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Leishmania express a functional Cdc20 homologue

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ABSTRACT

Our knowledge concerning the mechanisms of cell cycle regulation in organisms belonging to the *Trypanosometidae* family is limited. *Leishmania donovani* are parasitic protozoa that cause kala azar, a fatal form of visceral leishmaniasis in humans. Here we provide evidence that the *L. donovani* genome contains a Cdc20 homologue. Cdc20 is a regulator of the Anaphase Promoting Complex/Cyclosome (APC/C) that mediates ubiquitin-dependent proteasomal degradation of key cell cycle regulators in eukaryotes. We show that *L. donovani* Cdc20 protein (LdCdc20p) can complement a lack of yeast Cdc20 protein in *Saccharomyces cerevisiae* cells, validating the functionality of LdCdc20p. Furthermore, we demonstrate cyclic expression of LdCdc20p and that it contains an active RXXL destruction motif, a distinctive feature of proteins targeted for proteasomal degradation by APC/C. Finally, in line with the proteasome mediating LdCdc20p degradation, promastigotes exposed to proteasome inhibitor display elevated LdCdc20p levels. Taken together our data indicate that *Leishmania* regulate their cell cycle by ubiquitin-dependent proteasomal degradation mediated by the APC/C.

1. Introduction

Ubiquitin-dependent degradation of cell cycle regulators such as cyclins is required for cell cycle progress and is mediated by ubiquitin ligases such as the Anaphase Promoting Complex/Cyclosome (APC/C) [1,2]. The activity and regulation of APC/C, in particular the nature of its activating proteins, is highly conserved from yeast to human. APC/C is activated in a cell cycle specific manner from metaphase until the G1–S transition. Its activity is regulated by phosphorylation and by sequential association with two regulatory proteins – Cdc20 and Cdh1. Cdc20 is an essential protein that binds and activates APC/C in mitosis and is required for the degradation of mitotic APC/C substrates like cyclin A, cyclin B, securin and others [3]. Cdh1 is required for APC/C activation during G1 [4].

Organisms of the genus *Leishmania* (*Leishmania spp.*) are intracellular parasitic protozoa of the family *Trypanosometidae*. *Leishmania donovani* is the causative agent of kala azar, a fatal form of visceral leishmaniasis where parasites infect humans and reside in the liver, spleen and bone marrow [5,6]. Until recently, limited information was available concerning cell cycle regulation in *Leishmania* even though it is likely to influence development inside both vectors and hosts. However, now that genome sequences are available it is expected that conserved cell cycle genes will be identified, which can be evaluated functionally in subsequent studies.

The capability to perform RNA interference (RNAi) assays in *Trypanosoma brucei*, a member of the *Trypanosometidae* family, has facilitated identification and biochemical characterization of cell cycle related proteins in this parasite (reviewed in [7]). Proteins identified thus include: two cyclin-like proteins with short half-lives and a putative APC/C destruction box motif [8,9]; the regulatory protein kinases aurora [10] and polo [11]; two subunits of APC/C, APC1 and APC3 (CDC27) [12]. All of these proteins influence cell cycle progression from mitosis through cytokinesis. In addition, numerous cyclin dependent protein kinases (CDKs) and their activating cyclins have been identified and shown to play roles in cell cycle checkpoints [13–15]. Finally, in other studies two additional cell cycle activities have been identified, an E3 ligase that controls cell cycle during S phase and G2 comprising the Skp1–Cdc53/Cullin-F-box (SCF) protein complex [16] and an F-box protein termed CFB2 that was shown to be essential for *T. brucei* cytokinesis [17]. Taken together these data support the premise that *Trypanosometidae*, like all other eukaryotes, regulate cell cycle using ubiquitin-dependent proteasomal degradation mediated by APC/C and SCF complexes.

Here, we present evidence that *L. donovani* promastigotes express a Cdc20 (*Ld*Cdc20) homologue containing an active RXXL destruction box motif that exhibits a very short half-life and cyclic behavior. This finding corroborates that cell cycle regulation also in this parasite involves APC/C mediated ubiquitin-dependent proteasomal degradation.

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2. Materials and methods

2.1. Phylogenetic tree of Cdc20 proteins

Cdc20 protein sequences from 14 different organisms were collected from the NCBI and GeneDB (www.GeneDB.org/) databases. Sequences were aligned using ClustalW, and a neighbor-joining (NJ) bootstrapped phylogenetic tree was built using the Mega4 software (http://www.megasoftware.net).

2.2. Cloning of LdCdc20 and expression vectors

The LinJ24_V3.1790 (LdCdc20) ORF was amplified by PCR using *L. donovani* genomic DNA as template. For the *S. cerevisiae* complementation assay, the *Ld*Cdc20 ORF was cloned into yeast expression vector pDR197 between XmaI and BamHI sites [18]. The myc-tag sequence was added within the 5′ upstream primer in frame with the *Ld*Cdc20 ORF.

For expression in *L. donovani* cells the *Ld*Cdc20 ORF was cloned into pX-′GFP+ between the XmaI and BamHI sites, upstream and in frame with the GFP gene to generate *Ld*Cdc20–GFP. The pX-′GFP+ plasmid was a gift from Professor Steven Beverley at Washington University [19].

2.3. Leishmania cell culture, transfection and synchronization

A cloned line of *L. donovani* 1SR was used in all experiments [20]. Promastigotes were grown at 26 °C in medium 199 supplemented with 10% fetal calf serum [21]. *Ld*Cdc20–GFP plasmids were transfected using standard electroporation conditions [22] and transfected parasites were selected in 50 μg/ml of G418. Parasites were synchronized at G2/M phase using 5 μM flavopyridol (in DMSO), washed and released into fresh medium. Flavopyridol was a kind gift from Professor Jeremy C. Mottram at Glasgow University. Where indicated, 1 h prior to collection parasites were treated with proteosome inhibitor MG-132 (Calbiochem; 50 μM in DMSO).

2.4. S. cerevisiae complementation assay

S. cerevisiae strain W303α 1195 GAL1:CDC20 was transfected with a plasmid encoding myc-*Ld*Cdc20 or with an empty vector (pDR197) and grown on galactose minimal plates under the suitable selection conditions. Complementation was evaluated by transferring positive myc-LdCdc20 colonies to selective minimal glucose plates.

2.5. Flow cytometry

Flow cytometry of propidium iodide-stained promastigotes was done as previously described by Barak et al. [21]. Briefly, a 5 ml sample was taken from the synchronized cell culture (1×10^6–1×10^7 cells/ml), washed twice with phosphate buffered saline (PBS) and suspended in 90% ice-cold methanol. Once fixed the parasites were kept at −20 °C. Prior to analysis, parasites were treated with 20 mg/ml RNase for 1 h at 37 °C. Then, the DNA stain propidium iodide (20 μg/ml) was supplemented and DNA content analyzed using FACSCalibur (Becto Dickinson, San Jose, CA). Twenty thousand cells were analyzed from each sample. The G1, S and G2/M distribution of each sample was presented using the WinMDI 2.9 software.

2.6. Western blot analysis and microscopy

Pomastigotes cells were collected, centrifuged and resuspended in EB buffer (50 mM Tris–HCl pH 8, 250 mM NaCl, 20 mM EGTA, 50 mM NaF, 1% Triton X-100) containing protease inhibitor cocktail (sigma P8340) and leupeptin 5 μg/ml. Yeast protein was purified by TCA and glass bead precipitation. Each protein sample was boiled in Lamely buffer and the gels were equally loaded with 25 μg of total protein. The following antibodies were used in Western blot analyses: monoclonal mouse anti-myc (9E1O, Santa Cruz sc-40), polyclonal goat anti-ScCdc20 (Santa Cruz sc-6730), polyclonal rabbit anti-GFP (Santa Cruz sc-8334) and anti-Hsp90 [21].

For LdCdc20–GFP localization, $5–10 \times 10^6$ cells were placed on a slide, washed once with PBS and examined using a Leica DMEIR2 microscope.

3. Results

3.1. Cloning and functional analysis of LdCdc20

To better understand cell cycle regulation in *Leishmania*, we checked if there are *L. donovani* homologues of conserved essential genes involved in this process. Initially we searched the *L. infantum* genome sequence version 3 (www.genedb.org/genedb/linfantum) for homologues of the *S. cerevisiae* APC/C activators Cdc20 and Cdh1. Our sequence analyses indicated that the *L. infantum* genome contain one copy of a *Cdc20* homologue on chromosome 24 (*LiCdc20*; LinJ24_V3.1790; $1.3 \times e^{-43}$). Using this gene as a template, the *L. donovani* homologue was cloned (*LdCdc20*). Surprisingly, we could not identify a Cdh1 homologue based on sequences similarity.

Next we performed unrooted phylogenetic analysis using Cdc20p amino acid sequences from representatives of most eukaryotic phyla. The resulting phylogenetic tree possesses three distinct branches: the first contains only members of the *trypanosomatidae* family (Fig. 1); the second includes insects, vertebrates and mammals; and the third includes plants, yeast and fungi. Remarkably, according to our analysis human Cdc20p is closer to *Ld*Cdc20 than *S. cerevisiae* Cdc20p. The overall homology between leishmania to yeast and human Cdc20p is close to 30% with the most conserved motifs being the WD40 repeats.

To assess whether *Ld*Cdc20p can perform the typical function of Cdc20 proteins in mitosis, we examined its heterologous expression in *S. cerevisiae*. We used an *S. cerevisiae* strain whose *Sc*Cdc20p expression was under the control of a galactose promoter (GAL1:CDC20). These cells are able to grow with galactose as the sole carbon source, but upon shift to glucose medium they rapidly silence *ScCDC20*. Since *Sc*Cdc20 protein expression is essential for cell divi-

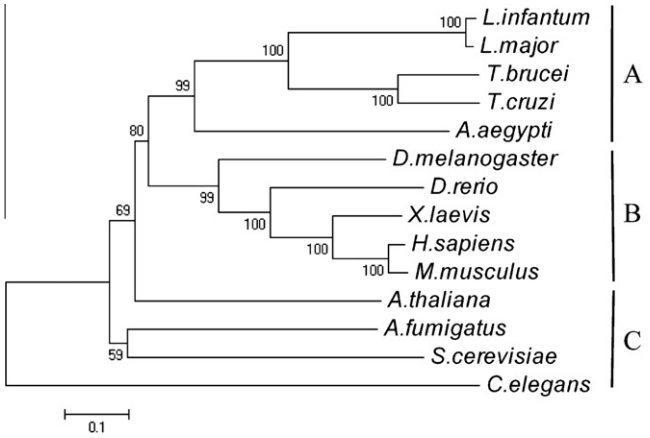

Fig. 1. Phylogenetic analysis of Cdc20 proteins from different organisms. A phylogenetic tree was constructed for 14 different Cdc20 proteins sequences using Mega 4 software. Numbers on branches indicate bootstrap distance values.

sion, these cells are unable to proliferate. As expected, yeast transformed with empty pDR vector grew on galactose plates but did not form colonies on glucose plates (Fig. 2A left panel). In contrast, yeast transformed with pDR encoding myc-LdCdc20 grew well on both glucose and galactose plates (Fig. 2A, right panel). Thus, in this yeast complementation assay, *Ld*Cdc20p can substitute for the essential yeast cell cycle regulator, *Sc*Cdc20p. Using antibodies against *Sc*Cdc20, we confirmed that endogenous *Sc*Cdc20p was not expressed in cells grown in medium where glucose was the only carbon source (Fig. 2B left panel). In addition, using anti-myc antibodies myc-*Ld*Cdc20p was shown to be expressed in both galactose and glucose media (Fig. 2B right panel). Taken together, these data demonstrate that *Ld*Cdc20p functions as a genuine Cdc20 homologue.

3.2. LdCdc20p exhibits cyclic expression during L. donovani promastigote cell cycle

To evaluate *Ld*Cdc20 expression we tagged the full length *LdCdc20* (that encodes a 90 kDa protein) at its carboxy terminus with Green Fluorescence Protein (GFP) to generate a 120 kDa *Ld*Cdc20–GFP reporter protein. As shown in Fig. 2C, this LdCdc20–GFP fusion protein is visible in the cytosol as well as in other compartments of promastigotes. The results indicate that parasite cells are viable and LdCdc20–GFP is not forming non-soluble inclusion bodies.

To examine if *Ld*Cdc20p exhibits a cyclic expression pattern we took advantage of flavopiridol, an ATP analog that inhibits CDK1 in mammalian and yeast cells [23–25] and is known to efficiently

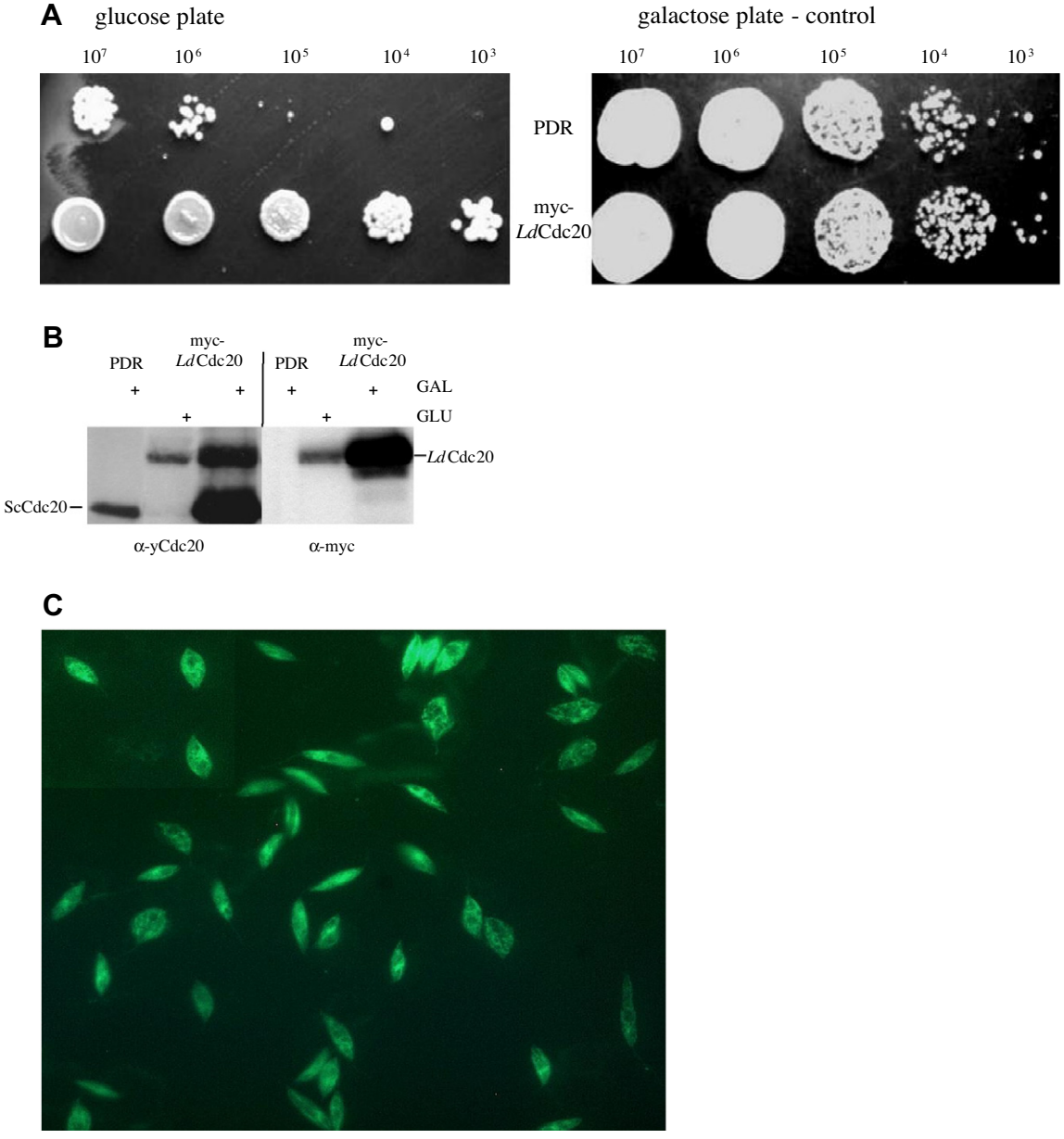

Fig. 2. Ldcdc20 complements a lack of *Sc*cdc20. *S. cerevisiae* GAL1:CDC20 cells were transformed with myc-*Ld*cdc20 or empty vector (PDR). (A) GAL1:CDC20 *S. cerevisiae* cells transformed with *Ld*cdc20 grow on glucose plates unlike cells transformed with empty vector. (B) Endogenous *Sc*cdc20p is absent in cells grown in glucose whereas myc-*Ld*cdc20 is expressed irrespective of growth media. Of note, anti-*Sc*cdc20 antibody recognized both *Ld*cdc20p and endogenous *Sc*cdc20p (90 and 55 kda, respectively). (C) *Ld*cdc20–GFP fusion protein is localized in the cytosol of promastigotes. Fluorescence analysis was performed using a fluorescent Leica DMEIR2 microscope.

synchronize *L. mexicana* and *L. donovani* promastigotes at G2/M [21,26]. Promastigotes expressing LdCdc20–GFP and wild type promastigotes (Ld1S) were incubated with 5 μM flavopiridol for 16–18 h, released from this G2/M arrest by transfer to fresh medium sampled at various time points and subjected to flow cytometry (FACS; Fig. 3A) or Western analysis (Fig. 3B, C). In agreement with an earlier study [21], FACS analysis of propidium iodide-stained log phase promastigotes confirmed that 90% of promastigotes were synchronized at G2/M yet almost all resumed cell cycle progression immediately after exposure to fresh medium (Fig. 3A upper panel). Importantly, Western analysis using anti-GFP antibodies revealed relatively low *Ld*Cdc20–GFP levels at G2/M that gradually increased 2 and 4 h after release (Fig. 3B, C). Addition of the proteasome inhibitor MG132 to samples of parasites 1 h before harvesting resulted in elevated *Ld*Cdc20–GFP levels, indicating that the proteasome is involved in regulating the steady state level of this protein. A high molecular weight variant of *Ld*Cdc20–GFP could be detected only 1 h after release and not at later time points.

Notably, expression of exogenous *Ld*Cdc20–GFP was observed to affect the rate of cell cycle progression. In general both normal (Ld1S) and transgenic (LdCdc20–GFP) parasites reached G1 within 2 h. However, the transgenic parasites progressed to G1/S within the following hour and took only an additional hour to fully shift into S phase, such that the majority of transgenic parasites were in S phase 4 h after release (Fig. 3A bottom panel). In contrast, normal promastigotes reached G1/S and S phase only 4 and 6 h after release, respectively (Fig. 3A top panel). This finding underscores a role for *Ld*Cdc20p in cell cycle regulation and validates the functionality of the chimeric protein.

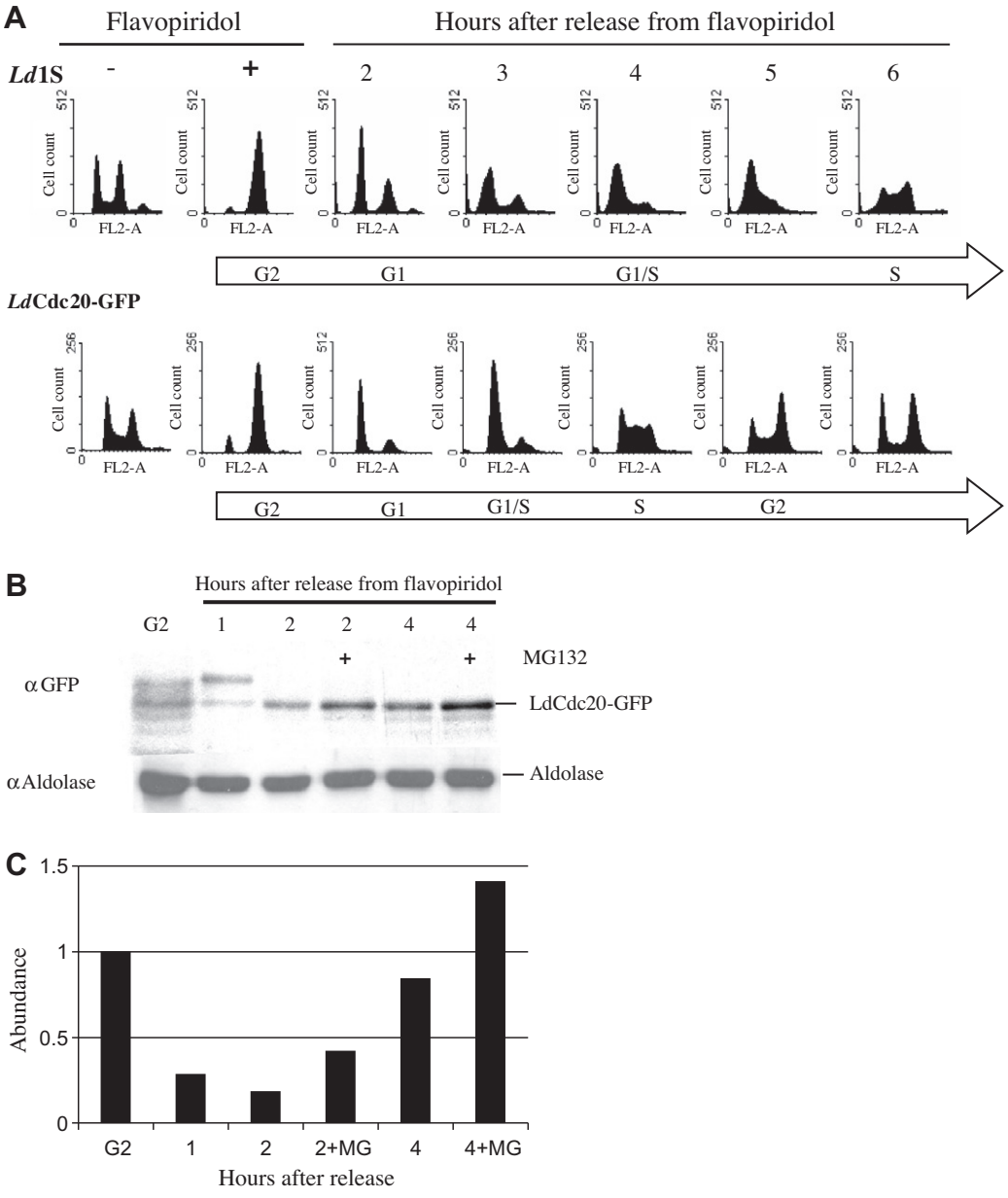

Fig. 3. *Ld*Cdc20–GFP exhibits cyclic expression. WT promastigotes (*Ld*1s) and promastigotes expressing *Ld*Cdc20–GFP were blocked at G2/M using flavopiridol (5 μM) and released into fresh medium. Samples were collected each hour for 4 h after release (as indicated) and subjected to (A) FACS or (B) Western analysis. Where indicated, samples were incubated for 1 h with the proteosome inhibitor MG-123 (50 μM) before Western analysis. (C) The relative density of each band. *Relative intensity* = the density of each LdCdc20 band divided by the density of its corresponding aldolase band. Graphs and blot represent 1 of 3 independent experiments.

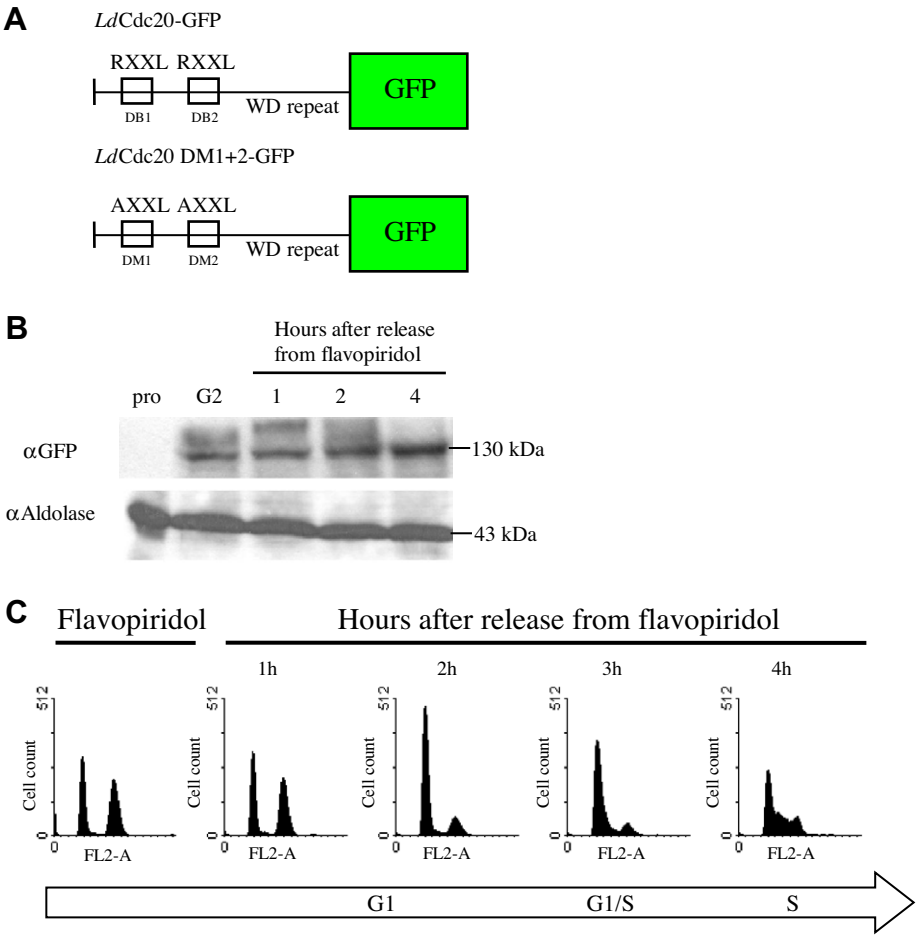

Fig. 4. *Ld*Cdc20p contains a functional RXXL destruction box motif. (A) A schematic view of *Ld*Cdc20–GFP and *Ld*Cdc20–DM1+2–GFP. (B, C) *Ld*Cdc20–DM1+2–GFP expressing cells were blocked at G2/M using flavopiridol (5 μM) and released into fresh medium. Samples were collected and subjected to (B) FACS or (C) Western analysis at the indicated times after release.

3.3. LdCdc20p possesses a functional RXXL destruction box motif

As described in the introduction, in higher eukaryotes Cdc20p is directed to proteasomal degradation by ubiquitination mediated by the APC/C complex. This ubiquitination occurs within a unique sequence termed the destruction box motif. Given that *Ld*Cdc20p displays cyclic expression (Fig. 3B), we searched for potential destruction box motifs (RXXL) within its amino acid sequence and identified two: one at 305-**R**S**LL**-308 and the second at 360-**R**VP**L**-363 (Fig. 4A).

To investigate if these potential destruction box motifs are functional, we performed site directed mutagenesis and examined the expression of the mutant proteins during the cell cycle and any effect on cell cycle progression. Specifically, the cationic arginine in one (R305A) or both (R305A and R360A) motifs was replaced with alanine to generate single (DM1) and double (DM1+2) mutant *LdCdc20–GFP* genes. Transfected parasites were synchronized using flavopiridol and subsequently, samples taken and subjected to Western blot or FACS analysis at the indicated time points following release from growth arrest. Notably, promastigotes expressing the doubly mutated *Ld*Cdc20–DM1+2–GFP were less sensitive to flavopiridol than wild type (Fig. 4C), such that after 16 h of exposure only half the cells were in G2, with the other half in G1. Nevertheless, once released these transgenic cells progressed rapidly through the cell cycle in a similar manner to promastigotes expressing wild type *Ld*Cdc20–GFP (compare with Fig. 3A). Importantly, in line with the putative motifs serving as functional destruction boxes, levels of the doubly mutated LdCdc20–DM1+2–GFP were high at the G2/M block and remained stable after release (Fig. 4B). In contrast, the singly mutated *Ld*Cdc20–DM1–GFP exhibited a cell cycle expression pattern indistinguishable from wild type *Ld*Cdc20–GFP (data not shown). One interpretation of these data is that LdCdc20p contains at least one functional destruction box DM2, suggesting that this protein is playing a regulatory cell cycle role.

4. Discussion

Ubiquitin-dependent proteasomal degradation mediated by the APC/C machinery is highly conserved among eukaryotes. Before the present study, this pathway had not been observed in *Leishmania* parasites. Cdc20 is an activator of APC/C during mitosis in higher eukaryotes. Here, we identify a Cdc20 homologue in *L. donovani* (LdCdc20, LinJ24_V3.1790) and establish its functionality. Firstly, despite the evolutionary distance between yeast and *Leishmania*, *Ld*Cdc20 complements a lack of *Sc*Cdc20 in *S. cerevisiae* yeast cells. Secondly, antibodies against ScCdc20 cross reacted with LdCdc20 protein. We noticed that the level of ScCdc20 may vary in cells that express large amounts of Cdc20 likely due to less efficient activity of the proteasome that could be manifested in higher levels of proteasome substrate. Despite the high levels of total Cdc20 protein cells were viable and most importantly, *CDC20* gene was silenced upon transferring the cells to glucose medium,

suggestion that the transfection did not alter the gene regulation. Thirdly, *Ld*Cdc20–GFP overexpression in promastigotes accelerates the cell cycle. Finally, as expected of a functional Cdc20 homologue, *Ld*Cdc20p exhibits cyclic expression and accordingly, was found to contain an active RXXL destruction box motif, the latter a feature of proteins targeted for destruction by APC/C. Taken together, these data support that the essential cell cycle role and basic mechanistic motifs of Cdc20 are extant in *Leishmania* parasites and therefore must have appeared early in the evolution of eukaryotes.

Our data supports the premise that the proteasome degradation pathway regulates *Ld*Cdc20 abundance. Namely, we show that exposure to the proteasome inhibitor MG132 causes *Ld*Cdc20p to accumulate in parasites and evidence that *Ld*Cdc20p contains a functionally active destruction box motif. Typically, Cdc20 proteins are degraded at early mitosis. We found that LdCdc20p levels were minimal during G2/M until mid G1 and that this protein accumulates at G1. A high molecular variant of LdCdc20–GFP appeared 1 h after the release from G/M arrest, which might represent a modified variant of LdCdc20–GFP. We suggest that this modification represent ubiquitination that was visible only 1 h after the release due to the experiment settings. Thus, it is likely that during that first hour the proteasome is not fully active or overloaded with substrates that accumulated during flavopiridol treatment, enabling visualization of an unstable ubiquitine modified LdCdc20–GFP variant.

We suspect that the timing of LdCdc20p degradation is less rigid because *Leishmania* lack a stringent mitosis checkpoint. Indeed, *L. donovani* do not have a stable genome and chromosome loss or duplication events are not rare [27]. In line with this, we observed cells with 4 N DNA content, and sometimes even more than 4 N, in populations of logarithmic phase promastigotes (data not shown).

*Ld*Cdc20 overexpression resulted in the promastigotes progressing more quickly through the cell cycle suggesting that high LdCdc20p levels override mitotic and even G1/S checkpoints. Based on this observation and our analyses indicating that the *Leishmania* genome does not contain a Cdh1 gene homologue, we conjecture that *Ld*Cdc20 plays a dual role in controlling both mitosis and G1 phases. In higher eukaryotes Cdh1 has an important role in G1 phase; Cdh1 facilitates degradation of specific G1 substrates and its own degradation at the G1/S boundary is a checkpoint marker [28]. We predict that endogenous *Ld*Cdc20p, as observed for *Ld*Cdc20–GFP, exhibits low levels at G2/M and accumulates at the G1/S checkpoint, and that enables normal S phase entry. Summarily, our data hint at the possibility *Ld*Cdc20 serves as the functional homologue of both Cdc20 and Cdh1 from higher eukaryotes, and therefore expression of ectopic LdCdc20–GFP resulted in accelerated cell cycle progression.

We evidence that *Ld*Cdc20 contains a conserved RXXL destruction box motif at amino acids 360–363 that is functional. This is a destruction motif known to be targeted by the APC/C complex. Van Hellemond et al [29] reported that *T. brucei* cyclin B1 homologues contain putative destruction box motifs but the functionality of these remains to be demonstrated. Our data establishing, for the first time, the functionality of a destruction motif in a member of the *Trypanosometidae* family reveals that APC/C-mediated cell cycle regulation appeared early during the evolution of eukaryotes.

Cell cycle research in *Typanosomatids* is an emerging field, its rapid growth enabled by the extensive knowledge gathered from studying higher eukaryotes. Much of the existing data concerning *Typanosomatids* is from RNAi knock down studies using *T. brucei* [30] but only a few regulatory proteins have been identified using this approach. Although *T. brucei* is close evolutionarily to *L. donovani* there are obvious differences in the life cycle and specializations of each parasite, therefore, more in depth studies must be carried out in each parasite. Better understanding of the cell cycle in *Typanosomatids* will not only expand our appreciation of the parasitic nature of these organisms but also could promote development of new therapies to manage associated human diseases, such as kala azar.

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