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Título: PAPEL DO TERRA NA REGULAÇÃO DA MANUTENÇÃO DOS TELÔMEROS E NA SENECÊNCIA REPLICATIVA EM *Leishmania major*

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Resumo do projeto

Esta proposta consistiu na continuação do projeto de pós-doutorado anteriormente executado pela Drª. Edna Gicela O. Morea. Os resultados desta primeira parte, publicados em PMID: 33548324, mostraram a transcrição das extremidades cromossômicas ricas em G em *Leishmania major*, utilizando análise in sílico de bibliotecas SL-RNA-Seq construídas a partir dos três estágios de desenvolvimento do parasito. Estes resultados foram validados por Northern blot, RNA-FISH e RT-qPCR. Foram identificados a transcrição de TERRA a partir de várias extremidades cromossômicas e que a expressão do TERRA depende do estágio de desenvolvimento do parasito e do tempo de duplicação da população. Também foi evidenciado que a maioria dos transcritos TERRA são poliadenilados e processados por *trans-splicing*. TERRA apresentou altos níveis de transcrição nas formas infectivas do parasito (metacíclicas > amastigotas) comparados aos promastigotas, as quais também apresentam abundância de TERRA R-loops especialmente na extremidade direita do cromossomo 29 (TERRA 29). Estes resultados indicam que em *Leishmania*, TERRA preserva características compartilhadas com outros eucariotos, contudo, somente a presença de TERRA R-loops são indicativos de seu envolvimento na manutenção da estabilidade telomérica no parasito. Adicionado a isso, estão as diferenças observadas no perfil de restrição telomérica entre as fases de desenvolvimento do parasito. Observamos maior facilidade na clivagem por enzimas de restrição das regiões subteloméricas de parasitos na fase metacíclica, comparado a amastigotas e promastigotas, sugerindo fortemente que nesta fase há diminuição na quantidade de base J nas extremidades dos cromossomos. Lembrando que diferente de outros tripanossomatídeos, em *L. major* a maior quantidade de base J é encontrada nas regiões subteloméricas / telomérica. Nesse caso, já foi extensamente reportado que a presença da base J em região subtelomérica inibe a clivagem do DNA por enzimas de restrição impedindo que se estime o tamanho dos fragmentos teloméricos no parasito. Além disso, a base J é considerada um marcador epigenético envolvido com a terminação da transcrição, pois sua presença inibe a ação da RNA polimerase II no local. Desta forma, nossos resultados sugerem fortemente que a presença de base J em região subtelomérica regula epigeneticamente a expressão do TERRA principalmente nas formas promastigotas recém transformadas. Na tentativa de responder a essa questão, este estudo visou a inibição da formação da base J. Assim, utilizamos CRISPR/CAS9 para obter parasitos nocaute para para enzimas envolvidas na síntese de base J e para o TERRA 29. Estes ensaios funcionais foram realizados com o intuito de se compreender a regulação dos transcritos do TERRA e a manutenção dos telômeros em um cenário de carência da base J.

1. Manuscrito em Preparação

Depletion of base J in *Leishmania major* triggers alterations in TERRA expression and telomere length

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Abstract

Leishmaniases affect millions nowadays. It is caused by more than twenty species of parasites of the *Leishmania* genus. Due to the lack of efficient treatment and disease control, many efforts have been made to uncover the parasite's biological peculiarities. Telomeres have been considered antiparasitic drug targets since they are crucial for genome maintenance and cell survival. In several eukaryotes, telomeres are transcribed in long ncRNA's, such as TERRA, which is likely to affect telomere regulation. In *Leishmania*, TERRA transcription seems to be controlled by the modified thymine Base J, which is 98% concentrated at telomeres. Base J blocks restriction enzyme digestion and is an epigenetic signal for RNA polymerase II transcription termination. Therefore, Base J can interfere with TERRA and telomere homeostasis. We created *L. major* cell lines depleted of base J by editing the genes encoding JBP1 and JBP2, which are involved in Base J synthesis. Although the double knockout of JBP1 (JBP1-/-) was lethal to the parasites, the double knockout of JBP2 (JBP2-/-) was successfully generated. JBP2-/- cells presented growth ratios different from wild-type parasites during continuous in vitro passages. Also, cell cycle analyses showed that JBP2-/- initially presented arresting in G2/M phases. After several passages, JBP2-/- cells changed their proliferation profile, returning to the control level and accumulating in the S phase of the cell cycle. When examined by optical microscopy, JBP2-/- cells in P7 did not show morphological differences. However, ultrastructural alterations such as intense cytosolic vacuolization, autophagosomes, blebs in the flagellum or flagellar pocket, and concentric membranal structures within the mitochondrion were detected by transmission electron microscopy. RTqPCR detected quantitative differences in TERRA mRNA expression in JBP2-/- cells that grew up to P21. They also showed down-regulation of mRNAs involved in DNA and telomere replication and damage repair, probably due to base J depletion, which triggers a readthrough of RNA pol II transcription termination. In addition, JBP2-/- cells also showed increased telomere length after a few passages, suggesting a new role for JBP2 in the parasite's telomere maintenance.

1. Introduction

Leishmaniases are endemic tropical diseases known to cause tegumentary and visceral manifestations, with high incidence in poverty-ranked areas, where approximately one million new diagnostics are expected yearly (1). Over twenty species of *Leishmania* can be transmitted to a mammalian host by insects of the Phlebotomine and Lutzomya families during the female blood meal (1,2), which ingest the infective amastigote forms. First, amastigotes inside the insect digestive system transform into non-infective but rather proliferative procyclic promastigotes form. Next, procyclics migrate to the proboscis and differentiate into metacyclic promastigotes, which are highly infective and can be transmitted to the vertebrate host, reinitiating a new transmission cycle (2,3). Neutrophils and macrophages further phagocytose metacyclics, and inside the phagolysosomes, they undergo a series of morphogenetic modifications leading to the formation of amastigotes. Amastigotes then multiply and reach the bloodstream, causing the initiation of clinical manifestations.

Since there is no effective treatment or vaccine for the different manifestations of leishmaniases, several studies on *Leishmania* spp. have been conducted to clarify aspects of parasite biology, such as genetic analysis and full sequencing of the parasite genome (4,5). In that context, telomeres arouse a great interest in the scientific community. Telomeres are the physical ends of eukaryotic linear chromosomes. They are an association between tandemly repeated DNA sequences and protein complexes at the end of chromosomes (6,7). Telomeric DNA comprises a double-stranded sequence (one rich in Cytosine, the other in Guanine) and a G-rich single-strand protrusion at the ends of the chromosome, known as 3' G-overhang (6,7). They are essential to cell cycle maintenance and other important cellular processes, e.g., cell aging, genome integrity, and maintenance of the nuclear arrangement (8–10).

Leishmania presents many peculiar specific telomeric features, making this nucleoprotein structure a potential target for developing new treatments against the parasite (11–13). One of these peculiar features is the substantial presence of the modified thymine β-D-glucopyranosyloxymethiluracil, or simply base J. First described in *T. brucei* and later in other trypanosomatids, base J is mostly found in the telomeric/subtelomeric regions (14–16). Moreover, in the *Leishmania* genus, 98 % of all genomic base J is located at telomere/subtelomere sequences (17). Base J is synthesized by two distinct thymidine hydroxylases, J-binding protein 1 and 2 (JBP1 and JBP2, respectively), and by a β-glucosyl transferase (18) in two steps: (*i*) hydroxylation of thymidine by either JBP1 or JBP2, at a specific location on the DNA; (*ii*) and glycosylation of the

intermediate HOMedU by a β-glucosyl transferase (18). JBP1 recognizes sequences of double-stranded DNA containing base J but fails to interact with free base J and nonmodified DNA and intermediates of base J synthesis (16). In contrast, JBP2, which contains a domain related to the SWI2/SNF2 family of chromatin remodeling proteins, can interact with nonmodified DNA (19). Based on these characteristics, in the most accepted model of synthesis and propagation of base J in the trypanosomatids genome, JBP2 regulates the initial sites of base J synthesis with further propagation and maintenance of base J by JBP1 (18).

Over the years, base J functions have been ruled out using different mutant lines for either JBP1 or JBP2 (20–23). Whereas *T. brucei* and *T. cruzi* can survive in a base J depletion scenario (18,21,24), i.e., JBP1 or/and JBP2 knockouts (KO), *Leishmania tarentolae* seems to require base J to survive since the knocking out of JBP1, lead cells to death (25). In contrast, ablation of JBP2 seemed to be initially tolerable but harmful upon a progressive loss of a significant amount of base J after several parasite duplications (26). In addition, the loss of base J can also be enhanced in the presence of bromodeoxyuridine (BrdU) in culture, leading cells to death (26). Thus, considering that most of base J in *Leishmania* is found at telomeres, one of its possible functions is to maintain cell homeostasis. However, in *L. tarentolae,* no telomeric phenotypes were shown in parasites maintained at early passages (17).

Interestingly, in *Leishmania major* and *L. tarentolae*, outside the telomeres, the remaining base J content has been localized at RNA polymerase II (Pol II) transcription termination sites (17). In agreement, van Luenen and collaborators (27) showed that the loss of base J is currently accompanied by massive readthrough of normal Pol II transcriptional termination sites. Therefore, the absence of internal base J is likely lethal for *L. tarentolae* due to the massive readthrough of transcriptional stops. In contrast, in *L. major*, the reduction of base J by using dimethyloxalylglycine (DMOG) also resulted in genome-wide transcriptional readthrough at convergent strand switch regions (cSSRs) and head-tail (HT) sites without triggering cell death (28). Thus, it seems that in *L. major*, base J regulates Pol II transcriptional termination.

Besides its genome-wide transcriptional activity, Pol II can transcribe long noncoding RNAs at telomeric/subtelomeric positions in different organisms (29,30). In most eukaryotes, including trypanosomatids, the telomeric-repeat containing RNA (TERRA) derives from the subtelomeres of the C-rich strand (31–33). In model organisms, TERRA transcripts are involved in telomere length regulation and replication, essential for telomeres maintenance, DNA damage response at telomeres, and telomeric chromatin assembly (34,35). Recent work from our group confirmed the expression of TERRA from several chromosome ends in *L. major* (36). This study

shows that metacyclic and amastigotes present higher TERRA transcription levels than procyclic promastigotes, and the number of transcripts changed after continuous in vitro passages. We also showed that in *L. major*, TERRA is transcribed by Pol II and processed by trans-splicing and polyadenylation coupled reactions.

Moreover, in the infective forms of *L. major* (metacyclics and amastigotes) and promastigotes, after many passages in vitro, the amount of base J diminished, facilitating the separation of the chromosome end termini by restriction enzyme digestion. It was already shown that the presence of base J at telomeres affects the telomeric restriction fragment (TRF) profile since it inhibits DNA cleavage by frequently cutting restriction enzymes that would help to separate telomeres from the rest of the chromosomes (17,37). That leads us to hypothesize that in *L. major*, shorter telomere fragments are associated with lower base J levels and, consequently, high TERRA transcription levels, suggesting that base J regulates TERRA expression (36).

Thus, we decide to test this hypothesis by depleting base J after inducing the knockout of JBP2. Our results showed that the double knockout of JBP1 (JBP1-/-) was lethal to the parasites, whereas the double knockout of JBP2 (JBP2-/-) was successfully generated. During continuous in vitro passages, JBP2-/- cells presented growth ratios different from wild-type and partial arrest in G2/M phases. After several passages, JBP2-/- cells changed their proliferation profile, returning to the control level and accumulating in the S phase of the cell cycle. When examined by optical microscopy, JBP2-/- cells did not show morphological differences in early passages. However, ultrastructural alterations such as intense cytosolic vacuolization, autophagosomes, blebs in the flagellum or flagellar pocket, and concentric membranal structures within the mitochondrion were detected by transmission electron microscopy. We also detected quantitative differences in TERRA expression in JBP2-/- cells grown up to a later passage and down-regulation of mRNAs involved in DNA and telomere replication and damage repair, probably caused by base J depletion. It is known that the absence of base J triggers readthrough of RNA pol II transcription, which probably disturbs mRNA maturation. Moreover, the knockout of JBP2 increased telomere length in *L. major* promastigotes after a few passages, suggesting a new role for JBP2 in the parasite's telomere maintenance.

2. Material and Methods

2.1 Parasites culture

In the present study, we used the *Leishmania major* strain (MHOM/IL/ 1980/FRIEDLIN) from the Oswaldo Cruz Institute collection, confirming that we worked with a genetically homogenous

population. As explained below, this strain was used as background for constructing the transgenic lines used in this article (Lm007, JBP2-/- and JBP2-/-; JBP1+/-). Promastigote forms were maintained in exponential growth at 26 °C in 1X M199 medium pH 7.3 (Cultilab), supplemented with 10 % (v/v) heat-inactivated fetal calf serum (Cultilab), 25 mM HEPES and 1 % (v/v) antibiotic/antimycotic solution (Cultilab). Promastigotes passages P7 to P21 represent the continuous cultivation in the exponential growth (every four days) of promastigotes. Metacyclics M7-M21 were obtained from stationary phase cultures (day ten of culture) of promastigotes passages P7-P21. Growth curves were constructed using different promastigotes passages (P7, P14, and P21).

2.2 Production of single guide RNAs (sgRNAs) and donor DNAs (dDNA) for CRISPR-Cas9 gene editing and transfections

A set of oligonucleotide sequences for the genes ID LMJF_09_1480 and LMJF_14_0040 (LmJBP1 and LmJBP2) and for the TERRA 29 was designed using information from the LeishGEdit website (5) (**Table 1**). The single guide RNA (sgRNA) requires amplification with a sgRNA scaffold primer (G00 primer), also designed from the LeisghGEdit website. Two sets of oligonucleotides were designed to specifically target the 5 ' and 3' ends of the genes encoding JBP1 and JBP2 and were used as donor DNA fragments. The primers contained a primer binding site for the pT plasmid containing a drug selection marker and a 30 nt homology arm intended for homologous recombination (5). Two DNA fragments containing antibiotic resistance genes (neomycin and puromycin) were used to amplify the donor template for JBP2 and JBP1 and to eliminate the possibility of heterozygotes' survival after knocking out. One fragment of plasmid with a third different selection drug (blasticidin) was used to amplify the donor template (DT) for JBP1. All the oligonucleotides used in this section are listed in Table 1.

The use of the CRISPR-Cas9 technique in this study follows approaches developed by Beneke and colleagues in editing the kinetoplast genome (4,5). *L. major* promastigotes episomally expressing the endonuclease Cas9 and T7RNA polymerase (T7RNAP) were previously generated (Lm007) by transfecting cells with the pTB007 plasmid (4). Lm007 promastigotes were transfected with specific JBP2 sgRNAs and dDNA to obtain a JBP2 knockout strain. To obtain JBP1 hemi-knockout, JBP2-/- promastigotes expressing the pTB007 plasmid were transfected with specific JBP1 sgRNAs and dDNA. Promastigotes of the three transgenic strains were plated on solid M199 media (see above) supplemented with 20 % FBS, containing the specific selection antibiotics. Approximately ten clones were isolated from each transfectant. Afterward, clones were

grown as in 2.1, and the success of each gene edition was confirmed by RT-PCR, Sanger (**Table 2**), and Nanopore Oxford long-read whole genome Sequencing.

2.3 Extraction of genomic DNA and telomeric Southern blot analysis

Lm007 and JBP2-/- procyclic promastigotes at passages P7 and P21 were harvested (1.0×10^8 cells), washed three times in sterile phosphate-buffered saline, and lysed in the presence of 10 µg/µl proteinase K overnight at 56 °C. Total genomic DNA was obtained using phenol: chloroform extraction (38) and DNeasy Blood and Tissue kit (Qiagen). The DNA samples were resuspended in 10mMTris- HCl, 1 mM EDTA pH 8,0, and stored at 4 °C. Genomic DNA was digested with 10 U *Rsa*I (Thermo Scientific) at 37 °C overnight to liberate chromosome end termini (39). DNA fragments were fractionated onto a 0.8 % agarose gel and transferred to nylon membranes (Amersham Hybond-N^+ membrane, Cytiva, Buckinghamshire, UK). Southern blots were hybridized using a DIG-labeled telomeric probe (DIG-TELC). The hybridization signals were developed by chemiluminescence after incubating the membranes with an anti-DIG serum (Roche) covalently coupled to alkaline phosphate, followed by incubation with CPD-Star (Roche). Afterward, the membrane was stripped from the DIG-TELC probe by heat exposure (60 °C) for 1 h, submerged in SDS 0.5 %, and hybridized with a DIG-labeled alpha-tubulin probe as a positive control. The signals were developed as described above.

2.4 RNA isolation and RT-qPCRs

Total RNA was obtained from approximately 5×10^7 of procyclic promastigotes from cultures of Lm007, JBP2-/- and JBP2-/-; JBP1+/- at passages P7, P14, and P21 using Trizol (Invitrogen). Each 1 µg of total RNA was treated three times with 1 U DNase I, RNase-free (Thermo Scientific), according to the manufacturer's protocol. Each sample's amount and purity were estimated by measuring OD260 nm in a spectrophotometer Epoch (BioTek). RNA samples were submitted to PCR amplification using primers that specifically amplify the *L. major* alfa-tubulin gene (GenBank Acc# AL359777) to check for genomic DNA contamination (data not shown). To examine the levels of selected mRNAs and TERRAs (**Table 3**), 100ng of DNase I treated total RNA was reversely transcribed in cDNA using iScript cDNA Synthesis Kit (BioRad) according to the manufacturer's protocol. Transcripts encoding telomere-associated genes and selected long noncoding RNAs TERRA were measured using real-time, quantitative PCR (RT-qPCR). All RT-qPCRs were performed in 10 µL reactions using iTaq Universal SYBR Green Supermix (BioRad), and cycle threshold (Ct) values were obtained in a StepOne Real-Time PCR system (Applied Biosystems) using default settings. The relative gene expression data were analyzed using the

$2^{-\Delta\Delta CT}$ method as previously described (40). Due to its stable expression among all groups investigated, RPN8 mRNA levels (LmF.32.0390) were used as an endogenous control as previously published (36,41). Samples from the control group (i.e., Lm007) were normalized to an arbitrary value of 1 and used as the negative control. The data were expressed as means of fold change over control (relative expression). P- values were obtained using a two-tailed non-parametric Wilcoxon Signed Rank Test, and $P < 0,05$ was assumed as statistically significant. Statistical analyses were performed using GraphPad Prism (version 8.0).

2.5 Assessment of DNA content by flow cytometry

Procyclic promastigotes (5×10^7 cells) from Lm007 and JBP2-/- at passages P7, P14, and P21 were harvested at 2,300 g for 5 min, washed twice by resuspension in 1 X PBS (phosphate buffered saline) followed by resuspension in 1X PBS to a final volume of 1 ml and fixed by the addition of 900 µl Methanol (absolute) in 1X PBS and incubation at -20 °C for 20 min. The fixed and permeabilized cells were harvested at 2,300 g for 5 min, resuspended in 1 ml 1 X PBS, centrifuged again before staining with 40 µg/ml propidium iodide and treated with 100 µg/ml RNAse A at 37 °C for 30 min. Samples were stored at 4 °C before analysis. About 20,000 events were collected with a flow cytometer (BD Biosciences) and analyzed using Flowjo. P- values were obtained using a two-tailed non-parametric Wilcoxon Signed Rank Test, and $P < 0,05$ was assumed as statistically significant. Statistical analyses were performed using GraphPad Prism (version 8.0).

2.6 Assessment of telomere length by Flow-Fish

Flow-fish was used to estimate parasite telomeres length quantitatively, according to already published protocols (42,43). Lm007 and JBP2-/- procyclic promastigotes ($5x10^7$ cells) from passages P7 and P14 were centrifuged at 2,500g for 5 min, and the pellets were washed twice with 1 ml of PBS 1X. Afterward, 200 µl of Hybridization buffer and 100 µl of hybridization buffer were mixed with the 18 mer PNA FITC-labeled telomeric oligo-probe (CCCTAA)3 (PANAGENE) (1:10,000) (200 µl final volume) and added to the control (i.e., Lm007) and to the mutant samples respectively. Samples were incubated at 82 °C for 10 min and stored at RT overnight. After overnight incubation, samples were washed and incubated at 40 °C for 10 min and treated with RNase A as in 2.6. Samples were incubated in the dark for 30 min, and approximately 20.000 events /samples were collected in the flow cytometer for analysis. Results were analyzed using Accuri C6 plus flow cytometer (BayBioscences, Kobe, Japan). Quantum FITC beads (Bangs Laboratories) were used to calculate the MFI (Mean Fluorescence Intensity) recorded for each sample. MFI was converted into equivalent MESF (Molecules Equivalent of Soluble

Fluorochrome), according to Baerlocher et al. (2006) (42). The results were represented as fluorescent histograms, and the morphometric parameters (FSC-area x FSC-height) allowed us to discriminate and select cells between controls and knockouts. P- values were obtained using a two-tailed non-parametric Wilcoxon Signed Rank Test, and $P < 0,05$ was assumed as statistically significant. Statistical analyses were performed using GraphPad Prism (version 8.0).

2.7 SEM and TEM

Lm007 and JBP2-/- procyclic promastigotes from passage P20 were washed twice with 0.1 M phosphate buffer saline (PBS) and fixed at 4 °C for 40 min with 2.5 % glutaraldehyde (GA) diluted in 0.1 M Na-cacodylate buffer (pH 7.2). For TEM analysis, the parasites were washed three times with 0.1 M sodium cacodylate buffer and post-fixed for 15 min with 1 % osmium tetroxide (OsO4), 0.8 % potassium ferricyanide and 5 mM CaCl2 in 0.1 M cacodylate buffer, pH 7.2. Then, samples were washed with the same buffer, dehydrated in an ascending acetone series, and embedded in PolyBed 812 resin as previously described (44). Ultrathin sections were stained with uranyl acetate and lead citrate and examined under a Jeol 1200 EX transmission electron microscope (Tokyo, Japan) at Centro Nacional de Biologia Estrutural e Bioimagem (CENABIO). Alternatively, for SEM analysis, fixed parasites adhered to glass coverslips were coated with 0.1% poly-L-lysine and post-fixed as described above. Then, samples were dehydrated in a crescent ethanol series (30-100 %), dried using the critical point method with CO_2, mounted on aluminum stubs coated with a 20 nm gold layer, and examined with a Jeol JSM6390LV scanning electron microscope (Tokyo, Japan) at Platform Rudolf Barth in Instituto Oswaldo Cruz, FIOCRUZ (45).

2.8 Inoculating mice with *Leishmania major*

Parasites were cultured until they reached the stationary phase (day 7). BALB/c Female mice at 6 weeks old were brought in from a mice facility and nurtured until 7 weeks 4 days when they were inoculated with 40 µl of PBS containing about 2×10^6 of parasites from Lm007 and JBP2-/- in suspension. Inoculation was performed in the right hind footpad of each mouse for all treatment groups using a needle and syringe. Feeding and water were provided ad libitum, with regular monitoring and changes in bedding material. Three mice were used per treatment, and lesion development was followed. Mice were euthanized 70 days post-inoculation. Both hind footpads were measured and used to retrieve parasites. The size of the lesion was determined. The study was conducted under the guidelines for the care and use of laboratory animals.

2.9. Nanopore Oxford long-read whole genome Sequencing

High-quality DNA was extracted using a DNeasy Blood and Tissue kit (QIAGEN). The genomic library was constructed using the Native Barcoding Kit, and the PromethION 2 Solo was used for sequencing. The library preparation and whole genome sequencing were performed according to the ONT standard protocol at Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA.

Mapping of the ONT (Oxford Nanopore Technology) long read to the reference genome and de novo genome assembly was achieved using the ONT sequencing machine output, fast5, which was converted into input files in the POD5 format. To generate raw Fastq reads, the POD5 file was subjected to base calling using Dorado, a high-performance open-source basecaller for Oxford Nanopore reads. After that, the quality of the reads was evaluated using NanoPlot with the default parameters.

3. Results and discussion

3.1 Construction of *L. major* JBP2, JBP1 and TERRA 29 knockouts

Aiming to create *L. major* knockout strains for the genes encoding JBP1 and JBP2 and for the TERRA transcribed from the right arm of chromosome 29 (TERRA 29), the CRISPR-Cas9 system was used according to the methodology described previously (4). Lm007 was used as the genetic background line for gene edition using CRISPR-Cas9 experiments and as a control group.

The modified nucleotide, β-D-Glucopyranosyloxymethiluracil (base J), is present in all trypanosomatids' telomeric or subtelomeric regions investigated so far (46). In *Leishmania*, about 98 % of these modified bases are in the telomeric repeats. Since high concentrations of this modified nucleotide inhibit DNA cleavage by restriction enzymes, possible participation of the base J in maintaining these structures is suggested (46). Also, base J is considered an epigenetic marker for the transcriptional termination of Pol II termination sites (47). We previously showed that in *Leishmania* spp., TERRA (Telomeric Repeat-containing RNA) could be transcribed from the subtelomeric C-rich strand towards the end of most parasite chromosomes. Its expression seems dependent on the amount of base J at the subtelomeric position, which changes during parasite development and after continuous in vitro passages. TERRA regulates telomere length and replication in most eukaryotes by interacting with telomeric proteins and the telomerase complex (48). In this way, we decided to create a scenario where base J levels were depleted by knocking out the genes encoding JBP1 and JBP2, proteins directly involved with its synthesis (18,27,36).

Specific primers were designed to obtain donor DNA cassettes and guide RNAs to construct the knockout lines for the genes that encode JBP1 and JBP2 and for TERRA 29. Donor

cassettes containing neomycin, puromycin, and blasticidin resistance genes were amplified by PCR using specific primers (**Table 1**) from pTPuro, pTNeo, and pTBlast plasmids (4). Parasites transfected with sgRNA's and specific donor DNA containing the puromycin and neomycin resistance genes, but aiming to ablate the JBP2 gene, remained viable after the recovery period in culture and were successfully selected as single knockout clones. Nine clones were selected by platting, and one was chosen to proceed with the experiments after preliminary analysis. *L. tarentolae* and *L. major* knockouts for JBP2 were previously reported as viable, showing a progressive loss of a significant amount of base J through several passages in culture (26). Therefore, we decided to conduct our investigations over time, using different culture passages (P7, P14, and P20-21), hypothesizing that strong phenotypes would be observed throughout time. The selected clone was preliminarily confirmed as JBP2-/- using PCR reactions with specific primers (**Figure 1A and Table 2**) and then by Sanger DNA sequencing. Both donor DNA cassettes were inserted and replaced the JBP2 gene at its specific locus (**Figure 1B-D**).

Primers for CRISPR-Cas9 and confirmation of mutants

Name	Sequences 5'- 3'	References
DS RV JBP1	ACAAACGTATTTCCTACGACTGCGAGGCCA	Current work
UpS FW JBP1	ACGGTTTTGAGTTTTGTGTAATTGGCGCCG	Current work
3' SgRNA JBP1	TTACTCTCCAATCTGCGGAA	Current work
5' SgRNA JBP1	CTAATCAGCCGCCGATAATC	Current work
JBP1 FW	AACGTATTTCCTACGACTGCGAGG	Current work
JBP1 RV	GAAGGACTTCAACTTTGGCGGCAC	Current work
DS RV JBP2	CAACAGAACATCCACATGGATTCGACGCCG	Current work
UpS FW JBP2	GGCGGGTACACCATCCTCCCTGCTATTCCG	Current work
3' SgRNA JBP2	ACAAGGGCGCGGCGTCTCTG	Current work
5' SgRNA JBP2	AGCAGCTCGTCATCGTATAG	Current work
JBP2 FW	GGTACACCATCCTCCCTGCTAT	Current work
JBP2 RV	CTCTCTGCACTTGTGCTGAGTAGG	Current work
DS RV TERRA29	ATGCTCTATGGTAGGCAGAGCAAGACACG	Current work
UpS FW TERRA29	TCTCGAAAGGTGGGTTTCGACGCGTTCGAG	Current work
3'TERRA_29sgRNA	AAAGGAGGGCAATCTTGCTG	Current work
5'TERRA_29sgRNA	CCAAGGGTGTGGGTTTACGG	Current work
LmCHR29_RV	GAATCAGTGGAGAGAAAAACGCATA	Current work
LmCHR29_FW	CAGCAAGATTGCCCTCCTTT	Current work

Table 1. List of primers for production and confirmation of JBP2, JBP1, and TERRA 29 mutants

On the other hand, parasites transfected with sgRNAs and specific donor DNA containing the puromycin and neomycin resistance genes aiming to ablate the JBP1 gene and TERRA 29 were

unviable. Similar results were previously reported for *L. tarentolae* and *L. major* JBP1 by Genest et al. (25). However, due to methodological differences and the fact that in other trypanosomatids (e.g., *Trypanosoma brucei*), the knockout of JBP1 does not result in lethality, we decided to include it in our experiments. Despite the lack of success at this first attempt, we applied a different strategy to ablate JBP1 activity partially and, therefore, base J content. Therefore, we used our established *L. major* JBP2-/- line as background for a second round of transfection aiming to knock out one allele of JBP1. We first confirmed that our *L. major* JBP2-/- was still bearing the pTB007 plasmid expressing the CAS9 and the T7RNApol (**Figure 1E**) and then transfected the cells with specific sgRNAs for JBP1 and donor DNA containing the blasticidin resistance gene. We could recover and select clones now expressing the genes encoding for JBP1 and the blasticidin resistance at the same locus, indicating that we successfully obtained a cell line presenting both a hemi-knockout for JBP1 in a JBP2-/- genetic background (**Figure 1F-H**). Oxford nanopore whole genome sequencing was done to check the occurrence of off-targets. The average coverage was about 99,4% to 99,9% of each chromosome for the knockout and the parental Lm007 lines **(Suppl. Fig. 1)**.

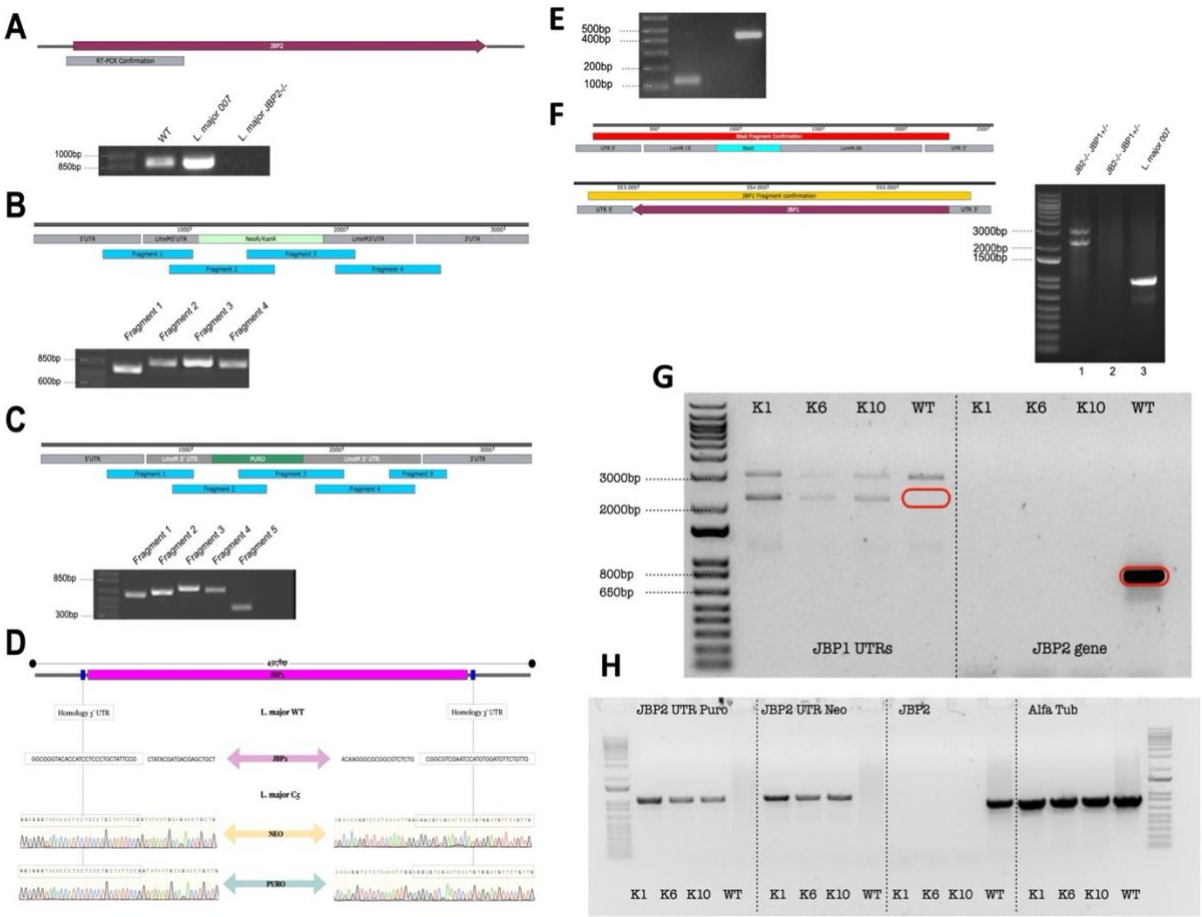

Figure 1. Confirmation of JBP2-/- and JBP2-/-; JBP1+/- lineages. (A) JBP2 gene map and location of the amplified fragment (above) by RT-PCR reaction (below) as preliminary confirmation of JBP2 gene deletion in transfected Lm007 cells. (B) Neomycin DNA donor map (above) and (C) puromycin DNA donor map (above). PCR products obtained from DNA extracted from transfected cells aiming to delete the JBP2 gene were fractionated on agarose gel (B-C) and sent for sequencing. (D) Diagram showing the insertion of donor cassettes into the JBP2 locus. (E) PCR reaction showing the amplification of T7RNAp and Cas9 from total DNA extracted from confirmed JBP2-/- cells. (F) Donor DNA cassette map containing the blasticidin resistance gene (above) and JBP1 gene map (below) with the location of fragments to be amplified in RT-PCR reactions. Using specific primers flanking the 5' and 3' UTR of JBP1, both inserted donor DNA (2,176bp - lower band) and JBP1 gene (2,954bp - upper band) were amplified by RT-PCR in non-clonal population (F) and three isolated clone populations (K1, K6, and K10) (G). Specific primers for JBP2 were used as a control. (H) Using genomic DNA from the three isolated clonal populations through differentiated plates and specific primers for the 5' UTR of JBP2 and for donor DNAs, the absence of the JBP2 gene and the presence of neomycin and puromycin resistance genes in the JBP2 gene locus in these three clonal populations were also verified, confirming that this new lineage maintained the pre-existing JBP2-/- mutation. Specific primers for JBP2 and Alpha-tubulin genes were used as negative and positive controls, respectively. All PCR/RT-PCR products were fractionated on 1 % agarose gel stained with ethidium bromide. WT, wild type; C5, JBP2-/- isolated clone population; K1, K6, and K10, JBP2-/- isolated clone populations; JBP1+/-.

Primers for Sequencing of Knockout Parasites

Name	Sequences 5'- 3'	References
NEO_1 UTR5' RV	ACAGAAGAGAGCTAGGACCT	Current work
NEO (1) FW	TCTCGTACATCCTCTGCTTA	Current work
NEO (1) RV	TTCGTCCAGATCATCCTGAT	Current work
NEO (2) FW	CTCCTGTCATCTCACCTTGC	Current work
NEO (2) RV	CCATTCTCCACAAGTTCGAC	Current work
NEO_2 UTR3' FW	TTCGAGTGTACTCTACCTGC	Current work
PURO_1 UTR5' RV	TTCGTGAGAGAAACCTGTAG	Current work
PURO (1) FW	GGTCACGACATATCACGAGC	Current work
PURO (1) RV	TCCTTAGGTCTATGTGGTGC	Current work
PURO (2) FW	GCTTGGACATTGGTAAGGTT	Current work
PURO (3) FW	GAAAGAAGTGCATCCGCATG	Current work
PURO (3) RV	CACCAACAGAAGCGGAGAT	Current work
PURO_2 UTR3' FW	AATACAGGCACGGTCCT	Current work
JBP2 UTR5' FW	CCTCTCTCTTTCGCAGCAAC	Current work
JBP2 UTR3' RV	ACACACACACACATACAAGAAA	Current work
PURO (2) RV	CCATCAGTGGTGAGAGAGG	Current work
JBP1 UTR5' FW	GATGCGTGTATCAGGTGAAT	Current work
JBP1 UTR3' RV	CAGACCTTCTCAACTGTGTC	Current work
BLAST RV 5'	CAGGCTGTGCACACATGG	Current work
BLAST FW 3'	TTCGGTACCGTCGAGAATGT	Current work

Table 2. List of primers for confirmation of JBP2 ablation by Sequencing

3.2 Cell growth analysis

The analysis of growth curves for Lm007, JBP2-/-, and JBP2-/-; JBP1+/- was performed at P7, P14, and P21 to observe the proliferative profile of mutant clones compared to the control (Lm007). Parasites from each culture were fixed and counted daily in a Neubauer chamber until

all cultures reached the stationary phase. According to the most accepted model, the enzymes JBP1 and JBP2 control the localization, level, and maintenance of base J in the genome (46). JBP1 recognizes and binds to genome regions containing base J, promoting hydroxylation of adjacent thymines, subsequently converted to base J. JBP2 recognizes specific chromatin regions (mainly telomeric) through its SWI2/SNF2 domain and, using ATP hydrolysis, promotes de novo hydroxylation of thymines. These residues are then converted to base J through a glycosyl transferase. Thus, JBP2 determines the region and sequences where base J will be produced, and JBP1 acts to amplify and maintain its levels. Therefore, as mentioned earlier, we expect a possible phenotype due to total deletion of the JBP2 gene and partial deletion of the JBP1 gene would be more prominent over time. Therefore, analysis at different checkpoints would be the best strategy to identify gradual differences in the phenotypes of JBP2-/- and JBP2-/-; JBP1+/- lineages related to the expected gradual loss of base J.

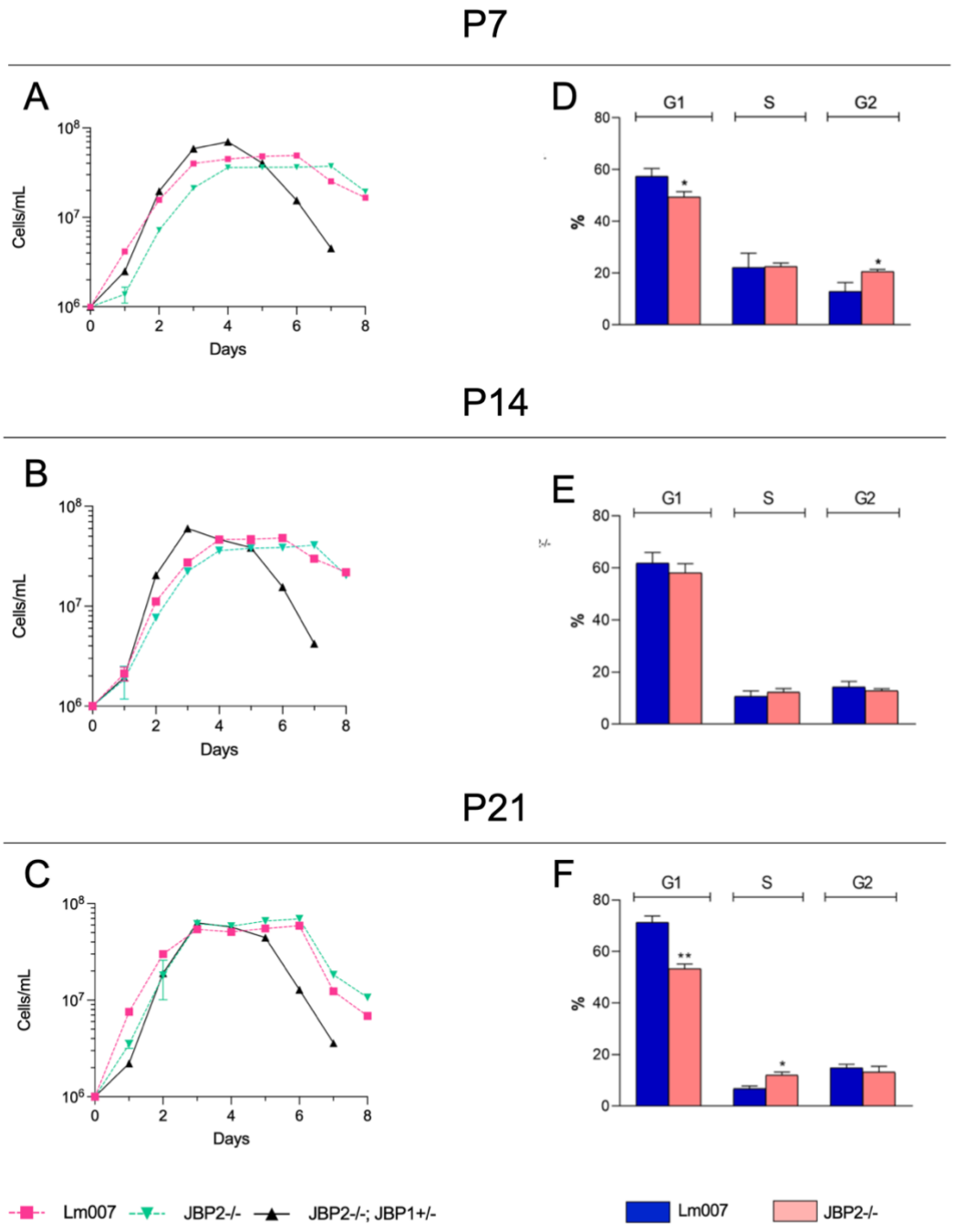

Figure 2. Growth curves for Lm007, JBP2-/-, and JBP2-/-; JBP1+/- (A-C) and analysis of DNA content for Lm007 and JBP2-/- (D-F). For the growth curves, parasites were counted daily for eight consecutive days until cell populations decreased during passages P7, P14, and P21. To assess DNA content/cell cycle progression, flow cytometry analysis was performed with parasites at the three passages above, which were collected during the exponential growth phase. Statistical assays were conducted in GraphPad Prism 9 software (t-test, $p<0.05$).

373 JBP2-/- showed lower population density than Lm007 cultures at P7 and P14 **(Figures**
374 **2A and 2B)**. Additionally, JBP2-/- cells at P7 showed a one-day delay in entering the stationary

phase **(Figure 2A)**. However, JBP2-/- at P14 not only entered the stationary phase on the same day as the control but also remained one day longer in the stationary phase before observing a population decline **(Figure 2B)**. No growth delay was observed for JBP2-/- at P21 at the stationary phase, although the growth rate was slightly slower than the control. On the other hand, the population density of JBP2-/- cells was higher after entering the stationary phase and during cell population regression **(Figure 2C)**. A unique pattern of rapid growth, without remaining in the stationary phase and abrupt population decline, was observed for the JBP2-/-; JBP1+/- lineage **(Figure 2A-C)**. Furthermore, a higher population density of cells was observed for this lineage at P7 and P14, suggesting that the gradual loss of J base interferes with cell proliferation.

In addition to the growth rate, we also analyzed the progression of JBP2-/- throughout the cell cycle by checking the DNA content of each culture during the log/proliferative phase **(Figure 2D-F)**. Promastigote forms were processed with DNA staining dye (propidium iodide) to assess the impact of JBP2 deletion on the parasite's cell cycle.

The data show a transient arrest of JBP2-/- cells in the G2/M phases compared to the control at P7 **(Figure 2D)**, which may explain the delay in entering the stationary phase and the less dense population observed. On the other hand, cultures at P21 presented a different profile **(Figure 2F)**. JBP2-/- had more cells in the S phase and fewer cells in the G1/G0 phase than the control. This profile corroborates what was observed during the growth curve analysis when JBP2-/- cells showed a denser population and no delay in entering the stationary phase **(Figure 2C)**. No statistical differences were observed in the JBP2-/- cell cycle profile compared to the control in the analyses at passage P14.

The present study is the first to demonstrate growth alterations for a JBP2 knockout line of *Leishmania* without additional treatment. Vainio and colleagues analyzed growth in culture for *L. tarentolae* JBP2-/- but in a different context (27). By adding bromodeoxyuridine (BrdU) to the culture, the growth of *L. tarentolae* JBP2-/- was drastically reduced over a long culture period (>6 months). It is worth noting that BrdU is a competitor of thymidine, which can be incorporated during DNA replication over cell divisions. The presence of BrdU impairs JBP1 from producing more base J since JBP1 cannot hydroxylate BrdU to HOMedU, the base J intermediate. Thus, we believe that the differences (mild effects on growth compared to the study by Vainio and colleagues) in our results are due to differences between experimental setups. However, the recovery in growth observed for *L. major* JBP2-/- still needs to be investigated. Cell cycle analyses for the JBP2-/-; JBP1+/- mutant lineage are ongoing and will complement these results.

3.3 JBP2-/- cells present a variety of ultrastructural alterations

Next, the impact of JBP2 disruption was investigated by SEM and TEM in early (P5) and late (P20) passages. Control (**Figure 3A**) and JBP2-/- promastigote cells (**Figures 3C and F**) were analyzed by SEM, revealing typical morphology and size, including an elongated shape and free flagellum in addition to the presence of round and intermediated forms in all studied groups. Dividing parasites were also noticed in control and JBP2-/- promastigotes. Control parasites processed for TEM also displayed a normal appearance of intracellular organelles, including the nucleus, mitochondrion, kinetoplast, and endoplasmic reticulum (**Figure 3B**). In contrast, JBP2-/- promastigotes show several ultrastructural alterations, including intense cytosolic vacuolization, the presence of autophagosomes, blebs in the flagellum and flagellar pocket, as well as the occurrence of concentric membranal structures within the mitochondrion, which can be suggestive of autophagy (**Figure 3D, E, and G**). However, no alteration could be observed in the nuclei and kinetoplast DNA (kDNA) in the control parasites and JBP2-/- cells (**Figure 3B, D, E, and G**). These results suggest that RNA pol II readthrough termination caused by the depletion of base J is very harmful to *L. major* promastigotes since it can induce cells to an autophagic state.

3.4 TERRA expression is altered in JBP2-/- and JBP2-/-; JBP1+/- cells

The expression of TERRA was evaluated by real-time quantitative PCR (RT-qPCR) for Lm007, JBP2-/-, and JBP2-/-; JBP1+/- cells at different passages. The target TERRA was selected based on Morea and colleagues (36), according to its differential expression in promastigotes using specific primers **(Table 3)**. The expression of TERRA transcripts from the left arm of chromosome 3 (Chr3 left), right arm of chromosome 10 (Chr10 right), left arm of chromosome 20 (Chr20 left), right arm of chromosome 24 (Chr24 right), and right arm of chromosome 29 (Chr29 right) were analyzed in the three lineages at P7, P14, and P21.

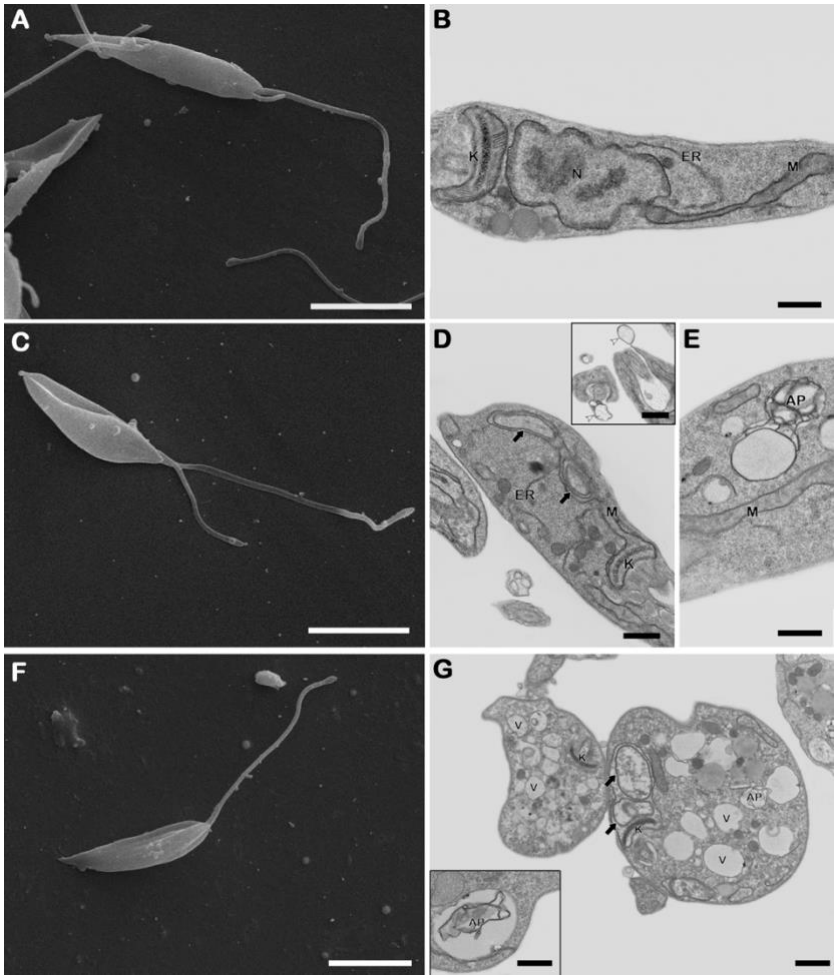

Figure 3. **Ultrastructural analysis of JBP2-/- promastigotes by SEM revealed cells induced to an autophagic state.** (**A, C, and F**) and TEM (**B, D, E, and G**) of control (**A, B**) and JBP2-/- promastigotes at passages P5 (**C-E**) and P20 (**F-G**). SEM images demonstrate typical promastigotes morphology in control and JBP2-/- cells, with most parasites presenting elongated morphology and free flagella besides round and intermediated-shaped forms and dividing cells. The TEM analysis shows control parasites (**B**) with the typical appearance of organelles, including the nucleus (N), mitochondrion (M), kinetoplast (K), and endoplasmic reticulum (ER). JBP2-/- cells (**D, E, and G**), in contrast, show intense cytosolic vacuolization (V), formation of autophagosomes (AP), and blebs in the flagellum and flagellar pocket (white arrowheads), as well as the presence of concentric membrane structures inside mitochondrion (black arrows), the suggestive phenotype of autophagy. Bars in **A**, **C,** and **F**: 5µm; bars in **B**, **D**, **E,** and G: 0.5µm.

Except for the TERRA transcribed from the right arm of chromosome 24, which showed a statistically significant decrease in its levels, no other differences were observed for the analyzed TERRAs in the JBP2-/- lineage compared to the Lm007 lineage at passage P7 (Figure 4). However, the JBP2-/-; JBP1+/- lineage showed an acute increase (>10X compared to the control) in the expression levels of TERRA from Chr3 left, Chr24 right, and Chr29 right when compared to parasites from the Lm007 and JBP2-/- lineages **(Figure 4)**.

In the analyses of parasites at P14, although all analyzed transcripts showed negative regulation, only TERRAs Chr3 left, and Chr24 right presented a statistically significant reduction in the JBP2-/- lineage. In contrast, all analyzed TERRA transcripts in the JBP2-/-; JBP1+/- lineage

showed statistically significant reductions compared to the Lm007 and JBP2-/- lineages, especially for TERRAs from Chr3 left, Chr20 left, and Chr29 right (~8-10 times compared to the control) and Chr24 right (~100 times compared to the control) (Figure 4). Interestingly, in the analyses of the three lineages at P21, although the investigated TERRA transcripts in the JBP2-/- and JBP2-/-; JBP1+/- lineages remain lower (statistically significant for TERRAs Chr3 left and Chr24 right in the JBP2-/- lineage, and for TERRAs Chr3 left, Chr24 right, and Chr29 right in the JBP2-/-; JBP1+/- lineage), there are indications of recovery of these levels at P21. To our knowledge, no TERRA expression in *Leishmania* under such experimental conditions has been reported previously.

Observation in yeast, mammalian, and mouse cells showed that deregulated TERRA expression or its changed location in the nucleus can affect telomere stability through various mechanisms, such as exacerbating conflicts between transcription and replication, encouraging trimming of chromosome ends, modifying telomeric chromatin, and facilitating homologous recombination (49,50). Therefore, it becomes clear that precise regulation of TERRA is crucial for preserving genome integrity. Furthermore, every TERRA transcript contains a repetitive sequence rich in guanine (G) at its 3' terminus, capable of creating hybrids between DNA and RNA (51). These structures, known as TERRA R-loops, actively participate in TERRA's functions and play pivotal roles in influencing telomere stability. In the present study, another criterion for selecting different TERRAs together with their transcription ratios was detecting TERRA R-loops structures from different chromosomes by Morea et al. (36).

While various regulators of TERRA have been described in humans (52–54), mice (49), and yeast (55), understanding its regulation in *Leishmania* species remains elusive. Notably, none of these organisms harbor base J in their genomes, which has been reported as a barrier to RNA polymerase II-mediated TERRA transcription (46). Thus, our study primarily aimed to investigate the relationship between the partial depletion of base J in the subtelomeric/telomeric regions from where TERRA is transcribed (36). In other organisms, TERRA expression is inversely correlated to telomere length. Interestingly, our results revealed intriguing differences in the expression patterns of selected TERRA transcripts between JBP2-/- and JBP2-/-; JBP1+/- lines at early passage P7 **(Figure 4)**. These two different lines exhibited opposite TERRA expression profiles for three out of the five transcripts analysed, with the JBP2-/-; JBP1+/- line showing upregulation of all TERRA tested. However, at passage P14 and P21, the selected TERRA transcrits showed downregulation in the JBP2-/-; JBP1+/- line, with a mild yet significant ($p<0.05$) recovery noted by passage P21 **(Figure 4)**. Given the complexity of interpreting the qPCR results under conditions of base J depletion, we subsequently assessed telomere size using Southern blot, Flow-Fish and

Oxford Nanopore whole genome sequencing to better understand and correlate base J ablation with TERRA expression.

Primers for RT-qPCR

Name	Sequences 5'- 3'	References
Chr3 left FW	CCCATCCATTCCTTTTCAGA	Morea et al. 2021
Chr3 left RV	ATCCAGTAAGGCTGCGAAGA	Morea et al. 2021
Chr10 right FW	CACACCGTGAACGCAAGGAAAC	Morea et al. 2021
Chr10 right RV	TCCGTGGTGGTGCGTTCT	Morea et al. 2021
Chr20 left FW	GCGCCGTGTATTTTCAGTCT	Morea et al. 2021
Chr20 left RV	ACTTCGCCCATCATATCAGC	Morea et al. 2021
Chr24 right FW	TCCTCCGATTCGTCTCTGTT	Morea et al. 2021
Chr24 right RV	GCACGCTACGCGAAAAGC	Morea et al. 2021
Chr29 right FW	ATGGGGATTAAGGGAAGCAC	Current work
Chr29 right RV	GAATCAGTGGAGAGAAAAACGCATA	Current work
RPN8 FW	ATGAACCGCCGCAAGCT	Morea et al. 2021
RPN8 RV	GGCGCGACGACGATCTTTGATT	Morea et al. 2021

Table 3. List of primers for RT-qPCRs

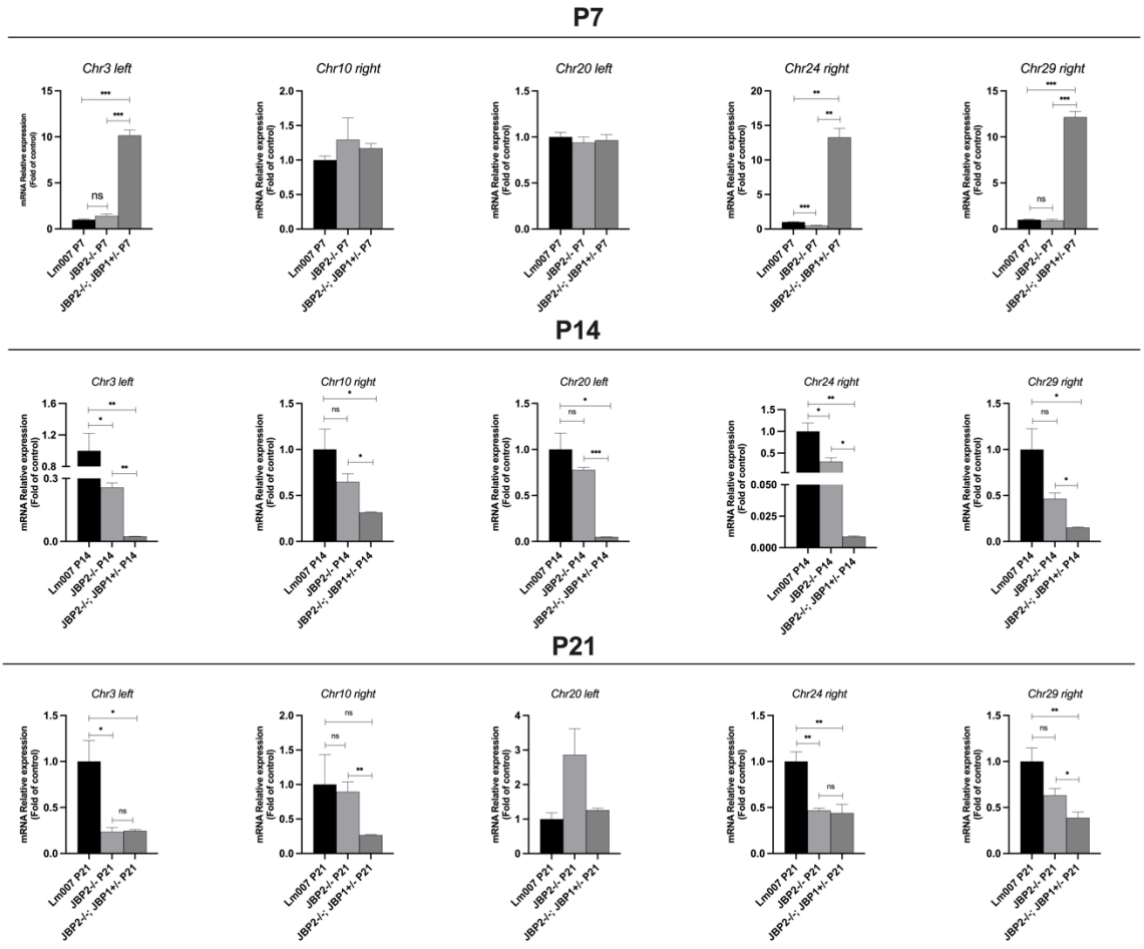

Figure 4. Alteration in TERRA transcription levels detected in JBP2-/- and JBP2-/-; JBP1+/- lineages. RT-qPCR analyses for TERRA transcripts were performed for parasites at passages P7, P14, and P21 compared to the control (Lm007). Data were analyzed using GraphPad Prism Version 9 software and the Student's t-test. Values of $p < 0.05$ were considered statistically significant. TERRA transcripts were selected for RT-qPCR from different chromosomal arms based on their expression in *Leishmania major* promastigote forms as previously published data in our group (36).

3.5 Telomeric profile analysis of the JBP2-/- lineage at different passages

Here, we investigated the telomere restriction fragment (TRF) profile of Lm007 and JBP2-/- promastigotes at different passages. It is worth noting that high levels of base J at the ends of *L. major* promastigote chromosomes inhibit DNA cleavage by restriction enzymes, generating subtelomeric/telomeric fragments ranging in size from 3.5-8 kb, as detected in Southern blots hybridized with a telomeric probe. Given that the JBP2-/- lineage should exhibit lower proportions of base J at subtelomeric/telomeric positions over divisions, we expected to observe hybridization in telomeric fragments of smaller sizes over different passages. In contrast, JBP2-/- cells from P7-P21 exhibited telomeres of a similar size to the Lm007 cells used as a control **(Figure 5A)**. In contrast, telomere length analysis using Flow-fish shows telomere elongation in JBP2-/- cells in P7 and P14 **(Figure 5B)**. However, Southern blot analysis did not detect this result using parasites from the same passages. Curiously, the Flow-fish analyses of JBP2-/- parasites at P21 showed no alterations in telomere length compared to the control lineage, Lm007. These results suggest i) that JBP2 regulates telomerase activity in the parasites and its absence triggers enzyme deregulation, leading to the addition of telomeric repeats uncontrollably at the chromosome ends, and ii) JBP1 can compensate for the absence of JBP2 and, over time is capable of recomposing the J bases lost in subtelomeric position and even help in telomerase regulation.

Telomere length alterations caused by JBP2 gene deletion in *L. major* promastigotes have never been shown. Vainio and colleagues (27) evaluated the telomere length profile and its nuclear distribution in *L. tarentolae* JBP2-/-, but no evident alterations were detected. However, the authors demonstrated that the absence of JBP2 triggers a decrease in base J levels at *L. tarentolae* telomeres. Despite its known role in base J synthesis, there is no information in the literature about a possible role of JBP2 in telomere homeostasis regulation in *Leishmania* spp.

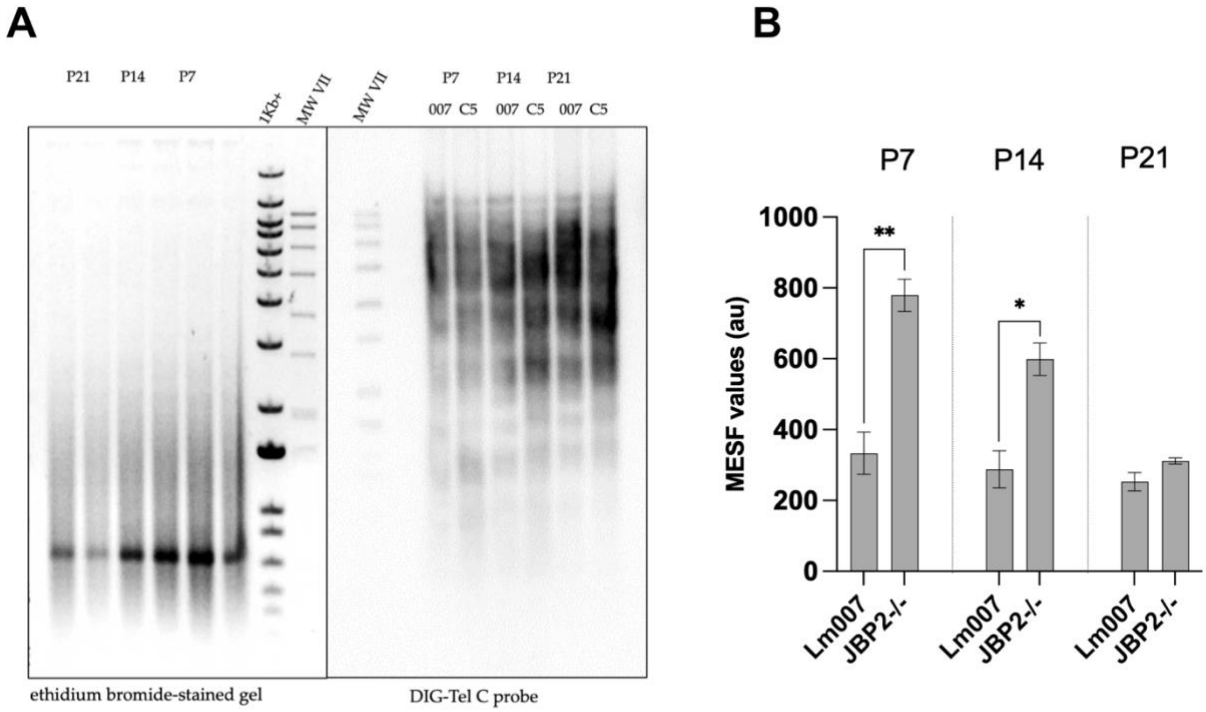

Figure 5. (A) Telomere length profile (TRF) of JBP2-/- and Lm007 cells at passages P7, P14, and P21. Genomic DNA from JBP2-/- and Lm007 cells was digested with RsaI (5U). DNA fragments were separated on a 0.8% agarose gel and hybridized with a DIG-labeled telomeric probe (5′-TTAGGG3-3′). Specific anti-DIG antibodies covalently linked to alkaline phosphatase (Roche) and CPD-Star (Roche) were used for chemiluminescent detection. **(B)** A FITC-labeled telomeric DNA oligonucleotide probe (CCCTAA)3 was used in the Flow-FISH analysis of JBP2-/- and Lm007 cells at passages P7, P14, and P21. The fluorescence intensity between samples was calculated using MESF. MWVII - DNA Molecular Weight Marker VII, DIG-labeled (Roche); 007 = Lm007; C5 = JBP2-/-. Data in (B) were analyzed using GraphPad Prism Version 9 software and Student's t-test. Values of $p < 0.05$ were considered statistically significant.

3.6. In vivo infectivity assay

In vivo infection assays showed a significant difference in lesion development between mice infected with the JBP2-/- lineage and those infected with the Lm007 lineage. All mice in the Lm007 treatment group developed visible lesions 50 days post-inoculation **(Figure 6A)**. In contrast, mice infected with the JBP2-/- lineage showed no visible signs of lesions even after 70 days post-inoculation **(Figure 6B)**. The sizes of lesions/inoculated and uninoculated paws were measured using Vernier calipers, and the difference was estimated as the lesion size (mm) **(Figure 6C)**. With an average size of approximately 5 mm, the lesion on the paw infected with Lm007 was approximately twice as large as that observed in paws infected with the JBP2-/- lineage.

The findings from the infectivity assay yielded unexpected results, highlighting the complexity of understanding the relationship between various factors and in vivo infectivity.

Despite notable variances observed in growth curves, telomere size, and TERRA expression, the limited existing literature presents challenges in directly correlating these changes with the observed lack of in vivo infectivity. Notably, significant ultrastructural disparities were identified in the promastigote form, yet caution is warranted as these distinctions may not directly align with the outcomes of the infectivity assay, given the omission of investigation into the infective metacyclic form. Consequently, our approach pivoted towards assessing the ability of the JBP2-/- promastigote form to transition into the infective state, opting to focus on the selection of metacyclic cells from stationary phase cultures.

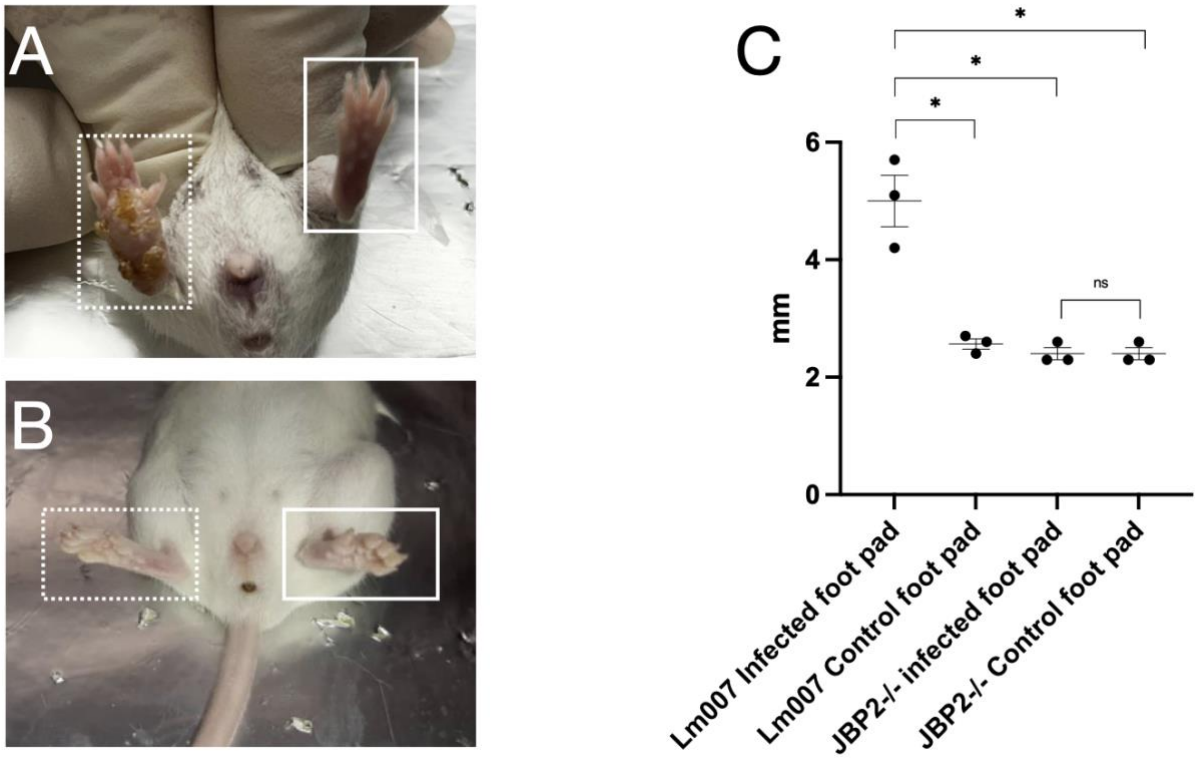

Figure 6. (A-B) Representative images of inoculated and uninoculated paws with parasites from the Lm007 and JBP2-/- lineages. Mice infected with the Lm007 lineage showed the first signs of lesion 50 days after infection. Seventy days post-infection, the animals were euthanized in a CO2 chamber, and the paws infected with the Lm007 (A) and JBP2-/- (B) lineages were collected for parasite recovery. **(C)** Size of lesions observed in mice infected with the Lm007 and JBP2-/- lineages. Statistical analysis was performed using GraphPad Prism 9 software, with the Student's t-test ($p<0.05$). Dotted squares = infected foot pads, regular squares = control foot pads (no injection of parasites).

This manuscript is in the preparation stage. Analyses of nanodrop telomere to telomere sequencing, Flow-FISH of JBP2-/-; JBP1+/-, and the ability of the mutants to transform into metacyclics are in progress.

Final remarks

The study conducted by Dr[a]. Edna Gicela O. Morea showed a comprehensive analysis of telomere dynamics and TERRA expression across the three forms of the *Leishmania major* parasite that provided a foundational framework for our research endeavors throughout the 34-month duration of the present investigation. The present study explored TERRA and telomeres, with a specific focus on the manipulation of the modified nucleotide base J production on *Leishmania major* genome. Continuing from Dr. Morea's publication, we explored TERRA and telomeres with a distinct focus. This investigation took a novel direction by delving deeper into the consequences of partial base J depletion on the parasite homeostasis. In contrast to prior studies concentrated mainly on the capability of *Leishmania* species to survive without the base J-producing enzyme JBP2, our inquiry sought to elucidate the nuanced effects of varying degrees of base J depletion by creating not only a *Leishmania major* JBP2 knockout line in our laboratory, but additionally engineering a second line partially knocked out of JBP1 using our own JBP2-/- *Leishmania major* line. Hence, new data on base J depleted *Leishmania major* growth / cell cycle profile, telomere size, ultra structure, TERRA expression, *in vivo* infectivity, and whole genome sequencing have been and are being generated. The on going analysis mentioned above will complete the manuscript aming for a impacting publication. Moreover, the mutant line lines created during this study may also be tuseful for studying different aspects of *Leishmania major* telomeres, such as control of telomerase activity, since the present results pointed to a elongation of telomeres on JBP2-/- line.

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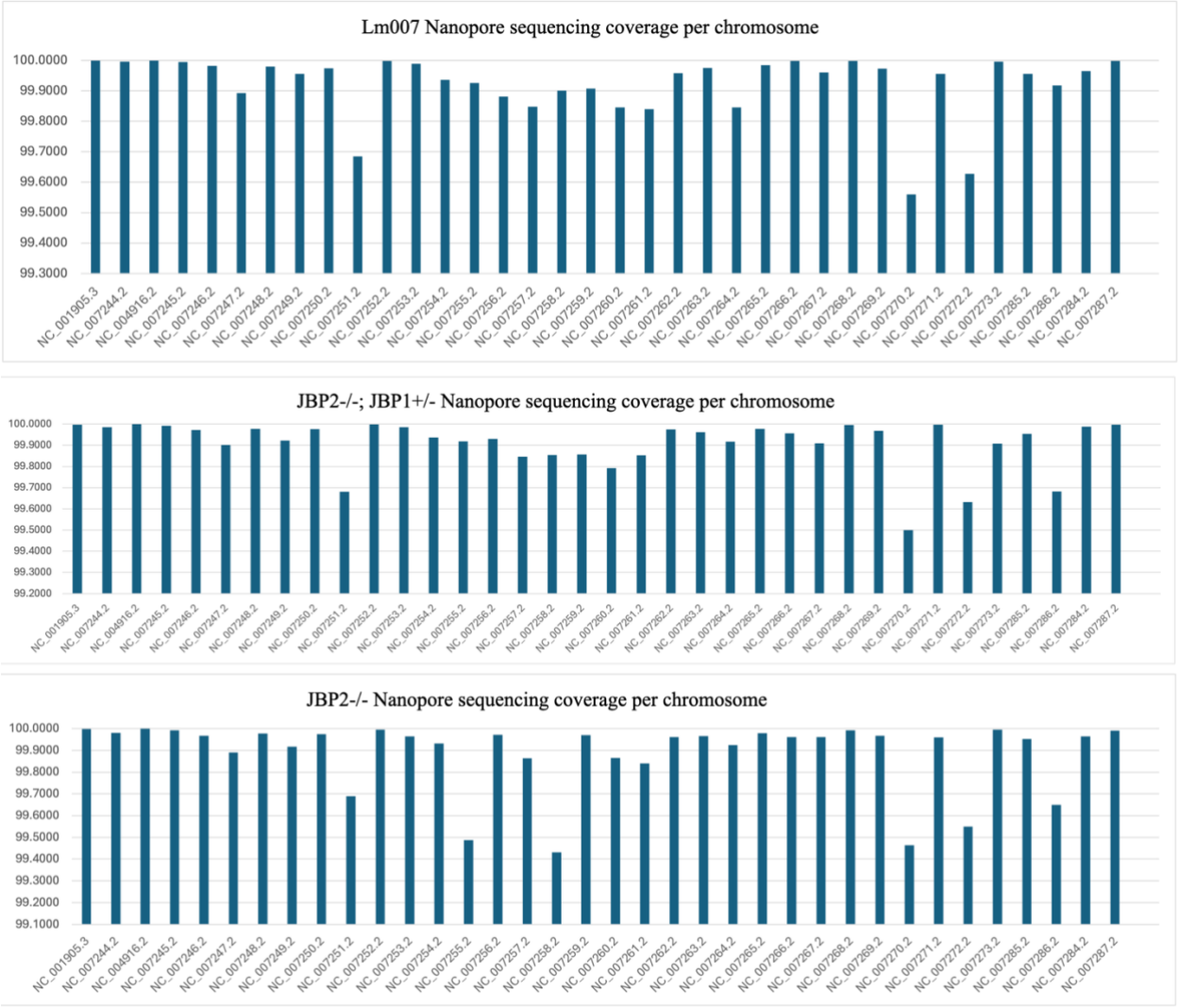
Supplementary Figure 1



Supplementary Figure1: Nanopore Oxford long-read whole genome Sequencing for each individual chromosomes for the parental line Lm007, JBP2-/-; JBP1+/- and JBP2-/- . The 36 chromosomes of *L. major* (MHOM/IL/ 1980/FRIEDLIN) are identified by the NCBI ID numbers.

2. Gestão de dados

Seguindo as normativas da FAPESP, os dados brutos, micrografias e fotografias de géis de eletroforese e membranas reveladas gerados durante o período de vigência do processo FAPESP 2021/04253-7 estão armazenados no drive do Laboratório de Telômeros. A versão refinada destes dados, tais como cálculos de expressão gênicas, alinhamentos de sequenciamento, gráficos e ensaios estatísticos estão também disponíveis no link: www.zenodo.org, sendo encontrados através do título do presente projeto. Os Arquivos em tabelas .xlx, tais como as análises dos RT-qPCR podem ser acessados com o software Microsoft Excel. Já os em extenção .pzf são compatíveis com o software GraphPad Prism e contem os gráficos gerados e as análises estatísticas. Os mapas contendo as sequencias dos genes e iniciadores utilizados neste trabalho possuem a extenção .dna e podem ser abertos no software SnapGene. Os arquivos referentes as análises de ciclo celular e Flow-Fish possuem a extenção .wsp e podem ser acessados com o software FlowJo. As eletromicrografias estão em formato .tiff e podem ser acessadas por diferentes leitores de imagem. Os dados do sequenciamento nanopore e suas análises realizadas até o presente momento estão em arquivos de extenção .fast5 (arquivos brutos), .fastq (para as sequências de nucleotídeos) e .bam (para as análises de cobertura e qualidade). Estes arquivos estão disponibilizados no link: https://labshare.cshl.edu/shares/martienssen/www-data/eernst-leishmania-20231114/.

3. Outras atividades

a. Participação em eventos científicos:

Os certificados estão apresentados no **anexo I** na ordem em que aparecem no texto.

i. Membro do comitê avaliador do XII Simpósio do Programa de Pós-Graduação em Ciências Biológicas Genética, do Instituto de Biociências, UNESP, campus de Botucatu, realizado no dia 9 de dezembro de 2021.

ii. 1st International Mini-Symposium on Trypanosomatids, realizado no dia 21 de junho de 2022.

iii. Escola FAPESP 60 Anos: Ciências Exatas, Naturais e da Vida, realizado entre os dias 7 e 10 de agosto de 2022.

iv. 51th Annual Meeting of the Brazilian Society of Biochemistry and Molecular Biology (SBBq) co-organized with the 46th Congress of the Brazilian Society of Biophysics (SBBf) / LAFESB, realizado entre os dias 5 a 8 de setembro de 2022.

v. Membro organizador do XIII Simpósio do Programa de Pós-Graduação em

Ciências Biológicas Genética, do Instituto de Biociências, UNESP, campus de Botucatu, realizado no dia 7 de novembro de 2022.

b. Resumos apresentados em eventos científicos:

Os certificados estão apresentados no **anexo II** na ordem em que aparecem no texto.

i. Role of TERRA in telomere regulation and replicative senescence in Leishmania major. Evento: Escola FAPESP 60 Anos: Ciências Exatas, Naturais e da Vida, realizado entre os dias 7 e 10 de agosto de 2022.

ii. Establishment of Leishmania major knockout lines to study telomeric dynamic through Base J levels and TERRA expression.

Evento: 51th Annual Meeting of the Brazilian Society of Biochemistry and Molecular Biology (SBBq) co-organized with the 46th Congress of the Brazilian Society of Biophysics (SBBf) / LAFESB, realizado entre os dias 5 a 8 de setembro de 2022

iii. Depletion of base J in Leishmania major triggers alterations in TERRA expression and telomere length.

Evento: The non-coding genome / EMBL Heidelberg, Alemanha, realizado entre os dias 11 e 14 de outubro de 2023. Apresentado pela supervisora Profª Dr. Maria Isabel Nogueira Cano.

c. Cursos:

Os certificados estão apresentados no **anexo III** na ordem em que aparecem no texto.

i. Curso de Extensão Universitária - Difusão do Conhecimento "Curso de Boas Práticas de Laboratório e Biossegurança", realizado entre os dias 17 e 21 de novembro de 2021, IBB-UNESP.

ii. Evento Desafios na educação e ciências no Brasil face ao negacionismo e aos cortes de financiamento", realizado no dia 23 de junho de 2022, IBB-UNESP.

d. Organização / participação em disciplinas de graduação e pós-graduação:

Os certificados estão apresentados no **anexo IV** na ordem em que aparecem no texto.

i. Organização e participação docente na disciplina Seminários em Genética, para o programa de pós-graduação Ciências Biológicas (Genética), ministrada entre 11 de agosto de 2022 e 24 de novembro de 2022, IBB-UNESP.

ii. Organização e participação docente na disciplina optativa Fundamentos em Biotecnologia, para o curso de graduação em Ciências Biológicas, ministrada entre 3 e 7 de julho de 2023, IBB-UNESP.

e. Participação em bancas de qualificação e defesa de mestrado e doutorado:

Os certificados estão apresentados no **anexo V** na ordem em que aparecem no texto.

i. Membro titular da banca do exame de qualificação de doutorado do aluno Lucas Benites Doretto, realizada no dia 31 de agosto de 2021 pelo programa de pós-graduação Ciências Biológicas (Genética), IBB-UNESP.

ii. Membro titular da banca do exame de qualificação de doutorado da aluna Beatriz Jacinto Alves Pereira, realizada no dia 10 de fevereiro de 2022 pelo programa de pós-graduação Ciências Biológicas (Genética), IBB-UNESP.

iii. Membro titular da banca de defesa de doutorado da aluna Marília de Paiva Camargo, realizada no dia 4 de fevereiro de 2022 pelo programa de pós-graduação em Biologia dos Sistemas – Área: biologia celular, tecidual e do desenvolvimento, ICB – USP.

f. Artigos publicados

Os certificados estão apresentados no **anexo VI** na ordem em que aparecem no texto.

Artigos de revisão:

i. **Assis, L.H.C.**; Andrade-Silva, D.; Shiburah, M.E.; de Oliveira, B.C.D.; Paiva, S.C.; Abuchery, B.E.; Ferri, Y.G.; Fontes, V.S.; de Oliveira, L.S.; da Silva, M.S.; et al. Cell Cycle, Telomeres, and Telomerase in Leishmania spp.: What Do We Know So Far? Cells 2021, 10, 3195. https://doi.org/10.3390/cells10113195.

ii. **Assis, L.H.d.C.**; de Paiva, S.C.; Cano, M.I.N. Behind Base J: The Roles of JBP1 and JBP2 on Trypanosomatids. Pathogens 2023, 12, 467. https://doi.org/10.3390/pathogens12030467.

Capítulos de livro:

i. Passos, A.d.O., **Assis, L.H.C.**, Ferri, Y.G., da Silva, V.L., da Silva, M.S., Cano, M.I.N. (2022). The Trypanosomatids Cell Cycle: A Brief Report. In: Wang, Z. (eds) Cell-Cycle Synchronization. Methods in Molecular Biology, vol 2579. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-2736-5_2.

ii. de Oliveira, B.C.D., **Assis L.H.C**., et al. (2022). Synchronization of Leishmania amazonensis Cell Cycle Using Hydroxyurea. In: Wang, Z. (eds) Cell-Cycle Synchronization. Methods in Molecular Biology, vol 2579. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-2736-5_10.

Trabalhos científicos em colaboração publicados na forma de pre-print

i. Ablation of telomerase reverse transcriptase in Leishmania major results in a senescent-like phenotype and loss of infectivity.

Mark Ewusi Shiburah, Beatriz Cristina Dias de Oliveira, Habtye Bisetegn, Débora Andrade Silva,

Luiz Henrique de Castro Assis, Rubem Menna Barreto, Marcos Meuser Batista, Maria de Nazaré Correia Soeiro, Benedito D. Menozzi, Helio Langoni, Juliana Ide Aoki, Adriano Capellazzo Coelho, Maria Isabel N. Cano. bioRxiv 2023.11.10.566596; doi: https://doi.org/10.1101/2023.11.10.566596. **LINK**

ii. The impact of knocking out the Leishmania major telomerase RNA (LeishTER): from altered cell proliferation to decreased parasite infectivity.

Beatriz Cristina Dias de Oliveira, Mark Ewusi Shiburah, **Luiz Henrique de Castro Assis**, Veronica Silva Fontes, Pedro Henrique Gallo-Francisco, Selma Giorgio, Marcos Meuser Batista, Maria Nazaré Correia Soeiro, Rubem Figueiredo Sadok Menna-Barreto, Juliana Ide Aoki, Adriano Cappellazzo Coelho, Maria Isabel Nogueira Cano. bioRxiv 2023.11.10.566567; doi: https://doi.org/10.1101/2023.11.10.566567. **LINK**

ANEXO I

XII Simpósio do Programa de Pós-Graduação em Ciências Biológicas – Genética

09 de dezembro de 2021

Certificado

Certificamos que **Luiz Henrique de Castro Assis** integrou a Comissão Avaliadora do XII Simpósio do Programa de Pós-Graduação em Ciências Biológicas – Genética, do Instituto de Biociências, UNESP, campus de Botucatu, realizado no dia 09 de dezembro de 2021, totalizando 8 horas.

Botucatu, 09 de dezembro de 2021

Profa. Associada Maria Isabel Nogueira Cano
Coordenadora do Programa de Pós-Graduação
em Ciências Biológicas - Genética

Instituto de Biociências
Unesp Botucatu

CERTIFICATE

I hereby certify that **LUIZ HENRIQUE DE CASTRO ASSIS** attended the 1st International Mini-Symposium on Trypanosomatids, which took place at Biosciences Institute (IB), São Paulo State University (UNESP), Botucatu campus, São Paulo, Brazil, on June 21st, 2022.

Marcelo Santos da Silva
organizer

unesp
UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Botucatu

escola FAPESP 60 ANOS 1962 - 2022
CIÊNCIAS EXATAS, NATURAIS E DA VIDA

CERTIFICADO

Certificamos que o trabalho intitulado:

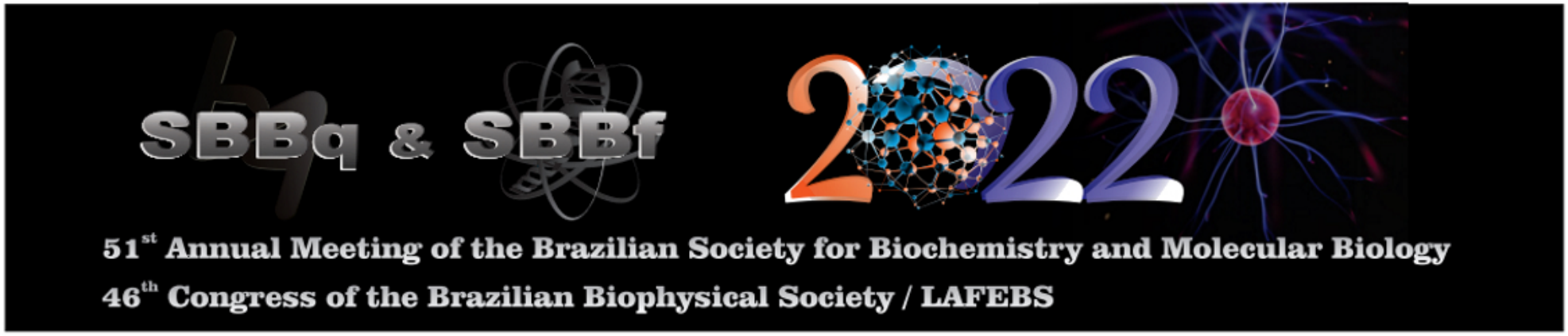
Role of TERRA in telomere regulation and replicative senescence in Leishmania major

Autoria de: **Luiz Henrique Castro Assis**

foi apresentado na sessão de pôster durante a **Escola FAPESP 60 Anos: Ciências Exatas, Naturais e da Vida**, realizada no Hotel Fazenda Dona Carolina, Itatiba - SP, de 7 a 10 de agosto de 2022.

Ronaldo Aloise Pilli
Vice-presidente do Conselho Superior da FAPESP

Euclides de Mesquita Neto
Coordenação Adjunta - Programas Especiais e Colaborações em Pesquisa - FAPESP



Águas de Lindóia, São Paulo
September 5th to 8th ,2022

Certificate

We hereby certify that the abstract entitled

ESTABLISHMENT OF LEISHMANIA MAJOR KNOCKOUT LINES TO STUDY TELOMERE DYNAMIC THROUGH BASE J LEVELS AND TERRA EXPRESSION

by authors: ASSIS, L.H.C, OLIVEIRA, B.C.D., CANO, M.I.N.

was presented during the 51th Annual Meeting of the Brazilian Society of Biochemistry and Molecular Biology (SBBq) co-organized with the 46th Congress of the Brazilian Society of Biophysics (SBBf) / LAFESB, from September 5-8 in the Convention Center of the Majestic Hotel in Águas de Lindóia, SP.

President of SBBq
Leda Quercia Vieira

President of SBBf
Rosangela Itri

ANEXO II

escola FAPESP 60 ANOS 1962 - 2022
CIÊNCIAS EXATAS, NATURAIS E DA VIDA

CERTIFICADO

Certificamos que o trabalho intitulado:

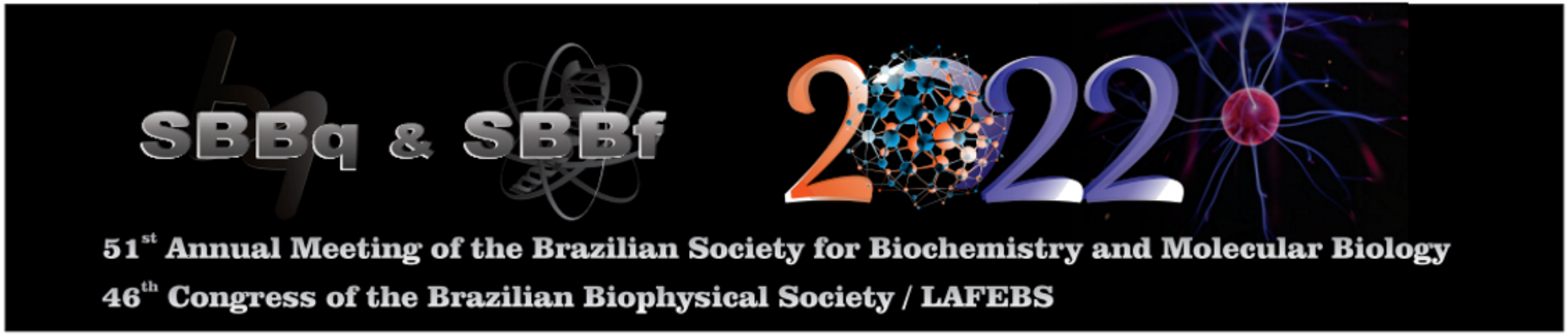
Role of TERRA in telomere regulation and replicative senescence in Leishmania major

Autoria de: **Luiz Henrique Castro Assis**

foi apresentado na sessão de pôster durante a **Escola FAPESP 60 Anos: Ciências Exatas, Naturais e da Vida**, realizada no Hotel Fazenda Dona Carolina, Itatiba - SP, de 7 a 10 de agosto de 2022.

Ronaldo Aloise Pilli
Vice-presidente do Conselho Superior da FAPESP

Euclides de Mesquita Neto
Coordenação Adjunta - Programas Especiais e Colaborações em Pesquisa - FAPESP



Águas de Lindóia, São Paulo
September 5th to 8th ,2022

Certificate

We hereby certify that the abstract entitled

ESTABLISHMENT OF LEISHMANIA MAJOR KNOCKOUT LINES TO STUDY TELOMERE DYNAMIC THROUGH BASE J LEVELS AND TERRA EXPRESSION

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President of SBBq
Leda Quercia Vieira

President of SBBf
Rosangela Itri

EMBL

Certificate

Maria Isabel Nogueira Cano

attended the

EMBO | EMBL Symposium: The non-coding genome

presented the poster: "Depletion of base J in Leishmania major triggers alterations inTERRA expression and telomere length"

on 11 - 14 October 2023, EMBL Advanced Training Centre

EMBL
European Molecular Biology Laboratory
Course and Conference Office
Meyerhofstr. 1
D-69117 Heidelberg, Germany

Heidelberg, 8 February 2024

ANEXO III

UNIVERSIDADE ESTADUAL PAULISTA

Pró Reitoria de
Extensão Universitária

CERTIFICADO

CERTIFICAMOS para os devidos fins que LUIZ HENRIQUE DE CASTRO ASSIS, CPF nº 068.202.096-65, participou no Curso de Extensão Universitária - na forma de Curso de Difusão do Conhecimento "Curso de Boas Práticas de Laboratório e Biossegurança", sob a coordenação do Prof. Dr. Rogerio Antonio de Oliveira, no período de 17/11/2021 a 21/11/2021, com duração/carga horária de 30 (trinta) horas a distância, promovido por Instituto de Biociências - IBB, tendo obtido conceito APROVADO e 75.00% de frequência.

Documento emitido em 14/07/2022

Código de autenticidade: 3E8C-C35F-D73E-B30B-0A7A-A68B-8D61-CDF5

Curso: Curso de Boas Práticas de Laboratório e Biossegurança
Boas práticas de Laboratório e Biossegurança
- C.H. Distância: 30 horas.

CERTIFICO QUE

Luiz Henrique Castro Assis

PARTICIPOU DO EVENTO **"DESAFIOS NA EDUCAÇÃO E CIÊNCIA NO BRASIL FACE AO NEGACIONISMO E AOS CORTES NO FINANCIAMENTO"**, MINISTRADO PELOS PROFS. RICARDO GALVÃO (IF-USP) E MARIANA MOURA (IEE-USP) E ORGANIZADO PELO PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS (GENÉTICA) E PELA AGÊNCIA DE DIVULGAÇÃO CIENTÍFICA E COMUNICAÇÃO (AGDC) DO INSTITUTO DE BIOCIÊNCIAS DE BOTUCATU - UNESP, EM 23 DE JUNHO DE 2022, CONTABILIZANDO 2 HORAS.

PROFA. ASSOCIADA MARIA ISABEL NOGUEIRA CANO
COORDENADORA DO PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS (GENÉTICA)

PROFA. ASSOCIADA ADRIANE PINTO WASKO
COORDENADORA DA AGÊNCIA DE DIVULGAÇÃO CIENTÍFICA E COMUNICAÇÃO (AGDC)

AgDC

IB Instituto de Biociências Unesp Botucatu

ANEXO IV

ATESTADO

ATESTAMOS que o Prof. Dr. Luiz Henrique de Castro Assis, RG MG12850787 - SSP-MG, ministrou/ministra aulas aos discentes do Programa de Pós-graduação desta Unidade Universitária, conforme abaixo descrito:

Programa: Ciências Biológicas (Genética)

Disciplina	Ano	Dt.Início	Dt.Término	Curso	Discentes	Hr/Aula
Seminários em Genética	2022	11/08/2022	24/11/2022	MD	19	30

Botucatu, 02 de fevereiro de 2024

Maria Victória Ramalho da Cunha
Assistente Administrativo II da Seção Técnica de Pós-Graduação
Instituto de Biociências de Botucatu

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

CERTIFICADO

Informo que o Dr. Luiz Henrique Castro de Assis, participou como docente assistente da disciplina optativa Fundamentos em Biotecnologia, ministrada aos alunos do curso de graduação em Ciências Biológicas, IBB-UNESP, no período de 03/07/2023 a 07/07/2023, totalizando 30h/aula.

Maria Isabel Nogueira Cano
Professora Titular
Responsável pela disciplina
Depto. Ciências Químicas e Biológicas, IBB-UNESP
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Botucatu, 19 de julho de 2023

Instituto de Biociências – DCQB
Rua Professor Doutor Antonio Celso Wagner Zanin, 250
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Tel (14) 3880 0388 maria.in.cano@unesp.br

ANEXO V

ATESTADO

Atestamos que o Prof. Dr. LUIZ HENRIQUE DE CASTRO ASSIS, RG nº MG12850787, expedido pela SSP/MG, do Departamento de Ciências Químicas e Biológicas do Instituto de Biociências de Botucatu - UNESP, participou das seguintes comissões examinadoras:

Programa: Ciências Biológicas (Genética)

Discente	Curso	Data	Tipo
DANIEL FERNANDES DA COSTA	Mestrado Acadêmico	20/12/2018	Defesa
LUCAS BENITES DORETTO	Mestrado Acadêmico	20/12/2018	Defesa
LUCAS BENITES DORETTO	Doutorado	31/08/2021	Qualificação
BEATRIZ JACINTO ALVES PEREIRA	Doutorado	10/02/2023	Qualificação

Botucatu, 02 de fevereiro de 2024.

Maria Victória Ramalho da Cunha
Assistente Administrativo II da Seção Técnica de Pós-Graduação
Instituto de Biociências de Botucatu

CPG | ICBUSP
COMISSÃO DE PÓS-GRADUAÇÃO

Universidade de São Paulo
Campus Armando de Salles Oliveira
Instituto de Ciências Biomédicas
spgicb@usp.br +55 (11) 3091-7439

D E C L A R A Ç Ã O

Declaro, que o(a) **Dr. LUIZ HENRIQUE DE CASTRO ASSIS**, **Pós-Doutorando do Laboratório de Telômeros do Instituto de Biociências da Universidade Estadual Paulista 'Julio de Mesquita Filho',** fez parte da Comissão Julgadora da Tese de Doutorado do(a) Senhor(a) **MARILIA DE PAIVA CAMARGO** junto ao Programa de **BIOLOGIA DE SISTEMAS – ÁREA: BIOLOGIA CELULAR, TECIDUAL E DO DESENVOLVIMENTO** deste Instituto. O trabalho apresentado tem como título: **"Regulação endócrina e parácrina do nicho espermatogonial em *Astyanax altiparanae* (Teleostei, Characidae): O papel do Amh"**. A Comissão Julgadora foi constituída pelos seguintes docentes: Profs. Drs**. Titular: LUIZ HENRIQUE DE CASTRO ASSIS, Titular: FABIANO GONÇALVES COSTA, Titular: PATRICIA PEREIRA COLTRI, e Orientadora: MARIA INES BORELLA.** A prova foi realizada no dia 04 de fevereiro do corrente.

São Paulo, 04 de fevereiro de 2022.

Profa. Dra. **ANA PAULA LEPIQUE**
Presidente CPG / ICB

ANEXO VI

pathogens

MDPI

Review

Behind Base J: The Roles of JBP1 and JBP2 on Trypanosomatids

Luiz Henrique de Castro Assis, Stephany Cacete de Paiva and Maria Isabel Nogueira Cano *

Telomeres Laboratory, Department of Chemical and Biological Sciences, Biosciences Institute, São Paulo State University (UNESP), Botucatu 18618-689, SP, Brazil
* Correspondence: maria.in.cano@unesp.br

Abstract: β-D-glucopyranosyloxymethiluracil (Base J) is a modified thymidine base found in kinetoplastids and some related organisms. Interestingly, Base J distribution into the genome can vary depending on the organism and its life stage. Base J is reported to be found mostly at telomeric repeats, on inactive variant surface glycoproteins (VSG's) expression sites (e.g., *T. brucei*), in RNA polymerase II termination sites and sub-telomeric regions (e.g., *Leishmania*). This hypermodified nucleotide is synthesized in two steps with the participation of two distinct thymidine hydroxylases, J-binding protein 1 and 2 (JBP1 and JBP2, respectively) and a β-glucosyl transferase. A third J-binding protein, named JBP3, was recently identified as part of a multimeric complex. Although its structural similarities with JBP1, it seems not to be involved in J biosynthesis but to play roles in gene expression regulation in trypanosomatids. Over the years, with the characterization of JBP1 and JBP2 mutant lines, Base J functions have been targeted and shone a light on that matter, showing genus-specific features. This review aims to explore Base J's reported participation as a regulator of RNA polymerase II transcription termination and to summarize the functional and structural characteristics and similarities of the remarkable JBP proteins in pathogenic trypanosomatids.

Keywords: trypanosomatids; Base J; J-binding proteins

Citation: Assis, L.H.d.C.; de Paiva, S.C.; Cano, M.I.N. Behind Base J: The Roles of JBP1 and JBP2 on Trypanosomatids. *Pathogens* **2023**, *12*, 467. https://doi.org/10.3390/pathogens12030467

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1. Base J in Model Trypanosomatids

Trypanosomatids comprise a diverse group of protozoan parasites of the class Kinetoplastids, among which are species of the *Trypanosoma* and the *Leishmania* genera, which possess dixenous development. Some of these trypanosomatids are pathogens of medical importance, causing diseases of a range of severity whose treatment and control methods are still precarious, urging the finding of new anti-parasitic drug targets [1]. Trypanosomatids, like other Kinetoplastida, present a single mitochondrion carrying circular and catenated DNA, the kinetoplast, that shows unique functionality and structure. Besides that, they also present some important features regarding genome organization and dynamics. Their genome is variable in size. The haploid genome can range from around 30 Mb in *Leishmania* sp. up to 55 Mb in *Trypanosoma cruzi*. Most of their genes are organized in large clusters, polycistronicaly transcribed and processed by *trans*-splicing [2–4].

Moreover, many aspects of transcriptional regulation are still under investigation, mainly due to the absence of canonical promoters in RNA polymerase II transcribed genes. Species such as *Trypanosoma brucei* and *T. cruzi* present a set of genes involved with virulence being transcribed at the subtelomeric position [5,6], transforming the chromosome ends into potential targets for anti-parasitic treatment. At the vicinity of the genes encoding virulent proteins (such as the VSG, variant surface glycoproteins, in *T. brucei*) and towards the end of the chromosome are the telomeres. They are arrangements of DNA and proteins crucial to cell cycle maintenance and important cellular processes such as cell aging, genome integrity, and nuclear arrangement [7,8]. Moreover, as in other eukaryotes, in trypanosomatids, subtelomeric, and telomeric sequences can be transcribed in long noncoding RNAs involved in telomere maintenance and, therefore, in genome stability and cell survival [9].

cells

MDPI

Review

Cell Cycle, Telomeres, and Telomerase in *Leishmania* spp.: What Do We Know So Far?

Luiz H. C. Assis [1,†], Débora Andrade-Silva [1,†], Mark E. Shiburah [1], Beatriz C. D. de Oliveira [1], Stephany C. Paiva [1], Bryan E. Abuchery [2], Yete G. Ferri [2], Veronica S. Fontes [1], Leilane S. de Oliveira [1], Marcelo S. da Silva [2,*] and Maria Isabel N. Cano [1,*]

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† Both authors contributed equally to the paper.

Abstract: Leishmaniases belong to the inglorious group of neglected tropical diseases, presenting different degrees of manifestations severity. It is caused by the transmission of more than 20 species of parasites of the *Leishmania* genus. Nevertheless, the disease remains on the priority list for developing new treatments, since it affects millions in a vast geographical area, especially low-income people. Molecular biology studies are pioneers in parasitic research with the aim of discovering potential targets for drug development. Among them are the telomeres, DNA–protein structures that play an important role in the long term in cell cycle/survival. Telomeres are the physical ends of eukaryotic chromosomes. Due to their multiple interactions with different proteins that confer a likewise complex dynamic, they have emerged as objects of interest in many medical studies, including studies on leishmaniases. This review aims to gather information and elucidate what we know about the phenomena behind *Leishmania* spp. telomere maintenance and how it impacts the parasite's cell cycle.

Keywords: *Leishmania* spp.; leishmaniases; cell cycle; telomeres; telomerase

check for updates

Citation: Assis, L.H.C.; Andrade-Silva, D.; Shiburah, M.E.; de Oliveira, B.C.D.; Paiva, S.C.; Abuchery, B.E.; Ferri, Y.G.; Fontes, V.S.; de Oliveira, L.S.; da Silva, M.S.; et al. Cell Cycle, Telomeres, and Telomerase in *Leishmania* spp.: What Do We Know So Far? *Cells* **2021**, *10*, 3195. https://doi.org/10.3390/cells10113195

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1. Introduction

Leishmaniases are among the poverty-related endemic diseases. They are well-known to cause a wide spectrum of clinical manifestations and their harsh incidences in East Africa, the Indian subcontinent, and Latin America, where approximately one million new diagnostics are expected yearly [1]. The disease is vector-induced and caused by more than twenty species of the *Leishmania* genus, protozoan parasites that belong to the Trypanosomatidae family [1]. The invertebrate host is a phlebotomine insect that is infected during a blood meal with amastigote forms. Inside the insect digestive system, amastigotes transform into procyclic promastigotes, which are noninfective but highly proliferative forms. Procyclics eventually migrate to the proboscis and differentiate into infective metacyclic promastigote forms [2,3]. The transmission to humans occurs when a preinfected insect (female phlebotomines) regurgitates during its blood meal infective but nonproliferative forms (metacyclic promastigotes) into the mammalian host skin. Afterward, metacyclics are phagocytosed by neutrophils or macrophages, and further inside the phagolysosomes, the parasites undergo a series of morphogenetic modifications, leading to the formation of amastigotes. The amastigotes then multiply and reach the bloodstream, causing the initiation of clinical manifestations. A new cycle of infection can occur when infected macrophages are ingested by other female phlebotomines (Figure 1).

Chapter 10

Synchronization of *Leishmania amazonensis* Cell Cycle Using Hydroxyurea

Beatriz C. D. de Oliveira, Luiz H. C. Assis, Mark E. Shiburah, Stephany C. Paiva, Veronica S. Fontes, Leilane S. de Oliveira, Vitor L. da Silva, Marcelo S. da Silva, and Maria Isabel N. Cano

Abstract

Leishmania spp. comprises a group of protozoan parasites that affect millions of people around the world. Understanding the main cell cycle-dependent events could provide an important route for developing specific therapies since some factors involved in cell cycle control may have low similarity relative to their homologs in mammals. Furthermore, accurate cell cycle-dependent analyses often require many cells, which can be achieved through cell cycle synchronization. Here, we described a useful method to synchronize procyclic promastigote forms of *Leishmania amazonensis* using hydroxyurea (HU) and the analysis of its DNA content profile. This approach can be extended to other trypanosomatids, such as *Trypanosoma cruzi* or *Trypanosoma brucei*, and provides an effective method for arresting more than 80% of cells at the G1/S phase transition.

Key words *Leishmania amazonensis*, Cell cycle synchronization, Hydroxyurea, DNA content profile

1 Introduction

Leishmania genus comprises more than 20 species of single-celled protozoan parasites, most of them showing human and veterinary medical importance. They are the causative agents of leishmaniasis, a neglected tropical disease with a large spectrum of clinical manifestations that affect millions worldwide [1, 2]. The parasite presents three main life forms in its developmental cycle: amastigotes (proliferative), procyclic promastigotes (proliferative), and metacyclic promastigotes (quiescent) [3, 4]. Procyclic and metacyclic promastigotes are both flagellates. They grow and suffer morphological transformations inside the gastric apparatus of the phlebotomine (insect vector). They can also be maintained in axenic cultures under controlled conditions, facilitating laboratory manipulation. Metacyclics are preadapted to infect the mammalian host.

Zhixiang Wang (ed.), *Cell-Cycle Synchronization: Methods and Protocols*, Methods in Molecular Biology, vol. 2579,
https://doi.org/10.1007/978-1-0716-2736-5_10,

Chapter 2

The Trypanosomatids Cell Cycle: A Brief Report

Arthur de Oliveira Passos, Luiz H. C. Assis, Yete G. Ferri, Vitor L. da Silva, Marcelo S. da Silva, and Maria Isabel N. Cano

Abstract

Trypanosomatids are protozoan parasites among which are the etiologic agents of various infectious diseases in humans, such as *Trypanosoma cruzi* (causative agent of Chagas disease), *Trypanosoma brucei* (causative agent of sleeping sickness), and species of the genus *Leishmania* (causative agents of leishmaniases). The cell cycle in these organisms presents a sequence of events conserved throughout evolution. However, these parasites also have unique characteristics that confer some peculiarities related to the cell cycle phases. This review compares general and peculiar aspects of the cell cycle in the replicative forms of trypanosomatids. Moreover, a brief discussion about the possible cross-talk between telomeres and the cell cycle is presented. Finally, we intend to open a discussion on how a profound understanding of the cell cycle would facilitate the search for potential targets for developing antiparasitic therapies that could help millions of people worldwide.

Key words Trypanosomatids, Cell cycle phases, DNA replication, Telomere maintenance, Organelle segregation, Synchronization

1 Introduction

Trypanosomatids (supergroup Excavata) are a group of single-celled eukaryotes, among which are the etiologic agents of some devastating neglected tropical diseases such as leishmaniases (caused by *Leishmania* spp.), Chagas disease (caused by *Trypanosoma cruzi*), and sleeping sickness (caused by *Trypanosoma brucei*). These diseases are vector-borne and have a widespread distribution across the globe, with a harsh incidence on East Africa, the Indian subcontinent, and Latin America, where approximately one million new diagnostics are expected yearly [1–3].

Leishmania spp., *T. cruzi*, and *T. brucei* present a heteroxenic life cycle, i.e., they have two hosts: a vertebrate (mammals) and an

Arthur de Oliveira Passos, Luiz H. C. Assis, Yete G. Ferri and Vitor L. da Silva have contributed equally to this chapter.

Zhixiang Wang (ed.), *Cell-Cycle Synchronization: Methods and Protocols*, Methods in Molecular Biology, vol. 2579,
https://doi.org/10.1007/978-1-0716-2736-5_2,