mol Biochem Parasitol* 128, 217–228.
- Shogren-Knaak, M. & Peterson, C. L. (2006). Switching on chromatin: mechanistic role of histone H4-K16 acetylation. *Cell Cycle* 5, 1361–1365.
- Shogren-Knaak, M., Ishii, H., Sun, J. M., Pazin, M. J., Davie, J. R. & Peterson, C. L. (2006). Histone H4-K16 acetylation controls chromatin structure and protein interactions. *Science* 311, 844–847.
- Siegel, T. N., Kawahara, T., Degrasse, J. A., Janzen, C. J., Horn, D. & Cross, G. A. (2008). Acetylation of histone H4K4 is cell cycle regulated and mediated by HAT3 in *Trypanosoma brucei*. *Mol Microbiol* 67, 762–771.
- Smith, C. M., Gaffken, P. R., Zhang, Z., Gottschling, D. E., Smith, J. B. & Smith, D. L. (2003). Mass spectrometric quantification of acetylation at specific lysines within the amino-terminal tail of histone H4. *Anal Biochem* 316, 23–33.
- Smith, A. T., Tucker-Samaras, S. D., Fairlamb, A. H. & Sullivan, W. J., Jr (2005). MYST family histone acetyltransferases in the protozoan parasite *Toxoplasma gondii*. *Eukaryot Cell* 4, 2057–2065.
- Sterner, D. E. & Berger, S. L. (2000). Acetylation of histones and transcription-related factors. *Microbiol Mol Biol Rev* 64, 435–459.
- Strahl, B. D. & Allis, C. D. (2000). The language of covalent histone modifications. *Nature* 403, 41–45.
- Suka, N., Luo, K. & Grunstein, M. (2002). Sir2p and Sas2p opposingly regulate acetylation of yeast histone H4 lysine16 and spreading of heterochromatin. *Nat Genet* 32, 378–383.
- Thomas, S., Green, A., Sturm, N. R., Campbell, D. A. & Myler, P. J. (2009). Histone acetylations mark origins of polycistronic transcription in *Leishmania major*. *BMC Genomics* 10, 152.
- Vonlaufen, N., Naguleswaran, A., Coppens, I. & Sullivan, W. J., Jr (2010). MYST family lysine acetyltransferase facilitates ataxia telangiectasia mutated (ATM) kinase-mediated DNA damage response in *Toxoplasma gondii*. *J Biol Chem* 285, 11154–11161.
- Waterhouse, A. M., Procter, J. B., Martin, D. M., Clamp, M. & Barton, G. J. (2009). Jalview Version 2—a multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25, 1189–1191.
- Wheeler, R. J., Gluenz, E. & Gull, K. (2011). The cell cycle of *Leishmania*: morphogenetic events and their implications for parasite biology. *Mol Microbiol* 79, 647–662.
- Yan, Y., Barlev, N. A., Haley, R. H., Berger, S. L. & Marmorstein, R. (2000). Crystal structure of yeast Esa1 suggests a unified mechanism for catalysis and substrate binding by histone acetyltransferases. *Mol Cell* 6, 1195–1205.
- Yan, Y., Harper, S., Speicher, D. W. & Marmorstein, R. (2002). The catalytic mechanism of the ESA1 histone acetyltransferase involves a self-acetylated intermediate. *Nat Struct Biol* 9, 862–869.
- Yun, M., Wu, J., Workman, J. L. & Li, B. (2011). Readers of histone modifications. *Cell Res* 21, 564–578.
- Zhang, K., Zheng, G. & Yang, Y.-C. (2007). Stability of Nmi protein is controlled by its association with Tip60. *Mol Cell Biochem* 303, 1–8.

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