Characterization of substrates of an S-phase cell cycle kinase of *Leishmania donovani* with emphasis on a Histone Acetyl Transferase

by

Anup Kumar Maity

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Saha Institute of Nuclear Physics, Kolkata

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Partha Saha

Partha Saha
Research Guide

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DEDICATIONS

*I dedicate this thesis to my beloved **Parents** for inspiring and instilling the importance of hard work, sincerity and higher education.*

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SYNOPSIS

Characterization of substrates of an S-phase cell cycle kinase of *Leishmania donovani* with emphasis on a Histone Acetyl Transferase

Introduction

In eukaryotic cell division cycle, duplication of the entire cellular genome takes place in S phase and its proper segregation into two daughter cells occurs at M phase. These two major events are separated by two preparatory or gap periods, namely G1 phase between M and S phases and G2 phase between S and M phases. In addition, a cell can go to an optional quiescent stage called G0 phase after the division where it remains until there is any external signal for re-entry into G1 phase. The events during the progress of cell cycle are regulated by plethora of posttranslational protein modifications, reversible protein phosphorylation mainly by the members of cyclin dependent protein kinases (Cdks), being the key mechanism among them [1]. Binary complex formation of constantly expressed catalytic subunits Cdks with periodically expressed cognate cyclin partners is the main mode of regulation of cyclin-Cdk activity. Cell cycle progression in mammals is regulated mainly by four classes of cyclins. Among them D- and E-type cyclins are involved in G1- and S-phases [2] whereas A- and B-types cyclins are critical for mitotic entry. The activity of Cdks further enhanced by Cdk activating kinase (CAK) and Cdc25 super family proteins through phosphorylation and dephosphorylation of specific residues respectively. When the initial cyclin-Cdk complexes are activated in response to external stimulus, they activate or inactivate the downstream target proteins through phosphorylation to direct coordinated entry into cell cycle [3].

Several Cdk related kinases (CRKs) and cyclins have also been identified in early branching pathogenic kinetoplastida parasites *Leishmania* and found to be involved in regulation of their cell cycle. The parasites undergo a biphasic life cycle alternating between promastigotes (flagellated and motile) in sand fly phlebotomies and amastigote (non-flagellated and non-motile) in human host. The parasites cause different types leishmaniases in 88 countries affecting more than 12 million people. Three main forms of leishmaniases caused by different species of the parasites are Cutaneous, Mucocutaneous and Visceral. Among them Visceral Leishmaniasis (VL), also known as Kala-azar, is fatal

if not treated in time and Northern Bihar in India is one of the worst affected region by VL. Unfortunately, the parasites are growing resistance to the available and widely used drugs including pentavalent antimony. Moreover, some of the presently effective drugs are expensive and have various side effects. Hence, a major focus of *Leishmania* research is to identify novel potential therapeutic targets and approaches. Interestingly, several cellular processes of the parasite are quite different from their mammalian hosts to take care of their unique requirements including transformation to alternating life cycle forms and concerted replication of nuclear and mitochondrial DNA. However, nothing much is known about these aspects of cell cycle regulation in *Leishmania*. Therefore, it is important to carry out in depth scientific research in this area.

In order to elucidate the regulatory mechanism of cell cycle progression in *Leishmania* parasites, an S-phase cell cycle kinase complex LdCyc1-CRK3 from *L. donovani* was characterized previously in our laboratory **[4, 5]**. LdCyc1 contains a conserved MRAIL motif that is responsible for interaction with proteins harbouring R/KxL like Cyclin binding (Cy) motif. Since no knowledge about the identities of such targets of LdCyc1-CRK3 in *Leishmania* is available, a screening was carried out to identify substrates of the S-phase kinase based on the presence of Cy motif as well as Cdk phosphorylation site (S/T-P-x-R/K) in the targets **[6]**. The study resulted in identification of three substrates of LdCyc1-CRK3 complex and one of the major goals of thesis is to further characterize the substrates.

In eukaryotes, chromatin remodeling through the dynamics of nucleosome assembly proteins histones regulates DNA replication, gene expression, cell proliferation and terminal differentiation. The reversible transient opening and closing of chromatin by specific posttranslational modifications of core histones are necessary to modulate the important cellular processes like DNA replication, repair and transcription. One of the major forms of modifications is the acetylation of specific lysine residues of the protruding tails of core histones such as H3 and H4. Interestingly, in the study of substrate search, the histone acetyl transferase from *L. donovani* LdHAT1 has been identified as a substrate of S-phase LdCyc1-CRK3 kinase, implicating its role in DNA replication related activities. In fact, the role of one of its human homologues Hbo1 in replication licensing has already been demonstrated **[7]**. Therefore, further characterization of the HAT and the effect of phosphorylation on its activity are studied in detail.

Aims and Objectives

Like in other eukaryotes, cyclin-Cdks play a pivotal role in controlling the cell cycle progression in trypanosomatid parasites. To characterize the cell cycle regulation in *Leishmania* parasites in details, an S-phase cyclin-Cdk complex, *viz.*, LdCyc1-CRK3, was identified and studied in depth in the laboratory previously. However, no knowledge about the substrates of the cyclin-Cdk complex in *Leishmania* is available. Therefore, it would be interesting to identify the targets of cyclin-Cdk in the parasite to characterize cell cycle regulation in greater details. In this regard, a screening for the potential substrates of the cell cycle kinase LdCyc1-CRK3 was performed resulting in the identification of three substrates. One of the major aims of my doctoral work is to authenticate and further characterize the identified substrates. One of the identified substrates is homologous to histone acetyl transferase (HAT). Since HATs regulate chromatin remodeling and the process is important for S-phase related activities such as DNA replication, one aim is to characterize the effect of phosphorylation by the S-phase cell cycle kinase on the activity of the HAT. Such studies could address important issues related to *Leishmania* parasites and also eukaryotic biology in general.

Summarized Result

Characterization of Substrates of the S-phase kinase LdCyc1-CRK3 from *Leishmania donovani*

Since R/KxL-type Cy motif and the phosphorylation target (S/T)Px(K/R) were shown to constitute a bipartite substrate recognition sequence of cyclin dependent kinases **[8]**, in order to identify the potential substrates of S-phase cell cycle kinase LdCyc1-CRK3 from *L. donovani* **[5]** 28 putative ORFs containing both the conserved Cy-motif and the Cdk phosphorylation site were screened **[6]**. Finally, three substrates of the kinase could be identified and the identified substrates were designated as Ld29.1050L (LmF 29.1050 like from *L. donovani*), Ld28.0070L and Ld14.0140L. Since the templates of the identified substrates were amplified from genomic DNA during screening, the presence of the messages was determined in the expressed sequences by PCR from a cDNA library to confirm that the ORFs were actually expressed in cells.

Next**,** in order to confirm the authenticity of the identified substrates and further characterize them, the proteins were expressed in bacterial cells with ^{6}His tag and purified.

In parallel, LdCRK3 was expressed in Sf9 insect cells and GST-LdCyc1 in bacterial cells and the active LdCyc1-CRK3 was prepared by complex formation between them. Expectedly, all the three identified substrates could be phosphorylated by GST-LdCyc1-CRK3 complex *in vitro* confirming the validity of the screening. Interestingly, the phosphorylation of all the proteins was inhibited significantly and specifically by a peptide (PS100) containing the RRLFG Cy-motif but not by a control peptide confirming the Cy-motif mediated substrate docking. Such Cy-motif dependent phosphorylation of the substrates could also be observed with LdCyc1-CRK3 complex isolated from *L. donovani* cells through immunoprecipitation by antibody against LdCyc1. To further prove the hypothesis that the identified substrates interact with LdCyc1 through their Cy-motif, interaction assays were performed between the GST-LdCyc1 and ^{6}His-tagged substrate proteins. It was shown that ^{6}His-tagged proteins specifically interacted with GST-LdCyc1, and expectedly, PS100 peptide almost completely abolished the interactions whereas the control peptide did not, confirming the Cy-motif dependent interaction between the proteins.

Among the three identified substrates, the derived amino acid sequence of Ld28.0070L did not show homology with any previously characterized protein except the predicted equivalent proteins in related *Leishmania* species, implying its unique role in parasite specific cell cycle related processes. On the other hand, the predicted primary structure of Ld29.1050L was shown to contain conserved domains of Ku70 protein **[9]**, a subunit of the versatile heterodimeric protein complex Ku70-Ku80 that was implicated in various nuclear processes like DNA repair, telomere maintenance and apoptosis **[10]**. The third identified substrate Ld14.0140L was found to contain a MYST (human Moz, Yeast Ybf2 and Sas2, and mammalian TIP60) histone acetyl transferase (HAT) domain along with an associated chromodomain **[9]**. The accession numbers of the nucleotide sequences data of the three substrates Ld14.0140L, Ld28.0070L and Ld29.1050L submitted to the GenBank™, EMBL and DDBJ databases are **HM120719, HQ264173 and HQ264174,** respectively.

Histone Acetyl Transferase activity of LdHAT1 is regulated by an S-phase cell cycle kinase from *Leishmania donovani*

LdHAT1 is one of the identified substrates of the S-phase cell cycle kinase LdCyc1-CRK3 from *L. donovani*, which contains a MYST (human Moz, Yeast Ybf2 and Sas2, and human TIP60) domain of histone acetyl transferases **[6]**. The HAT protein from *L.*

donovani is 97% identical to LmHAT1, which was grouped with the HAT1 from *T. brucei* and *T. cruzi* in a phylogenetic analysis **[11]**. Maximum homology is present along the C-terminal canonical MYST domain (amino acid 254-456 of LdHAT1), which contains the characteristic acetyl CoA binding motif and Zn-finger motif. As previously described **[6]**, the cyclin binding R/KxL-type Cy-motif **[12]** is located within the MYST domain in LdHAT1, though such a typical motif is absent in HsTIP60, DmMof and HsHbo1. However, a canonical Cdk target phosphorylation site (TPEK) is well-conserved within the MYST domain of LdHAT1 and in the other MYST family members.

Since phosphorylation of LdHAT1 by S-phase Cdk suggested the possibility of its involvement in cell cycle related periodic activities, its expression profile was analyzed during cell cycle progression of *L. donovani* promastigotes. The exponentially growing promastigotes were synchronized by hydroxyurea treatment and LdHAT1 was found to be expressed at a constant manner as observed by immunoblotting with anti-LdHAT1 antibody raised during the study.

In order to determine the identity of Cy-motif in LdHAT1 and further confirm its contribution on the activities of LdHAT1, site directed mutagenesis was carried out to change the conserved residues in the motif (^{290}RRLVV→RDDVV, LdHAT1ΔCy). To confirm the identity of Thr in the TPEK as target residue of phosphorylation, it was mutated to Ala, again by site directed mutagenesis (^{394}TPEK→APEK, LdHAT1-T394A). Then an interaction assay of GST-LdCyc1 was carried out with wild type LdHAT1 as well as its two mutants, LdHAT1ΔCy and LdHAT1-T394A. The wild type and LdHAT1-T394A proteins were found to interact with GST-LdCyc1, whereas the interaction with LdHAT1ΔCy was almost completely abolished proving the involvement of Cy-motif during direct interaction between the proteins. The result also confirmed the identity of Cy motif in LdHAT1 protein. Again, LdHAT1-T394A was found not to be phosphorylated by LdCyc1-CRK3, confirming that Thr-394 is the target for phosphorylation by the kinase complex. To further establish the involvement of Cy motif in the phosphorylation event, wild type LdHAT1 and the mutant LdHAT1ΔCy was used as substrates in a kinase assay of LdCyc1-CRK3 complex and it was found that the mutant was not phosphorylated with LdCyc1-CRK3 kinase. The result confirmed that LdHAT1 protein is phosphorylated at Thr-394 residue in a Cy motif dependent manner.

It was previously implicated that HAT1 from *T. brucei* could acetylate Histone H4 from the parasite **[11]**. Therefore, in order to characterize the histone acetylation activity of LdHAT1, *in vitro* assays were carried out using purified *L. donovani* histone H4 full

length protein expressed in bacteria. The incorporation of acetyl group in lysine residue of histone was detected by anti-acetyl lysine antibody. To further characterize the acetylation activity of LdHAT1 the same assays were performed with a peptide substrate derived from the N-terminus of *L. donovani* histone H4. To identify the lysine residue that was specifically acetylated by LdHAT1, three antibodies were raised against *L. donovani* histone H4 derived peptides acetylated on K4, K10 or K14 residue, respectively. The peptide acetylated by LdHAT1 could be detected only by anti-H4K10Ac antibody, but not with other two antibodies, suggesting that the acetyl transferase from *L. donovani* specifically acetylates H4K10 residue. Most interestingly, H4K10 acetylation activity of LdHAT1 was found to be inhibited when the HAT is phosphorylated by LdCyc1-CRK3, suggesting the down-regulation of histone H4 acetylation by the S-phase cell cycle kinase. Further, its two mutants LdHAT1ΔCy and LdHAT1-T394A also did not show any acetylation activity. This could happen due to likely perturbation of MYST domain in LdHAT1 implicating the contribution of the mutated residues in the enzymatic activity.

Conclusion

Phosphorylation is one of the major posttranslational modifiers where cyclin dependent protein kinases (Cdks) play key role by phosphorylation of several proteins to control cell cycle progression and cell division in eukaryotes **[1]**. Several cyclins and Cdks also have been identified in early branching protozoan parasite *Leishmania donovani* in the order of kinetoplastida that regulate the cell cycle progression like in other eukaryotes. One such kinase complex, LdCyc1-CRK3, specific to S-phase of cell cycle has been identified in our laboratory previously **[4]**. A screening was carried out in our laboratory which resulted in the identification of three substrates of the S-phase cell cycle kinase LdCyc1-CRK3 of *L. donovani*. However, information about their mode of interaction with the kinase complex and regulation of their function due to phosphorylation remained unknown. Therefore, further characterization of the identified substrates of LdCyc1-CRK3 was carried out in order to elucidate the targeting mechanism of the substrates to the kinase complex. Further investigation of functional modification of one of the substrate proteins due to phosphorylation was also carried out.

All the identified three substrates are expressed in *L. donovani* promastigotes as confirmed by RT-PCR. One of the identified substrates, viz. Ld28.0070L, is unique to the

parasite, and therefore, it may help to identify new therapeutic targets to combat the parasite infection. The other two proteins, *viz*., Ld29.1050L and Ld14.0140L, are homologous to Ku70 DNA binding protein and MYST family HAT, respectively. It was established that for the phosphorylation of the three substrates by LdCyc1-CRK3 kinase a Cy motif dependent interaction with MRAIL motif of LdCyc1 is essential. The inference was confirmed by peptide competition assay as well as by site directed mutagenesis of the putative Cy motif in one of the substrates, *viz.*, Ld14.0140L, which we term as LdHAT1.

It was confirmed that LdHAT1 protein is expressed at a constant manner during cell cycle progression of *L. donovani* promastigotes, raising the possibility of regulation of its activity by posttranslation modification. Site directed mutagenesis establishes not only the identity of Cy motif at ^{290}RRL in LdHAT1 but also phosphorylation site Thr-394 that are located within the conserved MYST domain. The LdHAT1 acetylates lysine residue specifically at position 10 but not at positions 4 or 14 of Ldhistone H4. Strikingly, the activity of LdHAT1 is inhibited as a result of its phosphorylation by LdCyc1-CRK3. The inhibition could be due to the disturbance in the catalytically important Glu-396 residue adjacent to the phosphorylation site Thr-394 residue. Moreover, mutations in LdHAT1 at R/KxL type Cy-motif and Threonine residue at TPEK phosphorylation site show inhibition of acetyl transferase activity, most likely due to perturbation of MYST domain. It will be interesting to study the effect of inhibition of LdHAT1 activity by the S-phase kinase on the cell cycle events of the parasites.

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INTRODUCTION

The conserved family of cyclin dependent protein kinases (Cdks) primarily regulates the cell cycle progression in eukaryotes through phosphorylation of various key proteins. Several Cdk related kinases (CRKs) and cyclins have also been identified in early branching pathogenic kinetoplastida parasites *Leishmania* which undergo a biphasic life cycle alternating between promastigotes (flagellated and motile) in sand fly phlebotomies and amastigote (non-flagellated and non-motile) in human host. The parasites cause different types leishmaniases in 88 countries affecting more than 12 million people. Three main forms of leishmaniases caused by different species of the parasites are Cutaneous, Mucocutaneous and Visceral. Among them Visceral Leishmaniasis (VL), also known as Kala-azar, is fatal if not treated in time and Northern Bihar in India is one of the worst affected region by VL. Unfortunately, the parasites are growing resistance to the available and widely used drugs including pentavalent antimony. Moreover, some of the presently effective drugs are expensive and have various side effects. Hence, a major focus of *Leishmania* research is to identify novel potential therapeutic targets and approaches. Interestingly, several cellular processes of the parasite are quite different from their mammalian hosts to take care of their unique requirements including transformation to alternating life cycle forms and concerted replication of nuclear and mitochondrial DNA. However, nothing much is known about these aspects of cell cycle regulation in *Leishmania*. Therefore, it is important to carry out in depth scientific research in this area, and in the next chapter, the relevant information available in the literature are reviewed critically identifying the lacuna in the field.

In order to elucidate the regulatory mechanism of cell cycle progression in *Leishmania* parasites, an S-phase cell cycle kinase complex LdCyc1-CRK3 from *L. donovani* was characterized previously in our laboratory. LdCyc1 contains a conserved MRAIL motif that is responsible for interaction with proteins harbouring R/KxL like Cyclin binding (Cy) motif. Since no knowledge about the identities of such targets of

LdCyc1-CRK3 in *Leishmania* is available, identification of substrates of the S-phase kinase was carried out based on the presence of Cy motif as well as Cdk phosphorylation site (S/T-P-x-R/K) in the targets. The study resulted in identification of three substrates of LdCyc1-CRK3 complex and one of the major goals of thesis is to further characterize the substrates. The results of such studies are described in Chapter 4.

Among the three identified substrates, one is a unique protein with no known homologues. Another one is similar to MYST family histone acetyl transferase (HAT) and the third one contains Ku-70 related conserved domain. Cellular expression of all the three substrates has been confirmed by RT-PCR. A Cy-motif containing peptide has been shown to specifically inhibit the interaction between the substrates and LdCyc1 as well as phosphorylation of the substrates by the LdCyc1-CRK3 kinase suggesting the involvement of Cy-motif mediated association during the phosphorylation events. Such a phenomenon was further confirmed by site directed mutagenesis study with the substrate homologous to HAT, which we term as LdHAT1.

In eukaryotes, dynamics of nucleosome assembly proteins histones and their associates play important roles in chromatin remodeling, which regulates DNA replication, gene expression, cell proliferation and terminal differentiation. The reversible transient opening and closing of chromatin by specific posttranslational modifications of core histones are necessary to modulate the important cellular processes like DNA replication, repair and transcription. One of the major forms of modifications is the acetylation of specific lysine residues of the protruding tails of core histones such as H3 and H4. Interestingly, the histone acetyl transferase from *L. donovani* LdHAT1 has been identified as a substrate of S-phase LdCyc1-CRK3 kinase, implicating its role in DNA replication related activities. In fact, the role of one of its human homologues Hbo1 in replication licensing has already been demonstrated. The observation that LdHAT1 is phosphorylated by an S-phase kinase raised a possibility of another mode of regulation of replication initiation by cyclin-Cdk. LdHAT1 has been found to contain a conserved MYST domain and a chromodomain for specific functions. In Chapter 5 of the thesis, further characterization of the HAT is described. It has been found that LdHAT1 acetylates histone H4 at K10 residue, and interestingly, its activity is reduced after phosphorylation by LdCyc1-CRK3. Therefore, the S-phase specific kinase LdCyc1-CRK3 may play an important role in the regulation of LdHAT1 activity through phosphorylation to control the cell cycle and life cycle specific chromatin dynamics in *L. donovani*.

REVIEW OF LITERATURE

CELL CYCLE IN EUKARYOTES

The cell cycle is a series of events in a growing cell that regulate replication of DNA and their segregation into two daughter cells to maintain genome integrity. Protein phosphorylation is the key regulatory posttranslational modification that controls the cell cycle progression and cell division in eukaryotic organisms. In eukaryotes, cyclin dependent protein kinases (Cdks) play a central role in the regulation of cell cycle progression through phosphorylation of different proteins **[1]**. Duplication of the entire genome in S phase and its proper segregation into two daughter cells at M phase are the two major events of the cell cycle. These two events are separated by two preparatory or gap periods, namely G1 phase between M and S phases and G2 phase between S and M phases (**Figure 2.1**). In addition, a cell can go to an optional gap period or quiescent stage called G0 phase after the division where it remains until there is any external signal for re-entry into G1 phase. As mentioned above, cyclins and Cdks play major role in the highly regulated process of cell cycle progression in eukaryotes. These two classes of proteins form a bipartite complex in which cyclins are the regulatory subunits responsible for substrates specificity and docking and Cdks are the catalytic subunits that phosphorylate the substrates. The Cdks are expressed throughout all phases of the cell cycle whereas cyclin expression is periodic due to cell cycle dependent regulation of both cyclin gene transcription and protein degradation. When the initial cyclin-Cdk complexes are activated in response to external stimulus, they activate or inactivate the downstream target proteins through phosphorylation to direct coordinated entry into cell cycle **[2]**. As a result, fine tuning by regulatory network promote the cascade of events at precise time to duplicate DNA and divide the cell.

Cyclins and Cyclin Dependent Kinases (Cdks)

Although cyclin dependent kinases (Cdks) play major role during cell cycle progression, they cannot function alone and for its activity cyclin binding is essential. Each cell cycle in human cell is regulated mainly by four classes of cyclins. Among them D- (D1, D2, and D3) and E-type (E1 and E2) cyclins are involved in G1-and S-phases **[3]** whereas A- and B-types cyclins are critical for mitotic entry. However, it has been found recently that fibroblasts can proliferate in absence of Cyclin A **[4]**.

As already mentioned, cyclin dependent kinases (Cdks) play an important role in the cell cycle progression in eukaryotes. Interestingly, Cdks not only regulate the cell cycle progression but also control other key cellular processes including gene transcription and neural differentiation. To know the diversity and versatility of Cdk function in biological processes, it is important to study their structure and regulation. Cdk activity oscillates with varying cyclin concentration levels in different phases of the cell cycle. In the late G1 phase Cdk4/6 forms complex with Cyclin D (**Figure 2.1** and **Table 2.1**) and activate the transcription of pro-cell cycle genes by inactivating transcription repressor retinoblastoma (RB) family of proteins through phosphorylation. At hypo-phosphorylated state, the retinoblastoma family of proteins such as Rb, p107, p130 etc. inhibit transcription through binding to the pro-cell cycle progression transcription activator E2F. In mammals, Cyclin D-Cdk4/6 activity is essential for E2F mediated transcription of important genes whose products are required for G1-S transition and S phase progression. Cyclins E and A are two of the most important genes that are expressed by E2F. Cyclin E binds to Cdk2 to promote G1-S transition and Cyclin A-CDk2 is involved in progression of the cell cycle events from S phase to G2 phase. Cdk1 is regulated by Cyclin B to

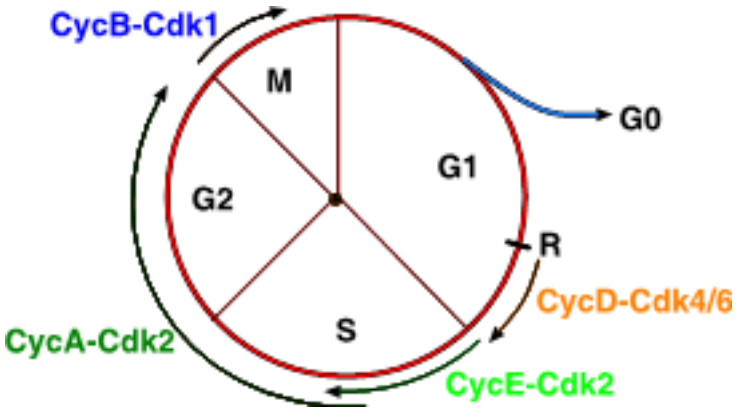


Figure 2.1: Periodic activity profile of different Cyclin-CDK complexes in *H. sapiens* during cell cycle progression.

Table 2.1: Role of different Cyclins and Cdks during eukaryotic cell cycle

Species	Cyclins	Cdks	Role/ Function
H. sapiens	Cyclin D	Cdk4/6	G1 phase progression
	Cyclin E	Cdk2	G1-S transition and replication initiation
	Cyclin A	Cdk2	S and G2 phases progression
	Cyclin B	cdc2 (Cdk1)	G2-M transition
	Cyclin A	cdc2	G2-M transition
	Cyclin B3	cdc2/Cdk2	G2 and M phases (in the Avian cells only)
S. cerevisiae	Cln1-3	CDC28	G1 phase progression
	Clb5,6	CDC28	S phase progression
	Clb1-4	CDC28	G2 and M phases progression
S. pombe	Cig2/Cig1	cdc2	S phase progression
	CDC13	cdc2	M phase progression

promote G2-M transition, and finally depletion of Cyclin B is critical for mitotic exit at the end of M phase.

Unlike humans, both budding yeast *Saccharomyces cerevisiae* and fission yeast *Schizosaccharomyces pombe* contain a single Cdk that regulates the entire cell cycle events through binding with different cyclins at different phases of the cell cycle (**Table 2.1**). CDC28 is the only Cdk in *S. cerevisiae* that binds to the G1 cyclins Cln1-3 for G1 activities. S-phase entry is ensured by the association of CDC28 with two cyclins Clb5/6. Other four cyclins, Clb1-4 control the mitotic events **[5]**. There is no clear G2 phase due to overlapping of S and M phases in *S. cerevisiae*. On the other hand, in *S. pombe* cdc2 is the only Cdk that regulates S phase through association mainly with cyclin Cig2 and also with Cig1, whereas for mitotic function it binds to CDC13 **[6]**.

Although different Cdk proteins are present in vertebrates rather than just one in yeast, only few are involved in the cell cycle regulation in higher eukaryotes. Other Cdks are not directly involved in cell cycle regulation, but may play auxiliary roles. Only cdc2 (Cdk1), Cdk2 and Cdk4/6 of mammals are functionally homologous to yeast cdc2/CDC28 and critically involved in cell cycle progression.

Regulation of Cyclin-Cdk Activity during Cell Cycle

By Phosphorylation and dephosphorylation: In addition to cyclin binding, Cyclin-Cdk complexes are also activated or inhibited by several phosphorylation and dephosphorylation events during cell cycle **[3]** (**Figure 2.2**). The Cdk activating kinase (CAK) and the Cdc25 phosphatase family of proteins are the two classes of proteins that regulate phosphorylation and dephosphorylation of the kinase complex, respectively. The CAK activates Cdks through phosphorylation at a conserved threonine residue – Thr-160 in Cdk2 or equivalent residue in other Cdks. The structural aspects of the activation process have been established by crystallography of a complex between Cdk2 phosphorylated at Thr-160 and a truncated version of Cyclin A **[7]**, which will be discussed in detail later in the chapter. In human cells, the Cdc25 proteins are encoded by a multigene family, consisting of three members - Cdc25A, Cdc25B and Cdc25C. Cdc25A promotes the G1-S transition by activating Cyclin D-Cdk4 or 6, Cyclin E-Cdk2 and Cyclin A-Cdk2 complexes through the removal of inhibitory phosphate groups on Thr-14 and Tyr-15 of Cdk2 or equivalent residues in other Cdks **[8-10]**. Activity of

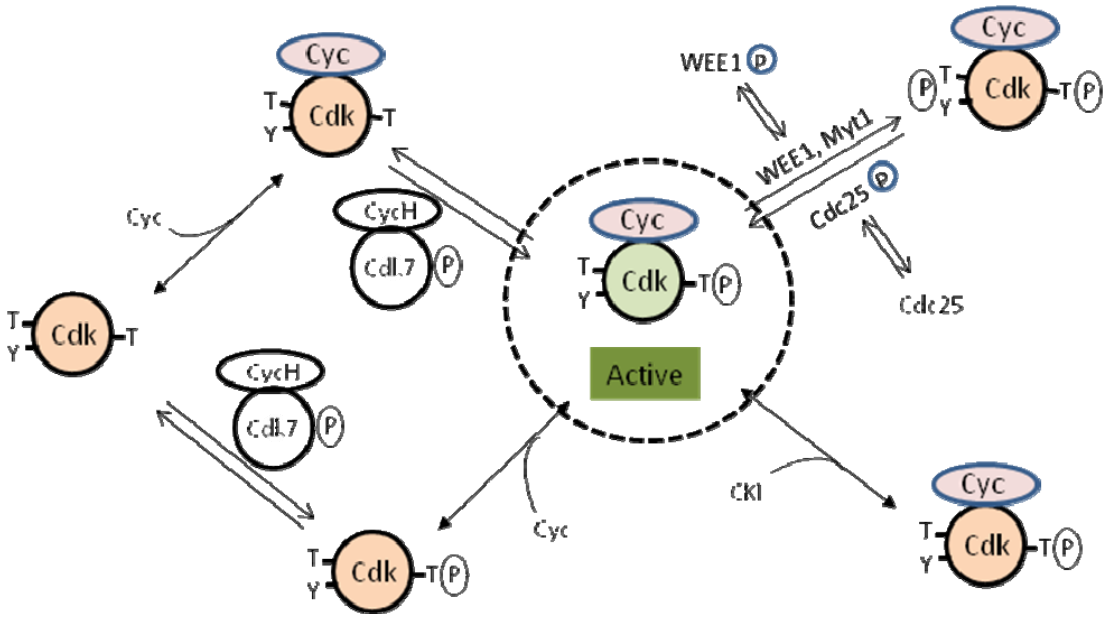


Figure 2.2: Different pathways of Cdk activation and inactivation. Threonine 160 or equivalent threonine phosphorylated Cdk-Cyclin complex in the center is the active state and four outward pathways leading to its inactivated states are shown. The kinases, phosphatases and Cdk inhibitors (CKI), which play important roles to control the reversible active-inactive status of Cdks are indicated.

Cdc25B starts at S-phase and peaks at G2-phase. The Cdc25B is first activated by Cyclin B1-cdc2 to generate a positive feedback loop that control mitotic entry **[11, 12]**. Both Cdc25B and Cdc25C play key roles for G2-M transition. Cdc25C dephosphorylates cdc2 of the Cyclin B1-cdc2 to promote the cell cycle progression through G2-M transition **[13]**.

By Cdk inhibitors: Kinase activity of Cyclin-Cdk complexes is also regulated by a group of functionally related small proteins called Cdk inhibitors. There are mainly two classes of Cdk inhibitors; one is the INK4 family of Cdk inhibitors consisting of p15, p16, p18 and p19 and other is the Cip/Kip inhibitors that includes p21, p27 and p73. The INK4 family of Cdk inhibitors is active during G1-phase of the cell cycle by specifically inhibiting Cdk6 complexes. On the other hand, the Cip/Kip families of inhibitors inhibit different Cyclin-Cdk complexes throughout the cell cycle. Both families of inhibitors can arrest the cells during G1-S transition by inhibiting the Cdk activities resulting in the hypophosphorylation of the tumor suppressor retinoblastoma family proteins **[2]**.

By Expression and degradation of Cyclins: For Cdk to become active it must form a bipartite complex with cyclin in different cell cycle stages (**Figure 2.1** and **Table 2.1**). Cyclins also help to specifically recognize substrates **[14-17]**. Although the expressions of Cdks are invariable during cell cycle progression, the cyclins expressions are periodic during cell cycle progression. Such periodic oscillation of cyclin concentration is mainly regulated at the level of gene transcription, protein degradation and protein translocation. Oscillations of cyclin concentration have been identified at mRNA as well as protein levels. In mammals, Cdk4 and 6 associate with Cyclin D in the late G1 phase to activate transcription factor E2F by phosphorylation of retinoblastoma (RB) family proteins **[18, 19]**. In the hypo-phosphorylation state, the transcription repressors, RB proteins strongly associate with E2F to suppress the transcription. When RB proteins are phosphorylated by Cyclin D-Cdk4/6 the unbound E2F transcriptionally activates many important genes like Cyclin E, Cyclin A etc. that are required for G1-S transition and S phase progression **[20-22]**. Cyclin E binds to Cdk2 and the complex phosphorylates RB proteins and enhances the transcription of Cyclin E and Cyclin A in a positive feedback loop. In *S. cerevisiae*, the Cln3-CDC28 complexes are activated by transcription factor SBF (consist of Swi6 and Swi4) to increase the transcription of other cyclins - Cln1 and Cln2 **[5, 23, 24]**. The Cln1 and 2 form complexes with CDC28 and stimulate the transcription of Cln1 and 2 genes in a positive feedback loop pathway.

Protein degradation is another pathway to regulate the availability of cyclins during cell cycle progression. The ubiquitin mediated protein degradation of Cyclin B is critical for both mitotic exit and preparation of the next cell cycle **[25]**. Anaphase promoting complex (APC) or cyclosome complex acts as an E3 enzyme to catalyze the transfer of ubiquitin molecule to a small sequence motif called "destruction box" of mitotic cyclins for degradation. The ubiquitin mediated degradation of Cyclin E requires phosphorylation by the associated Cdk subunit **[26, 27]**. Cyclin D degradation requires phosphorylation but not by the associated Cdk4 subunit **[28]**.

Another way of cyclin regulation is the translocation of cyclins during cell cycle progression. In vertebrates, Cyclin B remains in the cytoplasm during S and G2 phases of the cell cycle **[29]**. At the G2-M transition the cyclin B is translocated into the nucleus after dephosphorylation by Cdc25C **[30]**.

Structures of Cdks and Cyclins

Structure of Cdks: It is important to know the structure of Cdks to understand the mechanism of mode of their regulation and action. The catalytic subunits Cdks have specific binding sites for ATP as well as their protein substrates. Proper orientation of ATP and substrate proteins is critical for transferring the γ-phosphate of ATP to the side-chain hydroxyl group of serine or threonine residues of protein substrates through nucleophilic attack. Molecular size of the Cdk family members is approximately 35 kDa and their primary structures are 35 to 65% identical to cdc2 of *Schizosaccharomyces pombe* and Cdc28 of *Saccharomyces cerevisiae*. Three dimensional crystal structure of human Cdk2 with ATP has provided information on conserved structural signatures that may be present in all Cdks **[31, 32]**. Cdks contain three domains - a small N-terminal lobe composed of β-sheets, a large helical C-terminal lobe and a large PSTAIRE helix (**Figure 2.3**). The ATP fits well into the hydrophobic pocket within the cleft between the two lobes and the γ-phosphate group is oriented outward of the cleft-mouth. On the other hand, protein substrate binds to the upper-surface of C-terminal lobe at the entrance of the cleft-mouth to allow catalytic transfer of γ-phosphate to the hydroxyl oxygen of serine/threonine during active state of the kinases. For phospho-transfer reaction, Cdk2 activation requires two steps - first step is the removal of a large T-loop in front of the cleft-mouth that prevents the substrate binding at the C-terminal lobe through the phosphorylation of Thr-160 by CAK, whereas second step is to allow the ATP binding

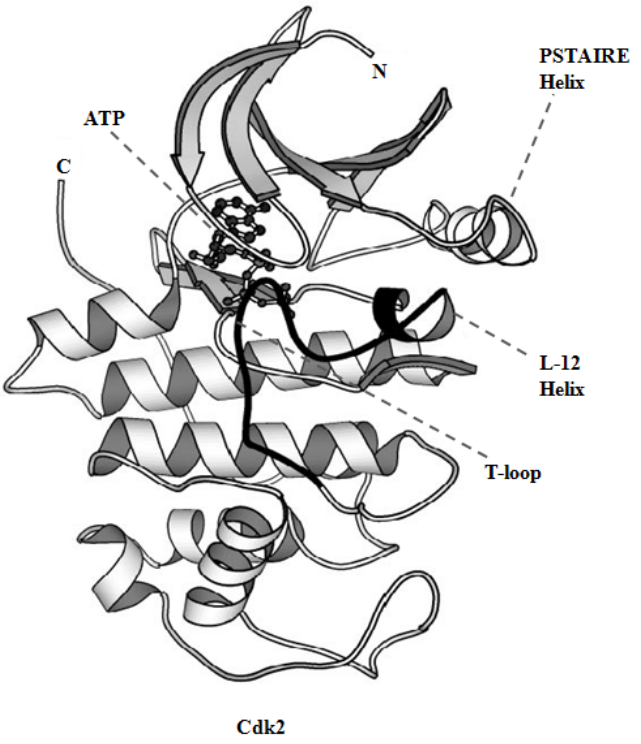


Figure 2.3: Structure of the complex of human Cdk2 and ATP. The smaller N-terminal lobe containing the beta sheet and the PSTAIRE helix is shown above the larger C-terminal lobe. The phosphate group of ATP (ball and stick representation) present in the active site in between the lobes is outward oriented. The T-loop (residues 146–170) is highlighted in black **[31]**.

through proper orientation of PSTAIRE helix by appropriate positioning of the helix-L12. Like PSTAIRE motif in Cdk2, other Cdks contain conserved helix motifs such as PCTAIRE, PISSLRE, PITALRE and PITSLRE **[33]**.

Structure of Cyclins: Unlike Cdks, sizes of the cyclins vary from 35 to 90 kDa. However, a stretch of approximately 100 residues called cyclin box, are conserved among different cyclins and responsible for Cdk association and activation **[34, 35]**. Crystal structure analysis of two cyclins, Cyclin A_t (truncated by deletion of 170 residues at N-terminal) (**Figure 2.4A**) and Cyclin H reveal that both contain two compact central domains of five helices and two helices - one at the N-terminal and the other one at C-terminals of proteins. The conserved MRAIL motif responsible for binding with substrate proteins containing R/KxL type Cy motif present in the N-terminal helix I (**Figure 2.4B)**. Helix-3 and helix-5 of the C-terminal compact domain of both Cyclin A_t **[36, 37]** and Cyclin H **[38, 39]** have been shown to be responsible for PSTAIRE motif mediated Cdk binding (**Figure 2.5**).

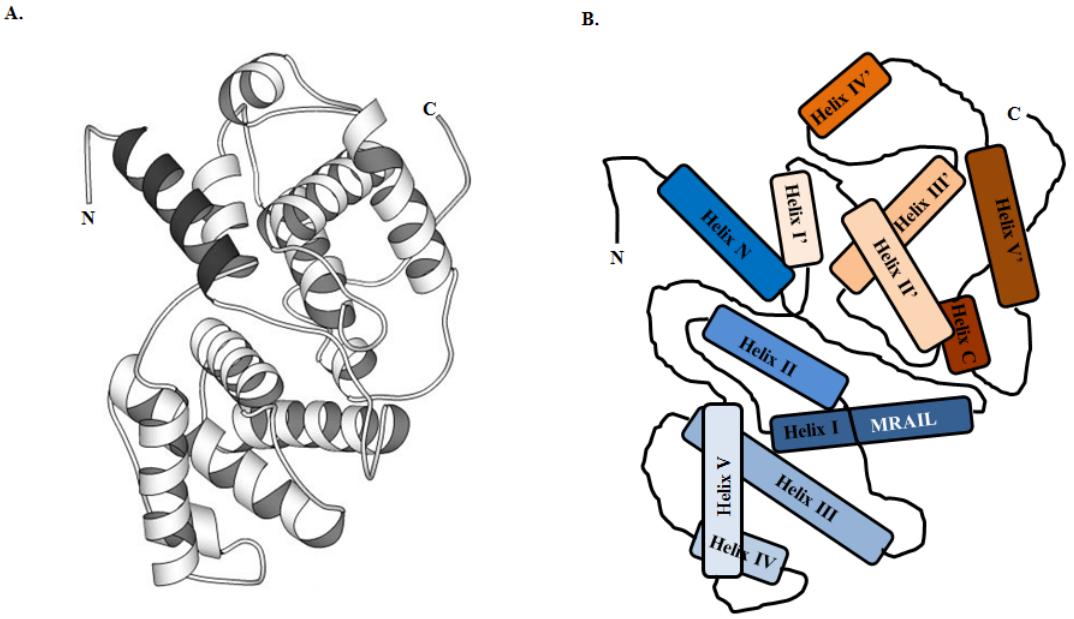


Figure 2.4: A. Structure of truncated human Cyclin A (lacking the N-terminal 172 residues). The cyclin box is a conserved domain present as 5-helix bundle in the lower half where helices 3 and 5 are responsible for Cdk2 binding. Similarly another 5-helix bundle lies above the cyclin box domain. Other two helices lie outside of the 10-helix core: the large N-terminal helix (helix N, *highlighted*) and the small C-terminal helix (hidden behind the second core domain) **[36, 37]**. **B. Schematic representation** of the same where MRAIL motif is indicated in the Helix I.

Substrates of Cdks

Although the importance of Cdk activity during different cell cycle phases is well known, there is a lack of in depth knowledge about their substrates whose phosphorylation is critical for cell cycle progression. Therefore, the identification of substrates of specific cyclin-Cdk complexes is very much essential to understand the detailed mechanism of cell cycle regulation. The transcription repressor pRB is one of the important substrates of cyclin-Cdks that is phosphorylated first by Cyclin D-Cdk4/6 at late G1 phase in response to mitogenic signal resulting in the initiation of cell cycle progression **[40]**. The hyper-phosphorylation state of pRB protein is maintained as it acts as substrate of Cyclin E-Cdk2 complex at the G1-S transition **[41]** followed by Cyclin A-Cdk2 complex during S phase **[42]**. On the other hand, during mitosis Cyclin B-Cdk1 complex primes the substrates like INCEP and BubR1 for further phosphorylation by another protein kinase, Polo like kinase (Plk1) **[43, 44]**. A large proportion of the known substrates are involved in transcriptional regulation like pRB, which is a substrate of Cyclin D-Cdk4/6 and Cyclin E-Cdk2 **[45, 46]**;

NPAT, which activates histone H2b promoter after phosphorylated by Cyclin E-Cdk2 and promotes S phase entry **[47, 48]** and CBP which is also activated by Cyclin E-Cdk2 **[49]**. Moreover, Cyclin E-Cdk2 seems to be important for centrosome duplication **[50]** where nucleoplasmin is a critical substrate **[51]**.

The requirement of R/KxL type Cy motif in the substrates that bind to a conserved hydrophobic region of Cyclins is well established **[52]** (**Figure 2.6)**, but the degree of contribution of the motif depends on the concerned Cyclin-Cdk complex **[53-55]**. The requirement of R/KxL motif for the substrates of cyclin D-Cdk4 has been found to be

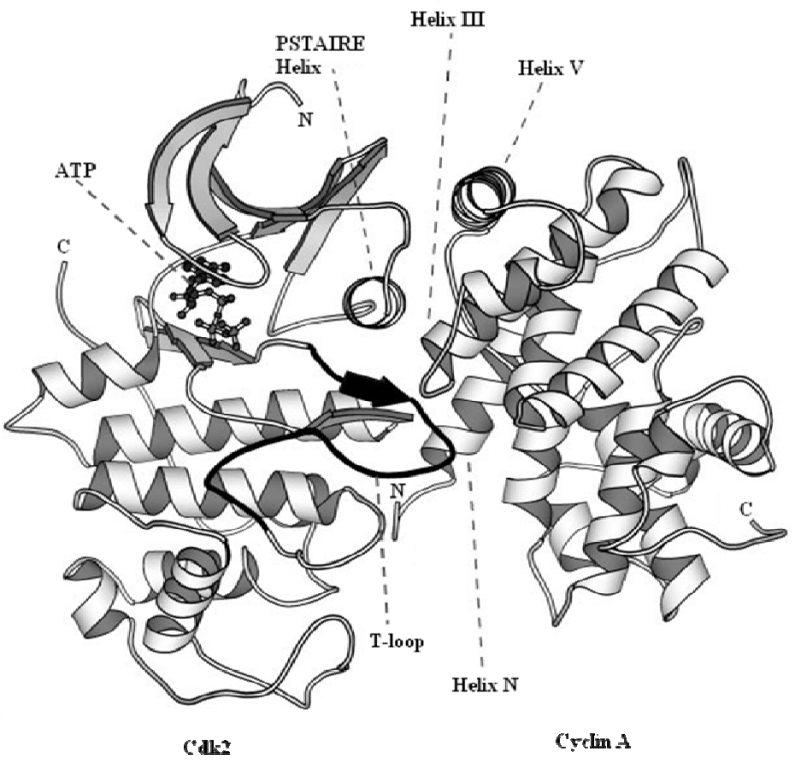


Figure 2.5: Structure of the truncated human cyclin A-Cdk2-ATP complex. On the left side, Cdk2 is shown in the same orientation as in **Figure 2.3** and the T-loop is highlighted in black. The truncated cyclin A is on the right and is rotated around the horizontal axis about 90^0 relative to its position in **Figure 2.4**. The interaction between Cdk2 and cyclin A occurs through the PSTAIRE helix of Cdk2 and helices 3 and 5 of cyclin A, and between helix N of cyclin A and the C-terminal lobe of Cdk2. Cyclin binding induces dramatic conformational changes in Cdk2 (compare with **Figure 2.3**). The L12 helix in the T-loop is replaced with a beta strand, allowing the PSTAIRE helix to move inward; this leads to the correct positioning of side chains involved in ATP phosphate orientation. The T-loop in the complex is flattened relative to its position in the Cdk2 monomer **[37]**.

Figure 2.6: Schematic presentation of substrate phosphorylation by Cdk2. The PSTAIRE domain of human Cdk2 (ATP bound) monomer form a complex with Cyclin A for its activation through phosphorylation of threonine residue at 160. Finally, RXL-type Cy motif containing substrates come close proximity of Cdk2 by interacting with MRAIL motif of Cyclin to get phosphorylation at serine or threonine residues.

more stringent due to the probable role of the substrate binding for stabilization of catalytically active conformation of the kinase complex **[55]**. Moreover, requirement of a Cy motif is more important for efficient phosphorylation of substrate peptides by cyclin E-Cdk2 than cyclin A-Cdk2 **[56]**. The Cy motif was first identified in the Cyclin-Cdk inhibitor p21 **[52]**, and later found to be present in other inhibitors (p27 and p57), activator Cdc25A **[57]** and substrates Rb, Cdc6 and others **[58-60]**. In *Xenopus*, tipin is a novel substrate plays critical role in recovery of stalled forks during DNA replication **[61]**.

Several studies based on large scale screening method have identified a large number of Cdk substrates mainly in budding yeast, *S. cerevisiae* and to a limited extent in other eukaryotes **[58, 62-67]**. In a screening using mitotic Xenopus extract as a source of protein kinases, twenty mitotic phosphoproteins have been identified most of which have been shown to be direct substrates of Cyclin B-Cdk1 **[58, 66]**. Another important approach to confirm the substrate phosphorylation by cyclin-Cdk complexes is the high throughput mass-spectrometric study **[63, 68, 69]**. Human homologues of nine novel *Xenopus* substrates have been demonstrated as phosphoproteins containing pSP/pTP peptide by mass spectrometric studies **[63, 70]** and another four novel *Xenopus* substrates are also phosphoproteins with pSP/pTP peptide that are likely to be much conserved target of Cdks. The consensus sequence motifs for Cdk phosphorylation are well established by screening oriented peptide libraries, where S/T-P and S/T-P-x-K/R are the minimal and complete motifs respectively **[54, 71]**.

Role of Cdks in cellular processes other than Cell Cycle Regulation

As Cyclin Activating Kinase (CAK): As mentioned before, in addition to complex formation with appropriate cyclin partners, activation of Cdks also requires

phosphorylation at conserved residues by Cdk-activating kinase (CAK). In higher eukaryotes, the components of CAK complex are Cdk and cyclin molecules as well. In mammals, catalytic subunit Cdk7 (also known as MO15) forms complex with Cyclin H for its CAK activity **[72]**. The Cdk7 homologue MCS6 in *S. pombe* acts as CAK upon association with cyclin partner MCS2 **[72]** (**Table 2.2**).

Table 2.2: Roles of Cyclin-Cdk complexes in cellular processes other than cell cycle regulation

Species	Cdks	Cyclin	CAK activity	CTD kinase activity
H. sapiens	Cdk7	Cyclin H	Positive **[72]**	Positive **[73]**
Vertebrates and Flies	Cdk8	Cyclin C	Unknown	Positive **[74-76]**
S. pombe	MCS6	MCS2	Positive **[72]**	Unknown
S. cerevisiae	Kin28	Ccl1	Unknown	Positive **[73]**
	Srb10	Srb11	Unknown	Positive **[77, 78]**
	CTK1	CTK2	Unknown	Positive **[79]**
	PHO85	PHO80	Positive **[80]**	Positive **[81]**

As Transcription regulator: In higher eukaryotes, all Cdks are not critical in the cell cycle regulation and several of them are involved in additional cellular processes such as regulation of gene transcription. In this context it can be mentioned here that the CAK complex Cyclin H-Cdk7 is also involved in the gene transcription and associated with general transcription factor TFIIH in human (**Table 2.2**). The complex phosphorylates the C-terminal domain (CTD) of RNA polymerase II during transcription **[73]**. Therefore, Cyclin H-Cdk7 complex plays an important role both in cell cycle regulation and gene transcription. In vertebrates and flies, Cyclin C-Cdk8 associates with RNA polymerase II and shows CTD kinase activity **[74, 75, 82]**. In *S. cerevisiae*, there are four CAK family proteins and one of them Kin28 is highly homologous to human Cdk7 and shows CTD phosphorylation activity upon association with cyclin Ccl1 **[73]**. The other three homologous CAK protein complexes Srb10-Srb11, CTK2-CTK1 and PHO80-PHO85 show CTD activity **[77-79]**, and among them, PHO85 complex also shows CAK activity. It could be mentioned here that like Cdk inhibitors, PHO81 acts as an inhibitor of PHO85 complex by binding to PHO80 cyclin **[81]**.

Cell Cycle Checkpoints in Eukaryotes

Successful completion of each and every event in the cell cycle is monitored by several surveillance mechanisms to decide upon initiation of the next events. Such mechanisms are called checkpoint pathways, which include specialized sensor proteins that can detect unresolved replication structure, DNA breaks or other abnormalities and translate into biochemical signals to activate appropriate signaling pathways involved in DNA repair and cell cycle arrest **[83]**. If the damage is too much to be handled by the cellular pathways, checkpoint mechanism can induce the elimination of the potentially hazardous cells through apoptosis. Deregulation of cell cycle checkpoint pathways is a universal alteration identified in human cancers. Several checkpoint pathways are involved in different phases of the cell cycle (**Figure 2.7**) and they are discussed briefly in the subsequent sections.

G1-S phase Checkpoint: Progression of cells through early G1, across the restriction point (R) into late G1 and then into S phase, requires the coordinated regulation of

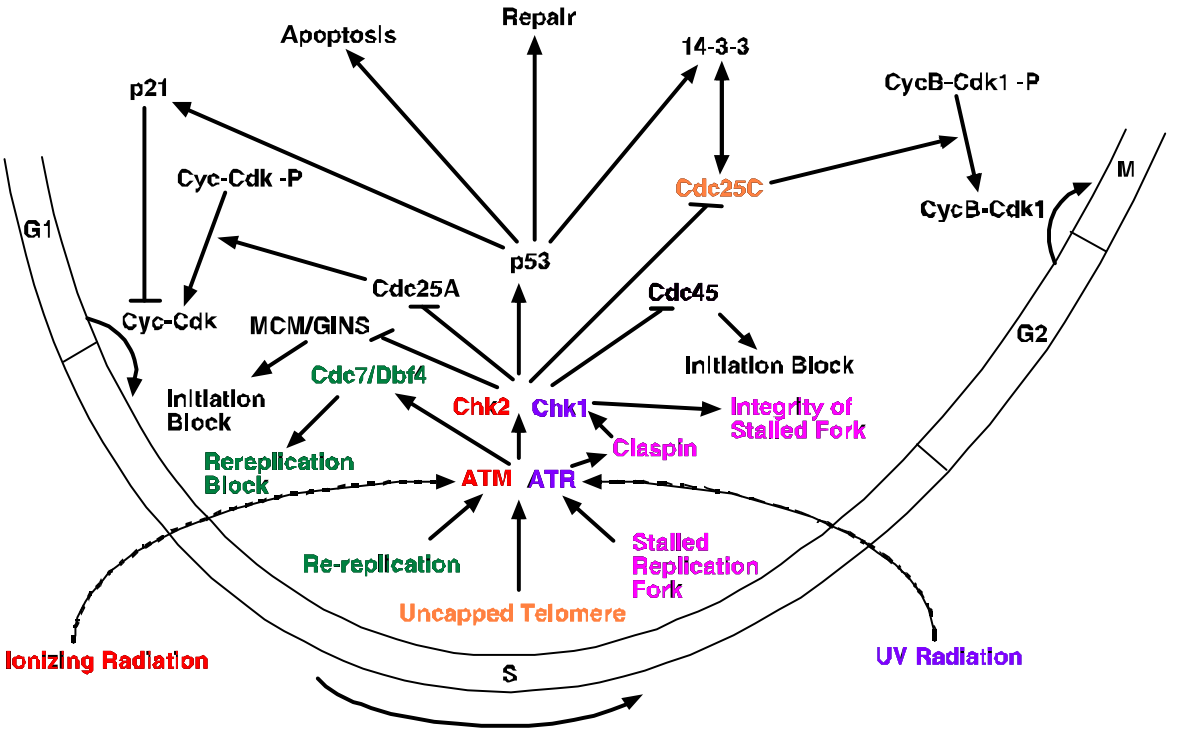


Figure 2.7: Checkpoint pathways in eukaryotes. Arrowheads imply activation of the processes and lines with blunt ends denote inhibition. Different colours indicate different checkpoints activation pathways where red, violet, green, pink and yellow colours represent Ionizing Radiation, UV Radiation, Rereplication, stalled replication fork and uncapped telomere pathways respectively.

multiple positive and negative factors **[84]**. In human, Cyclin D-Cdk4/6 complexes promote early G1 progression primarily by hyper-phosphorylating retinoblastoma (Rb) protein, which results in the release of E2F transcription factors from Rb-E2F complex inducing expression of late G1 and S phase specific genes like dehydrofolate reductase (DHFR), Emi1, Cyclin E and Cyclin A. Subsequently, Cyclin E/A-Cdk2 activity keeps the Rb protein in hyper-phosphorylated state for progression through S and G2 phases **[85]**. Therefore, tight regulation of Cyclin D-associated and then Cyclin E/A-associated kinase activities are required for coordinated cell cycle progression from G1 through S-phase **[86]**. In addition, cyclin E may bind to and activate Cdc2 for G1-S transition that may compensate the loss of Cdk2 function **[87]**. Cyclin A associated kinase activity is required to initiate DNA replication, prevent re-replication and mitosis entry. The anaphase promoting complex (APC) is active throughout the G1 phase through association with an activator Cdh1 (APC-Cdh1) that provides its substrate specificity **[88]**. Cyclin A protein does not function until the late G1-S phase transition due to ubiquitination by APC and subsequent proteolysis by the 26S proteasome. This APC-Cdh1 complex is inactivated by the binding of Emi1 to Cdh1 that give stabilization of Cyclin A and subsequent activation of Cyclin A associated kinase activity and inactivation of Cdh1 by phosphorylation for S-phase initiation **[89]**.

The G1-S checkpoints prevent transition of cells into S phase if the DNA is damaged. Cyclin E-Cdk2 is a key target for the DNA damage checkpoint due to its essential and rate limiting role in G1-S transition **[2]**. DNA damage inhibits the action of Cdk2 to stop the progression of cell cycle until the damage is repaired. If the damage is too severe to be repaired, the cell destructs itself by apoptosis. In mammalian cells, ionizing radiation induced double strand breaks in DNA activate checkpoint kinase ATM (Ataxia Telangiectasia Mutated), which in turn phosphorylates and activates Chk2 kinase. Similarly, DNA damage due to UV radiation activates ATR (ATM and yeast Rad3 related), which in turn stimulates Chk1 (**Figure 2.7**). The activated Chk1/Chk2-mediated phosphorylation triggers accelerated turnover of Cdc25A **[90]**. Cdc25A removes the inhibitory phosphate groups from Cdk2 molecules, and therefore, rapid degradation of Cdc25A prevents the activation of Cdk2 resulting in the G1-S arrest. An important endpoint of this checkpoint signaling is the inhibition of Cdk2-dependent loading of Cdc45 onto the DNA pre-replication complexes and thus initiation of S phase is prevented **[91]**.

Another important component of checkpoint pathway in cells during G1-S transition is the tumor suppressor protein p53 **[92]**. In normal non-stressed cells, p53 protein has short half-life due to MDM2-mediated rapid degradation of the protein after synthesis **[93]**. After exposure of cells to DNA damaging stresses, ATM/ATR directly and also the activated Chk2/Chk1 phosphorylate and stabilize p53 to induce cell cycle block, repair and/or apoptosis. One of the p53 regulated genes, the Cdk-inhibitor $p21^{WAF1/Cip1}$ plays a crucial role in G1-S arrest by inhibiting Cdks that are essential for entry into S phase (**Figure 2.7**) **[94-96]**. Both, the p53 mediated induction of G1-S checkpoint arrest and the Chk2/Chk1 kinases mediated checkpoint activities are activated by the same transducers ATR/ATR. In fact, phosphorylation of p53 by the activated effector kinases Chk2/Chk1 is an important step for its enhanced stabilization in response to DNA damage. However, as mentioned above, Chk2/Chk1 can induce G1-S transition arrest independent of p53 through phosphorylation mediated degradation of Cdc25A. Although ATM/ATR transduced signaling can phosphorylate key targets Cdc25A and p53 within minutes after DNA damage, the impact of signaling pathways regulated by Cdc25A and p53 on Cdk2 activity and G1-S blockage are separated in time due to the dependence of p53 signaling on transcription and translation.

S Phase Checkpoint: The S phase checkpoint may decrease the rate of or pause DNA synthesis to avoid normal cell cycle progression in cells containing damaged DNA. If the cells that are already in S phase face any DNA damage the initiation at new replication forks are halted and the progression of already fired forks are paused without dismantling the post-initiation elongation complex. The progression of DNA synthesis can be paused through ATR mediated mechanism if the elongating forks encounter any lesion. ATR is also activated because of stalled replication resulting from the depletion of nucleotide pool. Stalled replication fork generates stretches of single strand DNA, which accumulates higher amount of single strand binding protein RPA on chromatin. This attracts ATR interacting protein ATRIP (or homologue Ddc2 in *S. cerevisiae*) that in turn recruits ATR at the stalled fork. The cascades of downstream events are initiated with the recruitment and activation of Chk1 inducing G2-M arrest. Studies in *S. cerevisiae* has revealed that Chk1 is recruited in the stalled fork by the adaptor proteins Mrc1 and Tof1, which have been shown to be part of the normal replication fork and loaded after Cdc45 during origin firing. Claspin and TIM/Tipin are the homologues of Mrc1 and Tof1, respectively, in other eukaryotes and Claspin is hyperphosphorylated in ATR dependent

manner in response to fork arrest (**Figure 2.7**). It has also been shown that Claspin phosphorylation is required for its association Chk1 **[97]**. The function of the activated checkpoint pathways is to stabilize the stalled fork by preventing the disassembly of the components and one way this could be attained by jeopardizing the helicase action due to checkpoint activities **[98]**. The maintenance of the stalled fork is important because restarting the movement of the fork after effective removal of lesion is essential for the completion of DNA replication **[99, 100]**.

After DNA damage, the subunits of Mcm2-7 and the GINS protein Psf2 of the replication helicase complex Cdc45/MCM2-7/GINS can also be phosphorylated by Chk2, resulting in the interference of helicase activity and fork arrest or slowdown (**Figure 2.7**) **[101]**. Local phosphorylation of Mcm2-7 might modulate the coupling of the helicase with the polymerase there by regulating the amount of ssDNA exposed at stalled forks. This mechanism would provide a feedback loop for ATR activation **[99]**.

Several studies have suggested that checkpoint pathways play a role in preventing re-replication. The regions of rereplicated DNA may mimic double strand breaks that activate ATM kinase. The elongating fork may be stalled at such structure, which activates also ATR as a result of accumulation of stretches of RPA-coated single strand DNA. Re-replication in *Xenopus* egg extracts induced by the addition of Cdt1 protein was greatly enhanced by caffeine – an ATM/ATR inhibitor, indicating that the checkpoint pathways are activated in response to rereplication. Treatment of human cells with caffeine or with Chk1 inhibitor promotes rereplication by Cdt1 overexpression. Cdc45 accumulates on chromatin when checkpoint pathways are interfered by Caffeine-mediated inhibition of ATM and ATR, suggesting that checkpoint pathways inhibit origin firing before Cdc45 loading. Inhibition of rereplication by checkpoint pathways occurs through p53-mediated mechanism as re-replication due to overexpression of Cdt1 is observed in $p53^{-/-}$ cells. Rereplication in p53+ cells can be observed only if Mdm2, a p53 ubiquitin ligase is overexpressed. Inhibition of Cdk2 activity by p53-induced p21 is probably involved in preventing origin firing **[99, 102]**. Among the many proteins, Cdc7-Dbf4 is also one of the critical targets of the intra S-phase checkpoint. Recently it has been shown that ATR and ATM mediated direct phosphorylation of Dbf4 is critical in preventing rereplication in mammalian cells **[103]**.

G2 Checkpoint: Like G1-S and S phase checkpoints, G2 checkpoint is also active to prevent the passage of DNA lesions to daughter cells during mitosis. At this stage, entry

into mitosis is regulated by the activity of Cdc2 kinase. Maintenance of the inhibitory phosphorylations on Cdc2 is essential to prevent G2-M transition. ATM and ATR indirectly regulate the inhibitory phosphorylation status of Cdc2 in response to DNA damage by sharing all common upstream signaling pathways made up of the ATM/ATR transducer and Chk2/Chk1 effectors kinase **[104]**. ATM mediated phosphorylation and activation of Chk2 kinase are involved in G2 checkpoint pathways after genotoxic stress **[105, 106]**. The Chk1/Chk2 kinase phosphorylates Cdc25C phosphatase and regulates its sequestration in cytoplasm. Similar process is also affected by another kinase, Plk1 **[107]**. The activity of Plk1 is inhibited in G2 phase of human tumor cells exposed to IR and doxorubicin suggesting its importance in G2 checkpoint pathways **[108]**. Cdc25C dual specificity phosphatase removes the inhibitory phosphate groups from Cdk1 in nucleus to activate it for M-phase specific activities, and therefore, its sequestration in cytoplasm during G2-M transition keeps cyclin B-Cdk1 in an inactive state preventing the initiation of M-phase. Phosphorylation of Cdc25C by checkpoint kinases promotes its association with 14-3-3 protein which sequesters the phosphatase into cytoplasm. Notably, 14-3-3 is up-regulated by p53 further helping the cytoplasmic sequestration of Cdc25C. Like G1-S checkpoint, p53 also regulate the G2 checkpoint through transcriptional up-regulation of downstream target genes p21 and GADD45 (**Figure 2.7**). Similar to its regulation of the Cyclin D1-Cdk4/6 or Cyclin E-Cdk2 complexes at the G1-S checkpoint, p21 can bind to and inhibit the Cyclin B1-Cdc2 complex and inhibit cyclin activated kinase mediated Cdc2 activation **[108]**.

Another important trigger for G2-M checkpoint pathway is the uncapped telomere (**Figure 2.7**). The telomere devoid of the capping proteins TRF2 and POT1 activates ATM and ATR, respectively. The activated ATM and ATR in turn phosphorylate p53 along with Chk2 and Chk1 to prevent progression into mitosis with uncapped telomere **[109]**. Interestingly, recent experiments show that the mechanisms are different from canonical DNA damage response to ionizing radiation, which triggers cell cycle arrest through Cdc25A destruction. It has been observed that TRF2 depletion results in Chk2-mediated phosphorylation of Cdc25C leading to its export out of nucleus and subsequent degradation through proteasome mediated pathway. The G2-M arrest is further augmented by the down-regulation of Cdc25C expression by p53 **[109]**.

Mitotic Checkpoint: This checkpoint pathways monitor spindle microtubule structure, chromosome alignment on the spindle and chromosome attachment to kinetochores during

mitosis **[110]**. Mitotic checkpoint delays the onset of chromosome segregation during anaphase until any defects in the mitotic spindle are corrected. Unattached kinetochores are thought to be the source of the checkpoint signal and mechanical tension at the kinetochores dictates whether the checkpoint is initiated or not. Activation of the mitotic checkpoint prevents mitotic progression through inhibition of anaphase promoting complex activator, Cdc20 **[111]**.

Chromatin

Chromatin, which is a mass of genetic material composed of DNA and proteins, condenses to form chromosomes and remains compacted during maximum time of the cell cycle in eukaryotes. The basic unit of chromatin is the nucleosome that primarily packs the large eukaryotic genome to fit in the tiny nucleus. The major components of the nucleosome are histone proteins, DNA and a small amount of non-histone proteins. The first level of compaction is due to the wrapping of DNA into histone octamer cores to form nucleosomes, which are in turn packed into higher order structures of increasing

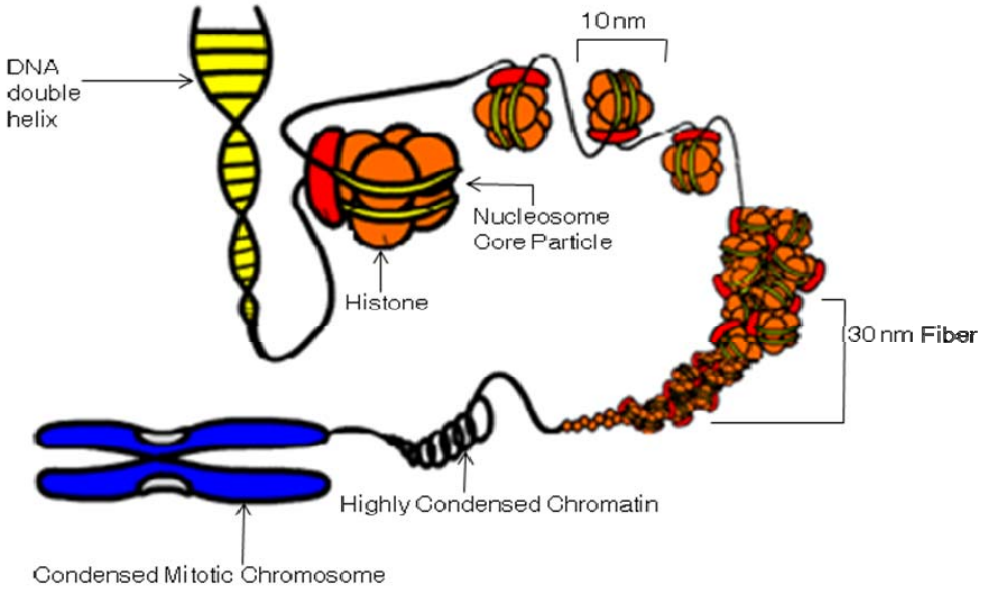


Figure 2.8: Step-wise compaction of a large DNA into condensed chromatin structure to fit into the small nucleus. The first level compaction of a large DNA is the formation of nucleosomes (~10 nm in length) by wrapping of histone octamer with dsDNA. The nucleosomes are further reorganized into a condensed chromatin fiber (~30 nm chromatin fiber) and finally into condensed mitotic chromosome (http://www.avs.uidaho.edu/KyleDunn_ChromaticIllustration.gif).

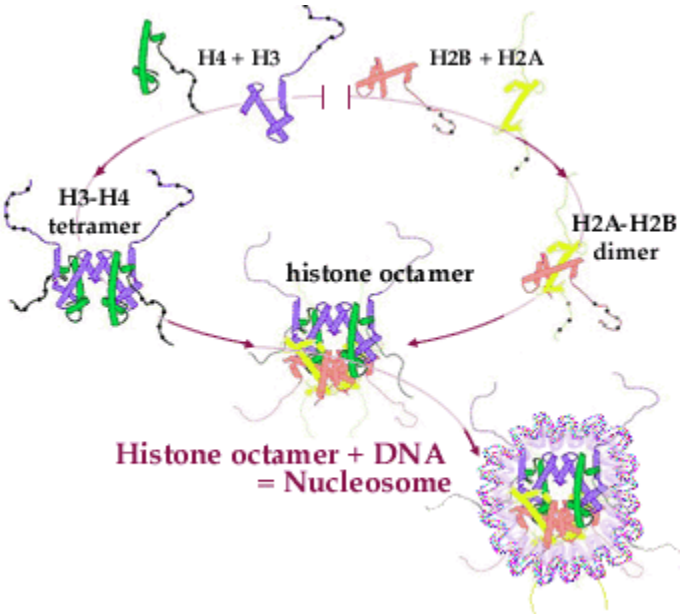


Figure 2.9: Schematic presentation of Nucleosome formation. First the two histones H2A and H2B formed a dimer. On the other hand two of each histones H3 and H4 formed the tetramer. Finally two dimer of histones H2A and H2B and one tetramer of histones H3 and H4 wrapped with large DNA to form a core nucleosome (http://www.nobelprize.org/educational/medicine/dna/a/transcription/pics/histone_octamer.gif).

complexity by effective organizations (**Figure 2.8) [112]**. Each nucleosome contains two copies of four core histones organized into a central $(H3/H4)_2$ tetramer and two peripheral H2A/H2B dimmers (**Figure 2.9**). A linker histone H1 connects the nucleosomes to increase the compaction in higher order structures. A crystallographic study shows that a stretch of 146 bp DNA wraps around the histone octamer in a nucleosome **[113]**. Timely opening up of the packed DNA structure is necessary for the processes like replication, damage repair and transcription to allow the access of necessary factors. Such chromatin remodeling is highly critical for normal cellular existence and a reversible and dynamic phenomenon.

Chromatin Remodeling

To access the chromatin for performing DNA template based functions its alteration is necessary. Such chromatin remodeling is affected by several enzymes and protein complexes through numerous mechanisms. Among them Swi/Snf complex belongs to a class of protein complexes that alter the DNA packaging in an ATP-dependent manner in various organisms **[114, 115]**. There are several other classes of chromatin remodelers

that change the chromatin structure by reversible covalent modification of histones. Histone proteins can be modified covalently through phosphorylation, ubiquitinylation, sumoylation, methylation and acetylation. Chromatin remodeling through various post-translational modifications of the protruding histone-tails of nucleosomal octamer controls the access of factors affecting transcription, replication and DNA repair. The modifications also provide recognition sites for the plethora of protein factors facilitating DNA repair and regulated flow of genetic information. Enriched proportions of the acetylated histones are associated with promoter regions and start sites of active genes compared to coding regions and silent chromosomes. Histone methylation at specific lysine residues have also been shown to be correlated to activation or silencing of specific genes or regions of genomes. Recently, several studies suggest the role of histone modifications in regulating the initiation of DNA replication. Studies in *Drosophila* and *Xenopus* have established the positive regulation of replication through histone acetylation **[116, 117]**. Direct involvement of MYST family histone acetylase HBO1 in regulation of replication licensing through the formation of pre-replication complex has been shown **[116, 118]**. The preference of open chromatin structures with enriched histone H3 methylation and acetylation at metazoan origin has also been established recently **[119]**. On the other hand, histone deacetylase Sir2 has been shown to interfere with pre-RC assembly in budding yeast regulating replication in a negative manner **[120, 121]**.

Histone Acetyl Transferases (HATs)

Histone acetyl transferases (HATs) acetylate the ε-amino group of lysine residues at the N-terminal tail of histone proteins by transferring the acetyl group from acetyl coenzyme A (Acetyl-CoA) **[122]**. In the context of nucleosome, the tail regions of a histone octamer that extend out of the globular domain are responsible for binding to the DNA through electrostatic interactions between positively charged histones and negatively charged DNA or nucleosome-nucleosome interactions **[123] [113]**. Acetylation in the lysine residues of histone by HATs neutralizes the positive charges reducing the histone-DNA **[124] [125]** or nucleosome-nucleosome **[126] [127]** interactions, signaling a conformational change **[128]**. Therefore, destabilization of nucleosome structure decreases its compaction and access to DNA increases for nuclear factors to start the DNA template dependent processes. Several HATs have been isolated and characterized from various organisms and they are divided into different families depending upon the sequence homology, such

as, GNAT (Gcn5-related N-acetyltransferase) super family, MYST family and p300/CBP. Since a major portion of experimental section of the thesis deals with characterization of a MYST family HAT, a more detailed discussion on the HAT family is presented below.

Histone Acetyl Transferases (HATs) of MYST Family

The MYST family is composed of a group of widely distributed but related histone acetyl transferases that are involved in diverse cellular activities including transcription activation, DNA repair, pre-replication complex formation and transcription silencing. The MYST (derived from human MOZ, yeast Ybf2 or Sas2 and Sas3 and mammalian TIP60) family members contain a characteristic MYST domain including the canonical acetyl CoA binding motif (A-motif) as well as a C_2HC Zn-finger. The MYST HATs also contain other conserved domains like chromodomain and plant homeodomain (PHD) for specific functions **[129, 130]**. One notable member of the family TIP60, a tumor suppressor, has been shown to be recruited at the DNA double strand break site through the interaction of its chromodomain with histone H3 trimethylated on lysine 9 (H3K9me3) resulting in the activation of ATM kinase and initiation of repair. The HAT activity of the TIP60 has also been shown to be regulated through phosphorylation by Cyclin B2-cdc2, though its significance in cellular processes remains unknown. Another important MYST HAT Hbo1 has been demonstrated to be essential for Cdt1-assisted loading of MCM to form pre-RC at eukaryotic replication origin **[131]**. Recently, one group shows that Hbo1 phosphorylation at the serine 57 residue by polo like kinase 1 (plk1) is necessary for pre-replicative complex formation and DNA replication licensing **[132]**.

DNA Replication initiation and HATs

Proper DNA replication followed by segregation of chromosomes during cell division is essential for appropriate assortment and propagation of genetic materials in the living world. Initial studies on enzymology of replication in bacteria *E. coli* along with related genetic and biochemical experiments in this and other bacteria and bacterio-phages have revealed many mechanistic details of the replication machinery. All organisms from bacteria to human require origin recognition proteins, helicase, primase, replicative polymerase, processivity factors and single strand binding proteins as the components of replication machine. Although this basic theme is conserved, the details are definitely

more complex in eukaryotic cells where the machinery has to replicate multiple chromosomes flawlessly only once in each cell division cycle **[133]**. In eukaryotes, DNA replication occurs during a short temporal period known as synthesis (S) phase and chromosome segregation occurs during a massive reorganization of cellular architecture at mitosis (M). These two major cell cycle events are separated through two gap phases: G1 between M and S phases and G_2 between S and M phases. To replicate the whole genome within the short period of S-phase, multiple initiations occur in a coordinated manner and once an origin is fired, that particular one cannot initiate replication again in the same cell division cycle. Therefore, the prevention of rereplication of genome happens mainly at the initiation stage of the cell cycle progression in eukaryotes. A major mode of regulation of replication initiation and other cell cycle regulatory events is phosphorylation and de-phosphorylation of various protein factors and cyclin dependent kinases (Cdks) mainly carry out the phosphorylation events. Detailed discussion on the regulation of cyclin-Cdks has been presented earlier in the chapter. As mentioned above, in eukaryotes, the DNA is compact in the form of chromatin during maximum time of the cell cycle. Therefore, chromatin remodeling is crucial for opening up the compact DNA that permits the accession of the initiation protein to form pre-replication complex at the origin. Histone acetyl transferase bound to origin (HBO1) is one of the covalent remodelers that acetylates the N-terminal protruding tail of histone H4 for MCM2-7 loading on to the origin to form pre-replication complex **[134]**.

Trypanosomatids

Trypanosomes remain a serious problem for human and other animals throughout the North-East Asia, Central South America and a large region of Africa. In the order of kinetoplastidae the trypanosomatidae family is divided into several genera, including *Trypanosoma*, *Leishmania*, *Crithidia*, *Leptomonas*, *Phytomonas* and many others. Among them *Trypanosoma* and *Leishmania* are causative agents of a variety of dreaded diseases in humans, whereas *Phytomonas* spp. organisms infect plants and *Crithidia fasciculata* are parasitic to insects **[135]**. In human, *Trypanosoma brucei* causes the African 'sleeping sickness', whereas 'Chagas' disease in South America is caused by *Trypanosoma cruzi*. Another Trypanosome, *T. evansi* primarily causes the disease 'Surra' in animals.

However, human was also found to be infected for the first time in October 2004 by *T. evansi* in Maharastra, India [WHO, 2005] **[136]**.

In the order of kinetoplastidae, the trypanosomatidae family of protozoan parasite *Leishmania* causes *Leishmaniases* and more than 350 million people in 88 countries of four continents covering South America, Africa, South Europe and Asia including Northern-Bihar in India are at risk. In 2002, approximately 2 million new cases have been reported including 59000 deaths. There are more than 20 species of organisms belonging to the *Leishmania* genus that cause the diseases. The main forms of leishmaniases caused by *Leishmania* spp. organisms are Cutaneous (CL), Diffuse Cutaneous (DCL), Mucocutaneous (ML), Visceral (VL) and Post Kala Azar Dermal leishmaniases (PKDL). CL is mainly caused by *L. major*, *L. tropica*, and *L. aethiopica* in the Old World **[137]**. In the New World, CL is caused by the *L. mexicana* species complex organisms, including *L. mexicana*, *L. amazonesis*, and *L. venezuelensis* **[138]**. DCL, caused by *L. amazonesis* and *L. aethiopica* is rare and not much harmful. The available drugs in the market are sufficient to cure DCL **[139, 140]**. In the New World, ML is caused mainly by *L. braziliensis* and *L. guyanensis*, whereas, *L. major*, *L. infantum* and *L. donovani* are responsible for Old World ML disease. The ML disease is more severe and fatal if not treated in time **[140]**. PKDL occurs in the VL patients after several months or years later due to existence of small amount of the parasite in the unaffected skin and peripheral blood monocytes even after treatment **[141] [142]**. PKDL is caused by *L. donovani* and *L. infantum* **[143]**. The HIV patients are prone to co-infection with VL due to malfunctioning of the immune system reducing the survival rates of the patients. HIV/*Leishmania* coinfection in HIV patients has been reported in about 30 countries. The co-infection is mostly caused by *L. donovani* and *L. infantum*, though co-infection by *L. braziliensis*, *L. aethiopica*, *L. tropica* and *L. major* have also been reported. Co-infection is somewhat controlled by the Highly Active Anti Retroviral Therapy (HAART) in few cases **[144] [145]**, but still remains a threat to human.

L. donovani, *L. infantum* and *L. chagasi* species are associated with most fatal form of the disease VL, and particularly *L. donovani* is responsible for VL in Indian subcontinent, parts of Asia and Africa. On the other hand, *L. infantum* and *L. chagasi* are involved for VL in the Central Asia and South America **[137]**. Annually around 6,00,000 new cases of VL, the most dangerous of the diseases, are reported. At present, 90% of the VL cases in the world are found in India, Bangladesh, Nepal, Sudan and Brazil. Unfortunately, the disease cannot be controlled efficiently by the available drugs, like

pentavalent Antimony, Amphotericin B etc due to growing resistance of the parasite against them. Besides, the drugs have many side effects. Hence, the development of new drug with minimum side effects is urgent to prevent the fatal disease.

Unique Characteristics of *Leishmania*

Leishmania parasites undergo a biphasic life cycle - promastigotes (flagellated and motile) in sand fly phlebotomies and amastigotes (non-flagellated and non-motile) in human host macrophages (**Figure 2.10**). The extra-cellular promastigotes are present within the intestinal tract of the insect vector of *Phlebotomus* and *Lutzomyia* genera and the intra-cellular amastigotes reside and multiply within phagolysosomal sac of the vertebrate macrophages. The promastigotes are long 15-30 µm in length and 2-3 µm in width and are motile due to the presence of a single long flagellum. On the other hand, amastigotes are smaller and ovoid in shape with about 2-6 µm in diameter and do not contain flagella. Like other eukaryotes, the parasites possess the similar sub-cellular structures like nucleus,

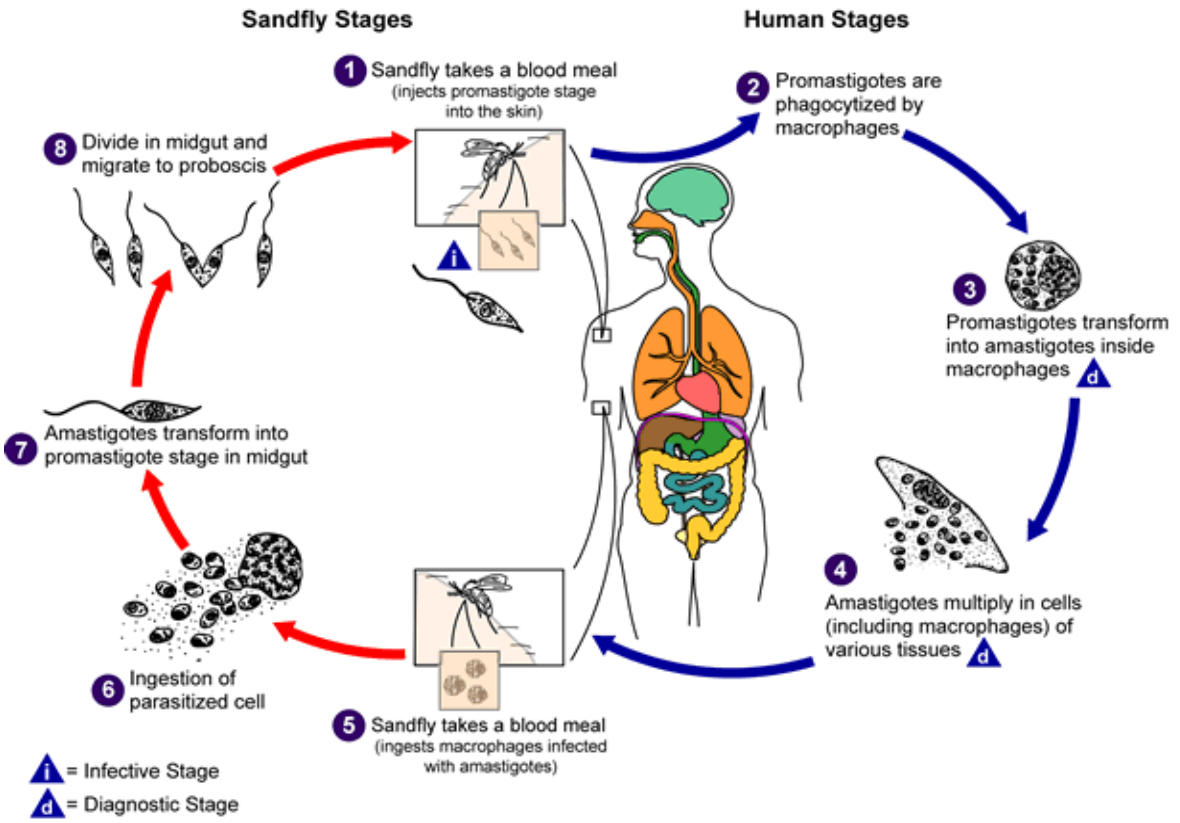


Figure 2.10: Life cycle of *Leishmania donovani* is separated in two alternating stages: promastigotes (flagellated and motile) in sandfly vector and amastigotes (non-flagellated and non-motile) in in human host (http://www.uni-tuebingen.de/modeling/images/Leishmania_Life Cycle.gif).

golgi apparatus, endoplasmic reticulum and unlike others, they contain a single copy of mitochondrion. The flagellar microtubules are responsible for cell movement, while the mitotic and the reservoir associated microtubules are involved in nuclear division and endocytosis **[146]**, respectively. In *Leishmania*, tubulin synthesis is developmentally regulated by post-transcriptional control **[147]**. The nucleus of *Leishmania* contains about 36 chromosomes ranging from 0.3-3.0 mega base pairs and their haploid genome size is ~34 mega bases. One of the unique features of the parasite is the presence of complex mitochondrial genome in the form of a huge network of catenated DNA called the kinetoplast DNA (kDNA). It comprises of 5000-10000 copies of heterogeneous minicircles of 0.5-2.5 kilo base pairs (kbp) and 20-50 copies of maxicircles of 20-40 kbp, all catenated together forming a compact and complex network structure **[148]**. Microscopically, kinetoplastids can be distinguished from other organisms by the presence of kinetoplast – a dense DNA-containing granule located within the single Mitochondrion. From the literature it is also known that the cellular processes of the parasite is quite different from their mammalian hosts due to involvement of many dissimilar proteins in its various pathways.

Regulation of Cell Cycle in Trypanosomes

Since the unicellular trypanosomatid parasites are eukaryotes, the cell cycle regulatory mechanism is broadly similar to the higher eukaryotes, but some differences could present due to some unique features essential for survival of the parasites in their host. One of the important unique features is that the parasite contains single copy of mitochondrion and unlike in other eukaryotes, duplication of the kinetoplast DNA and nuclear DNA is a concerted process. Moreover, the transformation between two distinct froms of biphasic life cycle is intricately related to cell cycle events **[149]**. Many cyclin-Cdks have been found in trypanosomes that are eukaryotic orthologues, suggesting that Cyclin-Cdks are major regulator of cell cycle progression in these organisms as well. Recently other cell cycle regulators like mitogen activated protein kinases, aurora kinases and polo-like kinases are also found in these parasites after the Trytrip genome sequencing **[150]**.

CRKs from Trypanosomes

Cdc2 related protein kinase (CRK) form *C. fasciculata* CRK (CfaCRK) was the first identified kinetoplastid CRK that was cloned **[151]**. The CfaCRK differs from other such

proteins due to the presence of two extra stretches of amino acids (66 aa and 79 aa) between the conserved domains and because of that, it is significantly larger in size (53 kDa) than usual 34 kDa. Like PSTAIRE motif in higher eukaryotes, PGAAIRE motif is responsible for binding of Cdks to cyclins. Many CRKs have also been identified from different trypanosomes like *Crithidia fasciculata, Leishmania mexicana, Trypanosoma brucei* and *Trypanosoma cruzi.* They are named Cdc2 related kinases (CRKs) because of their sequence similarities to Cdks from other organisms **[152]** and there are eleven CRKs in Trypanosomatids (CRK1-4, CRK6-12) **[153]**.

Among several CRKs in *L. mexicana*, one (LmmCRK1) has been detected with molecular mass of 34 kDa and 56% identity in amino acid sequence with human Cdc2, but has not been shown to complement Cdc2 of fission yeast *S. pombe*. This kinase contains PCTAIRE motif instead of PSTAIRE motif and is found in all life cycle stages of the parasite. The kinase activity has been checked by histone H1 phosphorylation *in vitro* with immunoprecipitated CRK1 from the promastigotes cell lysate **[154]**. But the exact function of the protein in the cell cycle regulation of the organism is not clearly known. LmmCRK3, another CRK in this organism, has also been found to contain all the characteristics of Cdc2. This gene is also present in single copy; encodes a 35.6 kDa protein with 54% identity with human Cdk2, and interestingly, the mRNA level is 5 fold higher in replicative promastigotes than in amastigotes and metacyclic form. Expectedly, the activity is associated with dividing forms only, not with non-dividing form (metacyclic form) **[155]**. LmmCRK3 is essential for survival of the parasite which has been proved by gene knockout experiments and the cell cycle arrest at G2-M by its potent inhibitor, flavopiridol, **[156]**. Recently, one CRK has been identified from *L. donovani* (LdCRK3) that is homologous to CRK3 from *L. mexicana*, binds to its cyclin partner, LdCyc1 **[157]** and phosphorylates the human histone H1 *in vitro* **[158]**.

In *T. brucei*, five CRKs (TbCRKs) have been identified. Among them, TbCRK1 is highly homologous to LmmCRK1 (72% identity). Other CRKs, TbCRK2 and TbCRK3 have also been identified and like LmmCRK1 they do not complement Cdc2 from *S. pombe*. All of them have amino acid substitutions in PSTAIRE motif **[159]**. TbCRK4 is homologous to CfaCRK **[160]** having two insertions in between catalytic domains. It is not sensitive to Cdk inhibitors and it is assumed that the two inserted domains alter the active site of the enzyme and substrate specificity. The fifth identified CRK form *T. brucei*, TbCRK5 is itself active towards histone H1 and myelin basic protein, without any cyclin partner.

One of the CRK from *T. cruzi*, TcCRK1 can bind to mammalian cyclins E, A and D3 **[161]**. Like TbCRK5, the TcCRK1 is also active in absence of cyclin partner. Moreover, it can undergo autophosphorylation during kinase assay indicating the involvement of other mechanism to regulate its activity **[161]**. Immunofluorescence studies show that TcCRK1 is highly concentrated in kDNA but also in nucleus and cytoplasm and invariable during cell cycle **[162]**. Three endogenous cyclins TcCYC4, 5 and 6 have been found to interact in a Yeast Two Hybrid (Y2H) assay where TcCRK1 has been used as bait. Among them TcCYC4 and 6 are homologous to TbCYC2 and *S. cerevisiae* PHO80 like proteins, respectively. TcCYC5 does not have any significant homology to any of these cyclins. Moreover, TcCYC4 and 6 can also interact with TcCRK3 suggesting roles of these proteins in regulation of both the CRKs **[162]**.

Cyclins from Trypanosomes

Cyclins are the regulatory subunit of CRKs in the trypanosomes also. The first identified cyclin in trypanosomes is TbCYC1 from *T. brucei*, homologous to human cyclin A and B **[163]**. It has been reported that the level of expression of TbCYC1 is up-regulated during differentiation but invariable in procyclic forms after release from hydroxyurea block **[164]**. Another cyclin from *T. brucei*, TbCYC2 encodes a 24 kDa protein, homologous to *S. cerevisiae* PHO80 cyclin. Gene knockout experiment shows that TbCYC2 is an essential gene and immunofluorescence microscopy shows that the protein is localized almost in all parts of the cell body of procyclic form. Moreover, Y2H experiments have shown that TbCYC2 interacts with TbCRK3, but no activity of this complex has been shown so far. Interestingly, it is evident from immunofluorescence study that the cyclin-CRK complex is localized in the cytoplasm throughout cell cycle of trypanosomatid **[165]**, though the functional significance of such an observation is unknown. The other cyclin, TbCYC3, is a 46.5 kDa protein, homologous to mitotic cyclin B and it contains a C-terminus destruction box like motif RGTLVVPRN instead of RXALGXLXN, located on N-terminus in higher eukaryotes **[165]**. Till date ten regulatory cyclin molecules (TbCYC2-11) have been identified in *T. brucei*. Out of them, TbCYC2, 4, 5 and 7 are homologous to PHO80 cyclin and may play a role in nutrient sensing. On the other hand, TbCYC6 and 8 shares a homology with mitotic cyclins and TbCYC9 is similar to cyclin C with possible role in transcription regulation **[166]**. Y2H assay suggests that TbCRK3 can interact with both CYC2 and CYC6 in *T. brucei* **[150] [166]**. RNAi technique

demonstrates that TbCYC6 is essential for mitosis and absence of the gene results in mitotic block in *T. brucei* **[166]**. Similarly, RNAi of TbCYC2 in *T. brucei* has resulted in G1 phase arrest in procyclic form **[167]**. TbCYC6-CRK3 activity is required for G2-M transition in *T. brucei* **[166]**. In *Leishmania donovani* an S-phase specific cyclin LdCYC1 **[157]** has been identified that interacts with CRK3 and this complex has a possible role in S-phase related activities **[158]**. Another cyclin from *Leishmania donovani,* LdCyc2 has also been identified but function is still unknown (Thesis of Dr. Santanu Roy).

Histone Acetyl Transferases (HATs) from Trypanosomes

Genome sequencing has revealed that four MYST family HATs are encoded by genomes of *Leishmania major* and *Trypanosoma cruzi* and three by that of *T. brucei*. In these organisms the tails of core histones have divergent sequences compare to other eukaryotes and unusual modifications of the histones have also been observed in several experiments **[168]**. It has been shown that one of the MYST HATs, TbHAT3 acetylates histone H4K4 though dispensable for growths **[169]**. Among the other MYST HATs, TbHAT1 is essential for telomeric silencing and its involvement in DNA replication has also been implicated. TbHAT2, the other identified MYST HAT, is required for H4K10 acetylation and growth **[169]**. Therefore, identification and characterization of HATs from *Leishmania* is crucial for understanding the regulation of the parasite cell cycle. Therefore, elucidation of any role of histone acetyl transferase from *Leishmania* on replication licensing is crucial to understand the cell cycle regulation of the parasite.

AIMS AND OBJECTIVES

Leishmaniases, caused by the protozoan parasite *Leishmania,* are serious health hazard in many tropical countries. Northern Bihar in India and the surrounding countries like Nepal and Bangladesh are particularly affected by the potentially fatal Visceral Leishmaniasis (VL), (also known as Kala-Azar), caused primarily by *L. donovani*. The situation is quite dreadful due to the growing resistance of the causative parasites against the available regime of drugs. It is further aggravated by serious side effects of many of the present drugs and their high cost. Hence, the development of new drugs with minimum side effects is urgent to cure and prevent such potentially fatal diseases.

As discussed in the previous chapter, *Leishmania* parasites undergo a biphasic life cycle with many unique features in the cellular processes. It is useful to characterize such unique cellular processes, so that the inhibition of such mechanism will control the proliferation of the parasite without creating much harm to the human host and this will help to find new potential therapeutic targets. On the other hand, many questions related to eukaryotic cell biology can be addressed using *Leishmania* model system as a lot of proteins that carry out the cellular processes are well conserved.

Like in other eukaryotes, cyclin-Cdks play a pivotal role in controlling the cell cycle progression. To characterize the cell cycle regulation in *Leishmania* parasites in details, an S-phase cyclin-Cdk complex, *viz.*, LdCyc1-CRK3, was identified and studied in depth in the laboratory previously. However, no knowledge about the substrates of the cyclin-Cdk complex in *Leishmania* is available. Therefore, it would be interesting to identify the targets of cyclin-Cdk in the parasite to characterize cell cycle regulation in greater details. In this regard, a screening for the potential substrates of the cell cycle kinase LdCyc1-CRK3 was performed resulting in the identification of three substrates. One of the major aims of my doctoral work is to authenticate and further characterize the identified substrates. One of the identified substrates is homologous to histone acetyl transferase (HAT). Since HATs regulate chromatin remodeling and the process is

important for S-phase related activities such as DNA replication, one aim is to characterize the effect of phosphorylation by the S-phase cell cycle kinase on the activity of the HAT. Such studies could identify some *Leishmania* specific substrate(s) and characterization some of the identified substrates could address important issues related to eukaryotic biology.

CHARACTERIZATION OF SUBSTRATES OF THE S-PHASE KINASE LDCYC1-CRK3 FROM *LEISHMANIA DONOVANI*

As discussed in the review of literature (Chapter 2), the cell cycle progression in eukaryotes is primarily regulated by the conserved family of cyclin dependent protein kinases (Cdks) through phosphorylation of several proteins including retinoblastoma (Rb) protein, components of origin recognition complex, Cdc6 and minichromosome maintenance (MCM) proteins **[59, 60, 170]**. In yeast, one catalytic Cdk subunit, cdc2 in *Schizosaccharomyces pombe* or Cdc28 in *Saccharomyces cerevisiae*, regulates the cell cycle progression by interacting with different cyclins at appropriate intervals **[171]**. On the other hand, more than one Cdk subunit is present in higher eukaryotes and they form complexes with periodically expressed cognate cyclin partners at appropriate stages of the cycle **[171, 172]** to regulate the cell cycle progression. In addition, phosphorylation and dephosphorylation on conserved threonine and tyrosine residues of Cdk subunits also regulate the activity of cyclin-Cdks **[171]**. Another important mode of regulation of cyclin-Cdk activity is the formation of complexes with small protein inhibitors (CKIs) and attempts are made to explore small molecular mimics having CKI activity as chemotherapeutic agents for cell proliferation disorders, such as cancer **[173, 174]**.

Several Cdk related kinases (CRKs) and cyclins have also been identified in early branching pathogenic kinetoplastida parasites, which are particularly affecting people in tropical and subtropical countries **[175]**. The parasites go through a biphasic life cycle involving two different morphological forms – the flagellated promastigotes in the sand fly vector and non-flagellated amastigotes in host macrophages **[176, 177]**. The morphological transformation between the different life cycle forms and the manifestation

of disease are intricately related to cell cycle regulation **[160]**, though the details of the targets of CRKs, the key regulators of cell cycle machinery, are still lacking.

Although the presence of CRKs and cyclins suggests that the basic mechanism of cell cycle regulation is conserved in kinetoplastida parasites, important variations may be present to serve their unique requirements. One such unique feature is the presence of a single copy of mitochondrion containing a network of kinetoplast DNA consisting of 5000 minicircles (0.5-2.5 kb) and 50 maxicircles (20-40 kb) **[135, 148, 178]**. Interestingly`, the replication of kinetoplast DNA takes place almost concomitantly with that of nuclear DNA during the S-phase **[135, 178]**. Another distinct feature of the organism is its nearly complete dependence of differential gene expression on post-transcriptional mechanisms in the absence of regulation at the transcriptional level **[160]**. Moreover, the cell cycle machinery has also to take into account the separation of basal bodies of flagella at appropriate time.

Recently, the characterization of an S-phase cyclin molecule LdCyc1 from *Leishmania donovani* that forms a complex with LdCRK3 has been reported from the laboratory. LdCyc1 protein **[157, 158]** contains the conserved substrate docking MRAIL-like motif, which is also found in other cyclins and responsible for the interaction with consensus R/KxL-like cyclin binding (Cy) motif present on the target proteins. The Cy motif was first recognized in the cyclin-Cdk inhibitor p21 **[52]**, and subsequently found to be present in other interactors including the inhibitors (p27 and p57), the activator Cdc25A **[57]** and the substrates like Rb, Cdc6 and others **[59, 60]**. Since LdCyc1 has been shown to contain the homologous substrate binding site, it is expected to interact with cellular targets containing Cy motif, though the identity of any such proteins in *Leishmania* was unknown till recently. Some of these proteins are likely to be unique as they may be involved in parasite specific mechanisms, and consequently, may be better therapeutic targets to stop proliferation of the parasite without affecting the host. In this context, three substrates of S-phase cell cycle kinase LdCyc1-CRK3 were identified in our laboratory based on a screening of putative ORFs carrying both the cycling binding Cy-motif and the Cdk phosphorylation site **[179]**. In this Chapter, the detail characterization of the identified substrates is described including the importance of R/KxL-type Cy motif for their phosphorylation by the cell cycle kinase.

Materials and Methods

Cells

Leishmania donovani promastigotes were derived from AG83 strain (MHOM/IN/83/AG83). For protein expression in insect cell system Sf9 cells derived from *Spodoptera frugiperda* were used. Various *Escherichia coli* strains used are the followings: XL1-Blue and DH10B for regular cloning and DNA manipulation; BL21DE3, BL21DE3- pG-JKE8 (BL21DE3 strain transformed with chaperon plasmid pG-JKE8 from TAKARA BIO INK producing groES-groEL chaperon proteins in presence of tetracyclin and dnaK-dnaJ-grpE chaperon proteins in presence of L-Arabinose) and T7shuffle (the strain helps to proper folding of desired proteins by disulphide bonding) for protein over expression; DH10BAC for generation of recombinant baculovirus.

Plasmids

The bacterial vector pBluescript II SK (+) (Stratagene) was used for some of the initial gene cloning and further manipulation. The bacterial expression vectors pRSET-C (Life Technologies), pGEX-5X-3 (GE Healthcare Lifesciences) and pET21b+ (Novagen) were used for protein expression. pXG and pXG-GFP+ were used for protein expression in *Leishmania* promastigotes; (kindly provided by Dr. Stephen M Beverley of Washington University School of Medicine, USA). A series of pFast-Bac (Life Technologies) vectors were used for generation of recombinant baculoviruses to express proteins in insect cells.

Chemicals and Reagents

Parasite Culture

Leishmania donovani promastigotes strain AG83 (MHOM/IN/83/AG83) was grown in M199 medium (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich and Life Technologies) and penicillin-streptomycin mixture at 22^0C with gentle shaking.

Insect cell (Sf9) culture

Sf9 insect cell was grown in Grace's Insect medium (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich and Life Technologies) and penicillin-streptomycin mixture at 27^0C in tissue culture flasks (Nunc, Thermo Scientific) for adherent monolayer culture.

Table 4.1: List of chemicals/reagents

Chemical /Reagents	Company
Agarose and acrylamide, bis-acrylamide, Penicillin-Streptomycin, Glycine, Tris, Tricine, IPTG, SDS, NP40, Glycerol, Imidazole, Bromophenol blue, Xylene cyanol, EDTA, EGTA, ATP, Lysozyme, PMSF, Imidazole, Salmon testes DNA, Urea, Guanidium.HCl, Ponceau S, Protease Inhibitor Cocktail , RNase A, PS100 and Somatostatin peptides, Sodium butyrate, Antibiotics (ampicillin, chloramphenicol, tetracycline, gentamycin), *Leishmania* culture medium M199, Grace's insect medium, Li_3-Acetyl CoA enzyme, FBS,	Sigma-Aldrich (USA)
Tricine, Ethidium Bromide	USB (USA)
RTS Wheat Germ Linear Template Generation kit and RTS100 Wheat Germ Continuous Exchange Cell-Free (CECF) system, Protease Inhibitor Cocktail	Roche Applied Science (USA)
glutathione beads, PVDF and nitrocellulose membranes.β-mercaptoethanol (β-ME), Triton X-100, Tween 20, DAPI	GE Healthcare (USA)
PCR purification and Gel extraction kit, Plasmid purification kit (mini and maxi), Pipette tips	Axygen (USA)
LB, Agar, PCR purification and Gel extraction kit, Plasmid mini and maxi purification kit	Himedia (India)
Taq, XT-5, pfx, pfu thermo-stable DNA polymerases, dNTPs, Mouse-anti-His and Rabbit-anti-GST IgG, Goat-anti-rabbit and Goat-anti-mouse IgG-HRP conjugated antibodies, Protein A agarose beads	Bangalore Genei (India)
PCR purification and Gel extraction kit, Plasmid mini and maxi purification kit, Pipette man	Eppendorf (Germany)
Cell-fectin Reagent, FBS	Invitrogen (USA)
Reverse Transcription kit and cDNA Library synthesis kit	Stratagene (USA)
Restriction enzymes and other DNA modifying enzymes	New England Biolabs (USA)
T4-DNA ligase	Sib Enzyme (Russia)
Restriction enzymes and other DNA modifying enzymes	Takara (Japan)
Ni-NTA agarose	Qiagen (Germany)
NaCl, Glucose, KCl, $MgCl_2$, Na_2HPO_4, NaH_2PO_4, KH_2PO_4	SRL (India)
DNA and Protein molecular weight marker	Fermentas (USA)
0.2 μm membrane filter	Sartorius (Germany)
FBS	GIBCO (USA)

Chemical /Reagents	Company
X-Ray film, Developer and Fixer	Kodak India Ltd (India)
30% H_2O_2	Ranbaxy (India)
Mouse-anti –phospho Thr-Pro IgG HRP	Cell Signalling (USA)
Normal and acetylated Ldhistone H4 peptide and their antibodies	Imgenex India Pvt. Ltd. (India)
Random hexamer	Life technologies (USA)
Oligomers and Primers, DNA sequencing	MWG biotech(Germany)
Oligomers and Primers, DNA sequencing	GCC (India)
[γ-^{32}P] ATP	BRIT (India)
Ammonium per-sulfate (APS), Methanol, Ethanol, Acetic Acid, Iso-propanol, HCl, DMSO	Merck (Germany)
TEMED	BDH Chemicals Ltd.
Concentrator unit	Pall Centricon (USA)
Concentrator unit	Viva Spin (USA)
0.2 μm membrane filter	Millipore (Germany)

Genomic DNA isolation from *L. donovani* promastigotes

The genomic DNA from *L. donovani* promastigotes (Ag83) was isolated according to Wilson *et al* **[180]** with some modifications. Briefly, the parasites were suspended in 500 μL of TE (10 mM Tris-HCl, 1 mM Na_2-EDTA) buffer containing 10 μg/mL RNase A, 50 μg/mL proteinase K and 0.5% (w/v) SDS and incubated at 65^0C for 15 minutes followed by overnight incubation at 37^0C. The DNAs were extracted thrice with equilibrated mixture of phenol-chloroform-isoamyl alcohol (25:24:1) and then precipitated from the aqueous phase with ethanol (70% v/v). The DNA precipitate was washed once with 70% ethanol, air-dried and suspended in TE buffer.

Preparation of the cDNA Library of *L. donovani*

Total RNA was isolated from 2 x 10^8 cells of *L. donovani* promastigotes with TRI-Reagent (Clontech). Then the population of mRNA was purified from total RNA with poly-dT column. The isolated mRNA was used for preparation of cDNA library as depicted in the **Figure 4.1** using cDNA library synthesis kit (Stratagene) following the

manufacturer's instruction. Briefly, 5 µg mRNA was added to the reaction mixture containing methylated dNTPs, linker-primer having XhoI restriction site and StrataScript RT enzyme for first strand cDNA synthesis. To synthesize second strand cDNA the reaction mixture was further incubated with RNase H and DNA polymerase I and one radio-labeled dNTP followed by blunting the cDNA termini with Pfu DNA polymerase. Then the cDNA was purified by phenol-chloroform-isoamyl alcohol mixture (25:24:1) and finally by ethanol precipitation. The purified cDNA was subjected to ligation with EcoRI adapter by T4-DNA ligase for restriction site generation followed by phosphorylation at the 5'end of EcoRI adapter. The sequences of linker primer and adapters used are shown below:

Linker primer

5´-GAGAGAGAGAGAGAGAGAGAACTAGT**CTCGAG**TTTTTTTTTTTTTTTTT-3´
"GAGA" *XhoI* Poly(dT)

EcoRI adapter 5´-**OH**-AATTCGGCACGAGG-3´
3´-GCCGTGCTCC-**P**-5´

Finally, the *XhoI* digestion released the *EcoRI* adapter and residual linker–primer from the 3´ end of the cDNA. These two fragments were separated on a gel filtration column containing Sepharose® CL-2B beads. The size fractionated cDNA was then precipitated and ligated to the pJG4-5 vector. The ligated DNA was then transformed into XL10-Gold cells and complexity of the library was determined by counting the colonies at suitable dilution. For amplification, the primary library was grown in the 2xLB-agar containing soft agar (SeaPrep agarose) by incubating at 30^0C for 45 hours. Subsequently the agar was removed by centrifugation and the bacteria were resuspended in 2x LB-glycerol. The amplified library was stored at -80^0C in suitable aliquots.

Confirmation of cellular expression of genes

The cDNA library of *L. donovani* promastigotes as prepared above was used as template in PCRs with XT-5 thermostable DNA polymerase with appropriate primer pairs (**Table 4.2**) to confirm the cellular expression of the identified genes.

Preparation of chemical competent *E. coli* cells for transformation

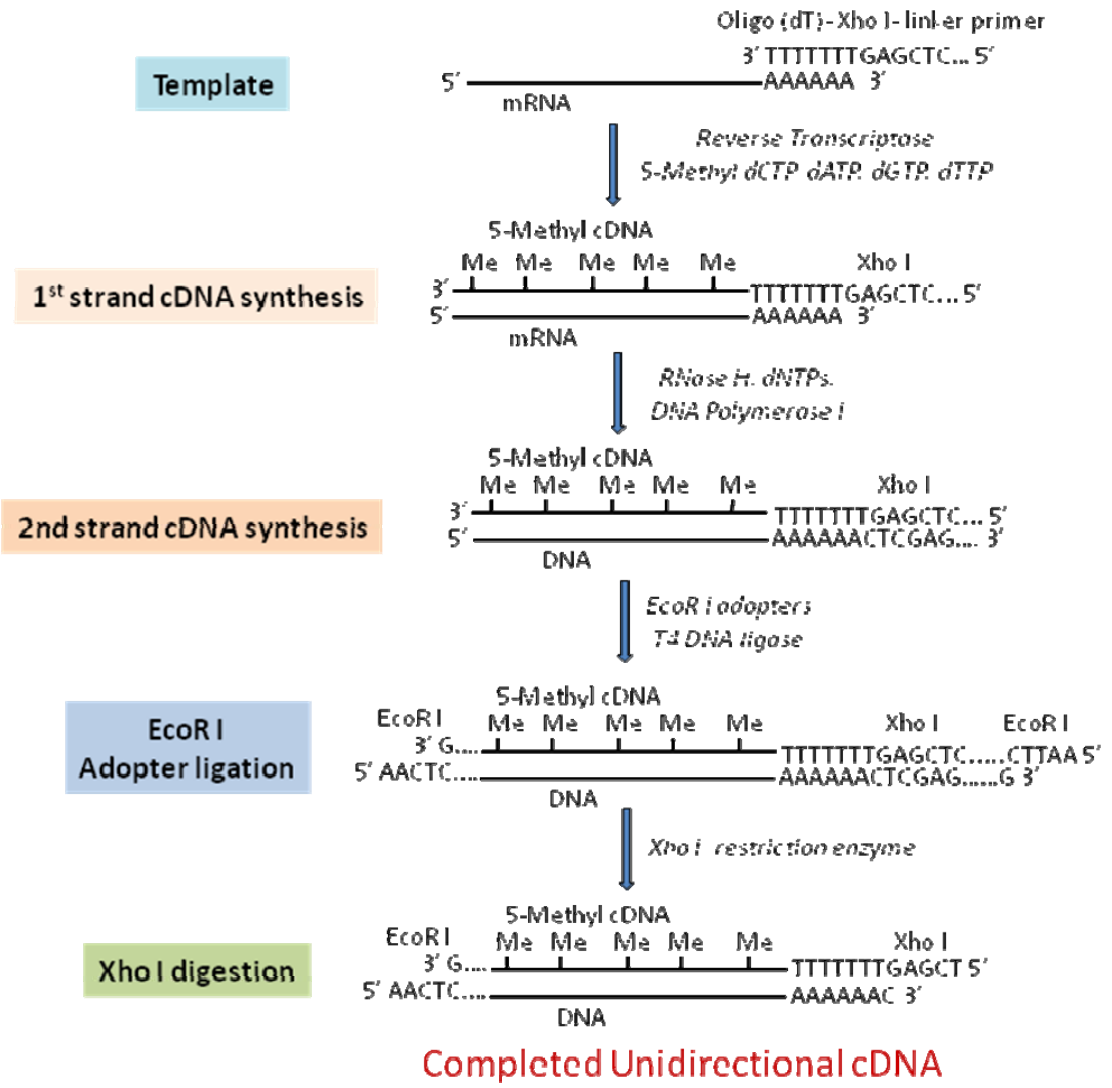


Figure 4.1: Schematic representation of cDNA library preparation. The cDNA library was prepared using Stratagene kit. The mRNA of *L. donovani* promastigotes was treated with 5-Methyl dCTP, dATP, dGTP and dTTP, oligo (dT)-XhoI linker primer and reverse transcriptase for 1st strand cDNA synthesis. The 2nd strand cDNA synthesis was done with DNA polymerase I after treating with RNase H. The EcoRI adapter was ligated with double stranded cDNA after phosphorylation of the 5' end. Finally, the XhoI digestion provided a unidirectional cDNA population that was cloned into pJG4-5 vector for preparation of cDNA library.

The preparation of *E. coli* competent cells for transformation by chemical method was prepared according to the method published before **[181]**. A single isolated bacterial colony from a freshly streaked LB-agar plate was used to inoculate 5 mL of LB medium and incubated overnight with vigorous shaking at 37^0C. The overnight culture was used to inoculate 500 mL of LB and allowed to grow with shaking at 37^0C till OD_{600} became 0.6. For the preparation of competent cells for transformation by chemical method, the pellet of

cells was suspended in Transformation buffer (TB; 10 mM PIPES, pH 6.7 containing 15 mM $CaCl_2$, 250 mM KCl, 55 mM $MnCl_2$, 100 mM RbCl and 3 mM hexamine cobalt chloride) and harvested by centrifugation at 2000 x g for 15 minutes at 4^0C. The cell pellet thus obtained was suspended in 0.3 volume (of the original culture) of ice cold TB, incubated on ice for 10 more minutes and centrifuged as before. The cells were finally suspended in 0.08 volume of TB containing 7% DMSO, divided into 200μL portions and stored in -80^0C freezer. Transformation by chemical method was done by incubating the cells with DNA for 20 minutes on ice followed by a heat shock at 42^0C for 90 seconds. Then the cells were incubated in LB without any antibiotic at 37^0C for an hour followed by spreading over LB-agar plate with appropriate antibiotic and incubated at 37^0C for overnight.

Table 4.2: Sequences of Primers used to check expression of the identified genes

Substrates	Primers	Sequences
Ld14.0140L	pET21b14.0140Nhe1+	5' GTCGTAGCTAGCATGTCGAGCAGTGCCGTCCC
	14.0140-	5' CTGGTGACGTTCCTTGATGT
Ld28.0070L	pET21b28.0070Nde1+	5' GAGGACCATATGCATGAATTGAGGCGATC
	28.0070-	5' CTTGCGGAGCCTGGTATCT
Ld29.1050L	pET21b29.1050Nde1+	5' GAGGACCATATGGATGAATTTGCAGAGTG
	29.1050-	5' AGCACCATTACTCATAGCTG

Preparation of electro-competent *E. coli* cells for transformation

For the preparation of electro-competent cells, the methods of growing and harvesting of the *E. coli* cells were similar to that for the preparation of chemical competent cells as described above. The collected cell pellet was washed once with equal volume of sterile ice-cold water and then with 0.5 volume of water. Finally, the cells were washed with 0.2 volume of 10% glycerol and suspended in 0.005 volumes of 10% glycerol, divided into aliquots of 40 μL each and stored in -80^0C freezer. The transformation by electroporation was done at 2.5 KV, 250 μF and 200 Ω in a 2 mm cuvette using a Bio-Rad GenePulser. Then the cells were

incubated in LB without any antibiotic at 37^0C for an hour and subsequently plated on LB-agar plate with appropriate antibiotic and incubated at 37^0C for overnight.

Cloning of the three identified genes in different vectors

The three identified substrates were cloned in different vectors for various experiments. For this purpose the products of first set of polymerase chain reaction (PCR) carried out for *in vitro* transcription and translation **[179]** were used as templates and XT-5 thermostable polymerase (Bangalore Genei) was used for amplification. The amplified DNA fragments and the suitable vectors were digested with appropriate restriction enzymes (NEB) followed by ligation with T4 DNA ligase (NEB). Names of the genes and the corresponding primer-pairs and vectors are listed in **Table 4.3**.

Table 4.3: Sequences of Primers used to clone the identified

Substrates	Primers	Sequences
Ld14.0140L	pET21b14.0140Nhe1+	5' GTCGTAGCTAGCATGTCGAGCAGTGCCGTCCC
	pET21b 14.0140Not1-	5' CTTCTGCGGCCGCACCGCCGGTTCGCGAGTGGG
Ld28.0070L	pET21b28.0070Nde1+	5' GAGGACCATATGCATGAATTGAGGCGATC
	pET21b 28.0070Not1-	5' GCTTCTGCGGCCGCGGCACCCTCACGGTCCATGG
Ld29.1050L	pET21b29.1050Nde1+	5' GAGGACCATATGGATGAATTTGCAGAGTG
	pET21b 29.1050Not1-	5' GCTTCTGCGGCCGCGGATGGCAGCGGAAAATGTT

Overexpression of recombinant proteins in *E. coli* cells

The recombinant vectors, that were designed to express fusion proteins with C-terminal ^{6}His tag, were used to transform either *E. coli* BL21 DE3 strain containing pG-JKE8 chaperon plasmid or *E. coli* T7-shuffle strain. A single colony of the transformed bacteria was used to inoculate 5 mL LB containing appropriate antibiotics and allowed to grow with shaking at 37^0C for overnight. Next day, the overnight culture was diluted into fresh LB at a ratio of 1:100 with the same antibiotics to initiate a fresh culture which was grown till the OD_{600} reached 0.6. Ld14.0140L protein was induced in BL21DE3-pG-JKE8 cells in presence of 5 ng/mL of tetracycline as chaperon inducer and 1 mM isopropyl-β-D-thiogalactopyranoside

(IPTG) for further 3 hours. Other two substrates, Ld28.0070L and Ld29.1050L were induced with 0.5 mM IPTG. The cells were harvested by centrifugation at 4^0C followed by washing once with phosphate buffer saline (PBS).

Purification of recombinant proteins from *E. coli* cells

The pellets of induced cells from 250 mL culture were resuspended in 5 mL of 50 mM Tris-HCl, pH 8.0, containing 500 mM NaCl, 10% Glycerol, 1 mM lysozyme, 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (Sigma-Aldrich, India) for lysis. The suspension was subjected to sonication after addition of 0.5% Triton-X100 and 15 mM β-marceptoethanol (β-ME). The extract was then centrifuged at 15,000 x g for 30 min at 4^0C and the supernatant was collected. The supernatant was added to 200 µL of Ni-NTA agarose and incubated in a rotating wheel at 4^0C for 3 hours followed by washing three times with the same buffer containing 20 mM immidazole and 20 mM β-ME. Finally the ^{6}His-tagged proteins were eluted with the same buffer containing 500 mM immidazole, dialyzed against 20 mM Tris-HCl (pH 8.0) containing 25 mM NaCl, 10% glycerol and protease inhibitor cocktail and kept at -70^0C freezer for long term storage.

Preparation of Recombinant Baculovirus of LdCRK3

To obtain the recombinant baculovirus, the LdCRK3 gene was cloned into pFastBac vector (Invitrogen). The recombinant donor plasmid (pFB-LdCRK3) was then used for transformation of DH10Bac *E. coli* cells carrying modified baculovirus genome as bacmid DNA and helper plasmid to provide protein factors required for transposition. Inside the DH10Bac cells, the gene of interest would get transposed from pFastBac vector to the Bacmid resulting in white bacterial colonies in blue-white selection in the presence of IPTG and Blue-O-Gal. Next, a few white colonies were selected and checked for the expected recombinant bacmids by PCR. Then the recombinant bacmid DNAs were isolated from the selected colonies and used for transfection of Sf9 cells using Cellfectin reagent (Invitrogen). For transfection, about 5 x 10^5 Sf9 cells were taken in 2 mL complete Grace's insect cell medium in a 35 mm tissue culture plate and allowed to attach for overnight at 27^0C. The recombinant bacmid DNA and 6 µL of Cellfectin reagent (Invitrogen) were taken separately in each of 100 µL of serum and antibiotic free medium then mixed together and incubated at 27^0C for 30 minutes, after which 800 µL of serum and antibiotic free medium was added to it. The attached Sf9 cells were washed with serum and antibiotic free medium and overlaid

with the bacmid-Cellfectin mixture and incubated for 5 hours at 27^0C. The entire process is schematically presented in **Figure 4.2**. After 5 hours, serum and antibiotic free medium was replaced with complete Grace's insect cell medium. After 72 hours, the medium carrying the recombinant baculovirus particles was collected and used for further infection of Sf9 cells. After several rounds of virus amplification the amplified virus particles were used for infection and the infected Sf9 cells were collected 48 hours post-transfection for protein expression. The expression of desired protein was confirmed by western blotting.

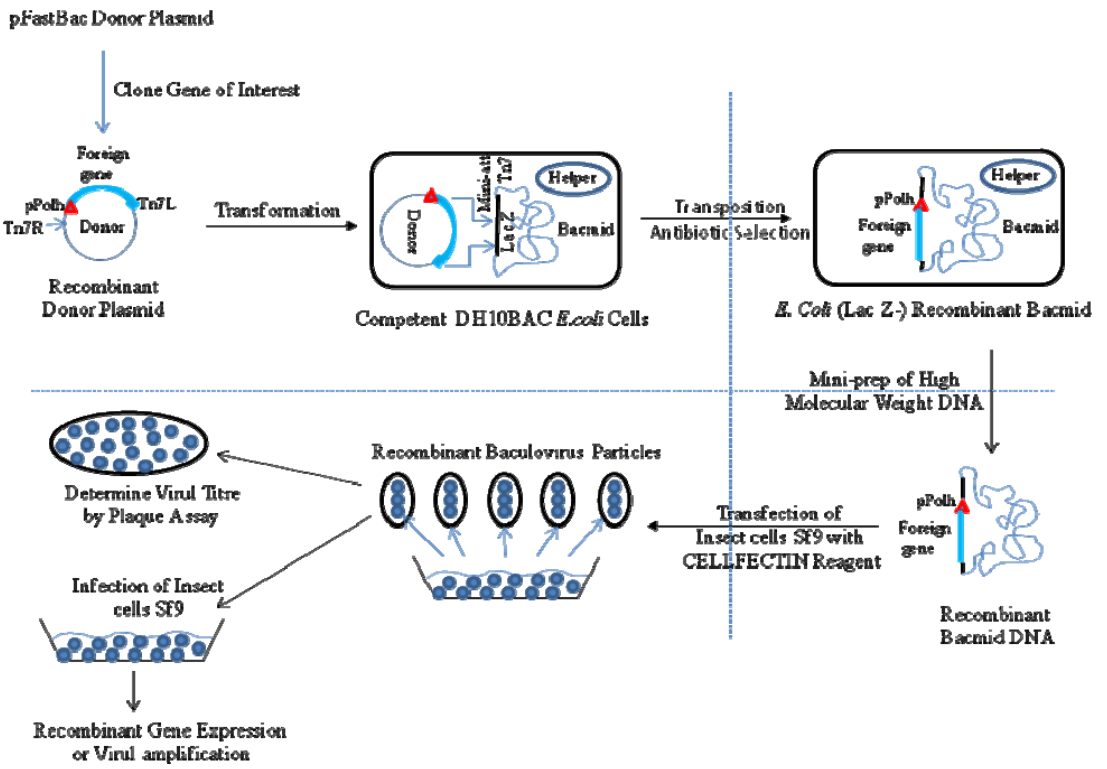


Figure 4.2: Schematic representation of recombinant bacmid preparation and protein expression using recombinant baculovirus. The gene of interest is cloned in pFB donor plasmid (Invitrogen) downstream of the polyhedron promoter sequence element, which is flanked by transposable elements Tn7R and Tn7L. Then the recombinant donor plasmid is transformed into DH10Bac *E. coli* competent cells for transposition into baculovirus genome, which carries appropriate transposon targets. The helper plasmid codes the transposase enzyme required for transposition. Upon successful transposition lacZ coding sequence within the viral genome is disrupted resulting in the white colonies in blue-white screening. Few white colonies are selected and recombinant bacmid DNA was isolated. This recombinant bacmid DNA was used for transfection of insect cells Sf9 with the help of cellfectin reagent. After 72 hours, recombinant baculovirus particles that come out of the cells and are used for further rounds of virus amplification in order to get a suitable viral titer to infect for protein expression.

Expression of GST-LdCyc1 and LdCRK3 in insect cell

Protein expression in insect cells was carried out using Bac-to-Bac Baculovirus Expression System (Invitrogen). To obtain the active kinase complex, the separate baculoviruses carrying GST-LdCyc1 and LdCRK3 ORFs were used to co-infect Sf9 insect cells grown in Grace's insect cell medium containing 10% fetal bovine serum at 27^0C. The complex was purified over glutathione Sepharose (GE Healthcare) column. The activity of the kinase complex was checked using histone H1 as substrate in a standard kinase assay. For some experiments, the active kinase complex between LdCRK3 expressed in insect cells and bacterially expressed GST-LdCyc1 was used.

Protein kinase assay

The kinase assay was usually carried out at 30^oC in 20 mM HEPES, pH 7.5 containing 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 2 mM DTT, 5 mM NaF, 50 μM ATP, 0.5 μg histone H1 or the expressed putative substrates and 2 μCi of [γ-^{32}P]ATP (3000 Ci/mmole) in a total volume of 15 μL. 0.2 μg of purified GSTLdCyc1-CRK3 complex was used in each reaction. The reaction was stopped by addition of Laemmli buffer and heated in a boiling water bath for 10 minutes. The products were analyzed by SDS-PAGE followed by autoradiography or phosphorimager analyses with Typhoon scanner (GE Healthcare).

Kinase competition assay

Kinase competition assays of all the three substrates were carried out similarly as described above with the addition of 75 μM each of Cy-motif containing PS100 small peptide **[52]** or Somatostatin (Sigma-Aldrich) control peptide into reaction mixture. The incubation time for Ld14.0140L, Ld28.0070L and Ld29.1050L were 6, 10 and 30 minutes, respectively. The amino acid sequences of the small peptides PS100 and Somatostatin are AC<u>RRLFG</u>PVDSE and AGXKNFFWKTFTSX, respectively.

Immunoprecipitation (IP) of LdCyc1

For IP of LdCyc1, 2 x10^8 *L. donovani* promastigotes were lyzed with 1 mL of 50 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 10% glycerol, 0.2% Triton-X-100, 1 mM PMSF and protease inhibitor cocktail (Sigma-Aldrich). The cell extract was pre-cleared with 5 μl of Protein A-CL Agarose (Bangalore Genie)

beads by rotating at 4^0C for 1 hour. The pre-cleared lysate containing 1 mg of total protein was incubated overnight at 4^0C with 10 µg of Rabbit-anti-LdCyc1 antibody **[158]** and 10 µl Protein A-CL Agarose beads. Finally the beads were washed thrice with the same buffer and used for kinase competition assay.

IP-Kinase competition assay

The IP-kinase competition assay was very much similar to the kinase competition assay as described above. In the IP-kinase competition assay the LdCyc1 kinase was first immunoprecipitated from *L. donovani* promastigotes with anti-LdCyc1 polyclonal antibody raised in rabbit **[158]**. Then the immunoprecipitated LdCyc1 was added in the kinase assay instead of purified GST-LdCyc1/LdCRK3 kinase.

Protein interaction assay

The interaction assays between LdCyc1 and the identified substrates were typically carried out by incubating 5 µg of bacterially purified substrate protein with 0.2 µg of either GST or GST-LdCyc1 proteins bound to glutathione beads at 4^0C for 1 hour in a total volume of 0.2 mL of 50 mM Na-phosphate (pH 8.0) containing 250 mM NaCl, 0.5% Triton-X100, 10% glycerol, 1 mM EDTA, 2 mM DTT and protease inhibitors. After incubation, the beads were pelleted down and the supernatant was kept aside for analysis as input control. Then the beads were washed with the same buffer for 5 times and the bound proteins were boiled in Laemmli buffer. The samples were analyzed by 12% SDS-PAGE followed by immunoblotting with mouse-anti-His tag and Mouse-anti-GST tag antibodies.

Protein interaction competition assay

For competition experiments, 75 µM of PS100 or Somatostatin (as control) peptides were added during the interaction assay. Subsequently, the beads were washed with the same buffer and the bound proteins were analyzed by immunoblotting with appropriate antibodies.

Results

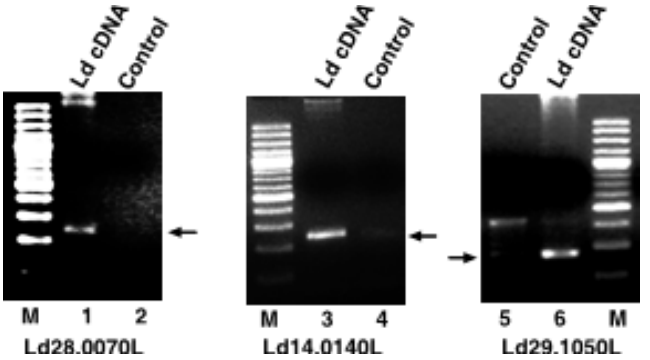
Three substrates of LdCyc1-CRK3 kinase complex are indentified

Since R/KxL-type Cy motif and the phosphorylation target (S/T)Px(K/R) were shown to constitute a bipartite substrate recognition sequence of cyclin dependent kinases **[54]**, in order to identify the potential substrates of S-phase cell cycle kinase LdCyc1-CRK3 from *L. donovani* **[158]** 28 putative ORFs containing both the conserved Cy-motif and the Cdk phosphorylation site were screened **[179]**. During the screening, the putative ORFs were amplified from genomic DNA of *L. donovani* for protein expression by *in vitro* transcription and translation and the expressed proteins were tested as substrates for the S-phase kinase complex. Finally, three substrates of the kinase could be identified and since the amplification and protein expression were carried out from *Leishmania donovani* genomic DNA taking the sequence information from *L. major* Friedlin database, the identified substrates were designated as Ld29.1050L (LmF 29.1050 like from *L. donovani*), Ld28.0070L and Ld14.0140L having the calculated molecular sizes of 103, 58.2 and 57.9 kDa, respectively **[179]**.

The identified substrates of LdCyc1-CRK3 kinase complex are expressed in *L. donovani*

It should be noted that the templates of the identified substrates were amplified from genomic DNA during screening. Therefore, the presence of the messages was checked in the expressed sequences to confirm that the ORFs were actually expressed in cells. As shown in **Figure 4.3A**, specific fragments were amplified for all the identified substrates from *L. donovani* cDNA library confirming that the genes were indeed expressed in the parasite. The sizes of the specific products for Ld14.0140L, Ld28.0070L and Ld29.1050L were 230, 240 and 280 bp, respectively (**Figure 4.3A**). To further confirm the identities of the expressed messages, the RT-PCR products were sequenced partially and aligned with sequences of the relevant portions of the clones obtained from the genomic DNA **(Figure 4.3B)**. The exact match of sequences of the RT-PCR products (cDNA) with the respective ORFs (Cloned) further proved that the cloned genes are expressed in cells. It could be mentioned here that the corresponding messages in *L. major* were previously shown to be expressed constitutively in promastigotes and amastigotes **[182]**.

A



B

Ld28.0070L

```
Cloned  ATGCATGAATTGAGGCGATCGGCCTCCAGCATCGCGACCGAGCTCTGGTCTCCTGATGAATTGT
cDNA    ATGCATGAATTGAGGCGATCGGCCTCCAGCATCGCGACCGAGCTCTGGTCTCCTGATGAATTGT

Cloned  ACAAGGCCGATCCCCAGGTCGATAGTTTGCAGCCGATTTTGAAGACGCTTGAAGTGGACTCCGA
cDNA    ACAAGGCCGATCCCCAGGTCGATAGTTTGCAGCCGATTTTGAAGACGCTTGAAGTGGACTCCGA

Cloned  TCGATTTTACGCGTTCCGTCTCTCACTGGTGCTGGCGCCGTTTGAGGCTCTCGAGAGATA 188
cDNA    TCGATTTTACGCGTTCCGTCTCTCACTGGTGCTGGCGCCGTTTGAGGCTCTCGAGAGATA 188
```

Ld14.0140L

```
Cloned  ATGTCGAGCAGTGCCGTCCCTGCGTACGAGGAGAAGCAAAAGGTTTACGCTCTCCTCAACGGTA
cDNA    ATGTCGAGCAGTGCCGTCCCTGCGTACGAGGAGAAGCAAAAGGTTTACGCTCTCCTCAACGGTA

Cloned  CCTTCCATGCGGCGATTGTGCTGGAGGTAGCAGAAGACGCAACAGAGGGCGGCTTTCTCTACTA
cDNA    CCTTCCATGCGGCGATTGTGCTGGAGGTAGCAGAAGACGCAACAGAGGGCGGCTTTCTCTACTA

Cloned  CGTTCGCTACGTGGAGCAGGACAGCAGGCTGGATCAATGGCTGCAGGCGAGCGACATCAAGGAA
cDNA    CGTTCGCTACGTGGAGCAGGACAGCAGGCTGGATCAATGGCTGCAGGCGAGCGACATCAAGGAA

Cloned  CGTCACCAG 201
cDNA    CGTCACCAG 201
```

Ld29.1050L

```
Cloned  ATGGATGAATTTGCAGAGTGGGGCGAGGGCACGACTGGTGTCGTTGCAGCAGACAGCACCTACA
cDNA    ATGGATGAATTTGCAGAGTGGGGCGAGGGCACGACTGGTGTCGTTGCAGCAGACAGCACCTACA

Cloned  CCGAGGGGCTCGACCTCGATGAGTTTCGTGAGCAGCTGTGGCAGCGCAATCAGCGCGATGCCGT
cDNA    CCGAGGGGCTCGACCTCGATGAGTTTCGTGAGCAGCTGTGGCAGCGCAATCAGCGCGATGCCGT

Cloned  TGTGTGCCTTGTTGACTGCAACGAGGGCATGTTCGGAGTGCTGCCAGCTGGCGAGTCGGCGAAG
cDNA    TGTGTGCCTTGTTGACTGCAACGAGGGCATGTTCGGAGTGCTGCCAGCTGGCGAGTCGGCGAAG

Cloned  ACGACTCAGACTGGTAAGAACGGGGAGGGTGCACCGGCGCACACTCGTGGTGTCACGAAACTCG
cDNA    ACGACTCAGACTGGTAAGAACGGGGAGGGTGCACCGGCGCACACTCGTGGTGTCACGAAACTCG

Cloned  CACTGCTCACCATTGGTCAGGGGGCAGCTATGAGTA 292
cDNA    CACTGCTCAGCATTGGTCAGGGGGCAGCTATGAGTA 292
```

Figure 4.3: A. Expression of the messages was confirmed by RT-PCR with ORF specific primer pairs from *L. donovani* cDNA library. Control, empty library vector pJG4-5 used as a template. **B. Alignment of Sequences** of RT-PCR products (cDNA) with that of the working clones obtained from genomic DNAs (Cloned) for the three ORFs.

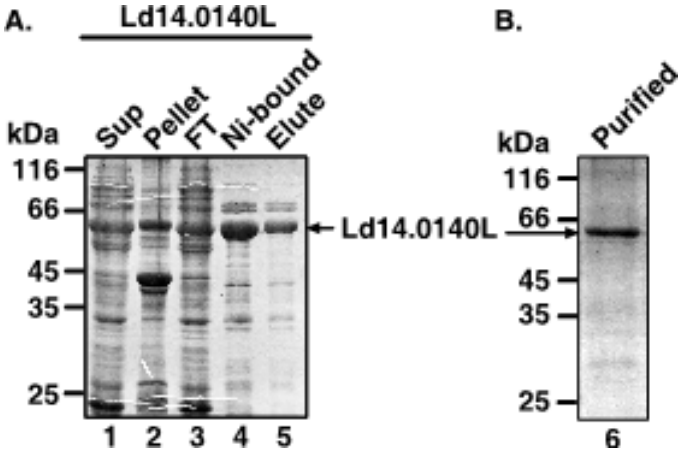


Figure 4.4: Purification of LdHAT1 protein. A. The over-expressed 6xHis tagged Ld14.0140L was purified from the bacterial cells over Ni-NTA agarose (lanes 1, Supernatant; 2, Pellet; 3, Flow through; 4, Ni-agarose bound and 5, Elute). **B.** The final purified LdHAT1 protein preparation (lane 6).

Over expression and purification of the identified substrates of LdCyc1-CRK3 kinase

As described above, three substrates of the S-phase kinase LdCyc1-CRK3 of *Leishmania donovani* were identified and during the screening they were expressed in small quantities using *in vitro* transcription and translation system. In order to further confirm the authenticity of the substrates and characterize them, it was decided to express the proteins in bacterial cells with ^{6}His tag, purify and repeat the kinase assay. For this purpose the three genes were cloned into pET21b vector separately to express the proteins with C-terminal ^{6}His tag and all three genes were successfully over expressed by IPTG induction as shown in **Figure 4.4A** and **Figure 4.5A**.

To get better solubility of the individual induced proteins, the different *E. coli* strains were tried to express the proteins. Finally Ld14.0140L (**Figure 4.4B)** was expressed in soluble form better in pG-JKE8 chaperon plasmid containing BL21-DE3 strain, whereas Ld28.0070L (**Figure 4.5B)** and Ld29.1050L (**Figure 4.5C)** gave better result in T7-shuffle strain. Some contaminating bands could not be removed from the preparation of Ld29.1050L after purification over Ni-Agarose (**Figure 4.5C**) and the partially purified protein was subsequently used for the assays.

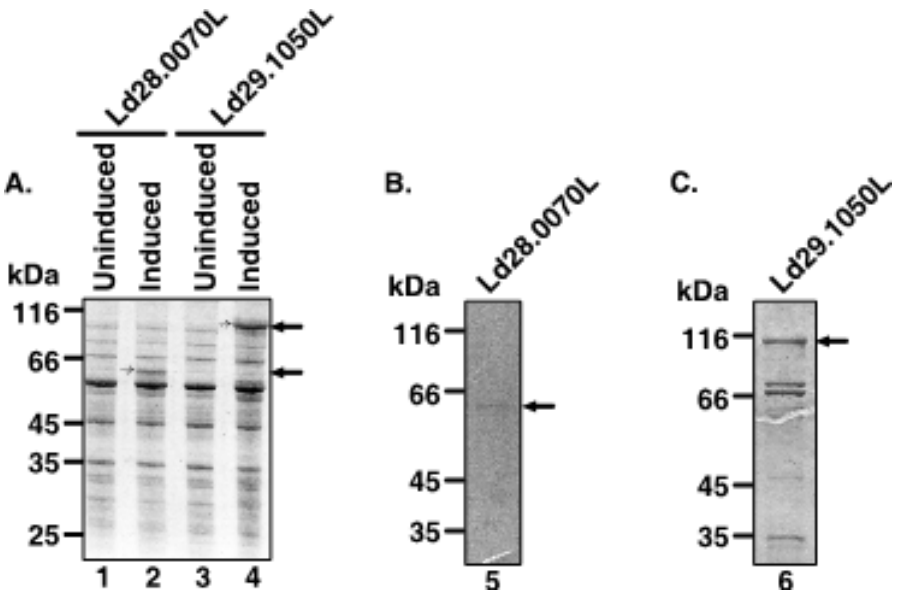


Figure 4.5: Purification of Ld28.0070L and Ld29.1050L proteins. A. Ld28.0070L and Ld29.1050L proteins were over-expressed in *E. coli* cells with IPTG (lanes 1, 3, Uninduced; 2, 4, Induced). B & C. The over-expressed 6xHis tagged proteins were purified over Ni-NTA agarose and lanes 5 and 6 indicate purified proteins of Ld28.0070L and Ld29.1050L, respectively. Ld29.1050L was partially purified as the preparation showed the presence of some contaminating bands. Arrows indicate desired proteins.

Bacterially expressed GST-LdCyc1 form active kinase complex with Sf9 expressed LdCRK3

In order to prepare the active kinase complex of LdCRK3 and perform interaction assays with the identified three substrates, the LdCyc1 protein purification was necessary. Therefore, the IPTG induced bacterially expressed LdCyc1, a GST-tagged protein, was purified over glutathione beads according to Banerjee et al **[157]**. The movement of glutathione beads bound GST-LdCyc1 protein band in SDS-PAGE corresponded to its theoretical molecular size of 61 kDa (**Figure 4.6**, lanes 4-5 and **Figure 4.7B**), and expectedly, the size of GST protein was 26 kDa as shown in **Figure 4.6**, lane 6. To obtain the active kinase complex, Sf9 insect cells were first infected with the baculoviruses carrying LdCRK3 ORF and the expressed 35 kDa LdCRK3 protein was allowed to form complex with glutathione beads bound bacterially expressed GST-LdCyc1 protein. The complex formation was confirmed by detecting the presence of LdCRK3 protein by immunoblotting with rabbit-anti-LdCRK3 antibody (**Figure 4.7A)** and that of GST-LdCyc1 protein by mouse-anti-GST antibody (**Figure 4.7B**). To check the activity of

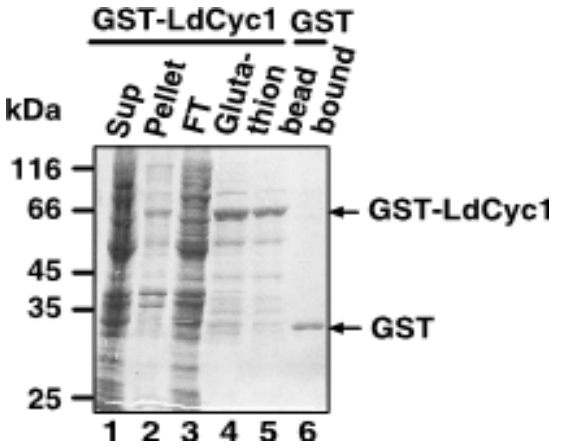


Figure 4.6: Purification of GST-LdCyc1 protein. GST-LdCyc1 protein was expressed in BL21DE3 *E. coli* cells and purified over glutathione beads (lanes 1, Supernatant; 2, Pellet; 3 Flow through; 4 & 5 glutathione beads bound GST-LdCyc1, and 6, GST).

kinase complex the protein kinase assay was performed with human histone H1 as substrate, and as shown in **Figure 4.7C,** it was phosphorylated by GST-LdCyc1-CRK3 kinase complex, confirming the preparation of an active LdCyc1-CRK3 kinase complex.

The bacterially expressed proteins act as substrates of LdCyc1-CRK3

In order to confirm the authenticity of the identified substrates, the kinase assays were carried out with the bacterially expressed purified ^{6}His tagged proteins, and expectedly, all three of them could be phosphorylated GST-LdCyc1-CRK3 complex (**Figure 4.8A**). Interestingly, the phosphorylation of all the proteins was inhibited significantly and specifically by a peptide (PS100) containing the RRLFG Cy-motif confirming the Cy-motif mediated substrate docking. The purified proteins could also be phosphorylated in a Cy-motif dependent manner by LdCyc1 associated kinase isolated from *L. donovani* promastigote extract by immunoprecipitation with anti-LdCRK3 antibody (**Figure 4.8B**), suggesting the proteins could be the authentic *in vivo* substrates.

Cy-motif dependent interaction between LdCyc1 and the identified substrates

To further prove the hypothesis that the identified substrates would interact with LdCyc1 through their Cy-motif, interaction assays were performed between the GST-LdCyc1 and ^{6}His-tagged substrate protein. As shown in the **Figure 4.9**, ^{6}His-tagged proteins specifically interacted with GST-LdCyc1, and expectedly, PS100 peptide almost completely abolished the interactions whereas the control peptide did not, confirming the

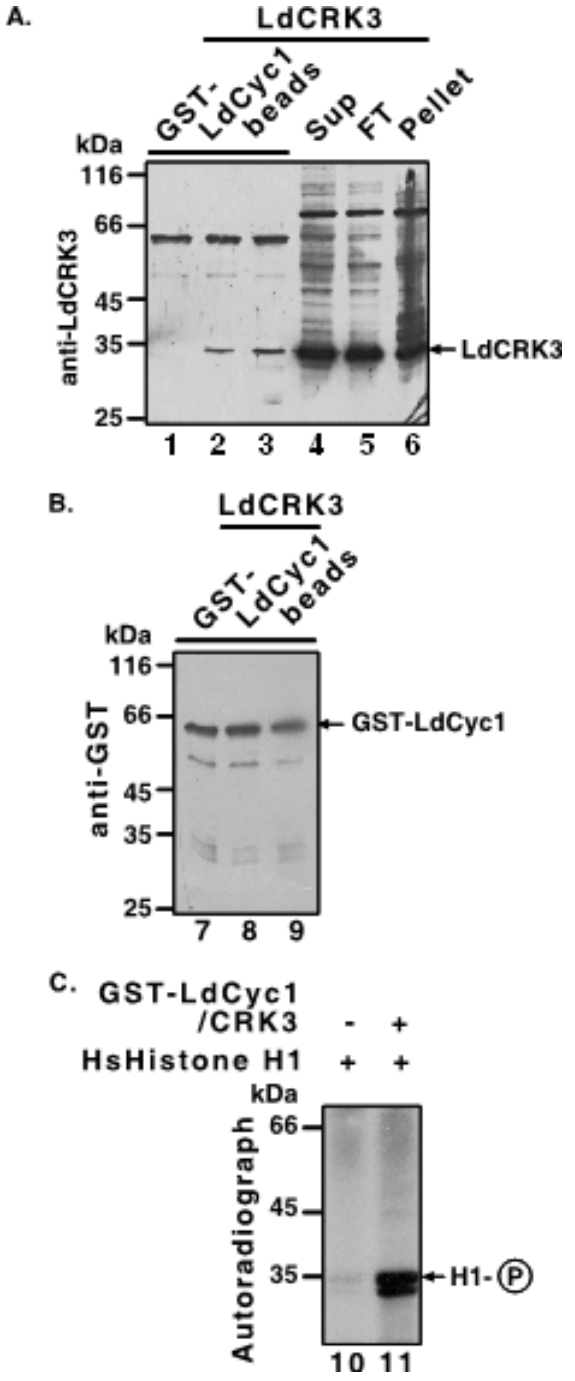


Figure 4.7: Preparation and characterization of GST-LdCyc1-CRK3 kinase. A. The extract of LdCRK3 baculovirus infected Sf9 cells was incubated with bacterially expressed and glutathione beads bound GST-LdCyc1 (lanes 1, glutathione bead bound GST-LdCyc1; 2 & 3, GST-LdCyc1+LdCRK3; 4, Super-natant; 5, Flow through; 6, Pellet of LdCRK3). Anti-LdCRK3 antibody was used for immunoblotting.

B. Immunoblotting was carried out with anti-GST antibody (lanes 7, glutathione bead bound GST-LdCyc1; 8 & 9, GST-LdCyc1+LdCRK3).

C. Autoradiograph of human histone H1 phosphorylation by purified GST-LdCyc1/CRK3 (lane 11) and lane 10 indicates without GST-LdCyc1/CRK3.

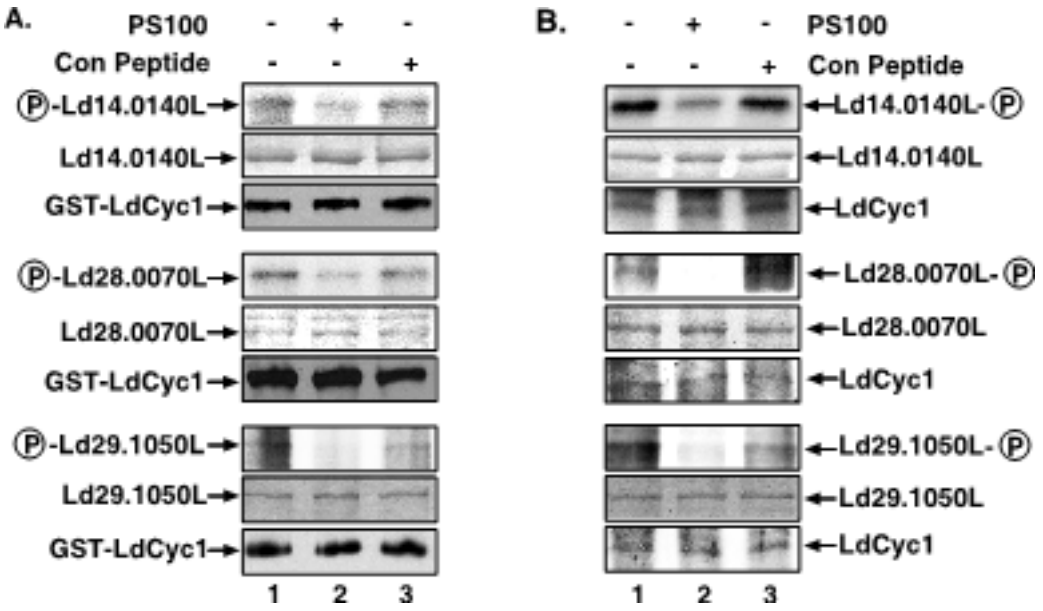


Figure 4.8: A. *In vitro* phosphorylation of purified proteins by GST-LdCyc1/CRK3 kinase complex. Cy-motif dependent phosphorylation of bacterially expressed putative substrates. For each substrate, phosphorimager scan of the phosphorylated bands were shown. Coomassie stained gel and anti-GST immunoblots (for GST-LdCyc1) were also shown to demonstrate the presence of equal amounts of substrates and kinase complex in different reaction tubes. **B. Cy-motif dependent phosphorylation by LdCyc1 complex immunoprecipitated from *L. donovani* extract.** Anti-LdCyc1 immunoblots were shown instead of anti-GST blots to confirm the presence of equal amount LdCyc1 in different

Cy-motif dependent interaction between the proteins. The data presented here, therefore, confirmed that the identified substrates could interact with LdCyc1, the cyclin partner of the cell cycle kinase complex in a Cy-motif dependent manner for being efficiently phosphorylated by the catalytic CRK3 subunit.

Homology of the three identified substrates with previously known proteins

Among the three identified substrates, the derived amino acid sequence of Ld28.0070L did not show homology with any previously characterized protein except the predicted equivalent proteins in related *Leishmania* species (**Figure 4.10**), implying its unique role in parasite specific cell cycle related processes. On the other hand, the predicted primary structure of Ld29.1050L was shown to contain conserved domains of Ku70 protein **[183]** (**Figure 4.11**), a subunit of the versatile heterodimeric protein complex Ku70-Ku80 that was implicated in various nuclear processes like DNA repair, telomere maintenance and apoptosis **[184]**. Notably, the Ld29.0150L protein and also its homologues from *L.*

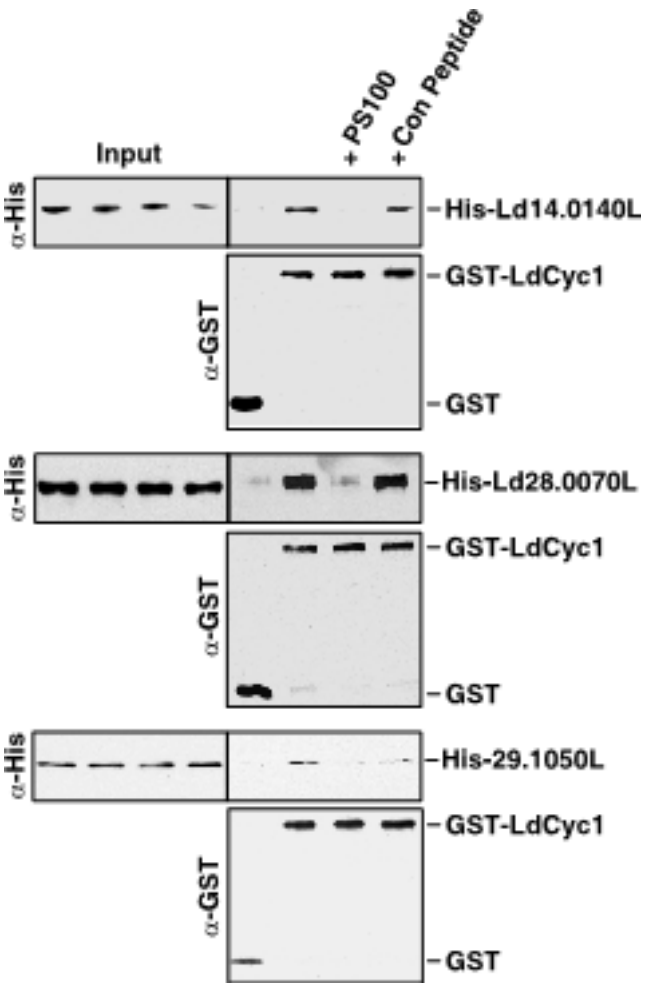


Figure 4.9: *In vitro* interaction between LdCyc1 and the substrate proteins through Cy-motif. Anti-His tag and anti-GST tag immunoblots were carried out to show the presence of ^{6}His-tagged substrates and GST or GST-LdCyc1 in the pulled-down complexes. Input, aliquots of supernatants were immunoblotted with anti-His tag antibody to demonstrate the presence of equal amount of substrates in all reaction tubes.

infantum and *L. braziliensis* were found to contain an extra stretch of 41 amino acids in the middle portion, which was not found in the *L. major* protein (**Figure 4.11**). The third identified substrate Ld14.0140L was found to contain a MYST (human Moz, Yeast Ybf2 and Sas2, and mammalian TIP60) histone acetyl transferase (HAT) domain along with an associated chromodomain **[183]** (**Figure 4.12**). The accession numbers of the nucleotide sequences data of the three substrates Ld14.0140L, Ld28.0070L and Ld29.1050L submitted to the GenBank™, EMBL and DDBJ databases are **HM120719, HQ264173 and HQ264174,** respectively.

Discussion

In the present study, three identified substrates of S-phase cell cycle kinase from *L. donovani* LdCyc1-CRK3 in a screening of the putative protein sequences available in *Leishmania* genome database based on the presence of cyclin binding Cy-motif and Cdk target phosphorylation site in them are characterized. All the three substrates are

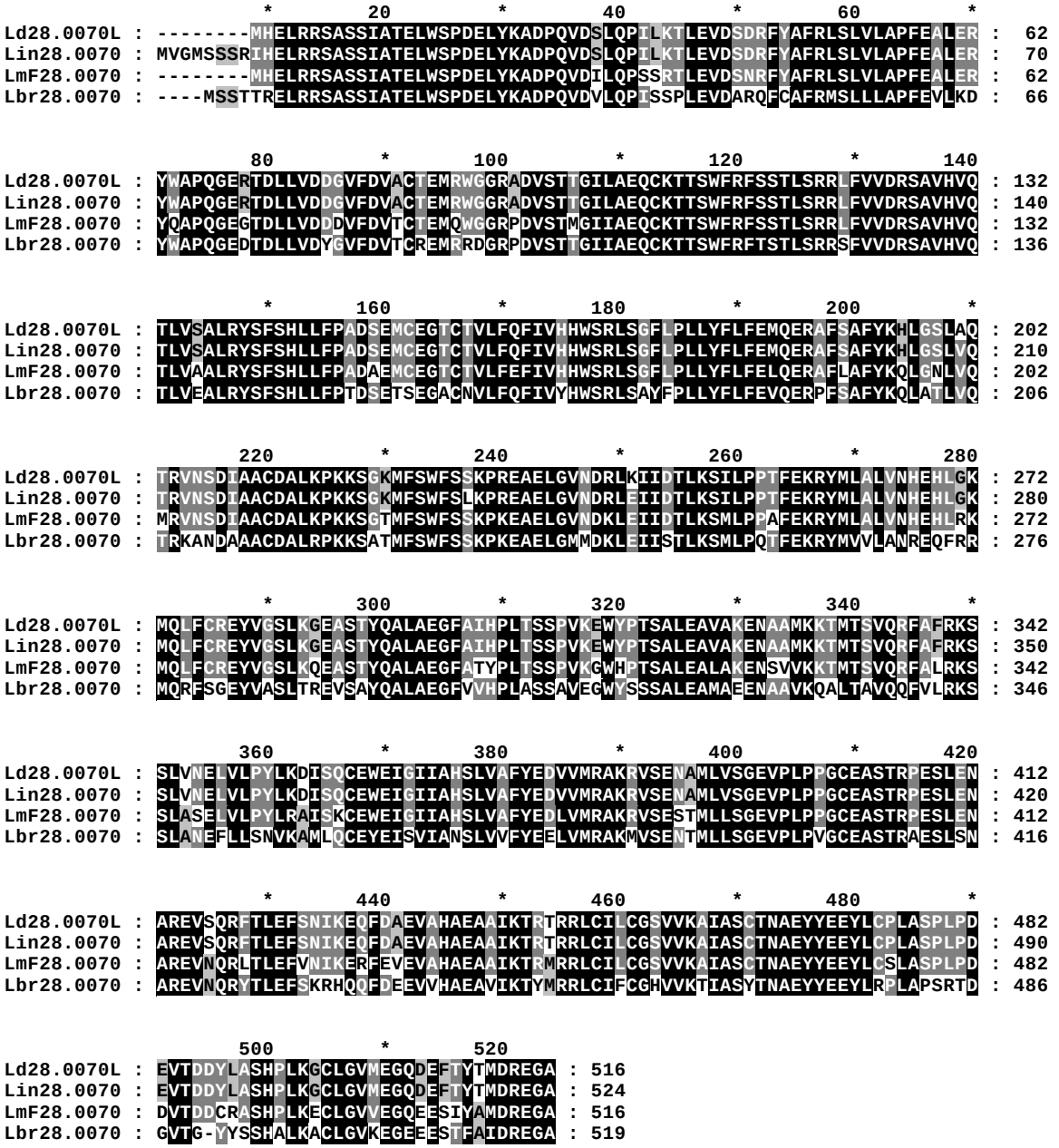


Figure 4.10: Alignment of the predicted protein sequences homologous to Ld28.0070L. Alignment was done using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index. html) followed by shading in Genedoc (http://www.psc.edu/biomed/genedoc/). The accession numbers of the sequences are as follows: Ld28.0070L, HQ264173; Lin28.0070, XP_001470042; LmF28.0070, XP_001684285; Lbr28.0070, XP_001566044. Ld, *Leishmania donovani*; Lin, *L. infantum*; Lm, *L. major*, Lbr, *L. braziliensis*.

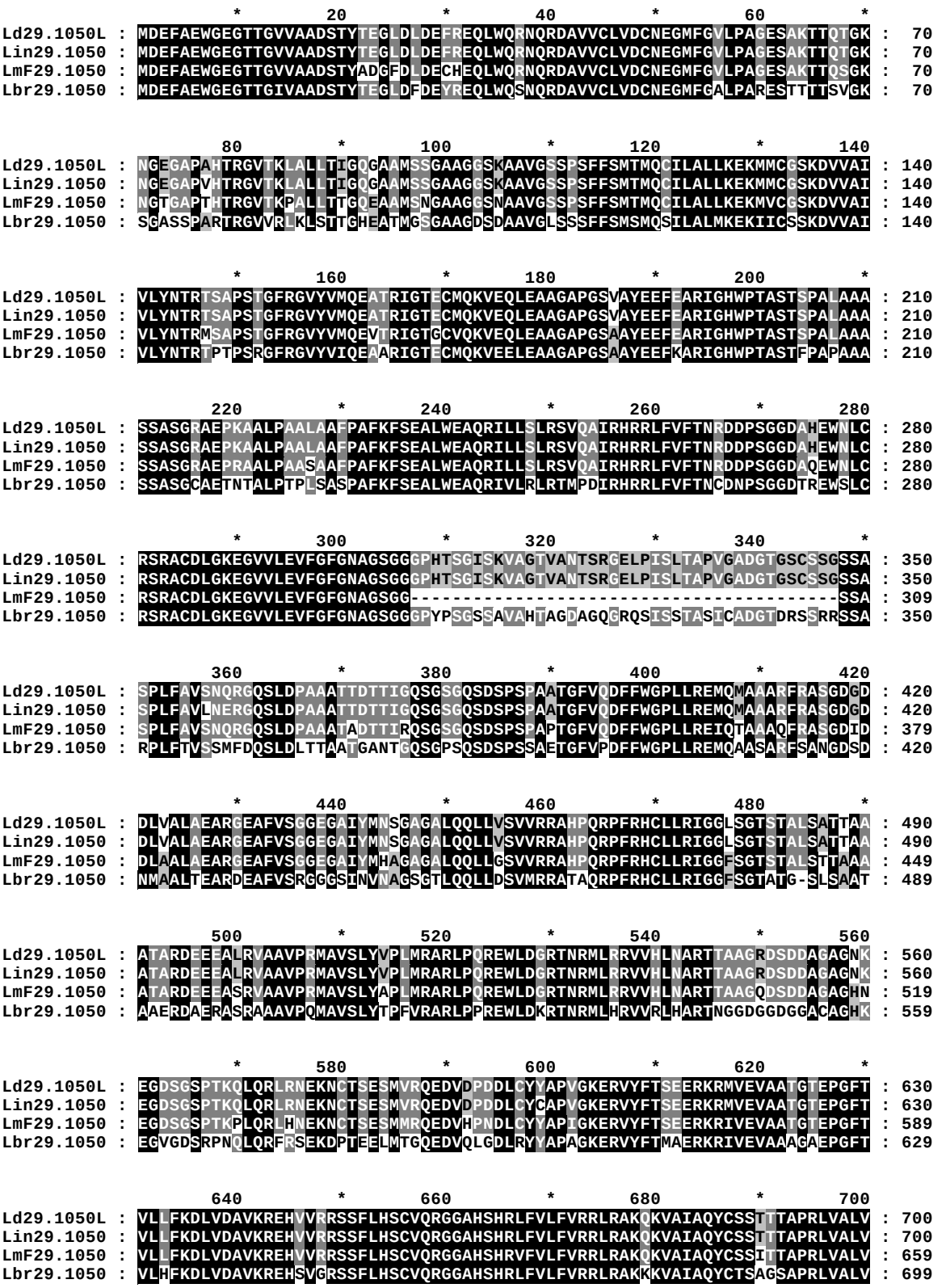


Figure 4.11: Continued to next page

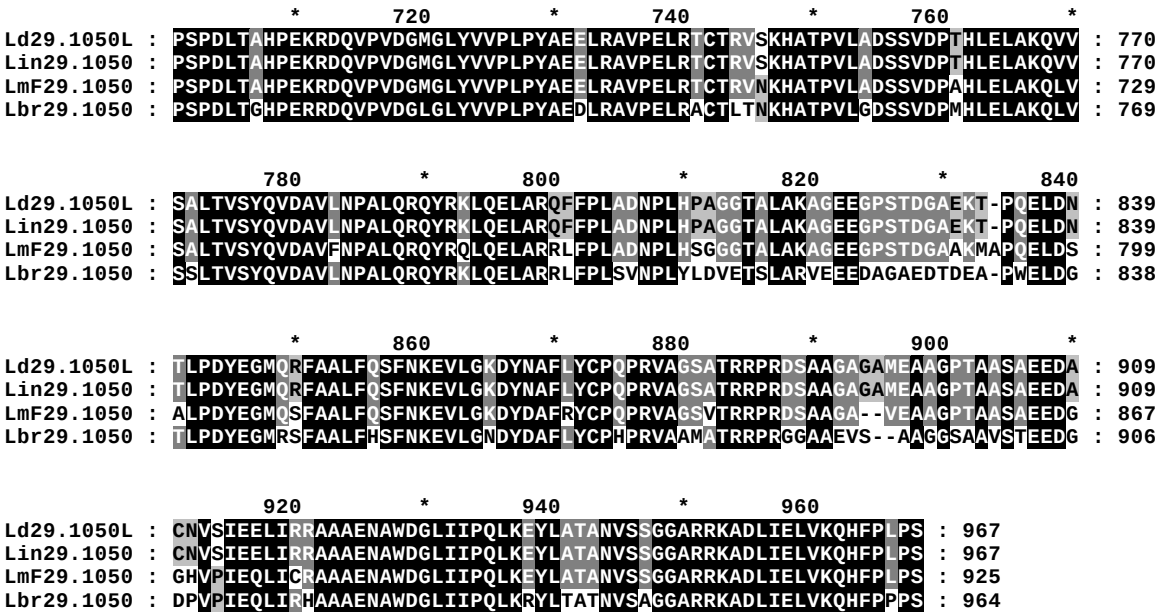


Figure 4.11: Alignment of the predicted protein sequences homologous to Ld29.1050L. The accession numbers of the sequences are as follows: Ld29.1050L, HQ264174; Lin29.1280, XP_001466633; LmF29.1050, XP_847769; Lbr29.1130, XP_001566458. The regions homologous to Ku-70 protein are indicated by blue bars.

expressed in *L. donovani* promastigotes as confirmed by the presence of specific messages in a cDNA library. The results described here have established the fact that the three identified substrates interact with the cyclin subunit LdCyc1 in a Cy-motif dependent manner for being efficiently phosphorylated by Cdk component CRK3. Similar phenomenon was also observed with LdCyc1 associated kinase complex immunoprecipitated from *L. donovani* promastigotes, implicating Cy-motif dependent phosphorylation by cyclin dependent kinase in *Leishmania* cells. The contribution of R/KxL type Cy motif in substrate recognition by different cyclin-Cdks is well established **[54, 56]**. An analysis of sequences of about 80 identified substrates of different mammalian cyclin-Cdks **[185]** have revealed that almost all the substrates contain R/KxL type motif (**Table 4.4**). Only a few of them do not contain Cy motif. However, slightly altered, but acceptable motifs could be present in them as previous study showed that some replacements in the conserved motif are allowed **[56]**. It has also been observed that the specificity of Clb5-Cdc28 of budding yeast in S-phase primarily depends on the substrate R/KxL motif **[186]**. Our studies established that R/KxL type Cy motif is operational in *Leishmania* parasites as well.

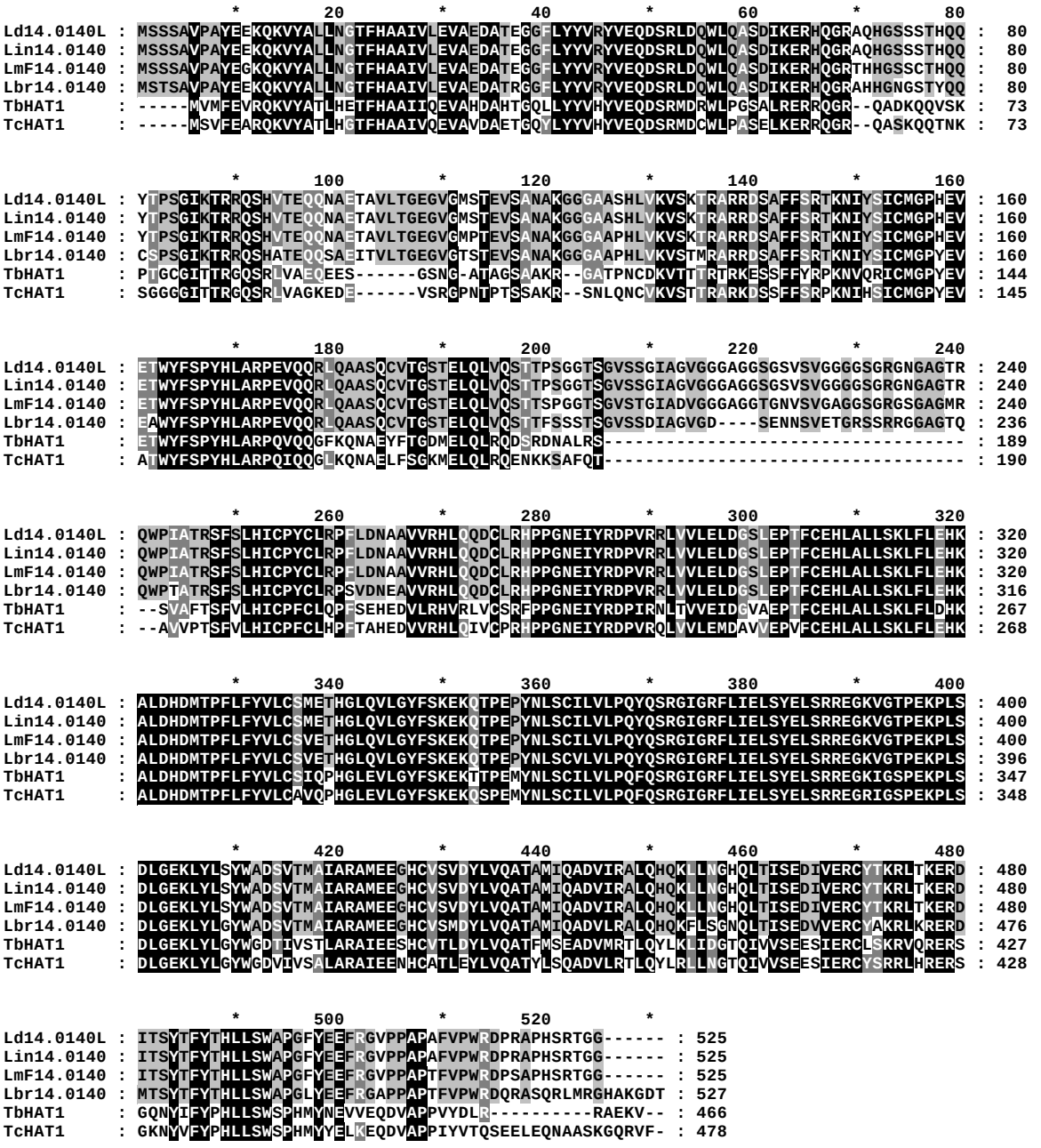


Figure 4-12: Alignment of the protein sequences homologous to Ld14.0140L. The accession numbers of the sequences are as follows: Ld14.0140L, HM120719; Lin14.0140, XP_001464197; LmF14.0140, XP_001687613; Lbr14.0140, XP_001563308; TbHAT1, AAN77136.1; TcHAT1, EAN92545. Tb, Trypanosoma brucei; Tc, Trypanosoma cruzi. The chromodomain is indicated by blue bar and MYST domain by grey bar.

Table 4.4: Substrates containing R/KxL motifs in human

SUBSTRATE	ENSG00000-	RXL MOTIF	KINASE
ACTOPAXIN	197702	**RVLYNL**	CDK1/CYCLIN B
ADENOMATOUS POLYPOSIS COLI (APC)	134982	**RRL**TSH	CDK1/CYCLIN B
AMPHIPHYSIN	078053	**KGLF**PE **RRL**D	CDK1; CDK5
AML/RUNX1 TRANSCRIPTION FACTOR	159216	**RIL**PPC	CDK1/CYCLIN B; CDK2/CYCLIN A
ANDROGEN RECEPTOR	169083	**RML**YFA **RQL**VHV **RKLKKL**	CDK1
B-MYB	101057	**RAL**VRQ **RWLRVL** **RQL**LGR	CDK 2/CYCLIN A
BCL2-ASSOCIATED AGONIST OF CELL DEATH	002330	**REL**RRM	CDK1
BARD1	138376	**RQL**DSM **RNL**LHD	CDK2/CYCLIN A & E; CDK1/CYCLIN B
BETA3 TUBULIN	198211	**RKL**AVN **RAL**TVP	CDK1
BRCA2	139618	**KLQLF**I **KNL**FDE **KVLF**KS **RRLF**MH	CDK
C/EBP BETA	172216	**RNLF**KQ **REL**STL	CDK1
CALDESMON	122786	**RRL**KEE	CDK1
CDC 14/ CLP1	172409	**RDL**PHF **RPL**PKN	CDC2
CDC20	117399	**RIL**DAP **RVL**SLT	CDK1
CDC25	158402	**RCL**DLS **RQL**REQ	CDK1/CYCLIN B
CDC6	094804	**RRLVF**D **REL**AKV	CDK2
CDK7	134058	**RDL**KPN **KKL**IF	CDK1; CDK2
CK2 (CASEIN KINASE2)	204435	ABSENT	CDK1
GAP JUNCTION PROTEIN CONNEXIN-43 (CX43)	152661	**RIL**LLG **KLL**DKV	CDK1/CYCLIN B
DISABLED-2 (DAB2) OR P96 OR DOC-2	153071	**KDLF**QV **KGL**SIQ	CDK1

SUBSTRATE	ENSG00000-	RXL MOTIF	KINASE
DNA POLYMERASE LAMBDA	166169	**RRLV**DV **RALRLL** **RLL**GLP	CDK2/CYCLIN A
DRC1	143536	**RLL**EQE **REL**YSY	CDC2
DYNEIN, CYTOPLASMIC 1, INTERMEDIATE CHAIN 1	158560	**RRL**HKL	CDK1/CYCLIN B
DYSTROPHIN	198947	**RRL**LDL **RLF**QKP **RRL**NFA	CDK1
ECT2	114346	**RRL**KET **RAL**R**RAL**	CDK1; POLO KINASE
FANCG	221829	**RSL**E**RVL** **RAL**LYLV	CDK1
FORKHEAD BOX PROTEIN M1	111206	**RRL**PLPV **KLLFG**E	CDK2/ CYCLIN E & A
FOXO1	150907	**RVL**GQNV **RPL**PHTV	CDK2
GM130 (GOLGI PROTEIN)	167110	**REL**KEQ **RRL**AHL	CDK1
GRASP 65	114745	**KAL**LKA **KPL**KLMV	CDK1; POLO KINASE
HCN1 (APC/C COMPONENT)	164588	**RAL**RIV **RLL**RL **RRAF**E	CDK1
HHR6A	077721	**RRL**MRD	CDK1; CDK2
HIRA	100084	**RRL**LSP **RLF**SVP	CDK2/CYCLIN A
HMG-1	189403	**KKL**GE	CDC2
HUCDC7	097046	**RRL**KK	CDK2/CYCLIN E & A; CDK1/CYCLIN B
KIF11 (EG5)	138160	**KTLFG**	CDK1
KINESIN FAMILY MEMBER 23/ ZEN-4	137807	**RPLGF** **RRL**EARL	CDK1/CYCLIN B
MAP4	047849	**KDL**VLL	CDK1
MARCKS	155130	ABSENT	CDK2/CYCLIN E
MCM2	073111	**RGL**LYD **RSL**RQL **RYL**SFRR	CDC7; CDK2; CDK1; CK2
MEF	102034	**REL**LR	CDK2/CYCLIN A
MYOD (MYOGENIC DIFFERENTIATION 1)	129152	**RRL**SKV	CDK1; CDK2
NBP-60/ LAMIN B RECEPTOR	143815	**RRL**KYR	CDK1
NDEL	166579	**REL**EA **RDL**RQE	CDK1; AURORA A KINASE
NEUROEPITHELIAL CELL TRANSFORMING 1	173848	**RPL**ARV **RKL**DLW	CDK; POLO KINASE
NIR2 CYTOKINESIS/ GOLGI CONTROL	110697	**RAL**LPK	CDK1

SUBSTRATE	ENSG00000-	RXL MOTIF	KINASE
NUCLEAR PROTEIN ATAXIA TELANGIECTASIA LOCUS	149308	**RRVLCF**	CDK2/CYCLIN E
NUCLEAR CASEIN KINASE AND CDK SUBSTRATE 1	069275	ABSENT	CDK1
NUCLEOPHOSMIN (NPM)/ B23/ NUMATRIN	181163	**KLL**SIS	CDK2/CYCLIN E
P107 (RETINOBLASTOMA-LIKE 1)	080839	**RRLFG**E **RRL**HGM	CDK2/ CYCLIN A
P21 PROTEIN (CDC42/RAC)-ACTIVATED KINASE 2	180370	**RLL**QTS **RAL**YLI	CDK1
P27KIP (CYCLIN-DEPENDENT KINASE INHIBITOR 1B)	111276	**RNLFG**	CDK2/CYCLIN E
POLO-LIKE KINASE 1	166851	**RSL**LEL **RDL**KLG	CDC28
PROTEIN PHOSPHATASE 1, REGULATORY SUBUNIT 2	184203	**RQL**ISK	CDK1/CYCLIN B
RAB4A	168118	**RQL**RSPRR	CDK1
REGULATOR OF CHROMOSOME CONDENSATION 1	180198	**RLLV**PK	CDK1/CYCLIN B
RETINOBLASTOMA PROTEIN PRB	139687	**RLL**KK **RVL**KRS	CDK4/CYCLIN D; CDK2/CYCLIN E
RIBONUCLEOTIDE REDUCTASE R2	171848	**RRIF**QE **REFLF**	CDK1; CDK2
SEPARASE	135476	**RILFV**L **RLF**SFR **RFL**DGQ	CDK1/CYCLIN B
V-SKI SARCOMA VIRAL ONCOGENE HOMOLOG (AVIAN)	157933	**RRVPR**V **RSL**HQE	CDK1
STATHMIN	117632	**KEL**EKR **KDL**SLE	CDK1; CDK2
STEM- LOOP BINDING PROTEIN	163950	**RKL**LIN **RHL**RQP	CDK2/CYCLIN A
SURVIVIN	089685	**KEL**EGW	CDK1- CYCLIN B
SWI5	175854	**RPL**PKS	CDC28
TOPK	168078	**RGL**SHS **RGL**KYL	CDK1/CYCLIN B
UPSTREAM BINDING FACTOR	108312	**RVL**GE **RFL**ESL	CDK2/CYCLIN A; CDK2/CYCLIN E
WARTS TUMOR SUPPRESSOR	131023	**RVL**KPE **RDL**VYV	CDK1/CYCLIN B
WEE1	166483	**RKLRLF** **RALF**TD	CDK1/CYCLIN B1; CDK2; POLO KINASE; CK2

Analysis of the primary structures of the identified substrates revealed the presence of one typical Cdk phosphorylation site (S/T)Px(K/R) in each of the proteins (**Figure 4.13**) along with R/KxL type Cy motif. In addition, Ld28.0070L protein was shown to contain two minimal consensus (T/S)P Cdk phosphorylation sites (^{17}SP and ^{477}SP), and several such minimal sites were found to be present in Ld14.0140L and Ld29.1050L proteins (**Figure 4.13**). Since many characterized Cdk substrates were shown to be phosphorylated at minimal consensus motifs **[65, 185, 187]**, some of T/S in such motifs in the identified substrates could also get phosphorylated in addition to the canonical consensus sites.

Among the three identified substrates, Ld28.0070L is unique to the parasite. 29.1050L is the Ku70 homologue in the parasite and phosphorylation by an S-phase kinase raises the possibility of its role in DNA replication related activities. In fact, the role of Ku proteins was implicated during the formation of pre-replicative complex at eukaryotic origins and G1-S transition **[188]**. Moreover, Ku70 was shown to be phosphorylated by the tissue specific cyclin A1-Cdk2 kinase in human **[64]**, suggesting

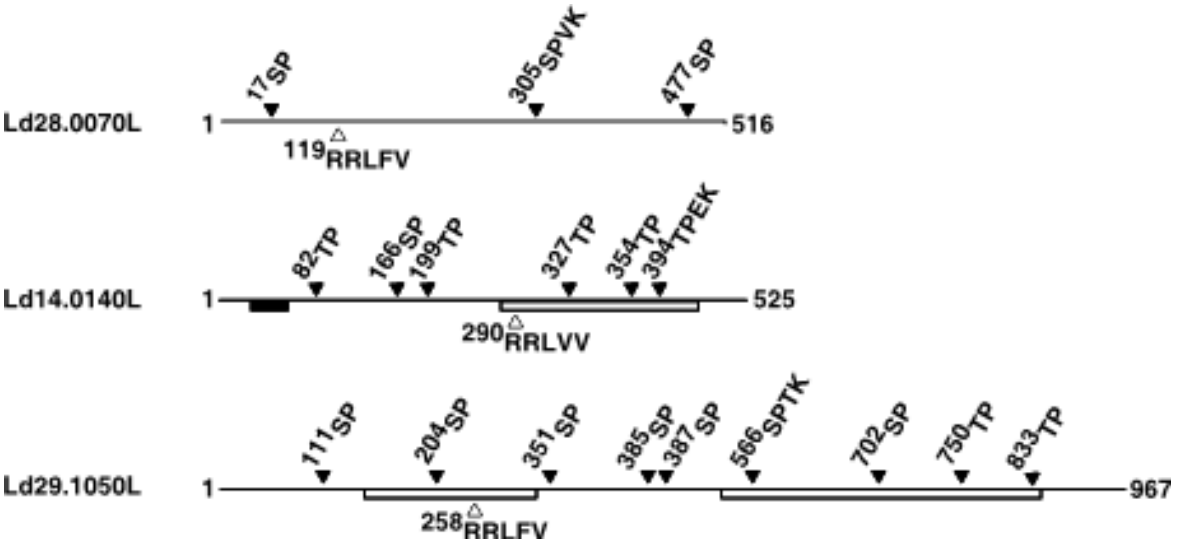


Figure 4.13: Schematic presentation of the three identified substrates of LdCyc1/CRK3. The potential phosphorylation sites are indicated by solid arrow-heads and the putative cyclin binding Cy-motifs are shown by hollow arrow-heads. The numbers indicate the position of the first residue in a motif. In Ld29.1050L, the empty bars denote the conserved Ku-70 related domains. In Ld14.0140L, the solid bar and the empty bar represent the conserved chromo-domain and MYST histone acetyl transferase domain, respectively.

that Ku70 homologue Ld29.1050L could be an authentic substrate of the *L. donovani* cell cycle kinase.

The third identified substrate Ld14.0140L contains a MYST domain and a chromodomain, implicating that the protein could be a member of MYST family of HATs. The members of MYST family of HATs were shown to be involved in key regulatory processes in eukaryotic cells. Recently, human MYST histone acetylase Hbo1 was shown to play critical role in replication licensing **[134]** and regulation of its activity through phosphorylation by Cyclin B-Cdk1 and Plk 1 was also implicated **[132]**. The activity of another MYST family HAT Tip60, which was found to play essential role in DNA repair and apoptosis, was suggested to be controlled through phosphorylation by Cyclin B-Cdk1 **[189]**. Interestingly, *Trypanosoma brucei* HAT1, which was found to be homologous to Ld14.0140L, was implicated to be essential for proper nuclear DNA replication in the organism **[169]**. Therefore, the phosphorylation of the HAT homologue by S-phase specific cell cycle kinase raised the possibility of regulation of chromatin remodeling during replication through the post-translational modification. In the next chapter, further characterization of the activity of Ld14.0140L and the effect of phosphorylation of the HAT homologue by LdCyc1-CRK3 on its activity are discussed.

HISTONE ACETYL TRANSFERASE ACTIVITY OF LDHAT1 IS REGULATED BY AN S-PHASE CELL CYCLE KINASE FROM *LEISHMANIA DONOVANI*

Large eukaryotic genome is compacted in a tiny nucleus constraining DNA template dependent processes including DNA replication, repair and transcription. Several protein factors are involved to overcome such constrains either by activity of ATPases or covalent modification of histone tail resulting in Chromatin remodeling to access the DNA template. These remodeling processes are reversible and can easily alter depending on the necessity of cell cycle progression in eukaryotes. The Swi-Snf complex and a number of others have been shown to involve in ATPase activity to remove tight DNA packaging for accessing several transcription factors. There are different types of post-translational modifications occur through acetylation, methylation, phosphorylation and ubiquitinylation of the N-terminal of histones that remodel the chromatin. These modifications can loosen the tight packing of chromatins to expose essential recognition sites for numerous protein factors facilitating gene transcription, DNA repair and replication. Apart from normal cellular processes, chromatin remodeling plays central role in epigenetics by methylating DNA bases and also in nuclear reprogramming of differentiated nuclei. Therefore, malfunction of these modifiers can causes an array of multisystem disorder.

Histone acetylation occurs at specific lysine residues and more abundantly in promoter regions of genes compared to their coding regions. It was reported that the histone acetyl transferases positively regulate the gene transcription and DNA replication through acetylation of specific lysine residue in histones. Recently it has been reported that MYST family histone acetyl transferase HBO1 is directly involved in replication licensing through acetylation of histone H4 and Cdt1 mediated loading of MCM on to the origin. On the other hand, histone deacetylase Sir2 negatively regulates DNA replication

by inhibiting formation of the pre-replicative complex. Like acetylation histones are also methylated at the specific lysine residues to regulate specific genes by either activation or silencing.

Histone acetyl transferases (HATs) enzymatically transfer the acetyl group from Acetyl Co-A to the ε-amino group of specific lysine residues of N-terminal tail of histones apart from globular domain and C-terminal tail. Lysine acetylation reduces the positive charge on amino group that in turn weakens the interaction with negatively charged DNA within nucleosome and also intermolecular interaction between nucleosomes. As a result DNA is more easily available for access of several protein factors. HATs belong to several families, namely, GNAT super family (consists of Hat1, Gcn5, PCAF, Hpa1 and Elp3), P300/CBP family, MYST family (consists of MOZ, Ybf2, Sas2, Sas3, TIP60, MORF, MOF, Esa1 and HBO1), Nuclear receptor co-activators and TAFIIIC family.

Like others, the MYST family is also involved in diverse cellular activities including transcription activation, DNA repair, pre-replication complex formation and transcription silencing. The MYST family is named derived from its founding members - human MOZ, yeast Ybf2/ Sas3, Sas2 and mammalian TIP60. These proteins are grouped together on the basis of their close sequence similarities. All members of this family contain an R/G-X-X-G-X-G/A segment, called canonical acetyl Co-A binding motif (A-motif) as well as a C_2HC Zn-finger in the characteristic MYST domain. Some of them also contain other conserved domains like chromodomain and plant homeodomain (PHD) for their specific functions. For example, chromodomain mediated recruitment of the tumour suppressor TIP60 – a MYST family histone acetyl transferase, at DNA double strand break site has been shown to activate ATM kinase for initiation of repair. The acetylation activity of TIP60 is also regulated through phosphorylation by Cyclin B2-cdc2, though its significance in cellular processes remains unknown.

It has been reported that four MYST family HATs are encoded by genomes of *Leishmania major* and *Trypanosoma cruzi* and three by that of *T. brucei*. As described in chapter 4, a putative HAT, which is highly homologous to TbHAT1, has been identified from *L. donovani*, during a search for potential substrates of previously characterized S-phase cell cycle kinase LdCyc1-CRK3 **[157, 158, 179]**. In the present chapter, the experiments to identify the R/KxL-like Cy-motif **[52]** through which the protein interact with LdCyc1 are described. It has also been shown that LdHAT1 gets phosphorylated by the kinase on a specific threonine residue and its acetyl transferase activity is modulated by such

phosphorylation suggesting a possible mechanism of regulation of chromatin remodeling by the S-phase cell cycle kinase.

Methods

Preparation of Mutants of LdHAT1 by Site Directed Mutagenesis (SDM)

The R/KxL type Cy motif (RRLVV) and the TPEK type phosphorylation motif present in the LdHAT1 were mutated by site directed mutagenesis (SDM) method where RL amino acids were mutated to DD in the Cy motif (^{290}RRLVV→RDDVV, LdHAT1ΔCy) and T was replaced by A amino acid in the TPEK phosphorylation site (^{394}TPEK→APEK, LdHAT1-T394A). For both mutations two sets of internal primers containing the mutated nucleotides were used and the procedure is depicted in **Figure 5.1**. For Cy motif mutation the two internal primers were (changed residues are marked red);

14.0140Mute1+:

5'CGAGATCTACCGCGACCCAGTGCGGGACGACGTCGTGTTGGAGCTGGATGG

14.0140Mute1-:

5'GCCATCCAGCTCCAACACGACGTCGTCCCGCACTGGGTCGCGGTAGATCTCG

On the other hand, for phosphorylation motif mutation the internal primers were:

14.0140 Mute2+: 5'CCGTGAGGGCAAGGTCGGCGCGCCGGAGAAGCCCCTCAGC

14.0140 Mute2-: 5'GCTGAGGGGCTTCTCCGGCGCGCCGACCTTGCCCTCACGG

Briefly, for each mutation, the first two PCRs were carried out with one end primer and one of the mutated primers using the plasmid pET21b-LdHAT1 as template with XT-5 proofreading polymerase (Bangalore Genie, India) followed by purification with DNA gel extraction kit (Axygen-India). The final PCR was carried out with the two end primers and mixture of the two purified first PCR products as DNA template. The cloning procedure is normal as described previously and mutations were checked by digestion with specific restriction enzymes, the target sites for which were generated due to mutation. Finally, the mutations were confirmed by DNA sequencing.

Restriction Digestion to confirm the LdHAT1 mutations

Both the mutants of LdHAT1 were checked by restriction digestion with appropriate restriction enzymes. The purified plasmids of LdHAT1ΔCy and LdHAT1-T394A were

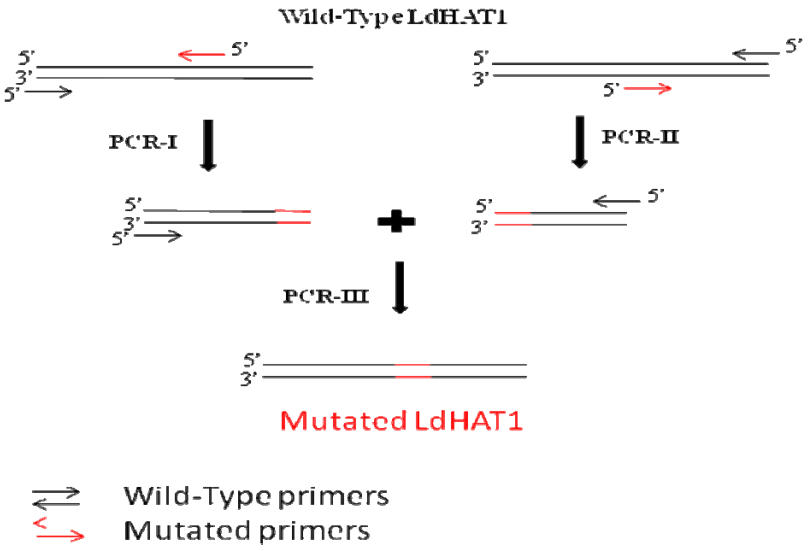


Figure 5.1: Schematic representation of Site Directed Mutagenesis (SDM). The wild-type LdHAT1 DNA template was first amplified by two PCRs separately; each reaction was carried out with one wild type end primer (black arrow) and other internal mutated primer (red arrow). Then the purified two DNA fragments were mixed together for a second round of PCR with the wild-type end primers.

incubated for 3 hours at 37^0C with (EcoRV and AatII) and at 50^0C with BssHII enzymes (New England Biolab), respectively. Control digestions were carried out with purified wild type LdHAT1 plasmid.

Over expression and Purification of LdHAT1 Mutants

To over-express the two mutant proteins, the appropriate recombinant plasmids were used to transform the chaperon plasmid containing strain BL21DE3-pG-EJK8 for better yield and solubility. The purification procedure was similar to that for the wild type protein, which was described before in chapter 4.

Protein Interaction Assay of Wild Type as well as Mutants of LdHAT1 with LdCyc1

To perform interaction assay of LdHAT1 and two mutants of LdHAT1, viz., LdHAT1ΔCy and LdHAT1-T394A with GST-LdCyc1, 5 µg each of bacterially purified wild type and two mutants of LdHAT1 proteins was incubated on a rotating wheel at 4^0C for 1 hour with glutathione beads bound to 0.2 µg of either GST or GST-LdCyc1 proteins in 50 mM Na-

phosphate (pH 8.0) containing 250 mM NaCl, 0.5% Triton-X100, 10% glycerol, 1 mM EDTA, 2 mM DTT and protease inhibitors. Subsequently, the beads were washed 6 times with the same buffer and the bound proteins were analyzed by immunoblotting with appropriate antibodies.

Preparation of GST-LdCyc1-CRK3 active kinase complex

To prepare GST-LdCyc1-CRK3 kinase complex, bacterially expressed GST-LdCyc1 was first bound to glutathione-Sepharose beads (GE Healthcare Lifesciences) and the bead bound GST-LdCyc1 was then incubated with an extract of Sf9 cells expressing LdCRK3 in the binding buffer (50 mM Tris-HCl, pH 8.0 containing 50 mM NaCl, 5 mM NaF, 1 mM Na_3VO_4, 0.1 mM EDTA, 0.1% T-X100, 10% glycerol, 2 mM DTT, 1 mM PMSF and protease inhibitors cocktail) on a rotating wheel at 4^0C for overnight. The beads were washed three times with the same binding buffer and the kinase complex was eluted in the elution buffer (50 mM Tris-HCl, pH 8.0 containing 10% glycerol, 10 mM reduced glutathione and 1 mM PMSF).

Phosphorylation of wild type and Mutant LdHAT1

The phosphorylation of wild type and two mutants of LdHAT1 were carried out as described previously **[179]**. Briefly, 0.5 µg each of wild type or the mutants of LdHAT1, viz., LdHAT1ΔCy and LdHAT1-T394A were added to the reaction mix containing 1x MKB (20 mM HEPES-KOH, pH 7.5 containing 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 5 mM NaF, 2 mM DTT), 50 µM ATP, 2 µCi of [γ-^{32}P] ATP (3000 Ci/mmole) in a total volume of 15 µL and incubated at 30^0C for 30 minutes. 0.2 µg of purified GST-LdCyc1-CRK3 kinase was used in each reaction. The reaction was stopped by addition of 4xLaemmli sample buffer and heated in a boiling water bath for 10 minutes. The products were analyzed by SDS-PAGE followed by autoradiography or phosphorimager scanning with Typhoon scanner (GE Healthcare Lifesciences).

Preparation of phosphorylated LdHAT1 (wild type and its two mutants) with non-radioactive ATP

The wild type and two mutants of LdHAT1 were phosphorylated by GST-LdCyc1-CRK3 bound to glutathione beads in presence of non-radioactive ATP as described above. After

completion of the reaction the supernatants were separated from the beads by centrifugation and followed by passing through the G-25 column (GE Healthcare Lifesciences) for purification. The purified phosphorylated proteins were used in the HAT assay.

Antibody generation against LdHAT1 full length protein

In order to raise antibody against LdHAT1, the purified soluble protein was provided to Imgenex India, who used four mice to generate polyclonal antibodies by a 77 day protocol. The titres of all the anti-sera were tested using ELISA by the company. The mouse polyclonal LdHAT1 antibodies along with their corresponding pre-immune anti-sera were further characterized by western blot analysis using two different amounts of crude *Leishmania* promastigote extract (10 and 20 μg) and 10 ng of purified antigen.

Cloning and over expression of Ldhistone H4

The *L. donovani* genome sequence was used for designing the primers for amplification of Ldhistone H4 and the target was amplified by PCR using the genomic DNA of *L. donovani* promastigotes as template. The PCR product and the plasmid vector (pET21b) were digested with appropriate restriction enzymes and ligated with T4 DNA ligase. The ligation product was used to transform XL1-Blue *E. coli* electro-competent cells. The cloned plasmid was then inserted into BL21DE3 *E. coli* chemical competent cells and a single isolated colony was selected for expression of protein with 1 mM IPTG.

Purification of Ldhistone H4

***Preparation of inclusion body of Ldhistone H4*:** The IPTG induced Ldhistone H4 protein from *E. coli* BL21-DE3 cell was purified according to the protocol developed by Karolin Lugar **[190]**. Briefly, the cell pellet (from 250 mL culture) were first suspended in 6 mL of 50 mM Tris-HCl, pH 8.0 containing 100 mM NaCl, 1 mM EDTA, 5 mM β-ME, 1 mM PMSF and protease inhibitor cocktail (wash buffer) by vortexing and then the suspension was subjected to sonication for 10 times with 30 seconds pulse followed by centrifugation. The resulting pellet was subsequently extracted with 6 mL of TW buffer (Wash Buffer containing 1.0% T-X100) twice to obtain the inclusion bodies of the histone proteins as the insoluble portion.

***Solubilization of Inclusion body of Ldhistone H4*:** The inclusion body of the protein was soaked in 50 µL of DMSO by mincing with spatula followed by solubilization in 1 mL of Unfolding Buffer (20 mM Tris-HCl, pH 8.0 containing 7.0 M Guanidium-HCl, 10 mM DTT) by stirring at room temperature. After complete solubilization of the inclusion bodies the mixture was centrifuged at high speed to collect the supernatant as crude denatured proteins mixture.

***Fractionation and isolation of histones from crude protein mixture by Sephacryl S-200 Column chromatography*:** The Sephacryl S-200 column was equilibrated with 100 mL of Buffer SAU-1000 (20 mM NaOAc pH 5.2, 7 M Urea, 1.0 M NaCl, 1 mM EDTA and 5 mM β-ME) at a flow rate 0.5 mL per minute. 1 mL of crude denatured protein mixture was applied to the equilibrated column and fractions were collected at a flow rate of 0.5 mL per minute. The collected fractions were analyzed by 15% SDS-PAGE followed by Coomassie blue staining and the desired fractions were pooled. The pooled fractions were dialyzed against double distilled H_2O containing 2 mM β-ME at 4^0C with four changes. The dialyzed sample was lyophilized and stored at -20^0C in suitable aliquots.

***Refolding of the unfolded histones*:** The lyophilized unfolded histone protein was resuspended again in 1.5 mL of Unfolding Buffer and subsequently dialyzed against Refolding Buffer (10 mM Tris-HCl, pH 7.5 containing 2.0 M NaCl, 5 mM β-ME and 1 mM EDTA) with three changes. The refolded Ldhistone H4 protein was analyzed by Bradford assay and SDS-PAGE.

Purification of Nucleosomes from *L. donovani* promastigotes

The nucleosomes of *L. donovani* promastigotes were isolated as previously described **[168]** with some modifications. Briefly, 2 x 10^9 Ag83 cells were lysed in 2.5 mL of lysis buffer containing 8% polyvinylpyrrolidone (PVP), 0.05% Triton-X100, 1 mM DTT, 1 mM PMSF and protease inhibitor cocktail (PIC) by homogenizer followed by centrifugation at 16,000x g for 15 minutes. The nuclear pellet was immediately washed with low salt buffer (10 mM Tris-HCl pH 8.0, 75 mM NaCl, 0.05% Triton-X100, 1 mM DTT, 1 mM PMSF and PIC). The nuclear pellet was resuspended in high salt buffer containing 10 mM Tris-HCl pH 8.0, 400 mM NaCl, 0.05% Triton-X100, 1 mM DTT, 1 mM PMSF and PIC by homogenizer followed by sonication 6 times with 30 seconds

pulse. The nuclear supernatant was separated from the pellet by centrifugation at 16,000 xg for 30 minutes and it was dialyzed against the buffer containing 10 mM HEPES pH 7.5, 50 mM NaCl, 1 mM DTT and 1 mM PMSF. The dialyzed supernatant was treated with micrococcal nuclease and characterized by 15% Triton-Acetic acid-Urea PAGE (TAU-PAGE) **[191]**.

Generation of antibodies against acetylated peptides derived from N-terminus of *L. donovani* histone H4

In order to raise the antibodies, three peptides derived from N-terminus of *L. donovani* histone H4 containing specific acetylated lysine (LdH4K4Ac: AKGK_{Ac}RSADAC; LdH4K10Ac: SADAK_{Ac}GSQKC; LdH4K14Ac: KGSQK_{Ac}RQKKC) were synthesized and conjugated to carrier protein keyhole limpet hemocyanin (KLH). For each peptide, two rabbits were immunized and the progress of immunization was monitored by ELISA. Specific antibodies were purified from the anti-sera having higher titre values through affinity column chromatography in a two-step process – first over a column containing a control non-acetylated peptide (AKGKRSADAKGSQKRQKKC) followed by a column containing the respective acetylated peptide. The specificities of the purified antibodies were checked by ELISA assay. The entire process was carried out by IMGENEX India, Bhubaneswar, India on contract basis. The specificities of the antibodies were further verified by dot blot analysis in our laboratory.

Table 5.1: Table for acetylated peptides of L. donovani histone H4

Name of Peptides	Sequences
N-terminal Ldhistone H4	AKGKRSADAKGSQKRQKKC
LdH4K4Ac	AKGK_{Ac}RSADAC
LdH4K10Ac	SADAK_{Ac}GSQKC
LdH4K14Ac	KGSQK_{Ac}RQKKC

Characterization of antibodies against acetylated peptides of Ldhistone H4 by dot blot analysis

Specificities of the three antibodies against acetylated lysine containing peptides derived from Ldhistone H4 were further checked by dot blot analysis. Briefly, the 100 ng each of the three acetylated peptides (LdH4K4Ac, LdH4K10Ac and LdH4K14Ac) along with a control peptide were spotted separately on PVDF membrane (GE Healthcare Lifesciences) after treated with methanol, H_2O and transfer buffer (0.3% Tris, 1.44% Glycine and 20% Methanol) followed by air-drying in 37^0C incubator. The pieces of membrane containing the spots were again treated with methanol, H_2O and transfer buffer followed by blocking with 3% BSA in phosphate-buffered saline containing 0.1% Triton X-100. Finally, the blots were treated separately with the three antibodies (anti-LdH4K4Ac, anti-LdH4K10Ac and anti-LdH4K14Ac) for immunoblot analysis.

Histone Acetyl Transferase (HAT) Assay

With Ldhistone H4 full length protein: HAT assay was performed in a volume of 20 µl containing the Assay buffer (50 mM Tris-HCl, pH8.0 containing 1 mM EDTA, 0.2% Triton-X100, 5% glycerol, 1 mM DTT, 1 mM PMSF and protease inhibitors cocktail), 10 mM Na-butyrate and 5 µl C^{14}-Acetyl Coenzyme A (50 nCi, 1 nmole) at 30^0C. 0.1 µg of bacterially purified Ldhistone H4 full length protein was used as substrate and 2 µg of LdHAT1 as enzyme in assays with different time intervals. The reactions were stopped by adding 5 µl of Laemmli's SDS-PAGE sample buffer and heated in a boiling water bath for 5 minutes. The boiled samples were analyzed in a 15% SDS-PAGE followed by western blotting and finally by immunodetection with anti-acetyl lysine polyclonal antibody. Anti-his tag monoclonal antibody was used for detecting the presence of equal amounts of the substrates in all reaction.

***With N-terminal L. donovani histone H4 short peptide*:** The same experiment was also performed with 2 µg each of three peptides derived from Ldhistone H4 N-terminal tail (**Table 5.1**) instead of full length protein. The reaction was carried out at fixed temperature, 30^0C for 1 hour. The reactions were stopped by adding 5 µl of Laemmli's SDS-PAGE sample buffer and boiled for 5 minutes. The samples were resolved by 18% Tris-Tricine SDS-PAGE for better resolution of smaller peptides **[192]**. Briefly, 18%

polyacrylamide (18%T, 5%C) in 0.75 M Tris-HCl, pH 8.45 containing 30% ethylene glycol and 0.1% SDS was used as resolving gel with 0.1 M Tris containing 0.1 M Tricine and 0.1% SDS as electrophoresis buffer. The resolved protein pattern was then transferred to PVDF membrane for 40 minutes. The blots were detected with three different anti-acetyl lysine antibodies raised against three different acetylated lysine containing peptides as well as their corresponding control antibodies. Coomassie blue staining of the gel were used for checking the presence of equal amount of substrate peptide in different reactions.

***With L. donovani nucleosome*:** The same experiment was also performed with 5 µg of *L. donovani* nucleosome (**Table 5**) instead of recombinant protein. The reaction was carried out at 30^0C for 1 hour. The reactions were stopped by adding 5 µl of Laemmli's SDS-PAGE sample buffer and boiled for 5 minutes. The boiled samples were analyzed in a 15% SDS-PAGE followed by western blotting and finally by immunodetection with anti-histone H4K4Ac, H4K10Ac, H4K14Ac and H4K10control polyclonal antibodies.

HAT assay with phosphorylated LdHAT1

The Ldhistone H4 small peptide HAT assay was carried out with normal as well as phosphorylated LdHAT1 as enzymes. The reaction products were analyzed using the similar procedure as described above with rabbit-anti-H4K10 acetylated polyclonal antibody and appropriate control antibody. The mouse-anti-phospho-T/P monoclonal antibody (Cell Signaling) was used for detection of phosphorylation at LdHAT1 protein by LdCyc1. The same experiment was also carried out with wild type and mutated LdHAT1 and their phosphorylated versions and analyzed by immunoblotting with appropriate antibodies.

Cell cycle analysis of LdHAT1

To synchronize *L. donovani* promastigotes, the growth of exponentially dividing cells was blocked with 10 mM Hydroxyurea (HU) for 36 hours followed by releasing the arrest by re-suspending the cells in equal volume of fresh growth medium and the cells were collected at different intervals (0, 2, 5, 8 and 10 hours). Cells from different time intervals were lysed in 50 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, 0.1% Triton-X100, 1 mM EDTA, 1 mM PMSF and protease inhibitors and the soluble extracts were analyzed by immunoblotting with antibodies against LdHAT1

(mouse polyclonal) and actin. Synchronization of cells was checked by both flow-cytometer analysis and fluorescence microscopy analyses which were described in details below.

Flow-Cytometer Analysis of *L. donovani* cells: Approximate $5x10^6$ exponentially growing *L. donovani* promastigotes were washed once with PBS and finally collected by centrifuging at 3000 rpm for 5 minutes. The cells were resuspended in ice-cold 70% ethanol with mild vortexing and kept at 4^0C for overnight. Next day the cells were centrifuged down at 5000 rpm for 5 minutes and ethanol was removed. The cells were washed twice with cold PBS. After that the cells were resuspended in PBS containing 200 µg/mL RNase A and incubated at 37^0C for 2 hours. Finally, the cells were analyzed in FACS-Caliber (BD-Bioscience) after treating with 40 µg/mL propidium iodide (PI) for 10 minutes in dark. To confirm the synchronicity of *L. donovani* promastigotes, similar treatments were done with cells from different time intervals.

Fluorescence Microscopy: For fluorescence microscopy, 1 x 10^5 *L. donovani* promastigote cells from exponentially growing phase were washed once with 500 µl PBS. The cells were fixed in 500 µl of freshly prepared 4% para-formaldehyde (w/v in PBS) for 20 minutes at room temperature and followed by washing with PBS twice. The cells were then resuspended in 500 µl PBS and spotted on a cover-slip (Corning) and dried in air for 30 minutes at 37^0C. The spotted cells were rehydrated in PBS and the cover slip was inverted on a drop of DAPI mounting solution on a glass slide and the stained cells were analyzed with a Zeiss Axio-observer Z1 inverted microscope. To confirm the synchronicity of *L. donovani* promastigotes, the same experiment was done with cells from different time intervals.

Results

LdHAT1 is a MYST family Histone Acetyl Transferase

One of the identified substrates of the S-phase cell cycle kinase LdCyc1-CRK3 from *L. donovani* was shown to contain a MYST (human Moz, Yeast Ybf2 and Sas2, and human TIP60) domain of histone acetyl transferases **[179]**, though further characterization of activity of the protein and its regulation by phosphorylation were not carried out. The HAT protein from *L. donovani* is 97% identical to LmHAT1, which was grouped with the HAT1 from *T. brucei* and *T. cruzi* in a phylogenetic analysis **[169]**. Therefore, we designate the 525 amino acid containing protein as LdHAT1, which is also highly homologous to other MYST family HATs from diverse organisms (**Figure 5.2** and **5.3**). Maximum homology is present along the C-terminal canonical MYST domain (amino acid 254-456 of LdHAT1), which contains the characteristic acetyl CoA binding R/Qx_2GxG/A motif (A-motif). Like other family members, on the N-terminus of the MYST domain the conserved C_2HC ($Cx_2Cx_{12}Hx_{3\text{-}5}C$) Zn-finger motif is also present in LdHAT1. As previously described **[179]**, the cyclin binding R/KxL-type Cy-motif **[52]** is located within the MYST domain in LdHAT1, though such a typical motif is absent in HsTIP60, DmMof and HsHbo1. However, a canonical Cdk target phosphorylation site (TPEK) is well-conserved within the MYST domain of LdHAT1 and in the other MYST family members

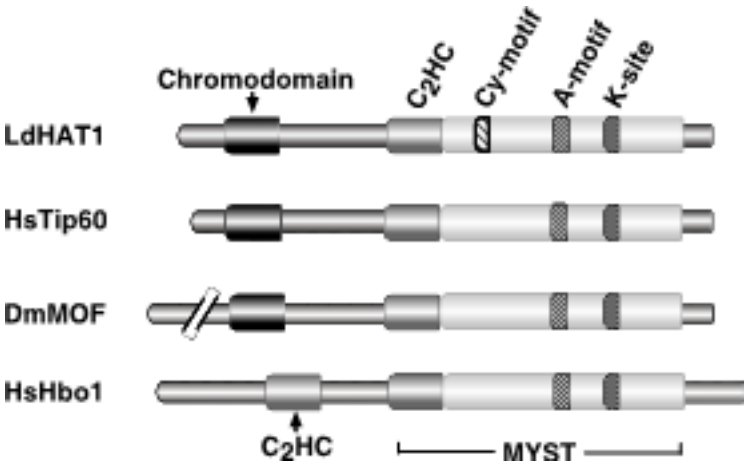


Figure 5.2: Homology of LdHAT1 with other MYST family HATs. Sequence homology was determined by Clustal-W. The accession numbers of the proteins used are as follows: LdHAT1, HM120719; HsTip60, BC064912; DmMOF, EF630377; HsHbo1, NM_007067. Ld, *Leishmania donovani*; Hs, Homo sapiens; Dm, *Drosophila melanogaster*.

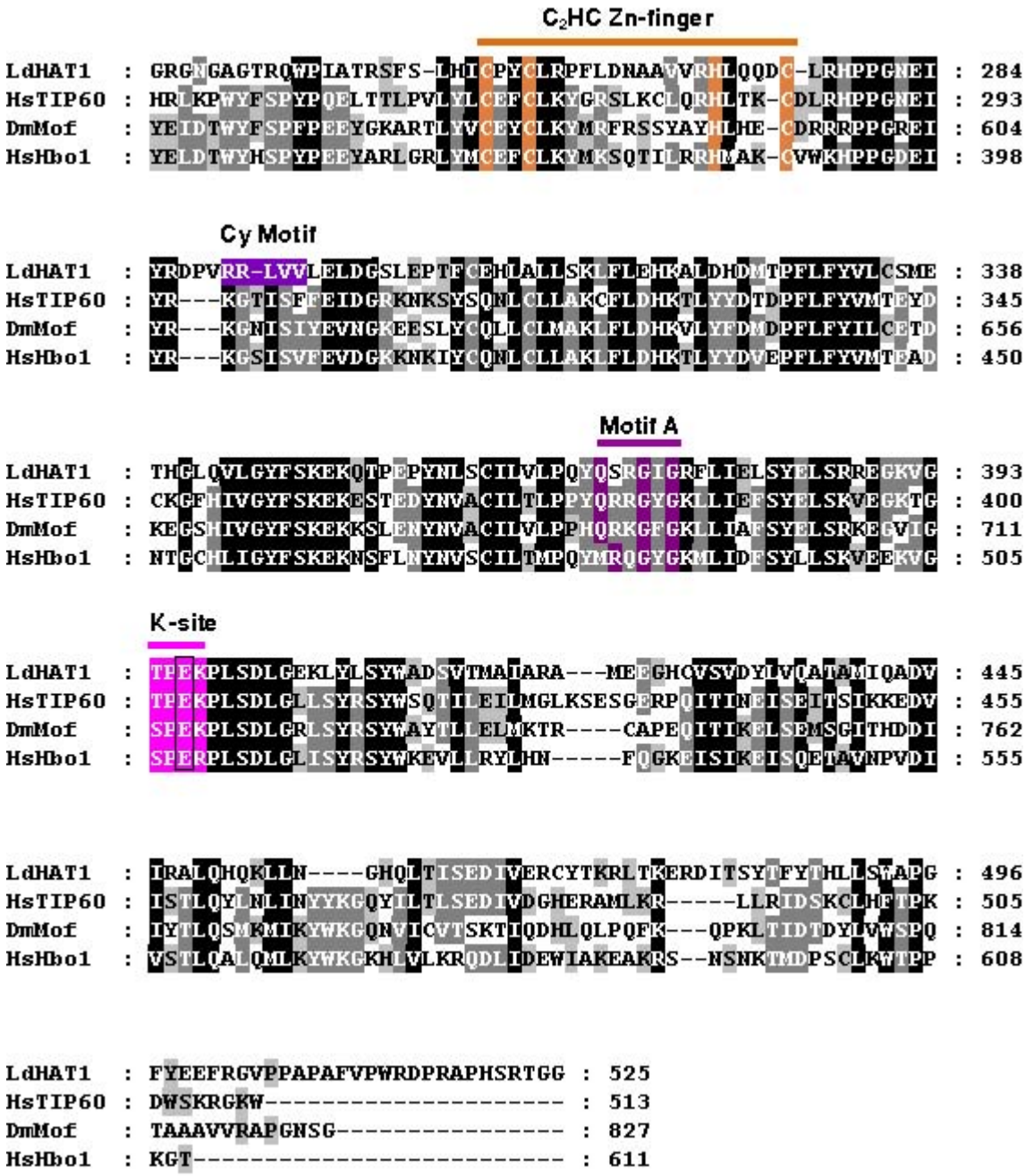


Figure 5.3: Alignment of MYST domains of *Lesihmania donovani* HAT1 (LdHAT1, HM120719), human TIP60 (HsTIP60, BC064912), Drosophilla Mof (DmMof, EF630377) and human Hbo1 (HsHbo1, NM_007067). The identified motifs were marked appropriately. Cy-Motif, RXL type cyclin binding motif; Motif A, Acetyl CoA binding motif; K site, Cdk phosphorylation site. Catalytically important Glu residue within the K-site is boxed.

(**Figure 5.2** and **5.3**). In addition to the canonical Cdk phosphorylation site, five minimal sites (T/S-P) are also present in the molecule in a scattered manner. Interestingly, catalytically critical Glu residue, corresponding to Glu338 in the prototype yeast Esa1 **[193]**, is located within the canonical Cdk target site (TPEK), implicating an interesting

regulatory mechanism if the Thr residue is actually phosphorylated by cell cycle kinases. Moreover, similar to HsTIP60 and DmMof, a Chromatin Organization Modifier (chromo) domain is located towards the N-terminus of LdHAT1. Chromodomain of heterochromatin protein HP1 was shown to interact with methylated lysine-9 residue of histone H3 to recruit the regulator at appropriate location **[194, 195]**. Chromodomain can also function as RNA interacting module to target the regulators to the specific chromosome site as was observed in case of DmMof **[196]**. The presence of chromodomain in LdHAT1, therefore, raises its possible role in crosstalk between methylation and acetylation of histones and/or RNA mediated chromatin remodeling in the parasites.

LdHAT1 expression was constant during Cell Cycle

Since phosphorylation of LdHAT1 by S-phase Cdk suggested the possibility of its involvement in cell cycle related periodic activities, its expression profile was analyzed during cell cycle progression of *L. donovani* promastigotes. The exponentially growing promastigotes were synchronized by hydroxyurea treatment and the synchronicity of the culture was tested by flow cytometric analysis and scrutiny of morphology of the cells from different time intervals. Uniform changes in morphology of the cell population with the progress of cell cycle along with corroborating flow cytometer analysis profiles ascertained the synchronicity of the promastigote culture (**Figure 5.4**). On the other hand, polyclonal anti-sera against the purified LdHAT1 were raised in mice and one of them was shown to detect a specific band of expected size in immunoblot analysis with the extract of *L. donovani* promastigotes (**Figure 5.5A**). The same anti-serum was used subsequently to analyze extracts from the synchronized cells. Analysis of protein extracts from such synchronously growing cells showed the presence of LdHAT1 protein at equal amounts in different cell cycle phases of *L. donovani* promastigotes (**Figure 5.5B**). Since the level of LdHAT1 found to be invariable during cell cycle, it would be interesting to study the effect of phosphorylation by the S-phase kinase on its activity.

Preparation of two mutants of LdHAT1

In order to further confirm the identity and contribution of Cy-motif for the activities of LdHAT1 and Thr residue in the TPEK as target residue of phosphorylation both the sites were mutated by site directed mutagenesis. The mutations at the putative Cy-motif

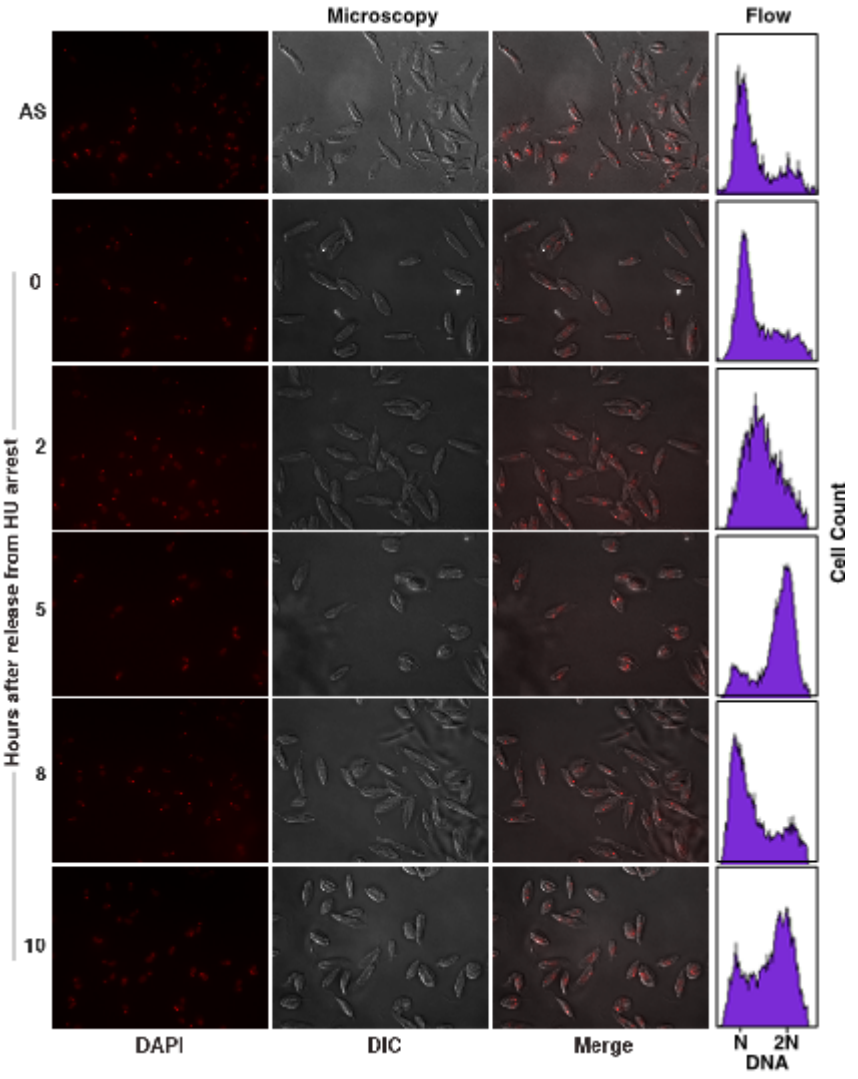


Figure 5.4: Synchronization of *L. donovani* promastigotes. Exponentially growing *L. donovani* cells were arrested at early S-phase with hydroxyurea (HU) and collected at different time points as indicated. The synchronization of the culture was ascertained by Flow cytometer (Flow) and DAPI stained fluorescence and DIC images (Microscopy) of the cells from each time points. DAPI stained nuclei and kinetoplasts were given a red pseudo-colour for better contrast in the representation. AS, asynchronous cell population; Merge, merged pictures of DAPI stained and DIC images.

(^{290}RRLVV→RDDVV, LdHAT1ΔCy) and at the putative cdk phosphorylation site (^{394}TPEK→APEK, LdHAT1-T394A) of LdHAT1 created two new restriction sites in the plasmids, Aat II and BssH II respectively. Therefore, the mutations in LdHAT1 were confirmed by restriction digestions. LdHAT1ΔCy plasmid gave two fragments of expected sizes 2.1 and 4.9 kbp upon double digestion with Aat II and EcoRV as shown in **Figure 5.6A**, lane 1, whereas the wild type plasmid gave a desired 7.0 kbp band (lane 2). Expectedly, the mobility of the undigested plasmids was similar (lanes 3 and 4). On the

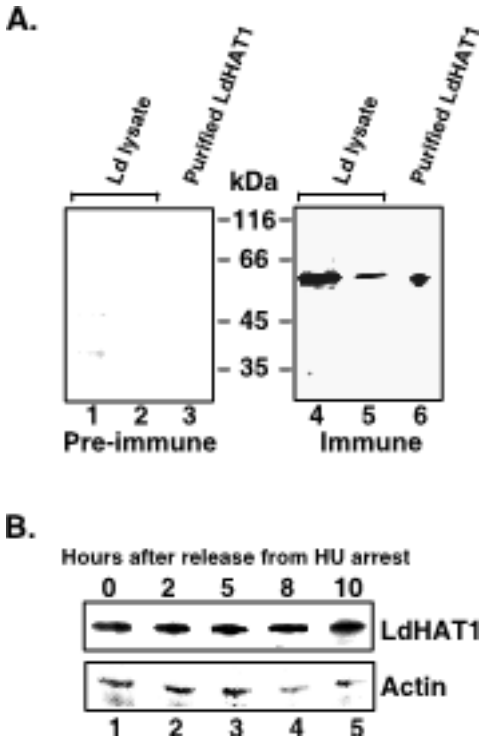


Figure 5.5: A. Characterization of anti-serum against LdHAT1. Anti-sera against the purified LdHAT1 were raised in mice and checked for specificity by immunoblot analysis using different amounts of crude *Leishmania* promastigote extract (lanes 1-2 and 4-5) and purified antigen (lanes 3 and 6). Anti-serum from one of the mice gave best result (represented in the figure above) and used for subsequent experiments. **B. Cell cycle expression of LdHAT1 protein.** Samples from the indicated time points after release from hydroxyurea arrest were analyzed by anti-LdHAT1 immunoblotting. β-actin was used as loading control. AS, asynchronously growing cells.

other hand, LdHAT1-T394A mutant plasmid produced two bands at 2.5 and 4.5 kbp upon single digestion with BssH II as shown in **Figure 5.6B**, lanes 1 and 3, and the wild type plasmid gave one band at 7.0 kbp (lanes 2 and 4). As before, the undigested plasmids moved similar distances (lanes 5 and 6). These observations confirmed that both mutations occurred at the desired sites of LdHAT1 coding sequence.

Purification of two mutants of LdHAT1

After IPTG induction, the mutant LdHAT1 proteins were purified over Ni-NTA agarose column following the same procedure used for wild type LdHAT1 and checked by Coomassie blue as shown in **Figure 5.7A** and **B**. The purity of all the three proteins that

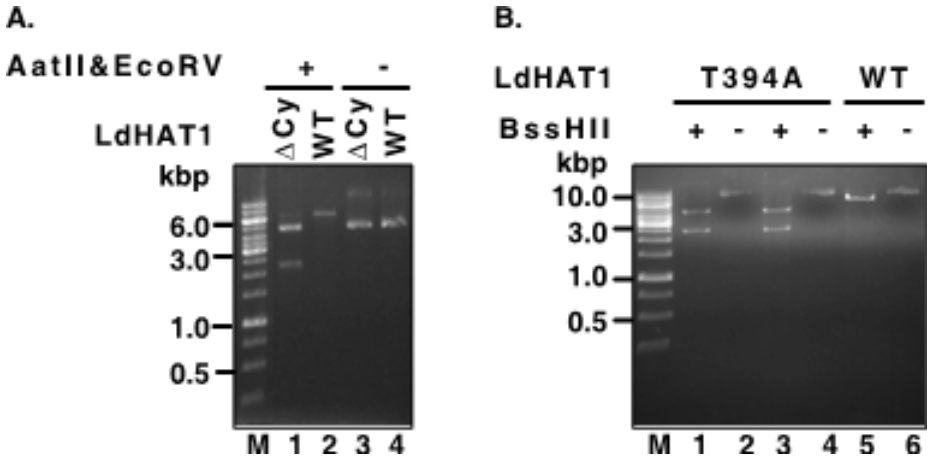


Figure 5.6: Confirmation of LdHAT1 mutantions by restriction enzyme digestion. **A.** The purified cloned plasmids, LdHAT1-ΔCy and LdHAT1-WT were digested with AatII and EcoRV restriction enzymes (lanes 1 and 2). Lanes 3 and 4 indicate two untreated plasmids respectively. **B**. Two purified cloned plasmids of LdHAT1-T394A and one wild type plasmid were digested with BssHII (lanes 1, 3 and 5). The respective undigested plasmids were analyzed in lanes 2, 4 and 6.

were used in the subsequent experiments was found to be high as there was no detectable contaminating band in Coomassie blue stained gel (**Figure 5.7C).**

LdHAT1 directly interacts with LdCyc1 through its Cy-motif

LdHAT1 was shown previously to interact with *L. donovani* S-phase cyclin LdCyc1 in a R/KxL-like Cy motif dependent manner by peptide competition assay **[179]**. To further confirm the contribution of Cy-motif in the interaction, the putative Cy-motif of LdHAT1 was altered (^{290}RRLVV→RDDVV, LdHAT1ΔCy) and the mutated protein was used in the interaction assay. As shown in **Figure 5.8A**, the wild type protein was found to interact with GST-LdCyc1, whereas the interaction with LdHAT1ΔCy was almost completely abolished, proving the involvement of Cy-motif during direct interaction between the proteins. The observation also confirmed the identity of an active Cy-motif in the molecule. The mutation at the putative Cdk phosphorylation site (^{394}TPEK→APEK, LdHAT1-T394A) of the protein did not affect the interaction (**Figure 5.8A**), confirming further the specific involvement of Cy-motif in the binding.

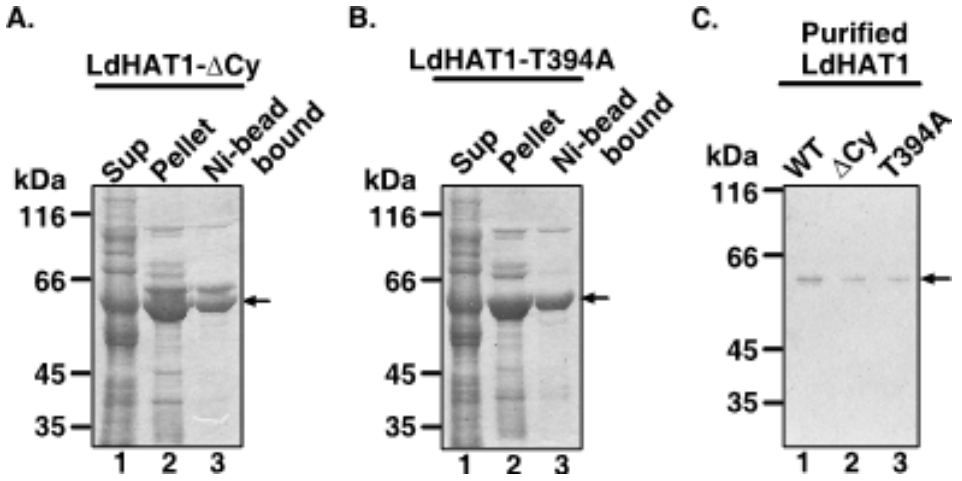


Figure 5.7: Purification of bacterially over-expressed 6xHis tagged LdHAT1 mutant proteins. Purifications of ^{6}His-LdHAT1-ΔCy (A) and ^{6}His-LdHAT1-T394A (B) over Ni-NTA agarose. Lanes 1, Soluble extract; lanes 2, insoluble pellet; lanes 3, Ni-bead bound proteins. Coomassie blue stained gels are shown. C. Coomassie blue stained gel of the purified preparations of the indicated proteins**.**

Cy-motif of LdHAT1 is required for its phosphorylation by S-phase cell division kinase

LdHAT1 was demonstrated to be phosphorylated *in vitro* by LdCyc1-CRK3 complex (**Figure 5.8B**) **[179]**. As the substrate docking on the cyclin moiety was shown to be important for phosphorylation, in order to investigate the effect of Cy-motif of LdHAT1 on its phosphorylation, LdHAT1ΔCy was used as substrate in a kinase assay of LdCyc1-CRK3 complex. As observed, LdHAT1ΔCy was not efficiently phosphorylated by the kinase complex compared to the wild type protein (**Figure 5.8C**, lanes 4 & 5). Since the mutation in Cy-motif of LdHAT1 was shown to disrupt its interaction with LdCyc1 (**Figure 5.8A**), the inhibition of the phosphorylation established the requirement of its docking through the Cy-motif on MRAIL-motif on LdCyc1 **[157]** for the phosphorylation on the target serine/threonine residue. LdHAT1 was also shown to contain a putative Cdk phosphorylation site on its C-terminal end. In order to confirm whether Thr-394 in the motif TPEK was phosphorylated by the kinase complex, the threonine residue was changed to alanine and the mutant LdHAT1-T394A was used as substrate. As shown in **Figure 5.8C**, the phosphorylation was completely abolished due to the mutation (lane 6) suggesting that the S-phase kinase LdCyc1-CRK3 targets Thr-394 for phosphorylation. It is interesting to note that Thr-394 is located very close to conserved catalytically critical Glu residue raising the possibility of regulation of HAT activity due to the incorporation

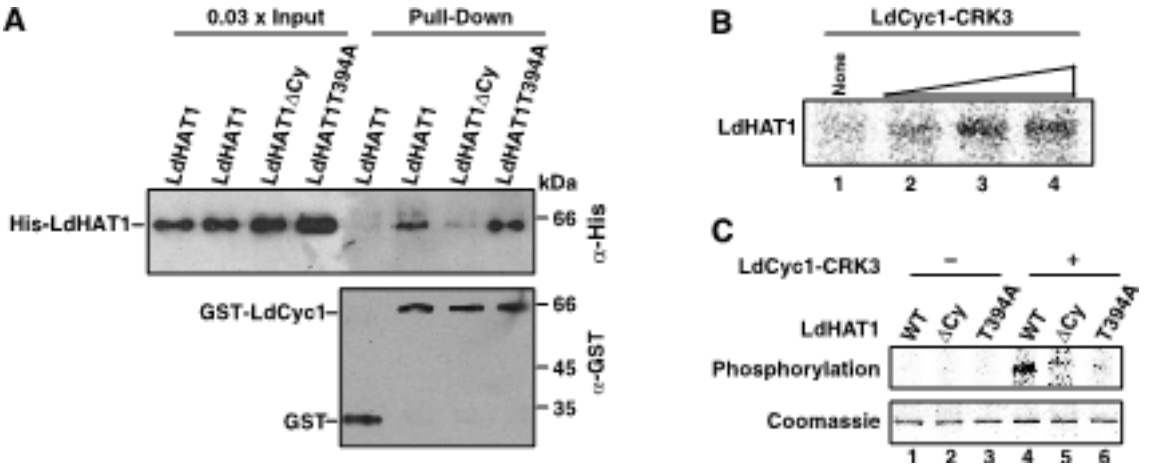


Figure 5.8: A. *In vitro* interaction between LdCyc1 and LdHAT1 through Cy-motif. Anti-His tag and anti-GST tag immunoblotting were carried out to show the presence of ^{6}His-tagged LdHAT1 protein or its mutants and GST or GST-LdCyc1 in the pulled-down complexes. Input, aliquots of supernatants were immunoblotted with anti-His tag antibody to demonstrate the presence of equal amounts of LdHAT1 or mutant proteins in all reaction tubes. **B. Phosphorylation of LdHAT1 by LdCyc1-CRK3 complex.** Phosphorylation of LdHAT1 by increasing amounts (0.1, 0.2 and 0.3 µg) of the kinase complex was determined by phosphorimager scanning of the SDS-PAGE analyzed reaction products. **C. Cy motif dependent phosphorylation of LdHAT1 by LdCyc1-CRK3 complex.** Phosphorylation by LdCyc1-CRK3 complex (0.2 µg) was carried out using wild type and mutated LdHAT1 as substrates as indicated.

of a phosphate group. Therefore, it is important to study the effect on the activity of LdHAT1 by phosphorylation of the Thr residue by the cell kinase.

Purification of *L. donovani* histone H4 from E. coli

The IPTG induced ^{6}His-tagged *L. donovani* histone H4 in *E. coli* cell (**Figure 5.9A**) was isolated as inclusion bodies and checked by Coomassie blue. Finally, the solubilized protein from inclusion bodies was purified by Sephacryl S-200 column chromatography (GE Healthcare Lifesciences) and checked by SDS-PAGE followed by coomassie blue staining. The purified protein was estimated by Bradford assay. The desired fractions were pulled together and confirmed by SDS-PAGE analysis (**Figure 5.9B).**

LdHAT1 acetylates *L. donovani* histone H4 at K10

It was previously implicated that HAT1 from *T. brucei* could acetylate Histone H4 from the parasite **[169]**. Therefore, in order to characterize the histone acetylation activity of

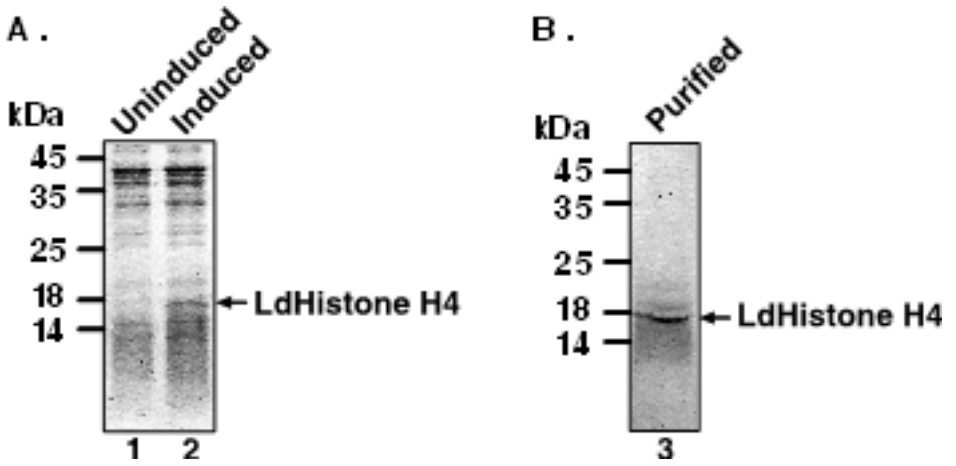


Figure 5.9: Purification of LdHistone H4 full length protein. A. The cloned LdHistone H4 was over-expressed in *E. coli* (lane 2) in presence of IPTG. Lane 1 indicates uninduced extract. **B.** Purified His tagged LdHistone H4 (lane 3). Arrows indicate the desired protein.

LdHAT1, *in vitro* assays were carried out using purified *L. donovani* histone H4 full length protein expressed in bacteria. The incorporation of acetyl group in lysine residue of histone was detected by anti-acetyl lysine antibody (Abcam). Acetylation activity gradually increases with time as shown in **Figure 5.10A** lanes 2-4. To further characterize the acetylation activity of LdHAT1 the same assays were performed with a peptide substrate derived from the N-terminus of *L. donovani* histone H4. To identify the lysine residue that was specifically acetylated by LdHAT1, three antibodies were raised against *L. donovani* histone H4 derived peptides acetylated on K4, K10 or K14 residue, respectively. Specificities of the antibodies were ensured by dot blot analysis which showed no cross-reactivity (**Figure 5.10B**). Once the specificities were confirmed, the antibodies were used to identify the lysine residue on the peptide derived from N-terminus of *L. donovani* histone H4 acetylated by LdHAT1. As shown in **Figure 5.10C**, the peptide acetylated by LdHAT1 could be detected only by anti-H4K10Ac antibody, but not with other two antibodies, suggesting that the acetyltransferase from *L. donovani* specifically acetylates H4K10 residue. In order to know the activity of LdHAT1 on endogenous histones, the HAT assay was carried out with isolated *L. donovani* nucleosomes. The nucleosome was purified and analyzed by TAU-PAGE and the band pattern was compared with that of previously published *T. brucei* nucleosome (**Figure 5.11A**) **[197]**. The *L. donovani* nucleosome preparation thus purified was used for HAT assay and specific increase in band intensity was observed specifically only with anti-histone H4K10Ac but not with H4K4Ac, H4K14Ac and H4K10control antibodies **(Figure 5.11B)**, indicating

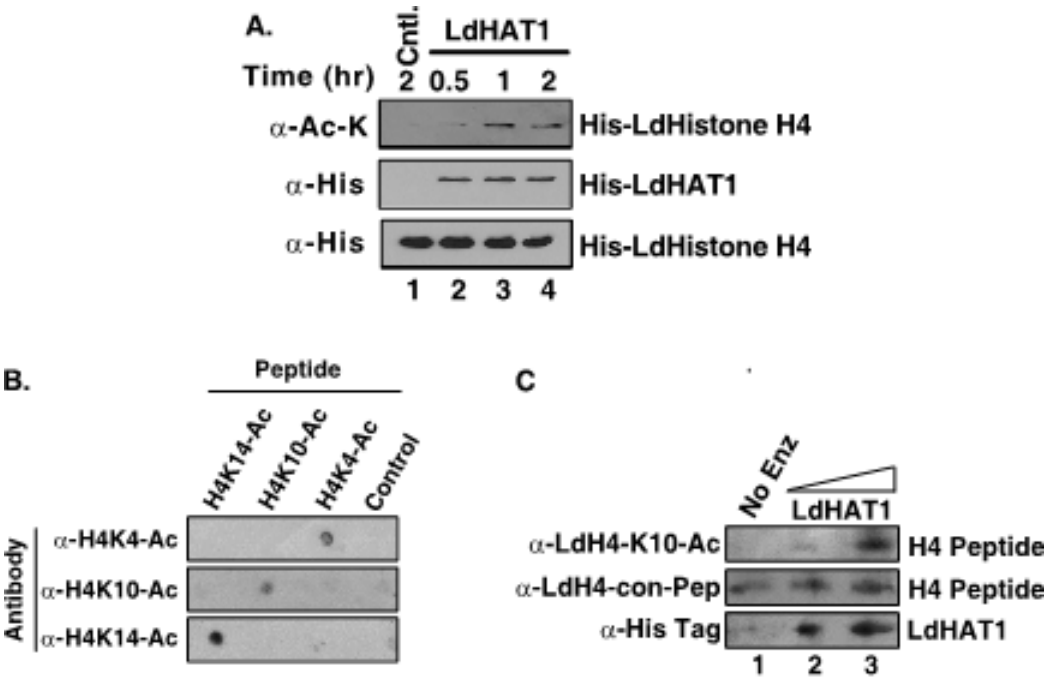


Figure 5.10: A. *L. donovani* histone H4 acetylation by LdHAT1. Purified *L. donovani* histone H4 full length protein was used as substrate in acetylation assay of LdHAT1 with increasing duration (lanes 2 to 4). Lane 1, control reaction without any enzyme. An anti-acetylated lysine antibody was used to detect the acetylation of LdHistone and anti-His tag antibody was used to show the presence of equal amounts of substrates and enzymes. **B. Specificities of antibodies against acetylated lysine containing peptide.** 100 ng of each of the indicated peptides were spotted on membrane and immunoblotting was carried out with the denoted antibodies against the peptides containing the specified acetylated lysine. **C. *L. donovani* histone H4K10 acetylation by LdHAT1.** Histone H4 N-terminus peptide was used as substrate and antibody (α-LdH4K10-Ac) against Acetylated H4K10 containing peptide was used to identify the specific product. The antibody against the non-acetylated peptide (α-LdH4-con-pep) was used to show the presence of equal amounts of substrate peptide in each reaction.

that LdHAT1 acetylates *L. donovani* histone H4 at K10 residue even in nucleosome fraction.

Phosphorylation of LdHAT1 by LdCyc1-CRK3 down-regulates its acetyl transferase activity

Since LdHAT1 was shown to be phosphorylated by S-phase kinase LdCyc1-CRK3, it would be interesting to investigate any possible effect of such phosphorylation on its histone acetylation activity. For this purpose, LdHAT1 protein was phosphorylated by S-

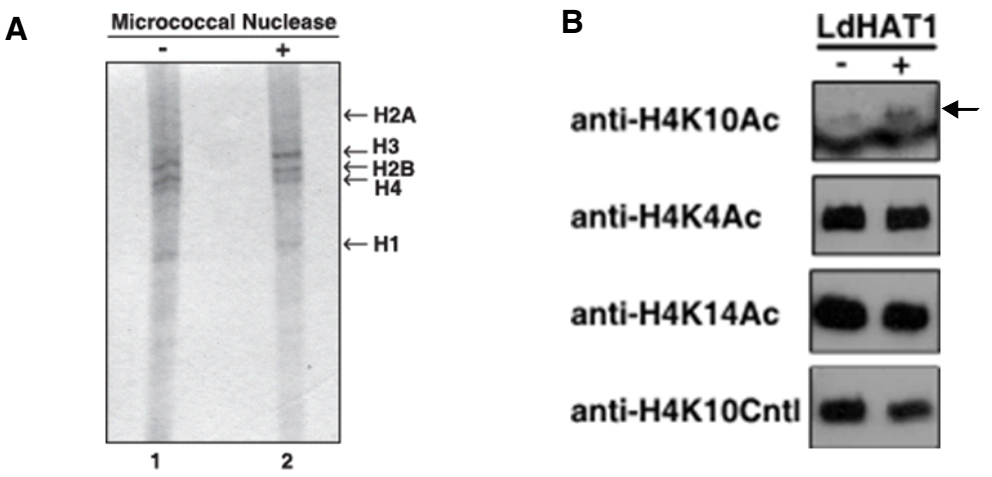


Figure 5.11: *A*cetylation of *L. donovani* nucleosomes by LdHAT1. Antibodies against N-terminal histone H4 acetylated peptides (anti-histone H4K10Ac, H4K4Ac, H4K14Ac) were used to detect acetylation of *L. donovani* nucleosome by LdHAT1. The antibody against the non-acetylated peptide (anti-LdH4K10cntl) was used to show the presence of equal amounts of histone H4 from the purified nucleosomes in each reaction.

phase kinase LdCyc1-CRK3 with non-radioactive ATP followed by removal of unincorporated ATP by Sephadex G-25 spun column (GE Healthcare LifeSciences). The status of phophorylation was confirmed by immunoblotting with anti-phospho-threonine-proline antibody (**Figure 5.12B**). It should be noted that T-P forms the minimal Cdk target phosphorylation motif and part of the canonical target S/T-P-x-R/K. Therefore,

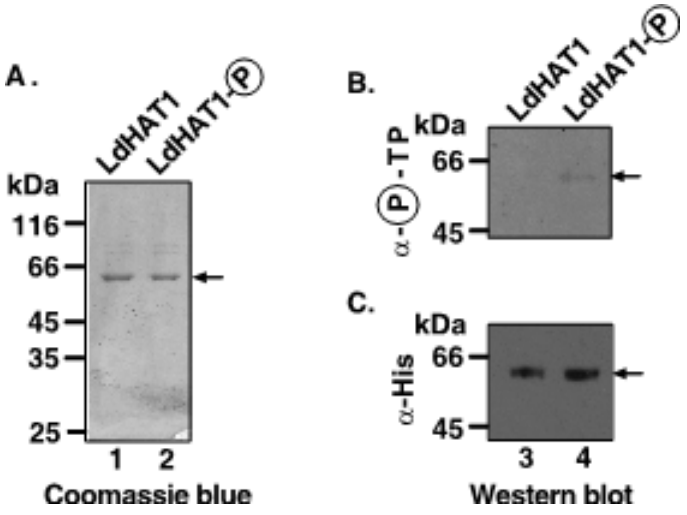


Figure 5.12: Phosphorylation of LdHAT1 by LdCyc1-CRK3 complex. **A.** SDS-PAGE followed by Coomassie blue stain. **B.** Phosphorylated LdHAT1 protein was detected by anti-PTP antibody. **C.** The anti-His tag antibody was used to ascertain equal amounts of LdHAT proteins. Lanes 1 and 3, unphosphorylated LdHAT1; 2 and 4, phosphorylated LdHAT1. Arrows indicate the desired proteins.

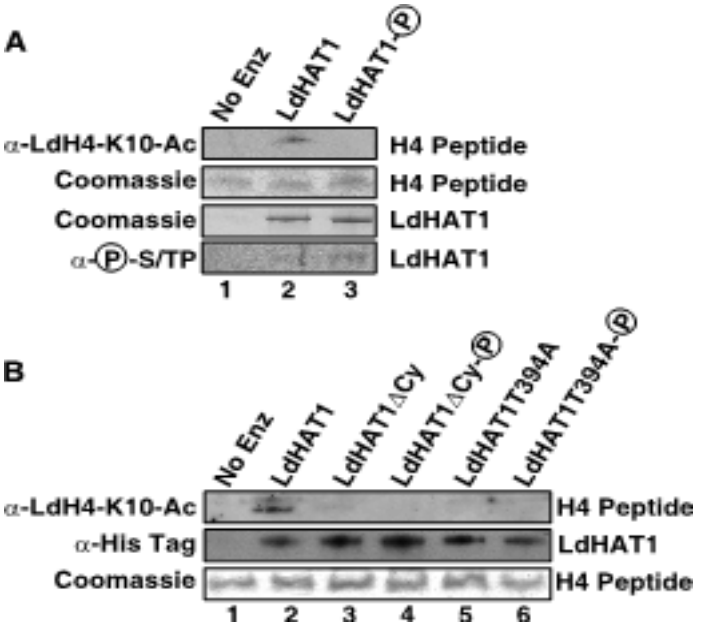


Figure 5.13: A. Inhibition of acetyl transferase activity of LdHAT1 due to phosphorylation by LdCyc1-CRK3 complex. Lanes 1, 2 and 3 indicate no enzyme, wild type LdHAT1 and LdHAT1- Ⓟ respectively. The anti-LdH4K10-Ac antibody was used to detect the acetylation of LdHistone H4 at K10 residue and anti-Ⓟ TP detects the phosphorylation status of LdHAT1. **B. Inhibition of acetyl transferase activity of LdHAT1 due to mutations at Cy-motif and Cdk phosphorylation target site within the MYST domain.** The anti-LdH4K10-Ac antibody was used to detect the acetylation of LdHistone H4 at K10 residue and anti-His tag antibody was used to detect equal amounts of LdHAT1 protein.

detection by anti-phospho-threonine-proline antibody indicated that the protein was phosphorylated by a Cdk. The presence of equal amounts of substrates was confirmed by coomassie blue staining (**Figure 5.12A**) and also by immunoblot analysis with anti-His tag antibody (**Figure 5.12C).** Subsequently, H4K10 acetylation activity of non-phosphorylated LdHAT1 was compared with that of LdHAT1 phosphorylated by LdCyc1-CRK3 obtained as described above. As depicted in **Figure. 5.13A**, the phosphorylated form of LdHAT1 did not show any H4K10 acetylation activity, suggesting the down-regulation of histone H4 acetylation by S-phase cell cycle kinase.

Mutations in MYST domain of LdHAT1 destroys its acetyl transferase activity

It was observed that the phosphorylation of LdHAT1 by LdCyc1-CRK3 inhibited its acetylation activity towards histone H4 at K10 residue. Moreover, its two mutants

LdHAT1ΔCy and LdHAT1-T394A also did not show any acetylation activity (**Figure. 5.13B**) due to likely perturbation of MYST domain in LdHAT1 implicating the contribution of the mutated residues in the enzymatic activity.

Discussion

LdHAT1 is one of the three identified substrates of S-phase kinase LdCyc1-CRK3 of *Leishmania donovani* and it belongs to MYST family of histone acetyl transferases. Studies described in the thesis have established that during cell cycle of *L. donovani*, LdHAT1 protein expresses at a constant manner. It acetylates specifically the lysine-10 of Ldhistone H4 but not the lysine at position 4 or 14. Interestingly, it was observed that the acetylation of Ldhistone H4 K10 inhibited by phosphorylation of LdHAT1 by S-phase kinase LdCyc1-CRK3. Moreover, mutations in LdHAT1 at R/KxL type Cy-motif and Threonine residue at TPEK phosphorylation site shows inhibition of acetylation of Ldhistone H4K10 most likely due to the perturbation of MYST domain. Several previous studies demonstrated the acetylation of K4, K10 and K14 residues of the N-terminal tail of histone H4 from *T. brucei* and *T. cruzi* **[168, 198, 199]**. Moreover, cell cycle dependent post-S-phase enhancement of K4, K10 and K14 acetylation of histone H4 was observed in *T. cruzi* **[200]**. Therefore, the observed inhibition of Ldhistone H4 K10 acetylation by LdHAT1 due to its phosphorylation by S-phase kinase in the present studies could correlate such cell cycle specific periodic acetylation.

Recently, human MYST histone acetylase Hbo1 was shown to play critical role in replication licensing **[134]** and regulation of its activity through phosphorylation by Cyclin B-Cdk1 and Plk 1 was also implicated **[132]**. The activity of another MYST family HAT Tip60, which was found to play essential role in DNA repair and apoptosis, was suggested to be controlled through phosphorylation by Cyclin B-Cdk1 **[189]**. Interestingly, *Trypanosoma brucei* HAT1, which was found to be homologous to Ld14.0140L, was implicated to be essential for proper nuclear DNA replication in the organism **[169]**. In these contexts, it will be interesting to study the effect of inhibition of the HAT activity on the S-phase events.

CONCLUSION

Protein phosphorylation is a major class of regulatory posttranslational modification and cyclin dependent protein kinases (Cdks) play a central role by reversibly phosphorylating different proteins to control cell cycle progression and cell division in eukaryotes **[1]**. Phosphorylation of retinoblastoma protein (pRB) by Cyclin D-Cdk4/6 is the key initial event for cell cycle progression in higher eukaryotes followed by subsequent phosphorylation of several proteins like Cdc6, ORC proteins, MCM by Cyclin E/A-Cdk2 in mammals. On the other hand, dephosphorylation of Cdk2/1 by a family of Cdc25 phosphatases also plays crucial role to maintain regulated progression of cell cycle.

The cell cycle progression in early branching protozoan parasite *Leishmania donovani* in the order of kinetoplastida is also regulated by several Cdks like in other eukaryotes. One such kinase complex, LdCyc1-CRK3, specific to S-phase of cell cycle has been identified in our laboratory previously **[157]**. Since there was dearth of knowledge about its substrates, a screening was carried out in our laboratory resulting in the identification of three substrates of the S-phase cell cycle kinase LdCyc1-CRK3 of *L. donovani*. However, information about their mode of interaction with the kinase complex and regulation of their function due to phosphorylation remained unknown. Therefore, the primary objective of my doctoral work was to further characterize the identified substrates of LdCyc1-CRK3 in order to elucidate the targeting mechanism of the substrates to the kinase complex. Investigation of functional modification of at least one of the substrate proteins due to phosphorylation was also a major aim of my work.

As mentioned, three substrates of S-phase cell cycle kinase LdCyc1-CRK3 from *L. donovani* were identified in a screening of the putative protein sequences available in *Leishmania* genome database based on the presence of cyclin binding motif and Cdk target phosphorylation site in them **[179]**. All the three substrates are expressed in *L. donovani* promastigotes as confirmed by RT-PCR. One of the identified substrates, viz. Ld28.0070L, is unique to the parasite, and therefore, its further characterization would be

interesting as this could lead to elucidation of parasite specific cellular mechanism regulated by a cell cycle kinase. Such study may help to identify new therapeutic targets to combat the parasite infection. The other two proteins, viz., Ld29.1050L and Ld14.0140L, are homologous to Ku70 DNA binding protein and MYST family HAT, respectively. Studies described here established that R/KxL type Cy motif mediated interaction of the substrates with LdCyc1 of the kinase complex are prerequisite for efficient phosphorylation. Therefore, like in other eukaryotes, in these parasites also substrates are loaded through Cy motif mediated interaction with cyclin subunit for efficient phosphorylation by the Cdk subunit. The inference was confirmed by peptide competition assay as well as by site directed mutagenesis of the putative Cy motif in one of the substrates, Ld14.0140L, which we term as LdHAT1. It would be interesting to determine the effect of such phosphorylation on cellular metabolism particularly of the parasites and eukaryotes in general.

LdHAT1 is one of the three identified substrates of S-phase kinase LdCyc1-CRK3 of *Leishmania donovani* which belongs to MYST family of histone acetyl transferases and is highly homologues to human Tip60, Hbo1, yeast Ybf2, Sas2/3 and drosophila Mof, mainly in the region of MYST domain. The protein is expressed at a constant manner during cell cycle progression of *L. donovani* promastigotes, raising the possibility of regulation of its activity by posttranslation modification. Like other two substrates, LdHAT1 also interacts with LdCyc1 in an R/KxL like Cy motif dependent manner for being efficiently phosphorylated by the kinase complex. Further studies through site directed mutagenesis establishes the identity of Cy motif at ^{290}RRL in LdHAT1 which is located within the conserved MYST domain. The threonine at position 394, which is very close to the catalytically important glutamine residue, is targeted by LdCyc1-CRK3 complex for phosphorylation. HAT activity of LdHAT1 was confirmed by acetylation of full length histone H4 from *L. donovani*. Based on acetylation assay using a substrate peptide derived from N-terminal tail of *L. donovani* histone H4, LdHAT1 acetylates lysine residue specifically at position 10 but not at positions 4 or 14. The identity of the target lysine is confirmed by detection of the product of acetylation assay with position specific anti-acetyl-lysine antibody. Since LdHAT1 is phosphorylated by LdCyc1-CRK3, effect of such phosphorylation on its acetylation activity was tested and strikingly the acetylation activity of LdHAT1 is inhibited as a result of its phosphorylation by LdCyc1-CRK3. The inhibition could be due to the disturbance in the catalytic mechanism because of the incorporation of phosphate group into Thr-394 residue that is juxtaposed to catalytically

important Glu-396 residue. Moreover, mutations in LdHAT1 at R/KxL type Cy-motif and Threonine residue at TPEK phosphorylation site shows inhibition of acetyl transferase activity, most likely due to perturbation of MYST domain. It will be interesting to study the effect of inhibition of LdHAT1 activity by the S-phase kinase on cell cycle specific periodic events.

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LIST OF PUBLICATIONS AND CONFERENCES ATTENDED

Publications

1. **Maity, A.K.**, Goswami, A., Saha, P. (2011) Identification of substrates of an S-phase cell cycle kinase from *Lishmania donovani.* **FEBS Letters.** 585, 2635–2639.

2. **Maity, A.K.**, Saha, P. (2012) The histone acetyl transferase LdHAT1 from *Leishmania donovani* is regulated by S-phase cell cycle kinase. **FEMS Microbiology Letters.** 336, 57-63.

Symposium and Conference Attended

1. **"International Conference on Recent Advancement in Physical and Chemical Biology",** Saha institute of Nuclear Physics, Kolkata, March 3-5, 2012**. Title of Poster:** Characterization of substrates of an S-phase cell cycle kinase of *Leishmania donovani* with emphasis on a Histone Acetyl Transferase

2. **"Workshop on Biocalorimetry",** Saha institute of Nuclear Physics, Kolkata, February 4-6, 2009.

3. **"International Training in Global Infectious Diseases (GID), US-India joint training program workshop on Intracellular Pathogens",** ISB, Hyderabad, January 21-24, 2008.

4. **"30th All India Cell Biology Conference & Symposium on *Molecules to Compartments: Cross-Talks and Networks*"** Dept. of Zoology, New Delhi. **Title of Poster:** Putative DNA Replication Initiation Protein LdORC1 from *Leishmania donovani*, February 2-4, 2007.

5. **"National Congress of Parasitology –2006"** Indian Institute of Chemical Biology, Kolkata, November 22-24, 2006. Participation

6. **"Molecular Mechanism of Disease and Drug Action-2005"** Saha Institute of Nuclear Physics, Kolkata November 16-18, 2005. Participation

Response to the comments of the thesis examiner

Both the examiners have recommended the thesis for the award of Ph.D. Degree. However, one of them has raised two minor issues and necessary addition and modifications have been included at the appropriate place as described in details below:

Minor Comment 1: It is mentioned that the potential substrates were cloned from LD genomic DNA. Cloning strategy was given for construction of a cDNA library, which was not used for cloning, but used only to detect the presence of transcripts. Although LD is known not to have introns, how can one be sure, that the clones obtained from genomic DNA are ACTUALLY present in expressed form as no sequence is provided or it is mentioned that the cDNA and genomic sequences were matched. Bands shown in PCR prove little unless they are characterized by sequencing.

Response: As suggested, the RT-PCR products from *L. donovani* cDNA library for the three identified substrates were sequenced from both ends. Then these sequences were aligned with their corresponding cloned sequences and it was found that they are identical with respect to cloned ones from genomic DNA. The description of sequence alignment is included in the thesis at page no. 45 and a new **Figure 4.3B** depicting the alignments is also included at page no. 46**.** The new figure and description address the concern of the examiner in this regard as the sequences of the PCR products exactly match with that of the respective clones from the genomic DNA.

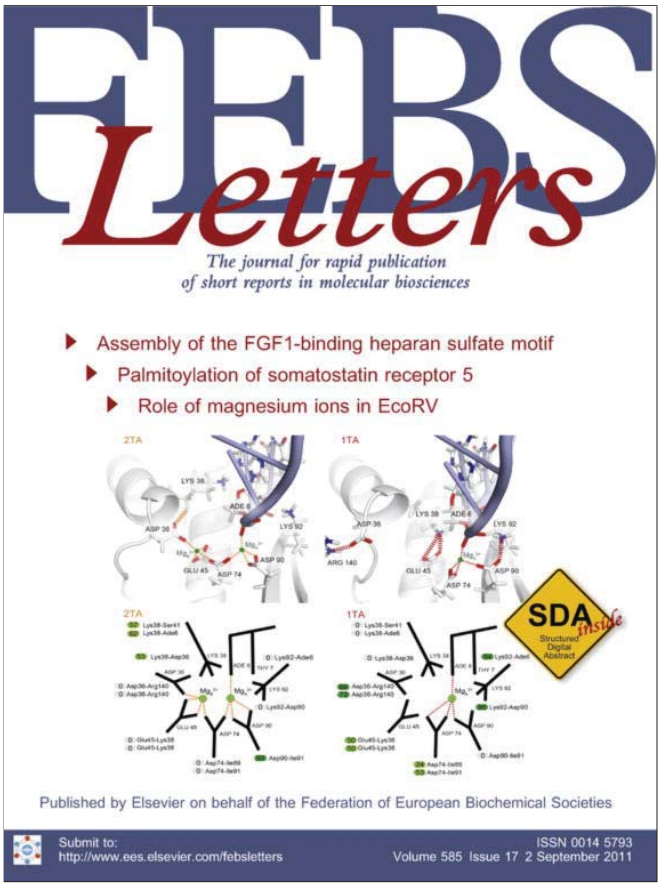
Minor Comment 2: Nowhere in this study there was any attempt to show the presence or use of any native protein. Anti-LDHAT antibody was made against the recombinant protein, it was not shown whether the protein specifically detect any native protein from LD.

Response: It was already reported in the thesis that the anti-LdHAT1 antibody which was raised against recombinant protein was used to detect native LdHAT1 protein from asynchronous as well as synchronous *L. donovani* parasites (**Figure 5.5A and B** at page no. 77). Moreover, it was found that the movements of the recombinant and native proteins are similar in SDS-PAGE as detected by immunoblotting with the antibody (**Figure 5.5A** at page no.77).

Minor Comment 2 (Contd): Even histone H4 was recombinant. Native histones can be extracted by a wide variety of methods. It would have been encouraging if it was shown that this newly identified protein at least acetylates a native protein.

Response: To address the concern, the nucleosome of *L. donovani* was purified (**Figure 5.11A** at page no. 83). After that the HAT assay was performed with the purified nucleosome and was detected with the antibodies raised against N-terminal lysine acetylated Histone H4 peptide from *L. donovani*. It was found that LdHAT1 acetylates native Histone H4 present in the purified nucleosomes at lysine 10 residue specifically (**Figure 5.11B** at page no. 83), similar to the result obtained with the substrate peptide. The methods of nucleosome preparation and HAT assay with purified nucleosome were described at page no. 68-69 and 71, respectively in the current modified version of the thesis.

Reprints



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Identification of substrates of an S-phase cell cycle kinase from *Leishmania donovani*

Anup Kumar Maity [†], Alakananda Goswami [†], Partha Saha [*]

Crystallography and Molecular Biology Division, Saha Institute of Nuclear Physics, Sector I, Block AF, Bidhannagar, Kolkata 700064, India

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ABSTRACT

Despite the importance of cyclin-Cdk related kinases (CRK) in regulation of cell and life cycle of kinetoplastida parasites, only limited knowledge about their substrates are presently available. Here, the potential substrates were searched for an S-phase LdCyc1-CRK3 complex from *Leishmania donovani* based on the presence of Cdk target phosphorylation site together with the cyclin interacting Cy-motif in genome-derived putative protein sequences. Three substrates could be identified with one of them being a unique protein with no known homologues. Another identified substrate is similar to MYST family of histone acetyl transferase and the third one contains Ku-70 related conserved domains. All the substrates interact directly with LdCyc1 and are phosphorylated in a Cy-motif dependent manner suggesting the importance of Cy-motif for their functions.

Structured summary of protein interactions:
LdCyc1 physically interacts with **Ld14.0140L** by pull down (View interaction).
LdCyc1 physically interacts with **Ld28.0070L** by pull down (View interaction).
LdCyc1 physically interacts with **Ld29.1050L** by pull down (View interaction).

1. Introduction

The cell cycle progression in eukaryotes is primarily regulated through phosphorylation of various key proteins by the conserved family of cyclin dependent protein kinases (Cdks) [1–3], and therefore, identification of their cellular substrates is extremely important for a comprehensive understanding of the Cdk regulatory network. However, less than a hundred Cdk targets have been reported in the literature so far, though several screening studies have provided lists of potential substrates in yeasts and other eukaryotes [4].

Several Cdk related kinases (CRKs) and cyclins have also been identified in early branching pathogenic kinetoplastida parasites that particularly affect people in tropical and subtropical countries [5]. The parasites undergo a biphasic life cycle involving two different morphological forms – the flagellated promastigotes in the sand fly vector and non-flagellated amastigotes in host macrophages [6,7]. The morphological transformation between the different life cycle forms and the manifestation of diseases are intricately related to cell cycle regulation [8], though the details of the targets of CRKs, the key regulators of cell cycle machinery, are still lacking.

Recently, we have reported the characterization of an S-phase cyclin LdCyc1 from *Leishmania donovani* which forms a complex with LdCRK3. LdCyc1 [9,10] contains the conserved substrate docking MRAIL-like motif, which is also found in other cyclins and responsible for the interaction with consensus RRL-like cyclin binding (Cy) motif present on the target proteins. The Cy-motif was first identified in the cyclin-Cdk inhibitor p21 [11], and later found to be present in other inhibitors (p27 and p57), activator Cdc25A [12] and substrates Rb, Cdc6 and others [1,3]. Since LdCyc1 contains the homologous substrate binding site, it is expected to interact with cellular targets containing Cy-motif, though the identity of such proteins in *Leishmania* is unknown. Moreover, some of these proteins are likely to be unique as they may be involved in parasite specific mechanisms such as coordinated replication of nuclear and kinetoplast DNA, duplication of single organelles like flagella basal bodies during cell cycle, and consequently, may be better therapeutic targets to stop proliferation of the parasite without affecting the host. In this context, we report here the identification of three substrates of S-phase cell cycle kinase LdCyc1/CRK3, one of them being unique to the parasites, based on a screening of putative ORFs carrying both the cycling binding Cy-motif and the Cdk phosphorylation site.

2. Materials and methods

2.1. Parasite culture

L. donovani promastigotes strain AG83 (MHOM/IN/83/AG83) was grown in M199 medium (Sigma–Aldrich) supplemented with

* Corresponding author. Fax: +91 33 23374637.
E-mail addresses: partha.saha@saha.ac.in, tharpa.saha@gmail.com (P. Saha).
† The first two authors contributed equally to this paper.

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10% FBS (Invitrogen) and penicillin–streptomycin mixture at 22 °C with gentle shaking.

2.2. PCR amplification of putative Open Reading Frames

The PCR were carried out with primer pairs for 28 putative ORFs (Supplementary Table 1) containing both the conserved cyclin binding motif RRL(F/L/V/I)(V/F/G/L) and the Cdk phosphorylation motif (S/T)PX(K/R) from *L. major* genome sequence database (www.sanger.ac.uk/Projects/L_major/) using *L. donovani* genomic DNA as template with Pfx (Invitrogen) or XT-5 (Bangalore Genei) DNA polymerase. Since *Leishmania* genome has a high GC content, for some reactions, addition of GC-melting MAGIC solution (Bangalore Genei) was necessary for obtaining specific product.

2.3. In vitro transcription and translation

The proteins from the amplified ORFs were expressed with N-terminal ^{6}His tag using T7 RNA polymerase based RTS100 Wheat Germ Continuous Exchange Cell-Free (CEFC) system (Roche Applied Science) following the manufacturer's instructions and purified over Ni–NTA agarose (Qiagen).

2.4. Expression of GST-LdCyc1 and LdCRK3 in insect cell

Protein expression in insect cells was carried out using Bac-to-Bac Baculovirus Expression System (Invitrogen). To obtain the active kinase complex, the separate baculoviruses carrying GST-LdCyc1 and LdCRK3 ORFs were used to co-infect Sf9 insect cells grown in Grace's insect cell medium containing 10% fetal bovine serum at 27 °C. The complex was purified over glutathione Sepharose (GE Healthcare) column. The activity of the kinase complex was checked using histone H1 as substrate in a standard kinase assay and subsequently used in kinase reactions with in vitro translated proteins. The equally active kinase complex between bacterially expressed GST-LdCyc1 and LdCRK3 expressed in insect cells was also prepared and used in the kinase reactions of bacterially expressed substrate proteins.

2.5. Protein kinase assay

The kinase assay was carried out at 30 °C for 30 min in 50 mM Tris–HCl (pH 8.0) containing 10 mM $MgCl_2$, 50 μM ATP, 5 μg histone H1 or appropriate amount of expressed putative substrates and 2 μCi of [γ-^{32}P] ATP in a total volume of 15 μl. The products were analyzed by SDS–PAGE followed by autoradiography or phosphorimager analyses with Typhoon scanner (GE Healthcare).

2.6. Protein interaction assay

The interaction assays between LdCyc1 and the identified substrates were typically carried out by incubating 1 μg of bacterially purified substrate protein with glutathione beads bound to 0.5 μg of either GST or GST-LdCyc1 proteins expressed in bacteria at 4 °C for 1 h in a total volume of 0.2 ml of 50 mM Tris–HCl (pH 8.0) containing 250 mM NaCl, 0.5% Triton-X100, 10% glycerol, 1 mM EDTA, 2 mM DTT and protease inhibitors. For competition experiments, 75 μM of PS100 [11] or Somatostatin (as control) peptides were added during the interaction assay. Subsequently, the beads were washed with the same buffer and the bound proteins were analyzed by immunoblotting with appropriate antibodies.

2.7. Confirmation of cellular expression of genes

A cDNA library was prepared from the total cellular RNA of *L. donovani* promastigotes using a kit from Stratagene and used as template in PCRs with XT-5 thermo-stable DNA polymerase (Bangalore Genei) with appropriate primer pairs for amplification of portions of the identified genes to confirm their cellular expression.

3. Results

3.1. Three proteins containing both Cy-motif and Cdk phosphorylation sites derived from Leishmania genome database could be expressed in vitro

Earlier, it was shown that RXL-type Cy-motif and the phosphorylation target site (S/T)PX(K/R) constitute a bipartite substrate recognition sequence of cyclin dependent kinases [13]. It was also observed that the specificity of Clb5-Cdc28 of budding yeast in S-phase primarily depends on the substrate RXL motif [14]. Therefore, in order to identify the potential substrates of S-phase cell cycle kinase LdCyc1/CRK3 from *Leishmania* [9,10], 28 putative ORFs containing both the conserved Cy-motif and the Cdk phosphorylation site were selected from the genome database of *L. major* for screening. The ORFs which were larger than 3 kb in length and/or

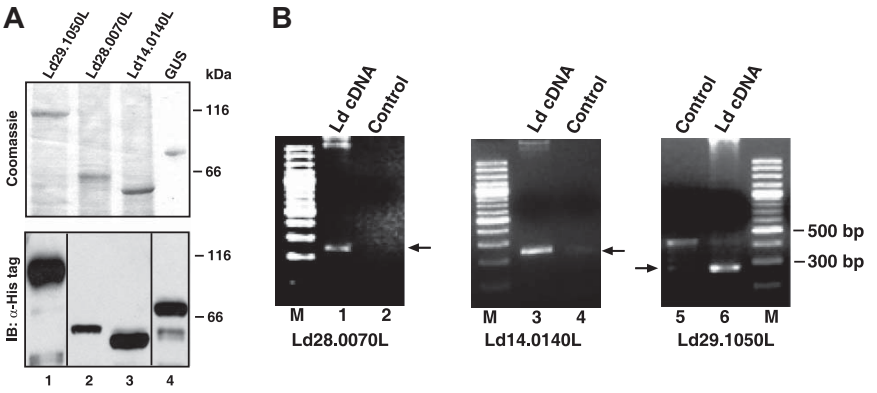

Fig. 1. Expression of the putative substrates of S-phase kinase of *L. donovani*. (A) The ORFs Ld29.1050L, Ld28.0070L, Ld14.0140L and a control protein GUS were expressed in vitro, purified and analyzed by SDS–PAGE followed by Coomassie Blue staining and anti-His tag immunoblotting. (B) Expression of the messages was confirmed by PCR with ORF specific primer pairs from *L. donovani* cDNA library. Control, empty library vector as template. Markers (M) in all panels are same and first intense band from the bottom is 500 bp.

contained putative trans-membrane domain were not included in the present studies to avoid the difficulties of amplification of large templates from the GC-rich genome and insolubility of the expressed proteins, respectively. Out of the 28 selected ORFs, 16 could be amplified specifically from *L. donovani* genomic DNA. Out of them, six could be further amplified in a second round of PCR for the preparation of templates of protein expression with ^{6}His-tag in vitro. The tagged proteins could be successfully expressed from three of the ORFs (29.1050, 28.0070 and 14.0140, the numbers are as per *L. major* genome database) in vitro using wheat germ cell extract (Supplementary Table I). The number of proteins that could be finally expressed in the present study is low most likely due to the difficulty of amplifying the targets from the GC-rich *Leishmania* genome. The success rate could probably be improved in future studies by using different combination of GC melting solutions during PCR. The expressed proteins were purified (Fig. 1A) and confirmed for the presence of ^{6}His tag with anti-His tag immunoblotting. Since the amplification and protein expression were carried out from *L. donovani* genomic DNA taking the sequence information from *L. major* Friedlin database, we designated the ORFs as Ld29.1050L (LmF 29.1050 like from *L. donovani*), Ld28.0070L and Ld14.0140L having the calculated molecular sizes of 103, 58.2 and 57.9 kDa, respectively. As shown in Fig. 1A, the expressed protein bands were detected at the expected positions. The corresponding ORFs (LdBPK_140140.1, LdBPK_280070.1 and LdBPK_291140.1) were also identified in the recently completed draft genome sequence of *L. donovani* (http://www.genedb.org/Homepage/Ldonovani_BPK282A1).

Since all the templates were amplified from genomic DNA, the presence of the messages was checked in the expressed sequences to confirm that the ORFs were actually expressed in cells. As shown in Fig. 1B, specific fragments were amplified for all the identified substrates from *L. donovani* cDNA library confirming that the genes were indeed expressed in the parasite. Notably, constitutive expressions of the corresponding messages were also shown previously in *L. major* promastigotes and amastigotes [15].

3.2. The in vitro expressed proteins act as substrates of LdCyc1/CRK3

To test whether the in vitro translated proteins act as substrates, the kinase assays were carried out with active GST-LdCyc1/LdCRK3 complex and it was found that all three of them could be phosphorylated by the kinase complex specifically (Fig. 2A). The observed multiple or broad phosphorylated bands could be due to breakdown of the proteins, some non-specifically associated proteins from the extract and/or phosphorylation at multiple sites. In order to further confirm the authenticity of the substrates, the kinase assays were repeated with the bacterially expressed purified ^{6}His tagged proteins, and expectedly, all three of them could be phosphorylated (Fig. 2B). Interestingly, the phosphorylation of all the proteins was inhibited significantly and specifically by a peptide (PS100) containing the RRLFG Cy-motif (Fig. 2B) confirming the Cy-motif mediated substrate docking. The purified proteins could also be phosphorylated in a Cy-motif dependent manner by LdCyc1 associated kinase (Fig. 2C) isolated from *L. donovani* promastigote extract through anti-LdCyc1 immunoprecipitation [10], suggesting the proteins could be the authentic in vivo substrates.

3.3. Cy-motif dependent interaction between LdCyc1 and the identified substrates

To further prove the hypothesis that the identified substrates would interact with LdCyc1 directly through their Cy-motif, interaction assays were performed between the GST-LdCyc1 and ^{6}His-tagged substrate proteins. As shown in the Fig. 3, ^{6}His-tagged proteins specifically interacted with GST-LdCyc1, and expectedly, PS100 peptide almost completely abolished the interactions,

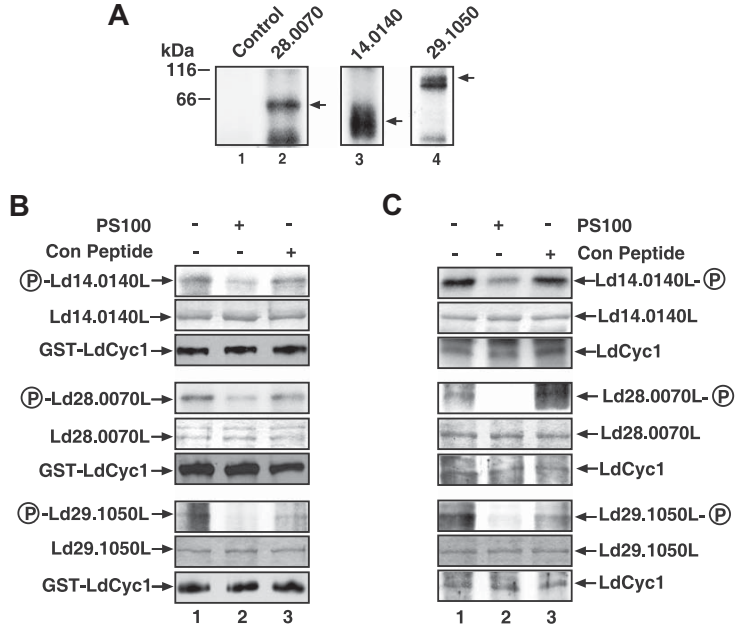

Fig. 2. (A) Phosphorylation of in vitro translated proteins by GST-LdCyc1/CRK3 complex. Control, phosphorylation using in vitro translated GUS protein. The arrows indicate the specific phosphorylated bands. (B) Cy-motif dependent phosphorylation of bacterially expressed putative substrates. For each substrate, a phosphorimager scan of the phosphorylated bands is shown. Coomassie stained gel and anti-GST immunoblots (for GST-LdCyc1) are also shown to demonstrate the presence of equal amounts of substrates and kinase complex in different reaction tubes. (C) Cy-motif dependent phosphorylation by LdCyc1 complex immunoprecipitated from *L. donovani* extract [10]. Anti-LdCyc1 immunoblots are shown instead of anti-GST blots to confirm the presence of equal amount LdCyc1 in different reactions.

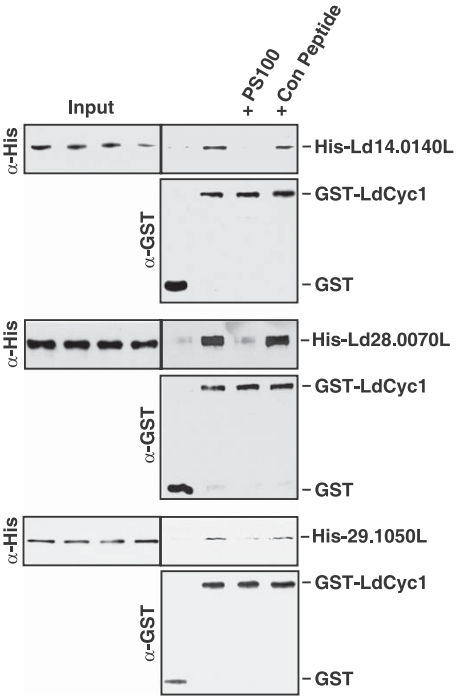

Fig. 3. In vitro interaction between LdCyc1 and the substrate proteins through Cy-motif. Anti-His tag and anti-GST tag immunoblots were carried out to show the presence of ^{6}His-tagged substrates and GST or GST-LdCyc1 in the pulled-down complexes. Input, aliquots of supernatants were immunoblotted with anti-His tag antibody to demonstrate the presence of equal amount of substrates in all reaction tubes.

confirming the Cy-motif dependent interaction between the proteins. The data presented here, therefore, confirmed that the identified substrates could interact with the cyclin partner of the cell cycle kinase complex in a Cy-motif dependent manner for being efficiently phosphorylated by the catalytic CRK subunit.

4. Discussion

In the present studies, three substrates of S-phase kinase LdCyc1/CRK3 of *L. donovani* were identified and among them the derived amino acid sequence of Ld28.0070L did not show homology with any previously characterized protein except the predicted equivalent proteins in related *Leishmania* species (Fig. 4 and Supplementary Fig. S1), implying its unique role in parasite specific cell cycle related processes. On the other hand, the predicted primary structure of Ld29.1050L was shown to contain conserved domains of Ku70 protein [16] (Fig. 4 and Supplementary Fig. S2), a subunit of the versatile heterodimeric protein complex Ku70-Ku80 that was implicated in various nuclear processes like DNA repair, telomere maintenance and apoptosis [17]. Recently, the role of Ku proteins was also implicated during the formation of pre-replicative complex at eukaryotic origins and G1–S transition [18]. Moreover, Ku70 was shown to be phosphorylated by the tissue specific cyclin A1/Cdk2 kinase in human [19], suggesting that Ku70 homolog Ld29.1050L could be an authentic substrate of the *L. donovani* cell cycle kinase. Notably, the Ld29.0150L protein and also its homologs from *L. infantum* and *L. braziliensis* were found to contain an extra stretch of 41 amino acids in the middle portion, which was not found in the *L. major* protein (Supplementary Fig. S2). The third identified substrate Ld14.0140L was found to contain a MYST (human Moz, Yeast Ybf2 and Sas2, and mammalian TIP60) histone acetyl transferase (HAT) domain along with an associated chromodomain [16] (Fig. 4 and Supplementary Fig. S3), suggesting that the protein could be a member of MYST family of HATs. The members of MYST family of HATs were shown to be involved in key regulatory processes in eukaryotic cells. Recently, human MYST histone acetylase Hbo1 was shown to play critical role in replication licensing [20] and regulation of its activity through phosphorylation by CycB/Cdk1 and Plk1 was also implicated [21]. The activity of another MYST family HAT Tip60, which was found to play an essential role in DNA repair and apoptosis, was suggested to be controlled through phosphorylation by cyclin B/Cdk1 [22]. Interestingly, *Trypanosoma brucei* HAT1, which was found to be homologous to Ld14.0140L, was implicated to be essential for proper nuclear DNA replication in the organism [23]. Therefore, the phosphorylation of the HAT homolog by S-phase specific cell cycle kinase raised the possibility of regulation of chromatin remodeling during replication through the post-translational modification.

In summary, three substrates of an S-phase cyclin/Cdk from *L. donovani* could be identified in the present studies with one of them being unique to the parasite. It would be interesting to determine the effect of such phosphorylation on cellular metabolism particularly of the parasites and eukaryotes in general.

Note

The nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the

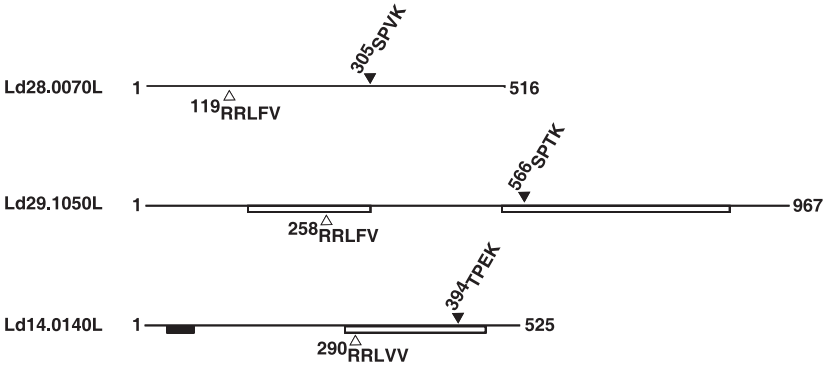

Fig. 4. Schematic presentation of the three identified substrates of LdCyc1/CRK3. The potential phosphorylation sites are indicated by solid arrow-heads and the putative cyclin binding Cy-motifs are shown by hollow arrow-heads. The numbers indicate the position of the first residue in a motif. In Ld29.1050L, the empty bars denote the conserved Ku-70 related domains. In Ld14.0140L, the solid bar and the empty bar represent the conserved chromo-domain and MYST histone acetyl transferase domain, respectively.

Accession numbers HM120719, HQ264173 and HQ264174 (for Ld14.0140L, Ld28.0070L and Ld29.1050L, respectively).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.06.017.

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RESEARCH LETTER

The histone acetyl transferase LdHAT1 from *Leishmania donovani* is regulated by S-phase cell cycle kinase

Anup Kumar Maity & Partha Saha

Crystallography and Molecular Biology Division, Saha Institute of Nuclear Physics, Kolkata, India

Correspondence: Partha Saha, Crystallography and Molecular Biology Division, Saha Institute of Nuclear Physics, Kolkata 700064, India. Tel.: +91 33 2337 0379; fax: +91 33 2337 4637; e-mail: partha.saha@saha.ac.in

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Abstract

Histone acetyl transferases (HATs) are important histone modifiers that affect critical cellular processes like transcription, DNA replication and repairs through highly dynamic chromatin remodelling. Our earlier studies recognized LdHAT1 as a substrate of the S-phase cell cycle kinase LdCyc1-CRK3 from *Leishmania donovani*. Here, we confirm through site-directed mutagenesis that RXL-like cyclin-binding (Cy) motif dependent interaction of LdHAT1 with LdCyc1 is essential for its phosphorylation at a canonical Cdk target site by the kinase complex. LdHAT1 acetylates K10 residue of a peptide derived from *L. donovani* histone H4 N-terminal tail. Interestingly, phosphorylation of LdHAT1 by the S-phase kinase inhibits its H4K10 acetylation activity, implicating an important mechanism of periodic regulation of histone acetylation during cell cycle progression.

Introduction

Chromatin remodelling through various post-translational modifications such as acetylation, methylation, phosphorylation and ubiquitinylation of protruding histone tails of nucleosomal octamer controls access of the factors affecting transcription, replication and DNA repair (Ehrenhofer-Murray, 2004; Osley, 2004; Peterson & Laniel, 2004; An, 2007). The modifications also provide recognition sites for the plethora of protein factors facilitating DNA repair and regulated flow of genetic information. By and large, histone acetylation on lysine residues is important to disrupt the tight packing of chromatins essential for the initiation of processes like transcription. Expectedly, higher proportions of the acetylated histones are associated with promoter region of active genes compared to coding regions and silent portions of genomes. Moreover, several recent studies demonstrate the role of histone modifications in regulation of initiation of DNA replication. Studies in *Drosophila* (Aggarwal & Calvi, 2004) and *Xenopus* (Danis *et al.*, 2004) have established the positive regulation of replication through histone acetylation. Direct involvement of the MYST family histone acetylase HBO1 in regulation of replication licensing through the formation of pre-replication complex has been shown (Miotto & Struhl, 2008). The preference of open chromatin structures with enriched histone H3 methylation and acetylation at metazoan origin has also been established recently (Rampakakis *et al.*, 2009; Karnani *et al.*, 2010). On the contrary, histone deacetylase Sir2 has been shown to interfere with pre-replicative complex (pre-RC) assembly in budding yeast regulating replication in a negative manner (Fox & Weinreich, 2008).

The MYST family is composed of a group of widely distributed but related histone acetyl transferases (HATs) that are involved in diverse cellular activities including activation of transcription, DNA repair, pre-replication complex formation and transcription silencing (Sapountzi & Cote, 2011). The MYST (derived from human MOZ, yeast Ybf2 or Sas2 and Sas3 and mammalian TIP60) family members contain a characteristic MYST domain including the canonical acetyl-CoA binding motif (A-motif) as well as a C_2HC Zn-finger. The MYST HATs also contain other conserved domains like chromodomain

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and plant homeodomain for specific functions. One notable member of the family TIP60, a tumour suppressor, has been shown to be recruited at the DNA double-strand break site through the interaction of its chromodomain with histone H3 trimethylated on lysine 9 (H3K9me3) resulting in the activation of ATM kinase and initiation of repair (Sun *et al.*, 2009). The HAT activity of the TIP60 has also been shown to be regulated through phosphorylation by cyclin B2/cdc2 (Lemercier *et al.*, 2003), although its significance in cellular processes is not known. Hbo1, another important MYST family HAT, has been demonstrated to be essential for Cdt1-assisted loading of minichromosome maintenance (MCM) proteins to form pre-RC at eukaryotic replication origin (Miotto & Struhl, 2010).

Genome sequencing has revealed that four MYST family HATs are encoded by genomes of *Leishmania major* and *Trypanosoma cruzi* and three by that of *Trypanosoma brucei* (Ivens *et al.*, 2005). The early branching trypanosomatid parasites including *T. brucei*, *T. cruzi* and *Leishmania* spp. cause potentially fatal diseases like sleeping sickness, Chagas disease and leishmaniases, respectively, affecting millions of people worldwide (Chatelain & Ioset, 2011). These parasites have many unique features in their biphasic life cycle such as concerted replication of nuclear genome and kinetoplastid DNA in a single copy of mitochondria, polycistronic message formation and nearly complete dependence on the post-transcriptional mechanism for differential gene expression (Gull, 2001; Hammarton *et al.*, 2003). In these organisms, the tails of core histones have divergent sequences compare to other eukaryotes (Alsford & Horn, 2004), and unusual modifications of the histones are also observed in several experiments (Janzen *et al.*, 2006; Mandava *et al.*, 2007). One of the MYST HATs TbHAT3 acetylates histone H4K4, although it is dispensable for growth (Siegel *et al.*, 2008). Among the other MYST HATs, TbHAT1 is essential for telomeric silencing, and its involvement in DNA replication has also been implicated. TbHAT2, the other MYST HAT, is required for H4K10 acetylation and growth (Kawahara *et al.*, 2008).

Recently, we have identified a putative HAT from *Leishmania donovani*, which is highly homologous to TbHAT1, during a search for potential substrates of a previously characterized S-phase cell cycle kinase LdCyc1-CRK3 (Banerjee *et al.*, 2003, 2006; Maity *et al.*, 2011). We term the protein as LdHAT1 and show by site-directed mutagenesis that it directly interacts with LdCyc1 through an RXL-like Cy-motif (Chen *et al.*, 1996). LdHAT1 gets phosphorylated by the kinase on a specific threonine residue, and its acetyl transferase activity is modulated by such phosphorylation, suggesting a possible mechanism of regulation of chromatin remodelling by the S-phase cell cycle kinase.

Materials and methods

Cell culture

Leishmania donovani promastigotes strain AG83 (MHOM/IN/83/AG83) was grown in M199 medium (Sigma-Aldrich) supplemented with 10% FBS (Invitrogen) and penicillin–streptomycin mixture at 22 °C with slow shaking.

Preparation of GST-LdCyc1-CRK3 active kinase complex

To prepare GST-LdCyc1-CRK3 kinase complex, bacterially expressed GST-LdCyc1 was first bound to glutathione–sepharose beads (GE Healthcare Lifesciences), and the bead-bound GST-LdCyc1 was then incubated with an extract of Sf9 cells expressing LdCRK3 in the binding buffer (50 mM Tris-HCl, pH 8.0 containing 50 mM NaCl, 5 mM NaF, 1 mM Na_3VO_4, 0.1 mM EDTA, 0.1% Triton X-100, 10% glycerol, 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl flouride (PMSF) and protease inhibitors) on a rotating wheel at 4 °C for overnight. The beads were washed three times with the same binding buffer, and the kinase complex was eluted with 50 mM Tris-HCl, pH 8.0, containing 10% glycerol, 10 mM reduced glutathione and PMSF.

Cloning and expression of LdHAT1

The complete ORF of LdHAT1 was cloned into pET21b vector, and the C-terminal ^{6}His-tagged chimera was expressed in pG-JKE8 (TAKARA)-transformed *Escherichia coli* strain BL21 cells (expressing GroEL and GroES chaperons for greater solubility of the over-expressed protein) by induction with 1 mM isopropyl β-D-1-thiogalactopyranoside at 37 °C for 3 h. Two mutants of LdHAT1, viz., LdHAT1ΔCy and LdHAT1-T394A were also cloned into pET21b vector and expressed as mentioned above. All ^{6}His-tagged proteins were purified over Ni-NTA agarose beads.

Protein interaction assay

To perform interaction assay between LdCyc1 and LdHAT1, 5 μg of bacterially purified LdHAT1 protein was incubated on a rotating wheel at 4 °C for 1 h with glutathione beads bound to 0.2 μg of either GST or GST-LdCyc1 proteins in 50 mM Na-phosphate (pH 8.0) containing 250 mM NaCl, 0.5% Triton X-100, 10% glycerol, 1 mM EDTA, 2 mM DTT and protease inhibitors. Subsequently, the beads were washed six times with the same buffer, and the bound proteins were analysed by immunoblot analysis with appropriate antibodies. Similar experiments were carried out with the mutant LdHAT1 proteins.

Cell cycle analysis

To synchronize *L. donovani* promastigotes, exponentially growing cells were blocked with 10 mM hydroxyurea (HU) for 36 h followed by releasing the arrest by re-suspending the cells in equal volume of growth medium, and cells were collected at different intervals. The synchronicity of cell cycle progression was confirmed by analysis in a flow cytometer (Supporting Information, Fig. S3). The population of cells from each time point was also examined by analysing the fluorescence and differential interference contrast (DIC) images of 4′,6-diamidino-2-phenylindole (DAPI)-stained cells captured by a Zeiss Axio-observer Z1 inverted microscope (Fig. S3). Cells from different time intervals were lysed in 50 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, 0.2% Triton X-100, 1 mM EDTA and protease inhibitors, and the soluble extracts were analysed by immunoblotting with antibodies against LdHAT1 (mouse polyclonal) and actin.

Kinase assay

The kinase assays were carried out with the increasing amount of GST-LdCyc1-CRK3 complex in 20 mM HEPES-KOH, pH 7.5, containing 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 5 mM NaF, 2 mM DTT, 50 μM [γ^{32}P]ATP (2.7 μCi/nmole) and 1.0 μg of LdHAT1 in a total volume of 15 μL at 30 °C for 30 min. For the assays with the mutated proteins, LdHAT1ΔCy and LdHAT1-T394A were incubated with 0.2 μg of kinase GST-LdCyc1-CRK3 in the reaction buffer. The reaction products were analysed by SDS-PAGE followed by phosphorimager scanning in Typhoon scanner (GE Healthcare Lifesciences).

Generation of antibodies against acetylated peptides derived from *L. donovani* histone H4

Three peptides derived from N-terminus of *L. donovani* histone H4–containing specific acetylated lysine (LdH4K4Ac: AKGK_{Ac}RSADAC; LdH4K10Ac: SADAK_{Ac}GSQKC; LdH4K14Ac: KGSQK_{Ac}RQKKC) were synthesized and conjugated to carrier protein keyhole limpet haemocyanin. For each peptide, two rabbits were immunized, and the progress of immunization was monitored by ELISA assay. Specific antibodies were purified from the anti-sera having higher titre values through affinity column chromatography in a two-step process – first over a column containing a control non-acetylated peptide (AKGKRSADAKGSQKRQKKC) followed by a column containing the respective acetylated peptide. The specificities of the purified antibodies were checked by ELISA assay. The entire process was carried out by IMGENEX India, Bhubaneswar, India, on contract basis. The specificities of the antibodies were further verified by dot blot analysis in our laboratory.

HAT assay

HAT assay was performed with 1.6 μM of ^{6}His-tagged LdHAT1 as enzyme in 50 mM HEPES-KOH, pH 8.0, containing 0.1 mM EDTA, 5% glycerol, 1 mM DTT, 10 mM Na-butyrate, 0.1 mM Li_3Acetyl-CoA and 50 μM of a peptide derived from *L. donovani* histone H4 N-terminus (AKGKRSADAKGSQKRQKKC) as substrate in a total volume of 20 μL. The reaction was carried out at 30 °C for 1 h, stopped by adding 5 μL of SDS-PAGE sample buffer, and the products were subjected to a modified Tris-Tricine SDS-PAGE for better resolution of smaller peptides (Schagger & von Jagow, 1987). Briefly, 18% polyacrylamide (18%T, 5%C) in 0.75 M Tris-HCl, pH 8.45, containing 30% ethylene glycol and 0.1% SDS was used as resolving gel with 0.1 M Tris containing 0.1 M Tricine and 0.1% SDS as electrophoresis buffer. Finally, the acetylated peptide was detected by immunoblotting with the antibodies raised against the peptides containing specific acetylated lysine residues as described above. The antibodies obtained after purification over non-acetylated peptides or Coomassie blue staining of the gel were used for checking the presence of equal amount of substrate peptide in different reactions.

Results and discussion

LdHAT1, a MYST family HAT, expresses at a constant level during cell cycle

One of the identified substrates of the S-phase cell cycle kinase LdCyc1-CRK3 from *L. donovani* was shown to contain a MYST (human Moz, Yeast Ybf2 and Sas2, and human TIP60) domain of HATs (Maity *et al.*, 2011), although further characterization of activity of the protein and its regulation by phosphorylation were not carried out. The HAT protein from *L. donovani* is 97% identical to LmHAT1, which was grouped with the HAT1 from *T. brucei* and *T. cruzi* in a phylogenetic analysis (Kawahara *et al.*, 2008). Therefore, we designate the 525 amino acid–containing protein as LdHAT1, which is also highly homologous to other MYST family HATs from diverse organisms (Fig. 1a and Fig. S1). Maximum homology is present along the C-terminal canonical MYST domain (amino acid 254–456 of LdHAT1), which contains the characteristic acetyl-CoA binding R/Qx_2GxG/A-motif (A-motif). Like other family members, on the N-terminus of the MYST domain, the conserved C_2HC ($Cx_2Cx_{12}Hx_{3-5}C$) Zn-finger motif is also present in LdHAT1. As previously described (Maity *et al.*, 2011), the

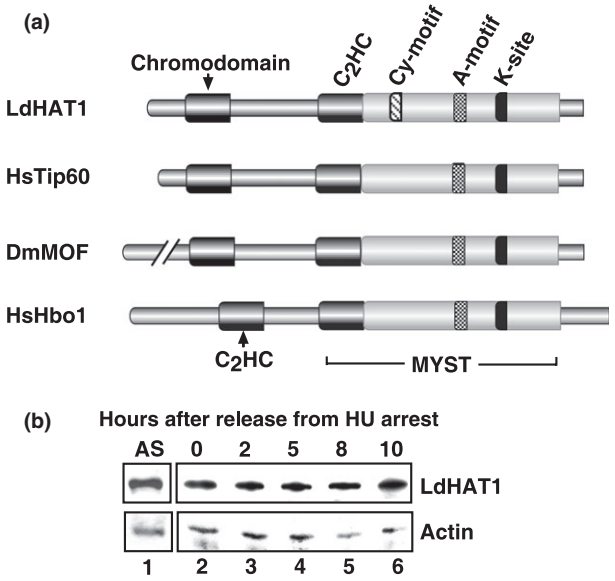

Fig. 1. (a) Homology of LdHAT1 with other MYST family HATs. Sequence homology was determined by CLUSTAL-W. The accession numbers of the proteins used are as follows: LdHAT1, HM120719; HsTip60, BC064912; DmMOF, EF630377; and HsHbo1, NM_007067. Ld, *Leishmania donovani*; Hs, *Homo sapiens*; Dm, *Drosophila melanogaster*. (b) Cell cycle expression of LdHAT1 protein. Samples from the indicated time points after release from HU arrest were analysed by anti-LdHAT1 immunoblotting. β-actin was used as loading control. AS, asynchronously growing cells.

cyclin-binding RXL-type Cy-motif (Chen *et al.*, 1996) is located within the MYST domain in LdHAT1, although such a typical motif is absent in HsTIP60, DmMof and HsHbo1. However, a canonical Cdk target phosphorylation site (TPEK) is well-conserved within the MYST domain of LdHAT1 and in the other MYST family members (Fig. S1). In addition to the canonical Cdk phosphorylation site, five minimal sites (T/S-P) are also present in the molecule in a scattered manner. Interestingly, catalytically critical Glu residue, corresponding to Glu338 in the prototype yeast Esa1 (Berndsen *et al.*, 2007), is located within the canonical Cdk target site (TPEK), implicating an interesting regulatory mechanism if the Thr residue is actually phosphorylated by cell cycle kinases. Moreover, similar to HsTIP60 and DmMof, a Chromatin Organization Modifier (chromo) domain is located towards the N-terminus of LdHAT1. Chromodomain of heterochromatin protein HP1 was shown to interact with methylated lysine[9] residue of histone H3 to recruit the regulator at appropriate location (Jacobs & Khorasanizadeh, 2002; Nielsen *et al.*, 2002). Chromodomain can also function as RNA-interacting module to target the regulators to the specific chromosome site as was observed in case of DmMof (Akhtar *et al.*, 2000). The presence of chromodomain in LdHAT1, therefore, raises its possible role in crosstalk between methylation and acetylation histones and/or RNA-mediated chromatin remodelling in the parasites.

As phosphorylation of LdHAT1 by S-phase Cdk raised the possibility of its involvement in cell cycle–related periodic activities, its expression profile was analysed during cell cycle progression of *L. donovani* promastigotes. Polyclonal anti-sera against the purified LdHAT1 were raised in mice, and one of them was shown to detect a specific band of expected size in immunoblot analysis with the extract of *L. donovani* promastigotes (Fig. S2). The same anti-serum was used subsequently to analyse extracts from the synchronized cells. Uniform changes in morphology of the cell population with the progress of cell cycle along with corroborating flow cytometer analysis profiles ascertained the synchronicity of the promastigote culture (Fig. S3). Analysis of protein extracts from such synchronously growing cells showed the presence of LdHAT1 protein at equal amounts in different cell cycle phases of *L. donovani* promastigotes (Fig. 1b). As the level of LdHAT1 found to be invariable during cell cycle, it would be interesting to study the effect of phosphorylation by the S-phase kinase on its activity.

LdHAT1 directly interacts with LdCyc1 through its Cy-motif

LdHAT1 was shown previously to interact with *L. donovani* S-phase cyclin LdCyc1 in a RXL-like Cy-motif-dependent manner by peptide competition assay (Maity *et al.*, 2011). To further confirm the contribution of Cy-motif in the interaction, the putative Cy-motif of LdHAT1 was altered (^{290}RRLVV→RDDVV, LdHAT1ΔCy), and the mutated protein was used in the interaction assay. As shown in Fig. 2a, the wild-type protein was found to interact with GST-LdCyc1, whereas the interaction with LdHAT1ΔCy was almost completely abolished, proving the involvement of Cy-motif during direct interaction between the proteins. The observation also confirmed the identity of an active Cy-motif in the molecule. The mutation at the putative Cdk phosphorylation site (^{394}TPEK→APEK, LdHAT1-T394A) of the protein did not affect the interaction (Fig. 2a), confirming further the specific involvement of Cy-motif in the binding.

Cy-motif of LdHAT1 is required for its phosphorylation by S-phase cell division kinase

LdHAT1 was demonstrated to be phosphorylated *in vitro* by LdCyc1-CRK3 complex (Fig. 2b) (Maity *et al.*, 2011). As the substrate docking on the cyclin moiety was shown to be important for phosphorylation, to investigate the

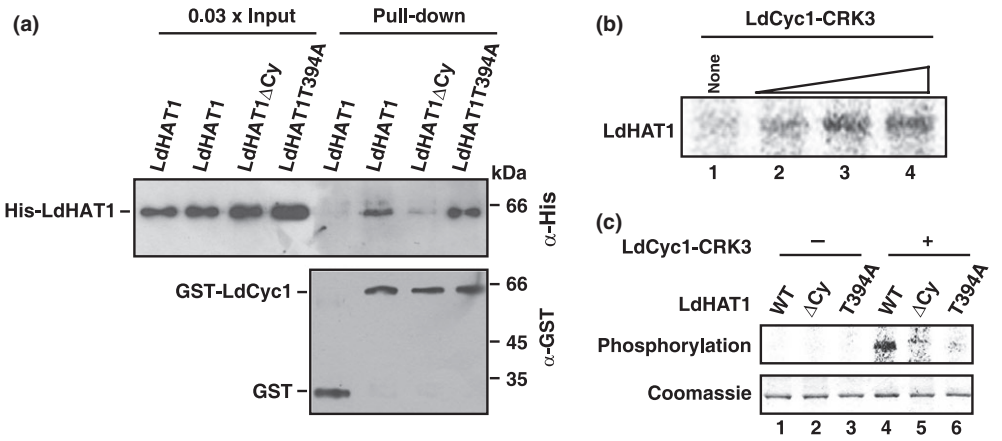

Fig. 2. (a) *In vitro* interaction between LdCyc1 and LdHAT1 through Cy-motif. Anti-His tag and anti-GST tag immunoblotting were carried out to show the presence of ^{6}His-tagged LdHAT1 protein or its mutants and GST or GST-LdCyc1 in the pulled-down complexes. Input, aliquots of supernatants were immunoblotted with anti-His tag antibody to demonstrate the presence of equivalent amounts of LdHAT1 or mutant proteins in all reaction tubes. (b) Phosphorylation of LdHAT1 by LdCyc1-CRK3 complex. Phosphorylation of LdHAT1 by increasing amounts of the kinase complex (0.1, 0.2 and 0.3 μg) was determined by phosphorimager scanning of the SDS-PAGE analysed reaction products. (c) Cy-motif-dependent phosphorylation of LdHAT1 by LdCyc1-CRK3 complex. Phosphorylation by LdCyc1-CRK3 complex (0.2 μg) was carried out using wild-type and mutated LdHAT1 as substrates as indicated.

effect of Cy-motif of LdHAT1 on its phosphorylation, LdHAT1ΔCy was used as substrate in a kinase assay of LdCyc1-CRK3 complex. As observed, LdHAT1ΔCy was not efficiently phosphorylated by the kinase complex compared to the wild-type protein (Fig. 2c, lanes 4 and 5). As the mutation in Cy-motif of LdHAT1 was shown to disrupt its interaction with LdCyc1 (Fig. 2a), the inhibition of the phosphorylation established the requirement of its

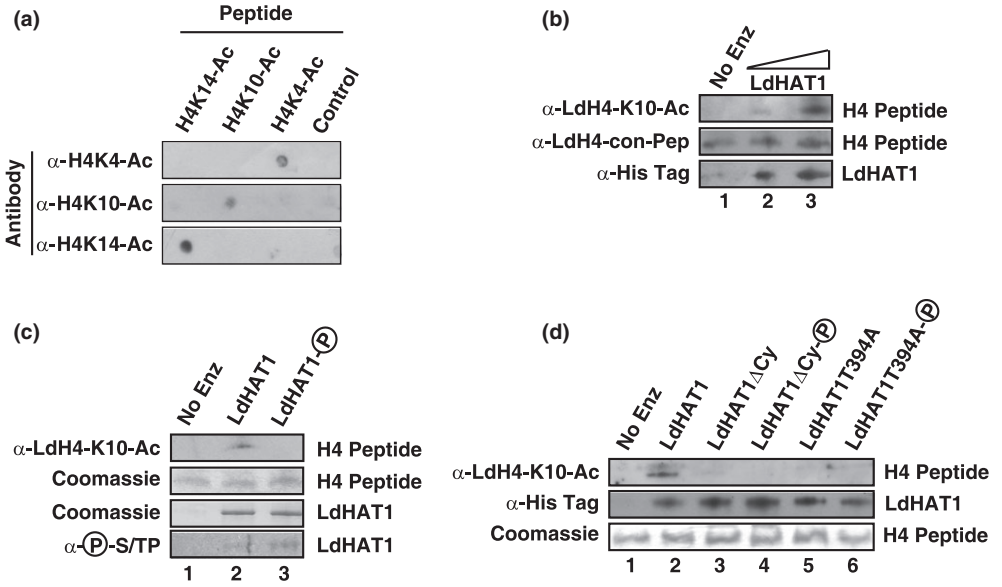

Fig. 3. (a) Specificities of antibodies against acetylated lysine containing peptide. One hundred nanograms of each of the indicated peptides was spotted on membrane, and immunoblotting was carried out with the denoted antibodies against the peptides containing the specified acetylated lysine. (b) *Leishmania donovani* histone H4K10 acetylation by LdHAT1. Histone H4 N-terminus peptide was used as substrate, and the antibody (α-LdH4-K10-Ac) against acetylated H4K10-containing peptide was used to identify the specific product. The antibody against the non-acetylated peptide (α-LdH4-con-pep) was used to show the presence of equal amounts of substrate peptide in each reaction. (c) Inhibition of acetyl transferase activity of LdHAT1 owing to phosphorylation by LdCyc1-CRK3 complex. (d) Inhibition of acetyl transferase activity of LdHAT1 owing to mutations at Cy-motif and Cdk phosphorylation target site within the MYST domain.

docking through the Cy-motif on MRAIL-motif on LdCyc1 (Banerjee *et al.*, 2003) for the phosphorylation on the target serine/threonine residue. LdHAT1 was also shown to contain a putative Cdk phosphorylation site on its C-terminal end. To confirm whether Thr-394 in the motif TPEK was phosphorylated by the kinase complex, the threonine residue was changed to alanine, and the mutant LdHAT1-T394A was used as substrate. As shown in Fig. 2c, the phosphorylation was completely abolished because of the mutation (lane 6), suggesting that the S-phase kinase LdCyc1-CRK3 targets Thr-394 for phosphorylation. It is interesting to note that Thr-394 is located very close to conserved catalytically critical Glu residue raising the possibility of regulation of HAT activity because of the incorporation of a phosphate group. Therefore, it is important to study the effect on the activity of LdHAT1 by phosphorylation of the Thr residue by the cell kinase.

Phosphorylation of LdHAT1 by S-phase kinase down-regulates its *L. donovani* histone H4-K10 acetylation activity

It was previously implicated that HAT1 from *T. brucei* could acetylate histone H4 from the parasite (Kawahara *et al.*, 2008). Therefore, to characterize the histone acetylation activity of LdHAT1, *in vitro* assays were carried out using a peptide substrate derived from the N-terminus of *L. donovani* histone H4. To identify the lysine residue that was specifically acetylated by LdHAT1, three antibodies were raised against *L. donovani* histone H4–derived peptides acetylated on K4, K10 or K14 residue, respectively. Specificities of the antibodies were ensured by dot blot analysis, which showed no cross-reactivity (Fig. 3a). Once the specificities were confirmed, the antibodies were used to identify the lysine residue on the peptide derived from N-terminus of *L. donovani* histone H4 acetylated by LdHAT1. As shown in Fig. 3b, the peptide acetylated by LdHAT1 could be detected only by anti-H4K10Ac antibody, but not with other two antibodies (data not shown), suggesting that the acetyltransferase from *L. donovani* specifically acetylates H4K10 residue. As LdHAT1 was shown to be phosphorylated by S-phase kinase LdCyc1-CRK3, it would be interesting to investigate any possible effect of such phosphorylation on its histone acetylation activity. To explore such a possibility, H4K10 acetylation activity of non-phosphorylated LdHAT1 was compared with that of LdHAT1 phosphorylated by LdCyc1-CRK3. As depicted in Fig. 3c, the phosphorylated form of LdHAT1 did not show any H4K10 acetylation activity, suggesting the regulation of histone H4 acetylation by S-phase cell cycle kinase. Intriguingly, LdHAT1ΔCy and LdHAT1-T394A mutants also did not show any acetylation activity (Fig. 3d) implicating the contribution of the mutated residues in the enzymatic activity.

Previous studies demonstrated the acetylation of K4, K10 and K14 residues of the N-terminal tail of histone H4 from *T. brucei* and *T. cruzi* (da Cunha *et al.*, 2006; Janzen *et al.*, 2006; Mandava *et al.*, 2007). Moreover, cell cycle-dependent post-S-phase enhancement of K4, K10 and K14 acetylation of histone H4 was observed in *T. cruzi* (Nardelli *et al.*, 2009). Therefore, the observed inhibition of histone H4K10 acetylation by LdHAT1 owing to its phosphorylation by S-phase kinase in the present studies could correlate such cell cycle–specific periodic acetylation. It will be interesting to study the effect of inhibition of the HAT activity on the S-phase events.

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Conflict of interest

The authors have no conflict of interest to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Alignment of MYST domains of *Lesihmania donovani* HAT1 (LdHAT1, HM120719), human TIP60 (HsTIP60, BC064912), *Drosophilla* Mof (DmMof, EF630377) and human Hbo1 (HsHbo1, NM_007067).
Fig. S2. Characterization of anti-serum against LdHAT1.
Fig. S3. Synchronization of *L. donovani* promastigotes.

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