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GlSir2.1 of *Giardia lamblia* is a NAD^+-dependent cytoplasmic deacetylase

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Abstract

The sirtuins are a group of well-conserved proteins widely distributed across all domains of life. These proteins are clustered in the class III of histone deacetylases and are distinctly characterized by their dependence upon NAD^+ to carry out the deacetylation of lysine residues in histone proteins (H3 and H4) and non-histones such as the transcription factor p53. The requirement of NAD^+ for sirtuin activity makes this group of proteins metabolic sensors, which are favored during caloric stress. Currently, it is known that these proteins are involved in numerous cellular processes that are fundamental for the proper functioning of cells, including control of the cell cycle and cellular survival. In spite of the importance of sirtuins in cell functions, the role that these proteins play in protozoan parasites is not completely understood. In this study, bioinformatic modeling and experimental characterization of the candidate G1Sir2.1 present in the genome of *Giardia lamblia* were carried out. Consequently, cloning, expression, purification, and *in vitro* evaluation of the recombinant GlSir2.1 protein's capacity for deacetylation were performed. This allowed for the identification of the NAD^+-dependent deacetylase activity of the

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identified candidate. Production of anti-rHis-GlSir2.1 polyclonal antibodies enabled the observation of a cytoplasmic localization for the endogenous protein in trophozoites, which exhibited a perinuclear aggregation and co-localization with acetylated cytoskeleton structures such as the flagella and median body. Currently, GlSir2.1 is the second sirtuin family member identified in *G. lambia*, with a demonstrated cytoplasmic localization in the parasite.

Keywords: Biochemistry, Bioinformatics, Cell biology, Molecular biology

1. Introduction

Giardia lamblia is an intestinal protozoan parasite in humans that is responsible for Giardiasis, one of the most common gastrointestinal illness in the world, which is characterized by the presence of diarrhoea, epigastric pain, nausea, vomiting, and weight loss (Ankarklev et al., 2010). It is estimated that this illness affects approximately 280 million people worldwide per year, especially children (Prucca & Lujan, 2009). *Giardia* is also considered an organism that underwent evolutionary divergence very early from the eukaryotic lineage. This makes it of great medical importance, as it is considered an excellent cellular model due to its metabolic simplicity, especially for the study of metabolic eukaryotic evolution (Gillin et al., 1996).

The NAD^+-dependent histone deacetylases, also known as sirtuins, are a family of proteins that are well conserved and widely distributed in nature, and they are present in the three domains of life: Archaea, Bacteria and Eukarya. This family includes proteins related to the silent information regulator 2 (SIR2), from which the family name comes and which was initially identified in yeast *(Saccharomyces cerevisiae)* (Michan and Sinclair, 2007). The sirtuins are clustered together in class III of the histone deacetylases (HDACs) and are responsible for the cleavage of acetyl groups in lysine residues on the N-terminal tails of histones and for the deacetylation of other non-histone proteins such as enzymes and transcription factors (Voelter-Mahlknecht and Mahlknecht, 2006). The distinctive characteristic and significant difference of sirtuins with respect to other HDACs is their dependence on nicotinamide adenine dinucleotide (NAD^+) to carry out its catalytic activity, which leads to the deacetylation of lysine residues and the generation of nicotinamide and O-acetyl-ADP-ribose (Greiss and Gartner, 2009).

The sirtuin family is subdivided into five classes (I, II, III, IV and U); classes I-IV correspond to eukaryotic sirtuins, while class U groups all prokaryotic sirtuins (Religa and Waters, 2012). In humans, 7 sirtuins (SIRT1-7) have been identified and feature a conserved sirtuin domain consisting of approximately 250 amino acids (Greiss and Gartner, 2009). However, their subcellular localization varies. Of these

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proteins, three are nuclear (SIRT1, SIRT6 and SIRT7), three are mitochondrial (SIRT3, SIRT4 and SIRT5) and the remaining one (SIRT2) is predominantly cytoplasmic. Presently, it is known that these proteins are involved in numerous and diverse cellular and metabolic processes that depend on the subcellular localization of the sirtuin and that are fundamental for proper cell function. These include metabolic regulation and homeostasis (Yu and Auwerx, 2009); transcriptional silencing; apoptosis; chromosome segregation; microtubule organization; genome stability; DNA repair (Religa and Waters, 2012); autophagy modulation (Ng and Tang, 2013), and progression of the cell cycle (Z. Wang and Sun, 2010). Dependence on NAD^+ for sirtuin deacetylase activity turns the sirtuins into metabolic state sensors and makes them the link between the cell's nutritional state and the post-translational regulation of metabolic effectors and gene expression (Z. Wang and Sun, 2010).

The role that these proteins play in protozoan parasites is a poorly explored field to date, with the exception of a few sirtuins of *Plasmodium, Trypanosoma, Leishmania* and *Entamoeba*. Of these sirtuins, it is known that the PfSIR2A of *P. falciparum* is involved in the transcriptional silencing of subtelomeric regions, which encode antigenic variants used by the parasite to avoid the host's immune system and are widely regulated by sirtuin activity. TbSir2rp1 of *T. brucei* is localized in the nucleus and utilizes the histones H2A and H2B as substrates, being important for DNA repair in this parasite (Zheng, 2013). Additionally, the sirtuin of *L. infantum* (LiSIR2rp1) is the first sirtuin that shows ADP-ribosyl transferase activity on α-tubulin, which causes the depolymerization and inhibition of the assembly of microtubules and produces deacetylated α-tubulin (Sereno et al., 2008)' (Religa and Waters, 2012). Therefore, it may be involved in the regulation of cell cycle progression. On the other hand, EhSir2a of *E. histolytica* is involved in tubulin deacetylation, regulating microtubules assembly during the cellular cycle (Dam and Lohia, 2010), as it happens with LiSIR2rp1. These diverse functions and roles in cellular processes make sirtuins interesting pharmacological targets for the control of protozoan parasites of medical importance such as *G. lamblia* and those mentioned before.

In regard to sirtuins of *G. lamblia*, five candidate sequences for the family have been reported by bioinformatic studies (Religa and Waters, 2012). Recently, the candidate GlSir2.2 (GL50803_10707) demonstrated NAD^+-dependent deacetylase activity and a nuclear localization in the parasite (Wang et al., 2016). This study reports the GlSir2.1 (GL50803_10708) functional identification and characterization as a NAD+-dependent deacetylase, with a typical cytoplasmic localization and a structural resemblance to human SIRT2. Therefore, it becomes the second member of the sirtuin family identified to date in *G. lamblia*.

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2. Materials & methods

2.1. Bioinformatic approach

The amino acid (aa) sequence of the GlSir2.1 candidate with the accession number GL50803_10708 (www.giardiadb.org) was employed in multiple alignments with sequences from human SIRT1-7 (Uniprot access: Q96EB6; Q8IXJ6; Q9NTG7; Q9Y6E7; Q9NXA8; Q86T7; Q9NRC8) and protozoan eukaryotes: *T. brucei* TbSir2rp1 (Q57V41), *P. falciparum* PfSIR2A (Q8IE47) and *L. infantum* LiSIR2rp1 (Q8I6E4). The multiple alignments construction and visualization were completed with the T-COFFEE (Tommaso et al., 2011) server and the Geneious (Kearse et al., 2012) software (version 6.1.6), respectively. Protein tertiary structures were predicted with the I-TASSER(Zhang, 2008) server. The predictive model was validated by means of a Ramachandran plot (Lovell et al., 2003). The visualization and comparison of structures were conducted with the UCSF Chimera (Pettersen et al., 2004) software (version 1.8.1).

2.2. Experimental approach

2.2.1. Cloning of GlSir2.1 of G. lamblia

The *GlSir2.1* fragment was amplified by PCR(Gibbs, 1990) with the enzyme *Pfu DNA polymerase* (Fermentas, USA), employing 100 ng of genomic DNA (gDNA) as a template of the WB strain of *G. lamblia* (WB clone 6) and the following primers: Forward, 5′-**CACC**ATGCCTGCTAATTAT-3′ and reverse, 5′-TCATTTACCTGAAGGCTC-3′. An initial denaturation of 95 °C was applied for 7 min, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 50 °C for 45 s and extension at 72 °C for 1.3 min. A final extension was performed for 7 min at 50 °C. The amplification product was cloned into the pET100/D-TOPO vector (Invitrogen), and the corresponding insert was verified by sequencing.

2.2.2. Expression and purification of the recombinant protein rHis-GlSir2.1

The recombinant plasmid pET100-GlSir2.1 was used to transform *E. coli* BL21 (DE3) cells (Invitrogen). The resultant clones were inoculated in LB liquid media supplemented with 100 μg/mL of ampicillin and 1 % (w/v) glucose until an OD 600 nm of 0.6. Protein expression was induced with IPTG (Isopropyl β-D-1-thiogalactopyranoside, Thermo Scientific) at a final concentration of 0.3 mM for 4 h at 18 °C. The cells were collected by centrifugation at 6000 rpm for 10 min and lysed by sonication in lysis buffer (50 mM NaH_2PO_4 pH 8.0, 400 mM NaCl, 100 mM KCl, 10% (v/v) glycerol, 0.5% (v/v) Triton X-100, 10 mM imidazole) supplemented with lysozyme (1 mg/mL) and without protease inhibitors. The soluble fraction was subjected to immobilized metal affinity chromatography (IMAC)

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employing the nickel nitrilotriacetic acid resin pre-equilibrated in lysis buffer (Qiagen). The resin was washed with lysis buffer supplemented with varying concentrations of imidazole (35, 75 or 150 mM). Protein elution was completed with the same buffer containing 300 mM imidazole. The expression and purification of the recombinant protein was evaluated by SDS-PAGE (Roy & Kumar, 2014) and western blot (Mahmood and Yang, 2012) employing the anti-6xHis monoclonal antibodies (Abcam, ab5000).

2.2.3. Immunodetection of rHis-GlSir2.1 by western blot

The samples were separated by SDS−PAGE and electro-transferred to a PVDF membrane (Millipore) by application of a 200 mA current for 2 h in transfer buffer (0.2 M glycine, 10 mM Tris/HCl pH 8.0, 10% (v/v) methanol). The membrane was blocked overnight with a solution containing 5% (w/v) skimmed milk in TBS (150 mM NaCl, 20 mM Tris/HCl pH 7.5). The immunodetection of recombinant proteins was performed using the primary anti-6xHis monoclonal antibodies (1:5000), the secondary anti-Mouse IgG antibodies conjugated to biotin (Sigma, B7264) (1:8000) in TBS and phosphatase alkaline conjugated to streptavidin (Promega) (1:3000) in TBS. NBT and BCIP (Promega) were used as substrates.

2.2.4. In vitro deacetylation assays

The NAD^+-dependent deacetylase capacity of the rHis-GlSir2 protein was evaluated with the commercial SIRT2 Deacetylase Fluorometric Assay (Cyclex) kit according to the manufacturer's instructions, employing 250 ng of recombinant protein with or without 0.8 mM of NAD^+. The assay was carried out in triplicate in a final reaction's volume of 25 μL. The excitation and fluorescence reading emission wavelengths were 488 and 521 nm, respectively. For fluorescence's detection, the *CFX96* Touch Real-Time PCR Detection System (BioRad) was used.

2.2.5. Polyclonal avian antibody production (IgYs)

To collect anti-rHis-GlSir2.1 IgYs from 19-month-old Babcock Brown hens, four inoculations were carried out with 150 ng of recombinant protein rHis-GlSir2.1 purified through inclusion bodies, which were obtained as follows (Niño et al., 2015): overexpression of the recombinant protein was induced in an *E. coli* strain BL21 (DE3) as mentioned before. The cell pellet was recovered by centrifugation at 6000 rpm for 10 min. Cell lysis was performed following the previously described procedure. Purification of inclusion bodies using denaturing agents followed a previously reported method (Sánchez-Lancheros et al., 2016). In addition, purified samples were run in a preparative 16 × 19 cm 10% SDS-PAGE. Guided by a pre-stained molecular weight marker, the band containing the protein of interest was identified and excised. The band was pulverized, and the recombinant rHis-GlSir2.1 antigen

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was eluted with deionized water at 37 °C under constant stirring. Protein content in the supernatant was quantified with the Bradford method. Antigen purity was assessed by 10% SDS-PAGE. The first inoculation consisted of a homogeneous mixture of antigen and Freund's complete adjuvant, while the three booster injections were homogeneous mixtures of antigen and incomplete Freund's adjuvant. Upon finalization of the immunization, the sera and avian antibodies (IgY) were purified through affinity chromatography employing the recombinant protein in nitrocellulose membranes (Smith and Fisher, 1984).

2.2.6. Cell culture and immunofluorescence

With the objective of determining the localization of the endogenous GlSir2.1 protein, trophozoites of *G. lamblia* WB strain were cultured in TYI- S-33 medium supplemented with 10% (v/v) bovine serum and 0.5 mg/mL bovine bile (Keister, 1983). The cells obtained by centrifugation were washed 3 times with PBS. The obtained cell pellet was resuspended in PBS (1500 parasites/μL), and the cells were attached to the surface of a glass slide (1 h at 37 °C). The cells were fixed with 4% (w/v) paraformaldehyde, blocked and permeabilized with 0.5% (v/v) Triton X-100 and 2% (w/v) BSA in PBS overnight at 4 °C. The fixed and permeabilized cells were incubated with the primary antibody, corresponding to the 1:25 dilution of the anti-rHis-GlSir2.1 sera (IgY) or unrelated serum and pre-immune serum (negative controls), and the anti-acetylated tubulin mouse-IgG (Sigma, T6793) monoclonal antibodies (1:1000) in PBS, for 3 h at 37 °C. Later the samples were incubated with the secondary anti-IgY conjugated to FITC antibodies (1:200) (Sigma, F4137) and the anti-IgG conjugated to Alexa 494 antibodies (1:1000) (Invitrogen, A11001) in PBS for 2 h in darkness at 37 °C. The nuclei were stained with 1 μg/μL 4′,6-diamidino-2-phenylindole (DAPI) (Tovar et al., 2003). The images were observed and recorded with a Nikon C1 fluorescence microscope plus confocal Nikon C1 Plus ECLIPSE Ti and were analyzed with the NIS Elements AR software.

3. Results

3.1. GlSir2.1 of *G. lamblia* shows typical structural characteristics of the sirtuin family

The bioinformatic search for putative sirtuin sequences in the *Giardia lamblia* genome identified G1Sir2.1 as a sirtuin candidate, with a corresponding sequence of 2043 bp, which encodes a putative protein of 680 aa with an estimated molecular weight of 74.6 kDa. The aa sequence analysis of GlSir2.1 through the CDD server (Marchler-Bauer et al., 2015) predicts the presence of a domain of approximately 270 aa localized at the N-terminal region of the candidate's primary structure that is conserved within the sirtuin family (Religa and Waters, 2012). Through

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this server, the following was predicted in this domain: binding sites for an acetylated peptide, NAD^+ and zinc. The multiple sequence alignment between sirtuins from human (SIRT1-7) and protozoan parasites (TbSir2rp1, PfSIR2A and LiSIR2rp1) with the Sir2 domain present in the candidate GlSir2.1 (aa 8−243) allowed for identification of conserved sequences in the GlSir2.1 protein, some of which are known to be involved in NAD^+ binding, such as the G-X-G motif, which is important for the interaction of the ribose groups of this molecule and sirtuin (Parenti et al., 2015). Another of the observed characteristics in the candidate is the presence of four conserved cysteine residues involved in zinc binding (motif: Cys-X_{2-4}-Cys-X_{15-40}-CysX_{2-4}-Cys) in this family of proteins (Moniot et al., 2012) (Fig. 1).

The tertiary structure prediction and the identification of structural domains in the putative protein, were achieved by structural analysis with the I-TASSER server and the GlSir2.1 N-terminal domain (aa 8−243). The quality of the model was validated by a Ramachandran Plot employing RAMPAGE (Lovell et al., 2003) and the RMSD and C-score (I-TASSER) values (Fig. 2). The obtained model shows two structural domains typical of the Sir2 family (Fig. 2A). The large domain is characterized by the presence of two Rossmann folds, which are present in NAD^+ binding proteins (Sanders et al., 2010), (North and Verdin, 2004). Additionally, the small domain is characterized by the presence of four cysteine residues involved in zinc

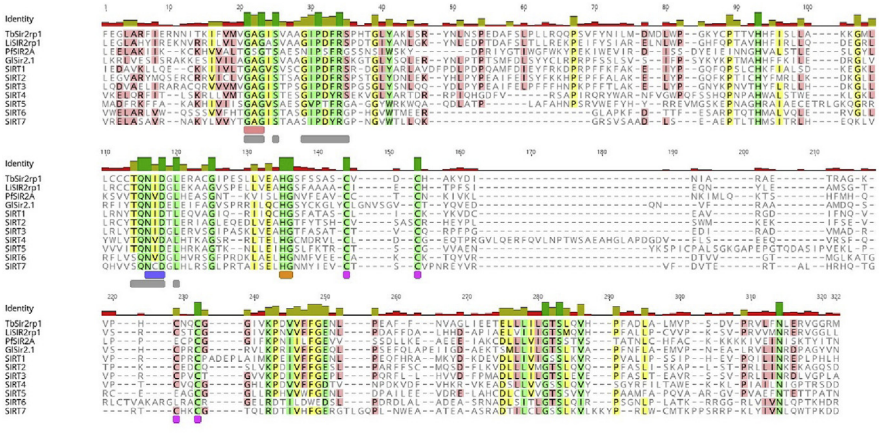

Fig. 1. The primary structure of the GlSir2.1 protein shows conserved characteristics of the sirtuin family. Multiple sequence alignment of GlSir2.1 N-terminal domain with human sirtuins (SIRT1-7), TbSir2rp1 (*T. brucei*), PfSIR2A (*P. falciparum*), and LiSIR2rp1 (*L. infantum*). The highly conserved predicted NAD^+ binding residues are in grey. Peach and blue: NAD^+ binding GAG and NVD motifs. Orange: HG motif. Pink: conserved zinc binding cysteine residues. The percentage of identity among sequences is proportional to the height of the bars. Color; Green: 100% similarity. Yellow: 80−90% similarity. Pink: 60−80% similarity. Without color: less of 60% similarity. The multiple sequence alignment was generated with T-COFFEE (Tommaso et al., 2011) and visualized using Geneious version 6.1.6 (Kearse et al., 2012).

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binding (North and Verdin, 2004). A hydrophobic pocket is located between these two domains, in which the substrates combine (NAD^+ and acetylated peptide) and the process of deacetylation is carried out (Fig. 2A and B) (Greiss and Gartner, 2009; Moniot et al., 2012; Religa and Waters, 2012), (Avalos et al., 2002).

To continue the bioinformatic characterization, an overlay of the candidate's structure on the human SIRT2's (PDB 1J8F) structure was carried out. This analysis enabled the identification of an elevated structural similarity between these proteins (Fig. 2C), which have a 34% identity at the primary structure level. The structural conservancy among sirtuins is a phenomenon for which the family is known (Sanders et al., 2010) and is consistent with the percentages of identity shown in this study.

3.2. GlSir2.1 of *Giardia lamblia* is an NAD^+-dependent deacetylase

The candidate's (GL50803_10708) open reading frame (ORF) was amplified by PCR from gDNA (Fig. 3A) and inserted into the pET100/D-TOPO vector. The recombinant vector (pET100-GlSir2.1) was transformed into *E. coli* BL21 (DE3) cells, which were induced with IPTG. The expression of the recombinant protein (rHis-GlSir2.1) was monitored by SDS-PAGE and western blot. Fig. 3B shows the differential expression of a band of approximately 70 kDa in total protein extracts in the induced and uninduced BL21 (DE3)-GlSir2.1 cells. The evaluation by western blot employing antibodies against the 6xHis tag present at the protein N-terminal, revealed that the band corresponds effectively to the rHis-GlSir2.1 protein (Fig. 3C), which was found, additionally, to be present in the soluble fraction

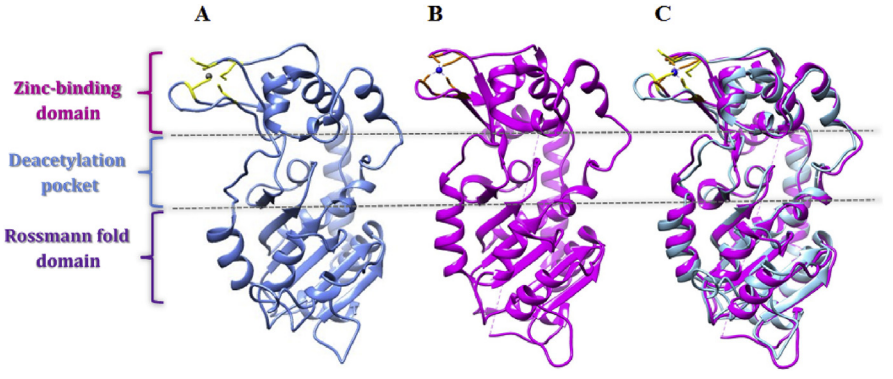

Fig. 2. The GlSir2.1 protein shows predicted structural characteristics of the sirtuin family. A: predicted structure of *G. lamblia* GlSir2.1 (aa 8−243), the C-terminal region was not modeled (C-score: -5 < 0.10 < 2, TM-score: 0.73 ± 0.11> 0.5, RMSD: 6.1 ± 3.8Å). Zinc-binding cysteine residues: yellow. Zinc: gray sphere. B: human SIRT2 tertiary structure (PDB 1J8F). Zinc-binding cysteine residues: orange. Zinc: blue sphere. C: overlap of the GlSir2.1 model with the tertiary structure of the human SIRT2 (PBD 1J8F) (RMSD between 251 atom pairs: 0.775). Images generated with UCSF Chimera (Pettersen et al., 2004).

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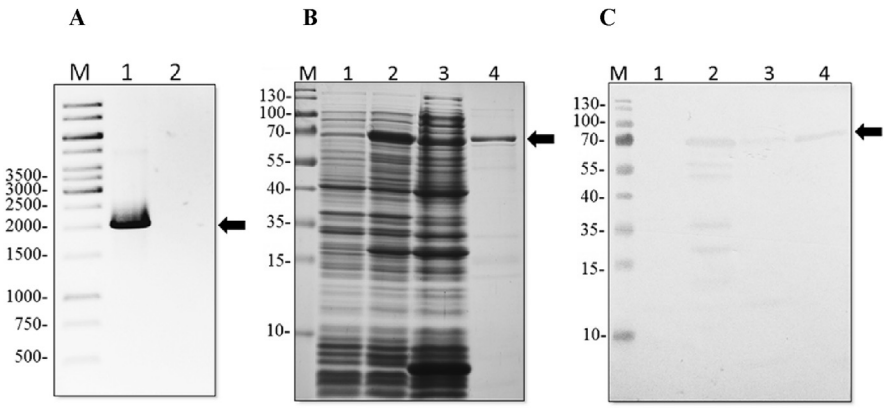

Fig. 3. The recombinant rHis-GlSir2.1 protein was successfully expressed and purified through IMAC. A: ORF amplification of *GlSir2.1* from genomic DNA (1), H_2O was used as negative control (2), Molecular weight marker, in base pairs (M). Agarose-TBE 1% (w/v). B-C: The soluble fraction of the BL21 (DE3) rHis-GlSir2.1 clones were subjected to IMAC. B: SDS-PAGE (10%) stained with Coomassie R250. C: Western blot using the anti-6xHis primary antibodies. Noninduced control cells (1). Induced rHis-GlSir2.1 cells (2). Soluble fraction of induced cells (3). rHis-GlSir2.1 eluate at 300 mM imidazole (4). Pre-stained molecular weight marker, in kDa (M). Black arrow indicates the ORF amplification or the rHis-GlSir2.1 protein.

(Fig. 3B and C line 3). Once the expression of the rHis-GlSir2.1 protein was obtained and verified, the soluble fraction was used to purify the recombinant protein by IMAC, which allowed the homogenous purification of the native rHis-GlSir2.1 protein (Fig. 3B and C line 4).

The determination of the NAD^+-dependent deacetylase capacity of the rHis-GlSir2.1 purified protein, was conducted with the SIRT2 Deacetylase Fluorometric Assay (Cyclex) kit. Excitation at 488 nm with fluorescence emission at 521 nm was used to measure the deacetylase activity of the evaluated protein. Fig. 4 shows the fluorescence emission obtained upon evaluation of 250 ng of the rHis-GlSir2.1 protein in the presence or absence of NAD^+. The results indicate that the recombinant protein is able to deacetylate the peptide substrate in the presence of NAD^+ (Fig. 4 line 3). However, in the absence of this substrate, its deacetylase capability is abolished (Fig. 4 line 2). Based on these results, it was concluded that the GlSir2.1 protein of *G. lamblia* belongs to the class III NAD^+-dependent histone deacetylases, also known as sirtuins.

3.3. GlSir2.1 of *G. lamblia* is a cytoplasmic protein

To determine the localization of the endogenous protein, polyclonal avian antibodies were developed. The anti-rHis-GlSir2.1 IgY polyclonal antibodies were used to carry out a western blot on BSA and the recombinant rHis-GlSir2.1 protein to confirm the specificity of the antibodies, which detected solely the recombinant protein, as expected (Fig. 5A). Then, the endogenous GlSir2.1 protein (74 kDa) was

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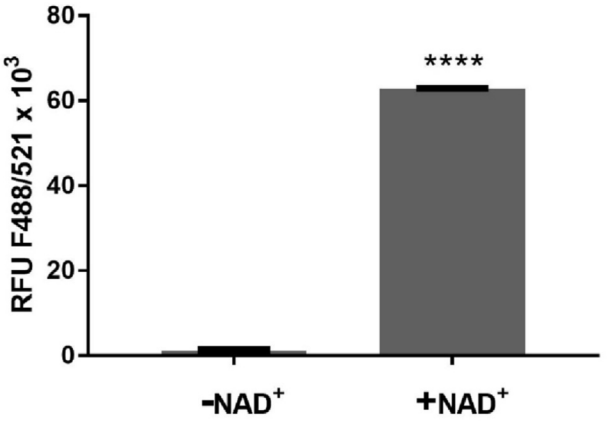

Fig. 4. The recombinant rHis-GlSir2.1 protein is a NAD^+-dependent deacetylase. The recombinant rHis-GlSir2.1 protein (250 ng) is able to carry out the deacetylation of the acetylated substrate in the presence of 0.8 mM NAD^+. Without NAD^+ the deacetylase capacity of the recombinant rHis-GlSir2.1 protein is significantly abolished ($p < 0.0001$). Asterisks indicate statistical difference. The assay was performed in triplicate.

immunodetected on soluble protein extracts from the parasite (Fig. 5B). In order to determine the localization of the GlSir2.1 protein, indirect immunofluorescence assay on fixed trophozoites of *G. lamblia* was conducted. This assay enabled the detection of a main signal in the cytoplasm and the perinuclear region of the parasite, with a possible association with cytoskeleton structures such as the flagella and the median body, which are rich in acetylated tubulin in *Giardia,* (Soltys and Gupta, 1994)' (Dawson, 2010) as it is showed through the use of antibodies against acetylated tubulin (Fig. 5C, lower panel).

4. Discussion

Sirtuins have been shown to be key mediators of the cell's energetic metabolic state and transcriptional regulation due to their direct deacetylase action on histones and activity on transcription factors such as $p53^{36}$, regulating cellular responses to caloric (Radak et al., 2013) and oxidative stress (Tong and Denu, 2010). The participation of sirtuins in the metabolism of protozoan parasites such as *P. falciparum*, *L. infantum, E. histolytica* and *T. brucei*, where they are found to be involved in processes such as the regulation of the expression of proteins involved in the evasion of the host's immune system, the regulation of the cytoskeleton by deacetylation of α-tubulin, and the repair of DNA damage, has allowed this group of proteins to be proposed as excellent anti-parasitic targets (Zheng, 2013).

In *G. lamblia*, bioinformatic studies have allowed the identification of five candidate sequences to NAD^+-dependent histone deacetylases in the genome of the parasite (Religa and Waters, 2012). Of these candidates, it was recently demonstrated that

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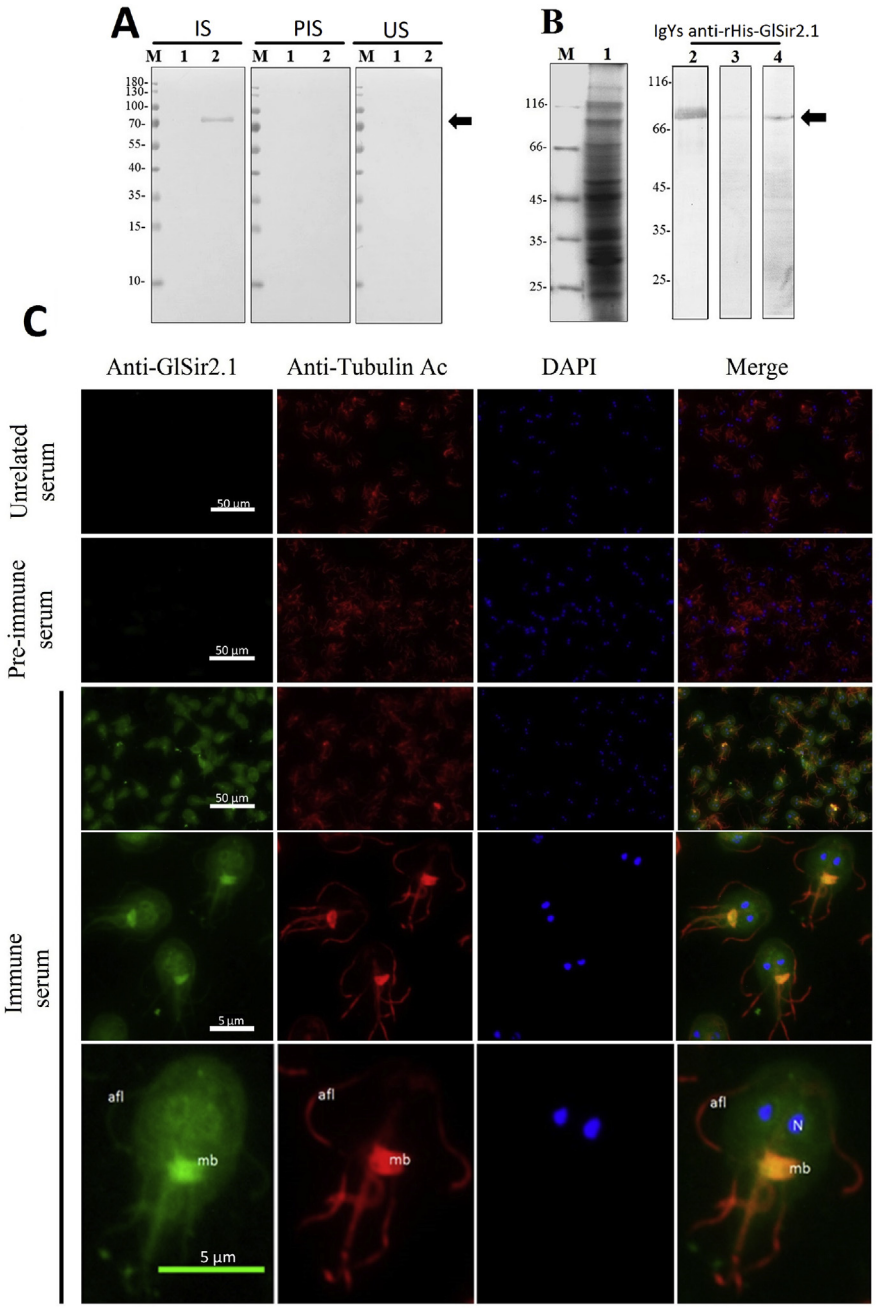

Fig. 5. GlSir2.1 of *G. lamblia* is a cytoplasmic protein. A: Evaluation of the obtained serums against 100 ng of the recombinant protein rHis-GlSir2.1 and 100 ng of BSA. IS; immune serum anti-rHis-GlSir2.1, dilution 1/5000. PIS: pre-immune serum, dilution 1:5000. US: unrelated serum, dilution 1:5000. (1); 100 ng of BSA. (2); 100 ng of rHis-GlSir2.1. (M); Pre-stained molecular weight marker, in kDa. B: Detection of the endogenous *Giardia lamblia* Sir2.1. SDS-PAGE- Coomassie. (M); Molecular weight marker, in kDa. (1); Total protein extracts of *Giardia* trophozoites (1×10^6 cells). Western blot. (2); 50 ng of rHis-GlSir2.1, dilution 1:250 of IgYs anti-rHis-GlSir2.1. (3−4); total protein extracts (1×10^6 cells), dilution 1:500 and 1:250 of IgYs anti-rHis-GlSir2.1. Black arrow indicates the rHis-GlSir2.1 or the endogenous protein. C: The unrelated, pre-immune and immune serums were employed in an indirect immunofluorescence assay with *G. lamblia* trophozoites. Green: endogenous GlSir2.1 localization. Red: acetylated alpha tubulin localization. Blue: nucleus localization (DAPI). White bars: 50 µm. Green bar: 5 µm. afl: anterior flagella. mb: Median body.

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GlSir2.2, a homologue of human SIRT1, is a protein with NAD^+-dependent deacetylase activity and a nuclear localization (Y.-H. Wang et al., 2016). In the present study, Glsir2.1 was identified as the second member of the sirtuin family in *G. lamblia*, which is structurally related to human SIRT2 and exhibits a predominantly cytoplasmic localization (Fig. 5). The NAD^+-dependent deacetylase activities of the remainder candidates are yet to be determined.

In regard to metabolic and cellular functions in *G. lamblia*, a recent study focused on determining the role of histone acetylation states in the encystation process. It showed through overexpression assays that none of the 5 mentioned sirtuin candidates, including GlSir2.1 and GlSir2.2, were found to be directly involved in the encystation of the parasite, which seems to be regulated by classical non-NAD^+-dependent histone deacetylases (Carranza et al., 2016). Additionally, the overexpression of GlSir2.1 is consistent with our results regarding the localization of the endogenous protein, which indicated a cytoplasmic distribution of GlSir2.1 in the trophozoites. Nevertheless, our results suggest a possible association between GlSir2.1 and cytoskeleton structures, such as the flagella and median body in *Giardia*. The latter has been proposed as a reservoir of tubulin, which can be important for the cell cycle. However, its cellular function has not been completely clarified (Dawson, 2010).

Our results, combined with those found for GlSir2.2(Y.-H. Wang et al., 2016), indicate the presence of multiple functional sirtuins in *G. lamblia*, which can display different metabolic functions in the parasite due to their differential localization (cytoplasmic Glsir2.1 and nuclear GlSir2.2(Y.-H. Wang et al., 2016)), such as is the case of human sirtuins. For instance, SIRT1 is a nuclear protein with a role in DNA repair, inhibition of apoptosis and cellular survival. Meanwhile, SIRT2 is a typically cytoplasmic protein involved in the deacetylation of α-tubulin (Baharia et al., 2015)' (North et al., 2003), regulation of the cytoskeleton and control of the cell cycle (Inoue et al., 2007). The structural similarity between GlSir2.1 and human SIRT2 (Fig. 2C) and its cytoplasmic localization let us postulate that, like SIRT2, GlSir2.1 could be involved in processes such as cytoskeleton regulation in the parasite (deacetylation of tubulin). In fact, it has been recently showed that KH-TFMDI, a class III histone deacetylase inhibitor, decreases the trophozoite growth and changes cell organization of *G. intestinalis* by altering its cytoskeleton structure (Huber et al., 2019). Along with our results, this evidence suggests a regulatory function of sirtuins on Giardia's cell structure, supporting the conceivable action of GlSir2.1 on tubulin acetylation according to the immunofluorescence analysis of the present study. The presence of sirtuins associated with cytoskeleton and tubulin in *L. infantum* (LiSIR2rp1) (Sereno et al., 2008), *E. histolytica* (EhSir2a) (Dam and Lohia, 2010), human (SIRT2) (North et al., 2003) and *G. lamblia* (GlSir2.1), suggests a conserved sirtuin interaction with this cellular structure in eukaryotes, which

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likely exists in other protozoan parasites such as *Plasmodium, Trypanosoma* and *Toxoplasma*.

The perinuclear location of GlSir2.1 suggests additional functions of this protein in relation to epigenetic control in *Giardia*. Moreover, a nuclear localization of this protein cannot be discarded under certain circumstances; in human it has been observed a nuclear translocation of SIRT2 from cytoplasm during the G2/M phase (Nahhas et al., 2007). The elucidation of the metabolic and regulatory processes of GlSir2.1, GlSir2.2 and the remaining three sirtuin candidates in *G. lamblia,* remain to be explored.

5. Conclusion

In the present study, the Sirtuin structural features of GlSir2.1 were identified using bioinformatic procedures. The functional identity and NAD^+-dependent deacetylase capacity of GlSir2.1 was confirmed through structural predicted models and *in vitro* enzymatic assays. Cytoplasmic and perinuclear subcellular localization of *G. lamblia* Sir2.1 was carried out with polyclonal antibodies developed in our laboratory. These findings demonstrate the existence of a second functional sirtuin in *G. lamblia.*

Declarations

Author contribution statement

Edian A. Herrera T.: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Luis E. Contreras, Aravy G. Suarez: Performed the experiments; Analyzed and interpreted the data.

Gonzalo J. Diaz: Contributed reagents, materials, analysis tools or data.

María H. Ramírez: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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