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Leishmania mexicana telomeres at high resolution: Ku80, TERT, and alternative lengthening mechanisms

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Abstract

Background Telomeres are known to be important for *Leishmania* biology but the mechanistic of how the process of telomere maintenance contributes to genome stability remains an unanswered question. Their maintenance is most commonly facilitated by the telomerase ribonucleoprotein complex that elongates telomeres countering their natural shortening due to the incomplete DNA replication in each cell cycle. In some organisms, telomere maintenance is achieved through telomerase-independent mechanisms, such as the Alternative Lengthening of Telomeres (ALT) pathways described in yeasts with dysfunctional telomerase and some rare telomerase-negative human cancer cells. Molecular markers for the ALT pathway include presence of the heterogeneous (in their length and sequence) telomeres, high level of telomeric exchange between the sister chromatids, increased expression of Rad51 and associated proteins, and occurrence of extrachromosomal telomeric repeats that can be present in either linear or circular form.

Results Here, we used third-generation sequencing techniques in combination with other approaches and analyzed telomeres of *L. mexicana* at unprecedented high-level resolution. We demonstrate that Ku80 ablation-driven telomere elongation varies between chromosomes, possibly due to the chromosome-specific recombination rates, which are sequence/content dependent and associated with the structure of the telomeric tandemly repeated sequence, TTAGGG. Moreover, this telomere length heterogeneity is accompanied by an increased level of C-circles, a subclass of circular telomeric DNA highly specific for ALT activity.

Conclusions Our findings underscore that *L. mexicana* promastigotes have an inherent ability to utilize ALT, and the loss of Ku80 and/or TERT further enhanced this trait. These proteins work together to maintain telomere integrity, inhibit recombination, and stabilize telomere lengths. Our data suggest that ALT may be a fundamental and readily activated feature of *Leishmania* biology, and that telomere regulation in this organism significantly differs from what has been observed in other eukaryotic model species, including iconic *T. brucei*.

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Keywords Leishmania, Ku80, Alternative telomere lengthening

Background

Leishmania is a genus of parasitic protists of the family Trypanosomatidae [1, 2]. Being transmitted by female phlebotomine sandflies, these flagellates cause leishmaniasis in vertebrates, including humans. This neglected tropical disease has three main clinical forms (cutaneous, mucocutaneous, and visceral) and the number of reported new cases exceeds 1 million a year [3, 4].

Telomeres are the protective “caps” of eukaryotic chromosome ends essential for preserving genome stability. Their maintenance is most commonly facilitated by the telomerase ribonucleoprotein complex that elongates telomeres countering their natural shortening due to the incomplete DNA replication in each cell cycle [5]. During telomere elongation, telomerase uses its RNA component (telomerase RNA, TR), which contain the template for the synthesis of un-replicated ends of telomeres using the reverse transcriptase activity of its catalytic protein subunit, telomerase reverse transcriptase (TERT) [6, 7]. In some organisms, telomere maintenance is achieved through telomerase-independent mechanisms, such as the Alternative Lengthening of Telomeres (ALT) pathways described in yeasts with dysfunctional telomerase [8] and some rare telomerase-negative human cancer cells [9, 10], targeted retro-transposition documented in *Drosophila* [11], or amplification of terminal satellite repeats in some species of Diptera [12]. Molecular markers for the ALT pathway include presence of the heterogeneous (in their length and sequence) telomeres, elevated level of telomeric exchange between the sister chromatids, increased expression of Rad51 and associated proteins, and the occurrence of extrachromosomal telomeric repeats that may be present in either linear or circular form (so called telomeric circles or t-circles). These can occur in double-stranded, partially single-stranded, or single-stranded circles. Among these, C-circles, a subclass of single-stranded or partially single-stranded circular telomeric DNA specifically enriched in C-rich strands, is a highly specific biomarker for ALT activity [13–16].

In addition to resolving the incomplete replication of chromosome termini (so called, “end-replication problem” [17]), telomeres block recognition of chromosomal ends as the unrepaired DNA breaks (“end-protection problem”) that would otherwise result in chromosome fusions and subsequent breaks of emerging dicentric chromosomes [18, 19]. This end-protection is mediated by the specific nucleoprotein structure formed by the telomere DNA-binding proteins that inhibit DNA damage signaling [20]. From the point of view of telomere function, the role of Ku70/Ku80 complex appears

paradoxical. Indeed, this key factor of non-homologous end-joining repair (NHEJ) pathway with a high affinity to double-stranded DNA breaks [21, 22] is involved in protection of telomeres against erroneous recognition of telomeres as double-stranded DNA breaks, at least in some groups of organisms, including vertebrates, yeasts, plants, and trypanosomatids [23, 24]. Surprisingly, the effects of Ku70/Ku80 loss are highly diverse among species. For example, in human and yeasts, Ku deficiency leads to the shortening of telomeres [25–27], while in plants, the loss of Ku results in elongation of telomeres [28, 29]. The latter phenotype was explained by de-repression of the ALT activity in the presence of functional telomerase. However, in the double Ku/TERT mutants, the critical telomere shortening observed in the TERT mutants was exacerbated [30, 31]. Ablation of Ku proteins in the presence of telomerase was not associated with the signatures of genome instability, such as appearance of chromosome end-to-end fusions and anaphase bridges, but manifested in the increased levels of C-circles, intermediates of telomere processing [32]) and elevated overall telomere heterogeneity [33–35]. In line with this, decrease in the Ku80 gene copy number was recurrently observed in ALT-positive pediatric osteosarcomas implying that depletion of Ku80 promotes ALT in that system [36].

Even more surprising are the diverse effects of Ku ablation among closely related organisms of the family Trypanosomatidae. While the loss of Ku in *Trypanosoma brucei* results in telomere shortening [37, 38], telomere lengthening was observed in *Leishmania mexicana* upon deletion of Ku80 [39]. Moreover, iconic *Blastocrithidia* spp. with all three stops recoded as sense codons [40], lost Ku-coding genes from their genomes entirely [41, 42]. These results may reflect the multiple roles of Ku proteins at telomeres [43]. In particular, they were documented to facilitate telomere repeat addition through direct interaction of Ku with TR, telomere healing, and protection from recombination and nucleolytic degradation [44–46].

We have recently reported that telomeres of *Leishmania mexicana* were elongated upon deletion of Ku80, yet underlying molecular mechanisms for this phenomenon remained unknown [39]. In this work, we applied third-generation sequencing techniques and analyzed telomeres of this species at unprecedented high-level resolution. We demonstrate that Ku80 ablation-driven telomere elongation varies between chromosomes, possibly due to the chromosome-specific recombination rates, which are sequence/content dependent. Moreover, this telomere length heterogeneity is accompanied by an

increased level of C-circles (a hallmark of ALT). A similar phenotype was also observed upon ablation of the catalytic telomerase subunit-encoding gene, *TERT*, as well as in the double *Ku80/TERT* mutants, indicating relative independence of the ALT induced by *Ku* deletion from the *TERT* status.

Methods

Axenic cultivation of *Leishmania mexicana*, species validation, growth curves, and cell cycle analysis

Leishmania mexicana MNYC/BZ/62/M379 promastigotes were grown in M199 medium supplemented with 2 µg/ml bioppterin, 2 µg/ml hemin (all from Sigma-Aldrich/Merck, St. Louis, USA), 25 mM HEPES (Lonza, Basel, Switzerland), 50 units/ml of Penicillin/Streptomycin (Life Technologies/Thermo Fisher Scientific, Carlsbad, USA), and 10% heat-inactivated fetal bovine serum (BioSera, Cholet, France) at 23 °C [47]. The species identity was validated as described previously using primers *GAPDH_dir* and *GAPDH_rev* [48, 49]. Growth dynamics and cell cycle were analyzed as described before [50, 51]. Multiple comparisons using a two-way ANOVA (Analysis of Variance) test were used to compare different conditions over the WT.

Genetic manipulations in *L. mexicana* and confirmations

The CRISPR-Cas9 *L. mexicana* line was established as described previously [52]. The ablation and episomal addback of *LmxM.29.0340* (encoding *LmxKu80*) were described in [39]. To ablate *LmxM.36.3930* (encoding *LmxTERT*) on the wild-type or *LmxKu80*-null backgrounds, a similar strategy with puromycin selection (resistance gene amplified from the pTPuro_v1 plasmid [52]) was employed. For primer sequences see Table S1. Successful ablation was verified by Southern blotting and hybridization using the 5' UTR, CDS, and PURO probes on *NcoI*-digested total genomic DNA from the mid-log phase grown cells, and whole-genome sequencing with Oxford Nanopore (ON) long reads. Early cell passage (passage 5) was used in all the subsequent experiments.

Gene expression analysis of *LmxM.29.0340*, *LmxM.36.3930*, and *LmxM.28.0550* by RT-qPCR

The expression of *LmxM.29.0340*, *LmxM.36.3930*, and *LmxM.28.0550* (encoding for *LmxRad51*) were analyzed as described previously [53] using primer pairs *Ku80_qPCR_F* and *Ku80_qPCR_R*, *TERT_qPCR_F* and *TERT_qPCR_R*, *Rad51_qPCR_F* and *Rad51_qPCR_R*, respectively, and normalized to the expression of *LmxM.07.0510* (encoding a putative 60 S ribosomal protein L7a, *LmxL7a* [54]) (Table S1).

High molecular weight genomic DNA Preparation and whole-genome long reads sequencing using Oxford Nanopore platform

Genomic DNA (gDNA) was isolated from 2×10^8 wild-type (WT), $\Delta Ku80$, *Ku80* Addback, $\Delta TERT$, and $\Delta TERT \Delta Ku80$ mid-log phase ($\sim 5 \times 10^6$ cells/ml) *L. mexicana* cells by phenol/chloroform method and assessed for integrity using electrophoresis in 1% agarose gel. Each sample was further purified by the Short Read Eliminator XS kit (PacBio, Menlo Park, USA) to remove short DNA fragments and quantified with a Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, USA). Approximately 1 µg of each purified gDNA sample was used for sequencing library construction at the Genomics core facility (CEITEC Masaryk University, Brno, Czechia) with the Native Barcoding Kit 24 V14 SQK-NBD114.24 (Oxford Nanopore Technologies, Oxford, UK). The libraries were sequenced on the PromethION Flow Cell (Oxford Nanopore Technologies). The base calling was done using both the default base caller Guppy2 [55] as well as Bonito, v. 0.8.1 [56] utilizing a previously published model for improving read quality in telomere sequences [57]. Raw reads are available from the SRA archive [58] under the BioProject accession number PRJNA1147409. The quality control of nanopore long reads was done with NanoPack tools v. 1.1.0 [59]. All parameters from Nanopack (mean read length, mean read quality, number of reads, N_{50} , etc.) are shown in Table S2.

De novo long reads genome assembly and annotation

Alignment of long reads to the reference genome of *L. mexicana* MNYC/BZ/62/M379 [47, 60] was performed using Minimap2 v. 2.28 [61] and output files were sorted and indexed using SAMtools v. 1.21 [62]. SAMtools descriptive statistics (reads, coverage, mean depth, mean mapping quality, etc.) are provided in the Table S3. Output files were manually inspected using IGV v. 2.8.9 [63] to confirm gene deletions and curate structural variants in other regions of interest. Additionally, long reads raw files from each cell line were assembled *de novo* using Flye v. 2.9.5 [64], polished twice with Pilon v. 1.24 [65], and annotated using Companion v. 2 web server [66] resulting in complete genomes comparable to the reference genome in the TriTrypDB release 68 [67] (Table S2).

Telomere length analysis from ONT long reads

Reads containing three or more tandemly arranged telomere repeats were extracted using the BBDuk2 tool (part of the BBTools suite [68]) with the following parameters: literal = TTAGGGTTAGGGTTAGGG, k = 18, hdist = 1. To identify reads corresponding to particular chromosome ends, we used 10 kb-long fragments extracted from the assembled chromosome ends capped with

telomere repeats in *L. mexicana* MNYC/BZ/62/M379 [60]. A total of 43 chromosomal ends were subjected to analysis. These 10-kb sequences were used as queries in BLASTn searches [69] against the set of extracted telomeric ONT reads. Reads were considered specific to a particular chromosome end if they matched with over 30% query coverage and had a query start position within the first 50 nt, ensuring unambiguous alignment of ONT long reads (BLASTn hits) to true chromosome termini. For each chromosome arm and genotype, the number and length of filtered telomeric reads were summarized. Telomere length was determined as the portion of each read extending beyond the region homologous to the query (i.e., the flanking sequence in the centromere to telomere direction), measured in Geneious R8.1 (<https://www.geneious.com>). The resulting BLASTn metrics and calculated telomere lengths were compiled into Table S4. Firstly, telomere length heterogeneity of all chromosomes together was assessed using two non-parametric tests, Wald-Wolfowitz one-sample runs test and Kruskal-Wallis H test to assess whether the distribution of values differed significantly across groups followed by (multiple) pairwise comparison (all groups included) analysis with Dunn's test and Bonferroni correction [70, 71] (Table S5). Secondly, chromosome-specific differences across groups were analyzed using Kruskal-Wallis H test, for significant results, a post-hoc Dunn's test was used to pinpoint specific group differences and significant *p*-values were adjusted with the Benjamini-Hochberg procedure to control the False Discovery Rate (FDR) (Table S6).

Additionally, the abundance of telomeric 5'-TTAGGG-3' repeats [72] was analyzed in 500 bp- left and right telomeric termini using a customized Python script (https://github.com/edubielalpizar/Ku80-TERT_MS_2025) (Table S7). To further support the recovery of terminal structures, they were manually inspected confirming that reads (and telomeric repeat motifs) were mapped unequivocally to unique chromosome termini. Notably, in many cases, ONT reads span tens of kilobases (over 80 kb at some chromosome ends) all the way into unique genic regions mapping through multiple annotated chromosome-specific ORFs and non-coding sequences, thus confirming their precise genomic placement. Statistical analyses and visualization were performed using RStudio v. 3.5.2 packages dplyr, FSA, multcomp and dunn.test, as well as ggplot2 v. 3.5.2 and Python v. 3.12.4 scripts [73, 74]. Details of the absolute- and normalized by coverage- counts and statistical analysis of the TTAGGG hexamers are provided in Table S7. The source code can be found at https://github.com/edubielalpizar/Ku80-TERT_MS_2025.

Analysis of structural variants

Structural variants (SVs) were analyzed using two open-source pipelines for long-read alignments: NanoVar v. 1.8.3 [75] and Sniffles v. 1.18 [76, 77]. SV hits from NanoVar ($N = \sim 3,268$) and Sniffles ($N = 4,529$) were filtered to remove variants with medium or low confidence scores and, in the case of Sniffles, SVs with 'imprecise break-point' flag, retaining only high confidence variants with scores from 12 to 15 (NanoVar) and 56 to 60 (Sniffles). Outputs from both pipelines were combined to remove duplicate SVs and those present in the parental line, resulting in 156 mutant specific SVs (109 - NanoVar and 47 - Sniffles) (Table S8). Manually curation was performed using IGV v. 2.8.9 tool [63] to characterize coding-, intergenic- or telomeric regions; in the former case, genes were annotated using TriTrypDB release 68 [67]. Translocations were mapped and visualized with Circos [78].

PETRA assay

The left end of chromosome capped by TTAGGG repeat in the *L. mexicana* genome assembly (GenBank accession number SRP126412 [47]) was analyzed by the Primer Extension Telomere Repeat Amplification (PETRA) assay [31] using high molecular weight genomic DNA prepared as above. Two sub-telomeric primers were designed at a specific distance from each other as a control for the corresponding shift in the lengths of PETRA amplification products (Table S1). Reactions were performed in two steps using Q5 High-Fidelity 2× Master Mix (New England Biolabs, Ipswich, USA). The first (extension) reaction with 1 µg gDNA and PETRA-T primer (Table S1) at 0.8 µM in total reaction volume of 25 µl was incubated for 5 min at 65 °C, 2 min at 55 °C, and 10 min at 72 °C. One µl from the extension reaction was used as a template for the PETRA PCR (30 s at 98 °C - 16 cycles of (10 s at 98 °C, 30 s at 60 °C, and 3 min at 72 °C) - 10 min at 72 °C) in 25 µl reaction volume with PETRA A and a specific sub-telomeric primers at 0.5 µM (Table S1). PETRA PCR products were separated by agarose gel electrophoresis, transferred onto the Hybond-XL nylon membrane (Cytiva/Danaher, Marlborough, USA) and hybridized with a telomere oligonucleotide probe huTEL4× (Table S1) that was radioactively labelled with [γ -P³²] ATP using T4 polynucleotide kinase (Thermo Fisher Scientific). Signals were visualized with a Typhoon phosphorimager (Cytiva) and evaluated using ClinX image analysis software (ClinX Science Instruments, Shanghai, China).

C-Circle Assay (CCA)

The CCA includes extraction of the total DNA encompassing C-circles, Rolling Circle Amplification (RCA), and detection by qPCR using specific primers [79].

A pellet of 10^8 *L. mexicana* cells was washed once with $1 \times$ PBS, resuspended in 50 μ l of lysis buffer (10mM Tris-HCl, 50mM KCl, 2mM MgCl₂, 0.5% SDS, pH 8.5, 50 mAU/ml proteinase K (Qiagen, Hilden, Germany) and shaken at 1,400 rpm for 1 h at 56 °C followed by 20 min incubation at 70 °C to inactivate proteinase. Samples were cooled down to room temperature and quantified using Qubit 4 Fluorometer. 32 ng of genomic DNA was incubated with 1 μ M of the G-rich primer (Table S1) for 5 min at 96 °C, cooled down to room temperature, and added to the Mastermix (4 mM DTT, $1 \times$ ϕ 29 buffer, 4 μ g/ml bovine serum albumin (all from New England Biolabs, Ipswich, USA), 1 mM dNTPs (VWR/Avantor, Radnor, USA), and 0.1% Tween-20 (Thermo Fisher Scientific) in the total volume of 20 μ l. The RCA reactions were performed with 7.5 U of ϕ 29 DNA polymerase (New England Biolabs) for 8 h at 30 °C followed by the inactivation step for 20 min at 70 °C. A reaction without ϕ 29 DNA polymerase was included as a negative control.

The RCA products were cleaned up with QIAquick PCR purification kit (Qiagen) and 2 ng of DNA was used for qPCR analysis with Telomeric_CCA_F/R primers (Table S1) at 200 nM in 10 μ l reaction. Abundance of a single copy *LmxM.33.0070* gene (encoding ascorbate peroxidase) [80] was used for RCA normalization (Table S1). The qPCR reactions were performed in technical triplicates for at least three biological replicates and quantified over the values obtained for reactions performed without ϕ 29 DNA polymerase.

Results

Establishment of the *LmxM.36.3930*^{-/-} (Δ TERT) and *LmxM.36.3930*^{-/-}*LmxM.29.0340*^{-/-} (Δ TERT Δ Ku80) *Leishmania mexicana* lines, validation and confirmation

To investigate molecular mechanisms governing telomere elongation upon deletion of Ku80 in *Leishmania mexicana*, we first established 2 additional lines referred to here as Δ TERT and Δ TERT Δ Ku80, in which the TERT-encoding *LmxM.36.3930* was ablated either on the wild-type or *LmxM.29.0340*^{-/-} (encoding Ku80) backgrounds. The successful knock-outs were validated using whole genome long reads Oxford Nanopore Technology (ONT) sequencing (Fig. 1A), RT-qPCR (Fig. 1B), and by Southern blotting followed by hybridization (Fig. S1A-B). Please note that in the Ku80 Addback, the coverage appears lower (alongside with the small gaps at both sides of this locus where reads do not align perfectly). This indicates that those reads do not actually belong to the analyzed locus but are mistakenly aligned here due to the sequence similarity. The gene expression analysis in Fig. 1B confirms successful add-back. Cell growth analysis revealed that Δ Ku80 *L. mexicana* cells divide slower compared to other lines, a phenotype that was partially restored in the Ku80 Addback line (Fig. S1C).

Our analysis of the cell cycle in *L. mexicana* confirmed data obtained for another *Leishmania* species, *L. major*, namely that ablation of TERT leads to the increased retention of cells in the G0/G1 phase of the cell cycle [81]. We also noted other changes, but they were not statistically significant (Fig. S1D).

Cross-talk between *LmxTERT* and *LmxKu80* at the level of gene expression

LmxTERT and *LmxKu80* appear functionally connected. Indeed, compared to the wild-type, the expression of *LmxKu80* was elevated when *LmxTERT* was ablated; and, conversely, the expression of *LmxTERT* was elevated under *LmxKu80* knock-out conditions (Fig. 1B). The exact mechanism of this interaction needs to be investigated further but we can speculate that *LmxKu80* can bind to the *tert* promoter to inhibit its expression as has been recently shown in other biological settings [82–84].

We also check the expression level of Rad51 (one of the main factors of involved in homologous recombination and break-induced replication [85, 86]) upon ablation of *LmxKu80*, *LmxTERT*, or both proteins. RNA-mediated recruitment of Rad51 to telomeres was implicated in human ALT cancer cell maintenance [87, 88]. In *T. brucei*, Rad51 was shown to interact with RNA-DNA hybrids and repair abundant DNA breaks at the single transcribed VSG locus [89, 90], while in *L. major*, conditional ablation of Rad51-related genes affected homologous recombination and, consequently, genome replication [16]. Expression of *LmxRad51* was upregulated upon deletion of either *LmxKu80* or *LmxTERT* and restored to the wild-type level in the Ku80 Addback line. Expression of *LmxRad51* was not upregulated when both *LmxKu80* and *LmxTERT* were deleted (Fig. 1B). This resembles previously reported results in *A. thaliana*, where in the absence of Ku proteins, ALT is triggered and Rad51 is upregulated to resolve ensuing recombination events [30, 33]. Conversely, when TERT is lost, telomere replication is compromised, leading to critically short telomeres that signal DNA damage and similarly activate ALT via Rad51 induction (ALT type I) resulting in recombination that includes telomeres and interstitial telomere sequences as ALT precursors. However, in the absence of both TERT and Ku80, Rad51-independent ALT type II is preferentially activated, since telomeres are not only eroded, but also unprotected against telomere-to-telomere recombination [91]. It must be noted that this hypothesis remains speculative and must be tested directly in the *LmxRad51* knock-out parasites in the future.

Ablation of *LmxTERT* and *LmxKu80* leads to activation of ALT: C-circles

We hypothesized that ablation of *LmxKu80* enhances ALT and validated it by analyzing C-circles. These are

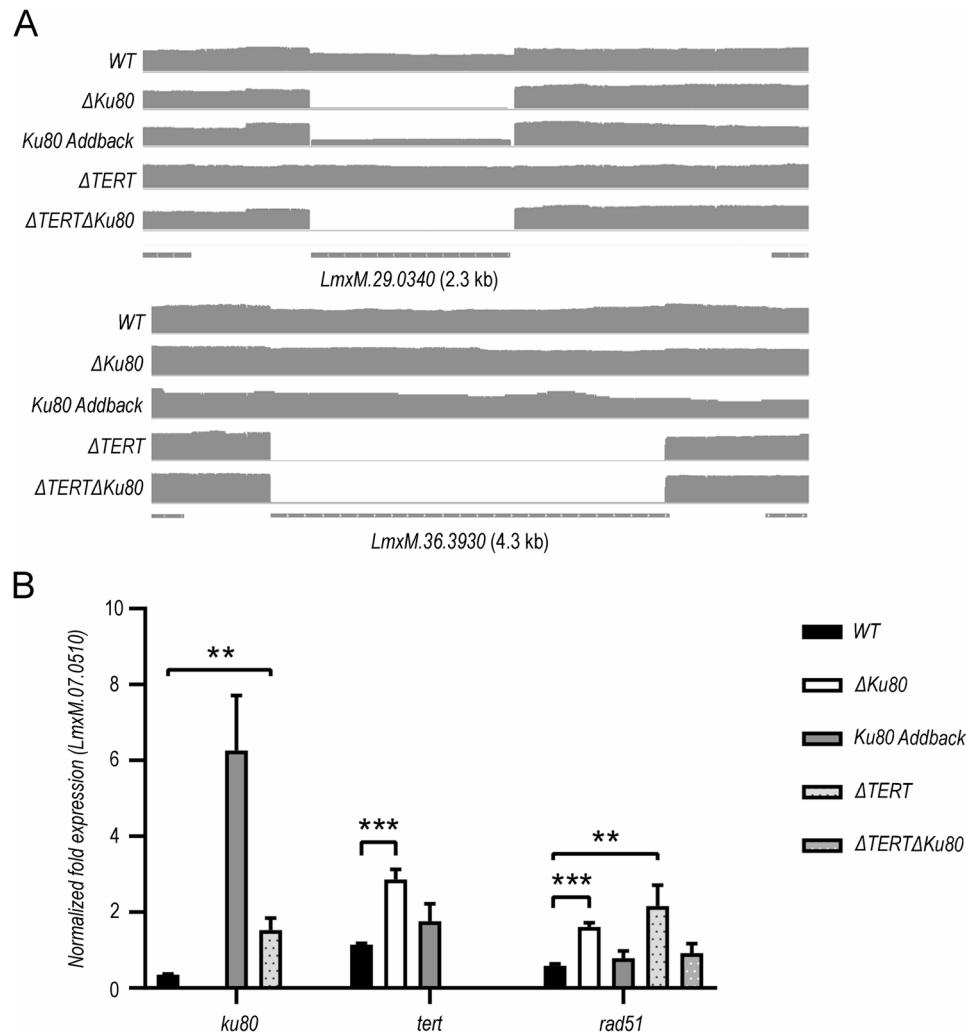


Fig. 1 Establishment and functional characterization of the $\Delta TERT$ and $\Delta TERT\Delta Ku80$ *L. mexicana* lines. **(A)** ONT long reads mapping to *LmxM.29.0340* (encoding *LmxKu80*) and *LmxM.36.3930* (encoding *LmxTERT*) loci of *L. mexicana* in WT, $\Delta Ku80$, Ku80 Addback, $\Delta TERT$, and $\Delta TERT\Delta Ku80$ lines. **(B)** Quantitative RT-PCR analysis of *LmxM.29.0340* (encoding *LmxKu80*), *LmxM.36.3930* (encoding *LmxTERT*), and *LmxM.28.0550* (encoding for *LmxRad51*) expression in WT, $\Delta Ku80$, $\Delta Ku80$ Addback, $\Delta TERT$, and $\Delta TERT\Delta Ku80$ lines of *L. mexicana*. The *p*-values for the unpaired Student's t-test are shown (** and *** denote *p*-values below 0.01 and 0.001, respectively). Data are summarized from three independent biological replicates. The error bars indicate standard deviation.

t-circles with a covalently closed, partially single-stranded C-rich strand capable of self-priming Rolling Circle Amplification (RCA) [92–94]. As shown in Fig. 2, the abundance of C-circles was significantly increased upon ablation of *LmxKu80*, a phenotype that was reverted in the addback line. Notably, increased C-circles' levels were also observed in the *Lmx* $\Delta TERT$ and *Lmx* $\Delta TERT\Delta Ku80$ lines, further confirming active ALT in these mutants and indicating that *LmxTERT* and *LmxKu80* are functionally connected in telomere maintenance.

Ablation of *LmxTERT* and *LmxKu80* leads to activation of ALT: heterogeneity of telomeres

Next, we decided to take advantage of the unprecedented high resolution of telomeric sequences yielded from the long read ONT data (Fig. S2A, Table S2) and analyze

telomere heterogeneity in *Leishmania* chromosomes upon deletion of *LmTERT* and *LmKu80*. Analysis of overall telomere length (of all chromosomes together) revealed extreme heterogeneity in all the cell lines under study, including the wild-type (Fig. S2B). To confirm this, a Kruskal-Wallis multiple comparison test of telomere length distribution revealed significant differences among the groups tested ($H(4) = 15.187$, $p = 0.004329$), indicating that at least one group was substantially different, warranting further investigation into the specific group differences. For this, pair-wise comparisons using post-hoc Dunn's test (*p*-values adjusted with the Benjamini-Hochberg method) showed that when the entire karyotype considered, telomere lengths in $\Delta TERT$ ($z = 3.2$, *p*-adjusted = 0.01) and $\Delta TERT\Delta Ku80$ ($z = 3.0$, *p*-adjusted = 0.01) were significantly higher than in the

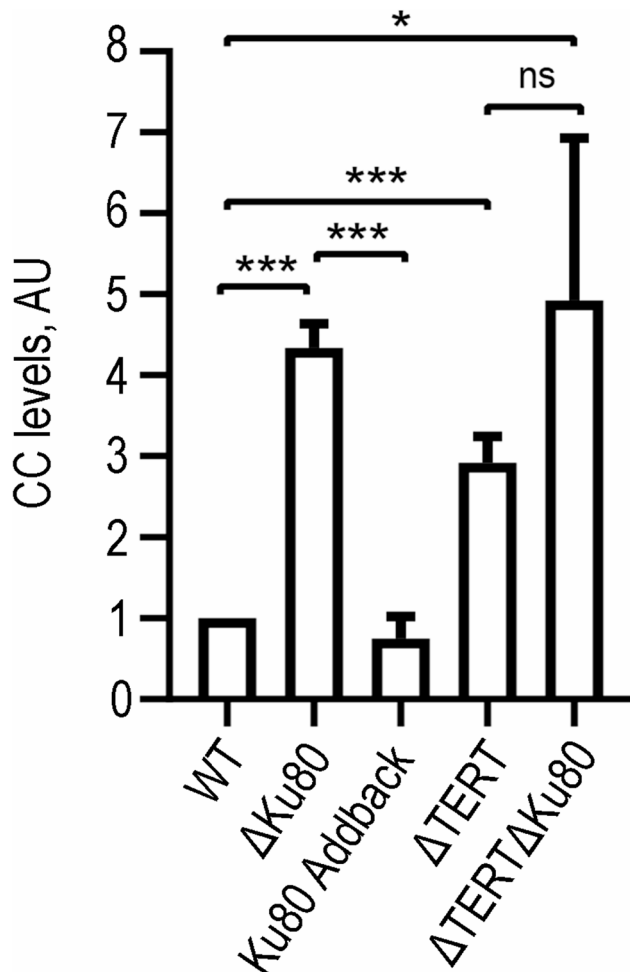


Fig. 2 C-circle assay for the WT, Δ Ku80, Ku80 Addback, Δ TERT, and Δ TERT Δ Ku80 *L. mexicana* cells. Data of at least three independent biological replicates are presented in arbitrary units normalized to the WT. *, **, and *** indicate unpaired Student's t-test *p*-values below 0.05, 0.01, and 0.001, respectively; ns, non-significant.

parental WT, while no other pairwise comparisons were significant, agreeing with a previous report, in which variability of the telomeric regions has been described [95]. This likely means that there is no unifying pattern that can be applied to all *L. mexicana* chromosomes and implies presence of chromosome-specific traits, as shown previously for *L. donovani* [96]. For example, our analysis of the left arm of the *L. mexicana* chromosome 19 demonstrated increase of the telomeric length upon ablation of *LmxKu80*, *LmxTERT*, or two proteins simultaneously. This phenotype was reverted when *LmxKu80* was added back (Figs. 3 A and 4) and correlated with the abundance of the 6-bp telomeric sequence TTAGGG in chromosome *LmxM.19* (Fig. 5). Notably and consistent with previous reports, organization of *Leishmania* spp. telomeres substantially differs from that in the best-characterized model trypanosomatid, *T. brucei* [97–99] or *T. congolense* [100], where the conserved telomeric region

is associated with Variant Surface Glycoprotein (VSG) loci. Conversely, in *Leishmania* spp., telomeric repeats were shown to be interspersed with telomeric satellites (referred to as *Leishmania* conserved telomere-associated sequence, LCTAS, first reported in *L. major* [101] but later shown to be present in other *Leishmania* spp [102, 103]). It has been proposed that these short non-coding repetitive sequences are maintained *via* terminal amplification, relying on replication slippage, which may operate with or without telomerase. As exemplified by chromosome 19 of *L. mexicana* (Fig. 3 A), the organization of telomeres in this species follows the same pattern with conserved satellite repeats (black arrows in Fig. 3 A) intermingling clusters of telomeric repeats (light grey triangles in Fig. 3 A). This complex telomere arrangement presents a limitation to the classical approaches of telomere length estimation, such as, for example, Telomere Restriction Fragment analysis [104]. Notably, the PETRA assay demonstrated the same trend (Fig. 3B). The observed discrepancies between the ONT and PETRA data (Fig. 3) can be explained by the technical limitations of the latter approach. Indeed, its results depend on the presence of ssDNA overhangs, which appear to be depleted in the telomeric sequences of *LmxΔKu80*, *LmxΔTERT*, and *LmxΔTERTΔKu80* mutants.

Prompted by the observations discussed above, we reconstructed telomeric structures at 43 chromosomal ends (belonging to 28 chromosomes) in all five analyzed cell lines. This detailed analysis (Fig. 4, Table S4) reconfirmed our conclusion on extreme telomere heterogeneity. To determine if telomeres became more heterogeneous upon ablation of *LmTERT* and *LmKu80*, we employed the Wald-Wolfowitz runs test that determines whether difference in the distributions of two analyzed samples is significant. Group A (WT) contained 1,019 observations, while groups B contained 670; 385; 1,155, and 1,768 observations for telomeres of the Δ Ku80, Δ Ku80 Addback, Δ TERT, and Δ TERT Δ Ku80, respectively. The test revealed totals of 766, 532, 924, and 1,177 runs for the above-mentioned cell lines that deviated from the expected number of runs under the null hypothesis of randomness. Statistical analysis showed that the difference was significant for Δ Ku80, Δ TERT, and Δ TERT Δ Ku80 lines of *L. mexicana* (*p*-values below 0.05 in all the instances) and not significant for Δ Ku80 Addback (*p*-value=0.067) (Table S5), indicating that ablation of *LmTERT* and *LmKu80* may result in appearance of overall more heterogeneous telomeres. Likewise, the non-parametric Kruskal-Wallis H test confirmed statistically significant differences among the distributions of telomere lengths across groups with *p*-value of 0.004 (Table S5). Following this statistical analysis considering all chromosomes together, we then performed a similar analysis per chromosome (left and right termini)

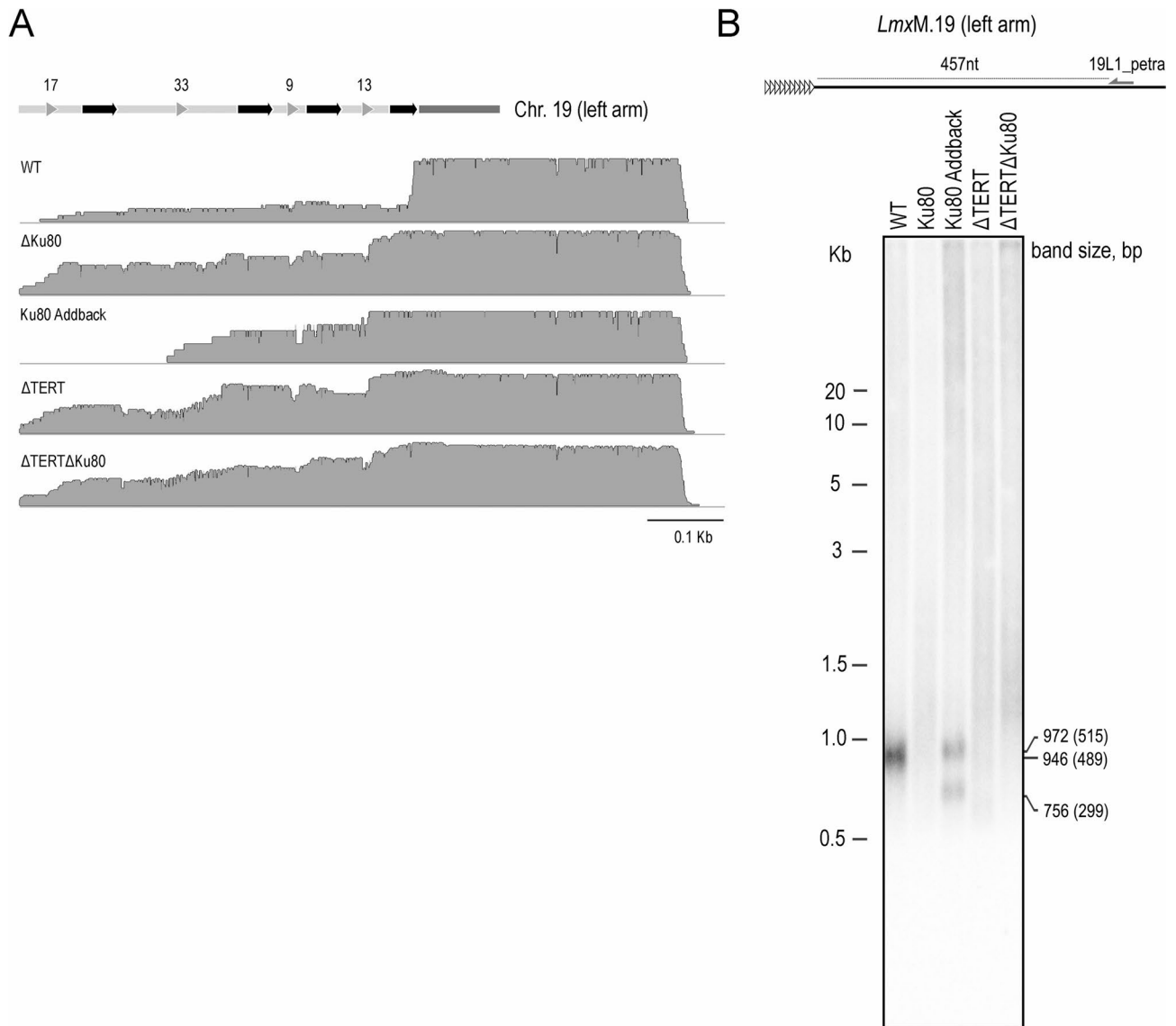


Fig. 3 Telomeres at the left arm of *L. mexicana* chromosome 19. **(A)** ONT reads coverage mapped to the reference genome in WT, ΔKu80, ΔKu80 Addback, ΔTERT, and ΔTERTΔKu80 lines of *L. mexicana* for the left arm of chromosome 19. Telomeric organization scheme is shown on top: light grey regions marked with triangles denote telomeric repeats (number of repeats is indicated above the scheme); dark grey regions are sub-telomeric loci; black arrows denote satellite repeats. **(B)** PETRA analysis of telomere lengths at left arm of chromosome 19 for the WT, ΔKu80, Ku80 Addback, ΔTERT, and ΔTERTΔKu80 *L. mexicana*. Band sizes are indicated in bp and the median average of the fragment sizes are shown in parentheses (observed value minus 457 nt of the linker).

and found significant differences (p -value < 0.05) in telomere length for 18 out of 28 chromosomes. From these, pairwise comparisons between groups (post-hoc Dunn's Test) pinpointed that in 14 chromosome ends (*LmxM.02*, 04, 07–09, 11, 13–15, 22, 27, 29, 31 and 34), telomeres were significantly more heterogeneous with respect to WT (Table S6). Notably, ΔTERT and ΔTERTΔKu80 showed the highest number of chromosomes (ten and nine, respectively) statistically different from those of the WT and chromosomes *LmxM.02*, 04, 07, 09, 15, and 22 were significantly more heterogeneous in both cell lines (Fig. 4). In summary, telomere change rate showed an

increasing trend in all groups in most chromosomes and is chromosome-end (left/right) specific.

Next, we identified the presence of the telomeric repeats TTAGGG (Table S7), a hallmark of telomeres in multiple organisms including trypanosomatids [72, 81, 105]. In our study, 1 kb-long chromosome ends (left and right) of all 34 chromosomes were manually inspected and confirmed to contain reads of good mapping quality (MQ over 60) verifying that these reads reached both telomeric ends, as annotated by the most recent (hybrid) reference genome assembly relying on both short- and long reads [52, 67]. This continuity from the telomeric



Fig. 4 Telomere lengths for 28 chromosomes of *L. mexicana*. Telomere length (x axes) in all cell lines (y axes) was reconstructed from ONT reads of 43 chromosomal ends covering 28 chromosomes. Where available, data for the left and right ends of chromosomes were combined. Black dots indicate statistically significant post-hoc Dunn's Test *p*-values adjusted with the Benjamini-Hochberg method. Non-significant values are shown in gray.

repeats into the gene-rich, uniquely mappable regions provides strong evidence that these TTAGGG telomeric sequences genuinely originated from the chromosomal ends and not from the internal telomere-like repeats or misalignments.

Analysis of structural variants in *L. mexicana* genomes

Comparison of the repertoires of structural variants (SVs) across five studied cell lines showed that the majority of them were either of low- or middle confidence or present in the wild-type and, thus, filtered out. In total, 156 high-confidence mutant specific SVs (57 translocations, 51 insertions, 32 deletions, 13 duplications, and 3 inversions) were mapped onto *L. mexicana* chromosomes

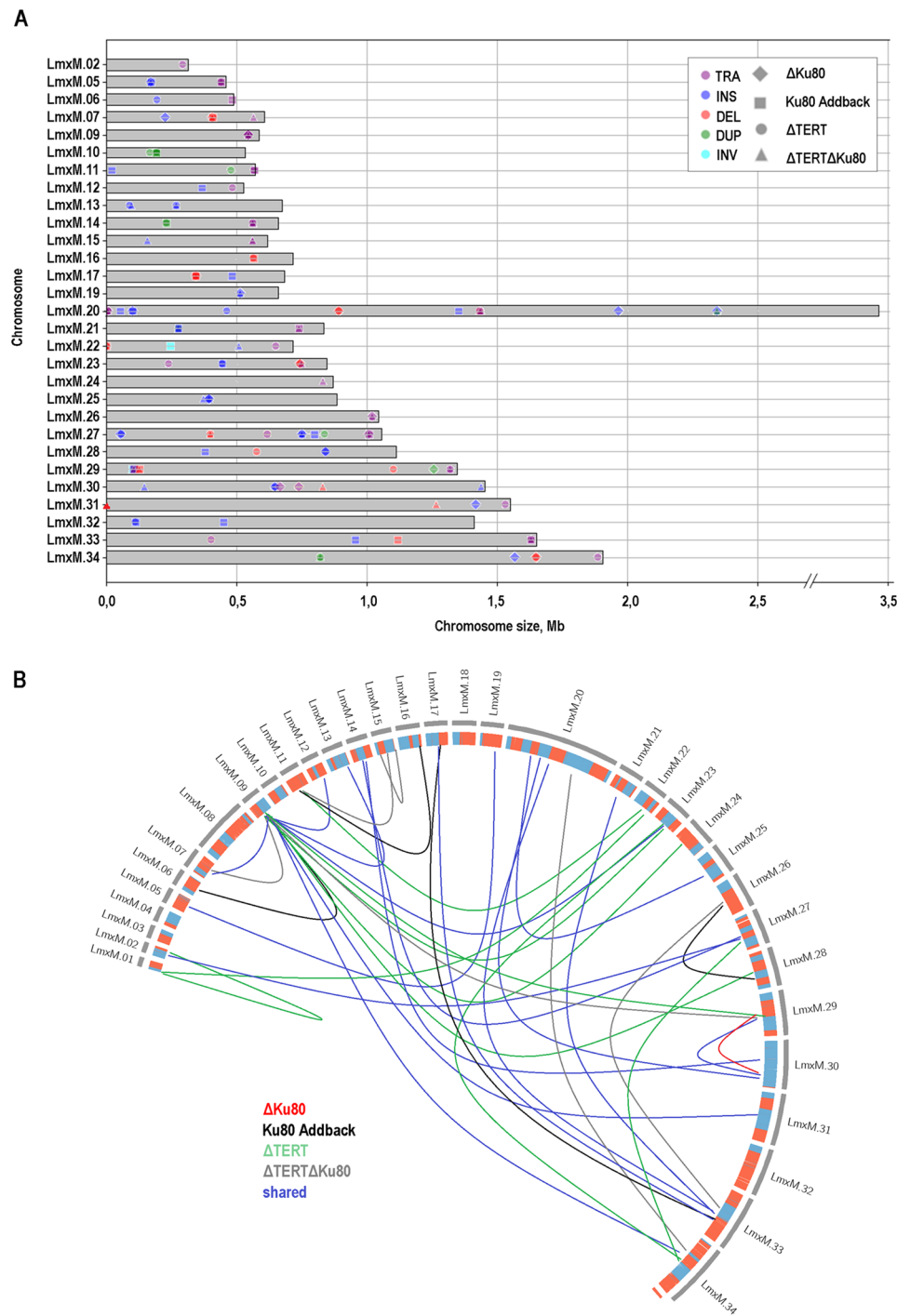


Fig. 5 Mutant specific structural variants in *L. mexicana*. **(A)** Schematic mapping of SVs that are not present in the wild-type genome on *L. mexicana* chromosomes. Please note that only chromosomes with identified SVs (translocations (TRA), insertions (INS), deletions (DEL), duplications (DUP), and inversions (INV)) are shown. Translocations are listed based on the calls of the first chromosome coordinates (when two chromosomes are involved, the second one is not called). Chromosome 20 in *L. mexicana* is a fusion of chromosomes 20 and 36 in other *Leishmania* spp. Other details are provided in the graphical legend. **(B)** Circos representation of genomic translocations in analyzed lines mapped onto parental background. Lines show translocations in each group (ΔKu80, red; Ku80 addback, black; ΔTERT, green; ΔTERTΔKu80, grey) and shared in more than one cell line (blue). Gray banners show relative size of *L. mexicana* chromosomes; blue and red denote genes mapped to the forward and reverse strands, respectively.

(Fig. 5A). Please note that the genome of this species consists of 34 chromosomes (in contrast to, for example, 36 chromosomes of *L. major* [106]), as chromosomes 8 and 29, as well as 20 and 36 are fused [107]. Insertions and translocation accounted for the majority of SVs with the latter localized mainly at the end of the chromosomes (Fig. 5B). The distribution of translocations showed 22 unique events in Δ TERT, 19 instances shared between Δ TERT and Δ Ku80 lines, followed by 9 and 7 cases present in Δ Ku80 and Ku80 Addback, respectively. The coordinates of these translocations showed that in most cases they involved intergenic regions or genes encoding hypothetical proteins.

Discussion

Telomeres are known to be important for *Leishmania* biology [102, 103, 105] but the mechanistic of how the process of telomere maintenance contributes to genome stability remains an unanswered question. In the present study, we demonstrate the upregulation of ALT pathway in *Leishmania mexicana* upon ablation of *LmxKu80* and *LmxTERT*. Using Southern hybridization, it has been previously shown that the absence of this protein results in the modest increase in telomere length [39]. Conversely, the ablation of its homolog in a related trypanosomatid species, *T. brucei* resulted in telomere shortening [37, 38]. A phenotype of telomere elongation upon Ku deletion, similar to that in *L. mexicana*, was also observed in *A. thaliana* [33]. In *Saccharomyces cerevisiae*, Ku proteins protect telomeres against nucleolytic degradation and facilitate TERT recruitment [25, 108], while loss of these genes leads to telomere shortening and increased recombination, with double knockout of Ku and TERT proved to be lethal [109, 110]. Conversely, in *Schizosaccharomyces pombe*, Ku proteins are dispensable for telomerase recruitment or telomere circularization; Ku/TERT double mutants remain viable, highlighting organism-specific dependencies on these proteins [111, 112].

Until now, a mutual relationship between Ku and TERT proteins in *L. mexicana* remained unclear, even though the alternative mechanisms of telomere lengthening have been reported [113]. Our findings reveal that knockout mutants for *LmxKu80* and *LmxTERT* exhibit chromosome end-specific telomere elongation that correlates with elevated levels of C-circles (a molecular marker of the ALT activity [92–94]), as previously reported in *T. brucei* [114]. These observations suggest that *LmxKu80* and *LmxTERT* play critical roles in telomere protection and suppression of ALT. Interestingly, the *LmxKu80* and *LmxTERT* double mutant line exhibited ALT activation comparable to that observed in single mutants, suggesting epistasis and a limited dependence on TERT in *L. mexicana*. This is different from *A. thaliana*, where the loss of Ku enhances telomere elongation exclusively in

the presence of TERT and where the double mutants display accelerated telomere attrition. Our data suggested that the absence of both proteins (TERT and Ku80) in *L. mexicana* is not a limiting factor for an efficient shift into ALT.

In addition, the ONT long-read sequencing data demonstrated that telomere elongation is not uniformly distributed across the genome. Only specific chromosome ends exhibited elongation in the mutants, indicating that ALT activation may depend on local chromatin context or specific telomere features. This variability might arise from differences in telomeric sequences, replication timing, chromatin accessibility, or the presence/absence or activity of telomere-binding proteins. A similar chromosome-specific telomere length variation was recently reported in *A. thaliana* [115].

Illumina platform has been previously shown to struggle with resolving repetitive structures or genes with highly similar sequences and arrayed in tandem [116, 117], underscoring the superior utility of long-read sequencing. In our study, alignment to a reference genome assembled with short-reads only led to a significantly lower resolution of telomeric ends (21 termini: 11 left and 8 right from 19 chromosomes, with chromosomes 12 and 23 resolved on both ends). For this reason, we employed ONT long-reads sequencing that revealed that the telomeres of *L. mexicana* consist of complex repeat arrays characterized by tandem blocks of canonical telomeric repeats interspersed with sub-telomeric satellite sequences, as previously observed in other *Leishmania* spp [101–103]. Nevertheless, it must be noted that the employed approach may also have drawbacks. Indeed, due to premature stop of reading the DNA fragment in ONT flow cells, the measurement of some telomeres might be shorter than they actually are. Ligating a terminal adaptor before sequencing and confirming its presence in the final read would ensure that the whole DNA fragment is sequenced, but the pipeline described above is technically challenging. Notably, classical techniques used to analyze telomeres in trypanosomatids did not have the resolution power of the current sequencing technologies employed in the present work. For instance, one of the first descriptions of LCTAS mapped to the *Leishmania* sp. genome showed that while all chromosomes possess both, the conserved and sub-telomeric regions, the hybridization signal was not uniform across chromosomes indicating that their structural arrangement was heterogeneous [102]. This agrees with our findings in *L. mexicana* showing that distribution of heterogeneous elongated telomeres is chromosome-dependent. Analysis of SVs also confirmed this conclusion as they were found only in 29 chromosomes, and, more importantly, some of them were predominantly mapped to the chromosomal ends.

The structural arrangement of telomeres in *Leishmania* spp. mirrors that found in ALT-positive organisms, such as telomerase mutants of *Caenorhabditis elegans* [118], *Physcomitrium patens* [119], or long-term *S. cerevisiae* ALT survivors [91]. The existence of such structures even in the wild-type *L. mexicana* suggests that ALT and TERT-dependent pathways co-exist in trypanosomatids. Such a structural plasticity may underpin the ability of these parasites to activate ALT rapidly following the loss of Ku or TERT.

Lastly, our results show the state of telomeres at a single timepoint, i.e. at the mid-log phase of promastigote growth. We recognize that telomeres elongation might be regulated throughout the parasite's life cycle [81, 103], and the state of telomere at different stages of *Leishmania* spp. development warrants further investigation.

In conclusion, our findings underscore that *L. mexicana* promastigotes have an inherent ability to utilize ALT, and the loss of Ku80 and/or TERT further enhanced this trait. These proteins work together to maintain telomere integrity, inhibit recombination, and stabilize telomere lengths. Our data suggest that ALT may be a fundamental and readily activated feature of *Leishmania* biology, and that telomere regulation in this organism significantly differs from what has been observed in other eukaryotic model species, including iconic *T. brucei*. This investigation used novel approaches based on the CRISPR/Cas system and third-generation sequencing filling some of the gaps of the telomere biology of *Leishmania* spp. and provided novel insights into telomere dynamics and the adaptation mechanisms of *L. mexicana*, paving the way for future investigations into the molecular underpinnings of telomere elongation, heterogeneity, and stability in this and related organisms.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12864-025-12154-z>.

Supplementary Material 1.
Supplementary Material 2.
Supplementary Material 3.
Supplementary Material 4.
Supplementary Material 5.
Supplementary Material 6.
Supplementary Material 7.
Supplementary Material 8.
Supplementary Material 9.
Supplementary Material 10.

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Author's contributions

V.Y. and J.F. conceived the study; E.A.-S., A.S., P.F., E.P., K.H., A.T.S.A. performed the analyses. E.A.-S., A.S., and P.F. prepared Figs. 1, 2, 3 and 4. All authors reviewed the manuscript.

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Data availability

Sequence data that support the findings of this study have been deposited at the National Center for Biotechnology Information under BioProject ID PRJNA1147409.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

V.Y. is an Associate Editor of BMC Genomics. Other authors declare that they have no competing interests.

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