

A Flap Endonuclease (TcFEN1) Is Involved in *Trypanosoma cruzi* Cell Proliferation, DNA Repair, and Parasite Survival

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ABSTRACT

FLAP endonucleases (FEN) are involved both in DNA replication and repair by processing DNA intermediaries presenting a nucleotide flap using its phosphodiesterase activity. In spite of these important functions in DNA metabolism, this enzyme was not yet studied in Trypanosomatids. *Trypanosoma cruzi*, the ethiological agent of Chagas disease, presents two dividing cellular forms (epimastigote and amastigote) and one non-proliferative, infective form (trypomastigote). The parasite survives DNA damage produced by reactive species generated in its hosts. The activity of a *T. cruzi* FLAP endonuclease (TcFEN1) was determined in the three cellular forms of the parasite using a DNA substrate generated by annealing three different oligonucleotides to form a double-stranded DNA with a 5' flap in the middle. This activity showed optimal pH and temperature similar to other known FENs. The substrate cut by the flap endonuclease activity could be ligated by the parasite generating a repaired DNA product. A DNA flap endonuclease coding sequence found in the *T. cruzi* genome (TcFEN1) was cloned, inserted in parasite expression vectors and transfected to epimastigotes. The purified native recombinant protein showed DNA flap endonuclease activity. This endonuclease was found located in the parasite nucleus of transfected epimastigotes and its over-expression increased both parasite proliferation and survival to H₂O₂. The presence of a flap endonuclease activity in *T. cruzi* and its nuclear location are indicative of the participation of this enzyme in DNA processing of flap fragments during DNA replication and repair in this parasite of ancient evolutive origin. J. Cell. Biochem. 118: 1722–1732, 2017. © 2016 Wiley Periodicals, Inc.

KEY WORDS: *TRYPANOSOMA CRUZI*; TcFEN1; DNA REPAIR; PARASITE PROLIFERATION AND SURVIVAL

FLAP endonuclease 1 (FEN1) is recognized as a central enzyme of DNA metabolism. In DNA, replication participates in processing intermediates of Okasaki fragment maturation through its endonuclease activity and it is pivotal in the long-patch (LP) DNA base excision repair pathway (BER) playing also key roles in DNA recombination, telomere maintenance, and spoiled replication fork rescue [Kao et al., 2002; Finger et al., 2012; Balakrishnan and Bambara, 2013]. It is considered a metallonuclease presenting three catalytic activities: flap endonuclease (FEN, main activity), gap endonuclease (GEN), and 5'-exonuclease (EXO) [Finger et al., 2012; Balakrishnan and Bambara, 2013]. Its main function is to remove any 5' DNA flap using its phosphodiesterase activity. Thus, FEN1 efficient and specific function is essential for high-fidelity DNA replication and for the maintenance of genome stability in

eukaryotes. In spite of its importance, the presence, enzymatic activity, and cellular location of FEN1 was not yet studied in the ancient hemoflagellate protozoan parasite *Trypanosoma cruzi*.

T. cruzi, an early divergent protozoan, is the etiological agent of Chagas' disease, an endemic pathology in Latin America that is considered among the world's most neglected diseases [WHO, 2012]. In Latin America 28 million people are at risk of exposure to this infection with an estimated total of 8 million cases in 21 endemic countries and 20,000 deaths per year. Identification of *T. cruzi* infected insect vectors and of more than 300,000 persons carrying the parasite in the USA [Bern and Montgomery, 2009] (together with the globalization of Chagas disease through immigration [Schmunis and Yadon, 2010] have converted this infection in a worldwide problem. There are no vaccines or effective drugs for treatment of

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this disease in its chronic manifestation and parasite molecular targets are badly and urgently needed.

T. cruzi presents an indirect life cycle, with hematophagous Triatominae insects as vectors, and mammalian including man, as hosts. The parasite presents different cellular forms such as extracellular replicative epimastigotes in the insect vector, non-replicative and infective trypomastigotes forms in the insect and in the mammalian hosts and intracellular, replicative amastigote forms inside infected mammalian cells [Tyler and Engman, 2001; Piacenza et al., 2009].

Trypanosome nucleus present particular characteristics probably derived from its ancient origin. Thus, though its chromatin is organized in nucleosomes [Hecker et al., 1994], it does not condense to chromosomes during cell division [Solari, 1980] and its histones are divergent [Galanti et al., 1998]. DNA replication and cell cycle regulation present conserved as well particular features. Most of the typical DNA replication proteins such as PCNA, Orc1/Cdc6 and topoisomerases as well as kinases and cyclins are present though showing particular features [Fragoso et al., 1998; Calderano et al., 2011; Li, 2012; Tiengwe et al., 2014; Calderano et al., 2015].

Both, replicative and non-replicative, infective forms of the parasite are exposed to oxygen and nitrogen reactive species (ROS/RNS) in its insect and mammal hosts, damaging its DNA [Cardoni et al., 1997; Graca-Souza et al., 2006; Piacenza et al., 2009; Cabrera et al., 2011]. In mammals, a DNA base excision repair mechanism is well established [Dianov et al., 1998; Robertson et al., 2009] and the parasite is able to repair oxidative DNA damage basically using the same pathway [Cabrera et al., 2011; Sepúlveda et al., 2014; Machado-Silva et al., 2016; Ormeño et al., 2016]. Thus, the infection is not fully eliminated and the vertebrate host will serve as a parasite reservoir, establishing a chronic infection [Peluffo et al., 2004].

Considering the pivotal role of flap enzymes in DNA synthesis and repair, we hereby show the identification, partial characterization, enzymatic activity, subcellular localization, and involvement in parasite proliferation and survival of TcFEN1, a key enzyme in eukaryotic evolution.

MATERIALS AND METHODS

CELL CULTURES

T. cruzi epimastigotes (Dm28c strain) were cultivated at 28°C in LIT medium (Liver Infusion Tryptose: 5 g/L liver extract, 3.97 g/L NaCl; 0.395 g/L KCl; 3.12 g/L HPO₄Na₂, 2 g/L glucose, supplemented with 10% fetal bovine serum (FBS), 20 µg/ml hemin, 100 U/ml penicillin, and 100 µg/ml streptomycin [Sepúlveda et al., 2014]. Trypomastigote and amastigote parasite forms were obtained from infected H9C2 (2-1) cardiomyocyte cell cultures (ATCC CRL-1446, *Rattus norvegicus* rat) maintained in DMEM supplemented with 10% active FBS at 37°C in 5% CO₂ as described for RAW cells [Sepúlveda et al., 2014]. Cultures were performed according to the instructions of the Biosafety Committee, Faculty of Medicine, University of Chile and following national (Bioseguridad 1ra edición, 1994, Comisión Nacional de Investigación Científica y Tecnológica, CONICYT, Chile) and international (Manual de Bioseguridad en Laboratorios, OMS, Ginebra 2005) guidelines.

T. cruzi HOMOGENATES

T. cruzi homogenates from epimastigote, trypomastigote, and amastigote cellular forms were obtained by cell lysis in 10 mM Tris-HCl pH 8, 200 mM KCl, 1 mM EDTA, 1 mM DTT, 0.25% Nonidet P-40, 20% glycerol buffer containing protease inhibitors (Roche). The samples were then centrifuged at 25,000g for 10 min at 4°C and the supernatant was obtained. The protein concentration was quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher), according to manufacturer's instructions.

flap ENDONUCLEASE ACTIVITY ASSAY

The most efficient substrate to test the activity of FEN1 enzymes from different species is a 5'-flap structure, consisting of three single annealed DNA strands oligonucleotides, forming a double-flap structure [Kao et al., 2002]. One strand is a continuous 54 mer oligonucleotide, while the other two are shorter (35 and 26 mer, respectively) forming a flap between them when annealing with the 54 mer oligo. The 35 mer oligo presents a 5' flap of 7 nucleotides, while the 26 mer oligo contains a 1-nucleotide flap on the 3'-tail adjacent to the 35 mer oligonucleotide 5'-flap.

To determine the flap endonuclease activity the 35 mer oligo was labeled at the 5' end with P γ ³² using the DNA 5' End Labeling System kit (Promega) and annealed with the 54 and 26 non-labeled oligos. 0.5 pmoles of this substrate was incubated with 18 µg parasite homogenate proteins or 30 ng of the native purified recombinant enzyme in 50 mM Tris pH 8.0 and 50 mM MgCl₂ for 10 or 30 min at 30°C. Assays were stopped by adding formamide loading buffer (96% v/v formamide, 20 mM EDTA, 5 mM Tris pH 7.5, xylene cyanol 0.05% p/v, bromophenol blue 0.05% p/v) and incubation for 10 min at 75°C. After heating at 95°C for 5 min, the samples were separated by electrophoresis in 16–20% acrylamide gels using denaturing conditions (7M urea) at constant 65W for 30 min. Labeled oligos were detected using a phosphorimager device (BioRad). As a negative control the untreated labeled oligo was used.

To estimate the optimal pH of the DNA flap activity, the following buffer conditions were applied: 50 mM acetate pH 5.0; 50 mM MES pH 5.5 and 6.0; 50 mM HEPES pH 7.0 and 8.0; 50 mM glycine pH 9.0 and 10.

For cleavage-ligation assays, the 26 mer oligo was labeled at the 5' end with P γ ³² using the DNA 5' End Labeling System kit (Promega) and annealed with the 54 and 35 non-labeled oligos. 0.5 pmoles of this substrate was incubated with 18 µg epimastigote homogenate, 50 mM Tris pH 8.0, 50 mM MgCl₂, 10 mM ATP, 1 mM DTT for 60 min at 30°C, in the presence or absence of 10 U of exogenous T4 ligase (Promega) during the last 20 min of the reaction. Ligation is evidenced by the linkage of the labeled 26 mer plus the 35 mer oligo, generating a labeled 54 mer oligo.

tcfen1 pTREX PLASMID CONSTRUCTIONS

A *tcfen1* DNA coding sequence was amplified from genomic *T. cruzi* DNA (strain Dm28c) by PCR using Platinum Taq High Fidelity polymerase (Invitrogen) and the primers forward (5'-GTCTAGAATGGGGATCTTGGGTCTTTCG -3') and reverse (5'-GGGAAGCTTTTCTTAACCACTTTTGTGCC-3'). Those primers present restriction sites for *Xba*I and *Hind*III enzymes in the sense and in the antisense sequences, respectively. The restriction

digestion created cohesive ends for oriented ligation into the plasmid pTREX-*his-gfp* expression vector, generating the construct pTREX-*his-tcfen1-gfp*. With this vector, a fusion protein with an 8 histidine tag in the N-terminal and with the green fluorescent protein tag (GFP) in the C-terminal region, respectively (HIS-TcFEN1-GFP) was produced. Furthermore, by using the same PCR reaction and restriction enzymes, the *tcfen1* DNA coding sequence was inserted in the plasmid pTREX-*gfp-his*, generating the construct pTREX-*tcfen1-gfp-his*. This last vector allowed the expression of a fusion recombinant protein TcFEN1-GFP-HIS, possessing the amino terminal end free while the GFP domain and the 8 histidines tag are located at the carboxy terminal. The correct insertion of *tcfen1* DNA sequence in each plasmid was confirmed by PCR, enzymatic digestion, and automatic DNA sequencing (data not shown).

TRANSFECTION AND OVEREXPRESSION OF HIS-TcFEN1-GFP AND TcFEN1-GFP-HIS PROTEINS IN *T. cruzi* EPIMASTIGOTES

Epimastigotes in the exponential phase of growth were electroporated with pTREX-*his-tcfen1-gfp* or pTREX-*tcfen1-gfp-his* constructs and with the corresponding empty control vectors. Briefly, parasites were washed in sterile PBS and resuspended in electroporation buffer (120 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄, 25 mM Hepes, 2 mM EDTA, 5 mM MgCl₂, pH 7.6). Afterwards 4×10^7 parasites were separately incubated with 50–100 µg of each plasmid. The electroporation was performed at 0.3 kV and 500 µF in two pulses separated by 30 sec maintaining the parasites on ice. Transfected epimastigotes were immediately transferred to 20% FBS LIT medium and, after 24 h, 250 µg/ml of G418 antibiotic was added and the antibiotic concentration was increased to 500 µg/ml at 72 h. Transfection efficiency was assessed by fluorescence microscopy. Due to the low percentage of fluorescent parasites, transfected epimastigotes of the different cultures were separated by FACS on a FACSAria II (BD Biosciences) equipment, obtaining 95% or more fluorescent parasites. These parasite populations were further employed.

The expression of the generated fusion proteins was assessed by 10% SDS-PAGE and Western Blot, using a primary anti-GFP antibody, and subsequent detection by chemiluminescence using a secondary antibody conjugated to horseradish peroxidase (Jackson Immuno Research Laboratories), employing the Supersignal West Pico kit (Thermo Scientific).

HIS-TcFEN1-GFP AND TcFEN1-GFP-HIS RECOMBINANT PROTEINS PURIFICATION

Recombinant TcFEN1 proteins were generated in transfected pTREX-*his-tcfen1-gfp* or pTREX-*tcfen1-gfp-his* *T. cruzi* epimastigotes as described above. Both recombinant proteins were purified from epimastigote homogenates by affinity chromatography using a HisPur Ni-NTA resin (Thermo Scientific) in native conditions, following the manufacturer's recommendations.

LOCALIZATION OF HIS-TcFEN1-GFP AND TcFEN1-GFP-HIS IN *T. cruzi* EPIMASTIGOTES

The localization of TcFEN1-GFP recombinant proteins expressed in *T. cruzi* epimastigotes was analyzed by GFP

fluorescence of the fusion proteins. Parasites were fixed in 70% cold methanol for 30 min, washed in PBS and nuclear and kinetoplast DNA were stained with 4',6-diamino-2-phenylindole (DAPI). Samples were evaluated by fluorescence microscopy using 430 ± 20 and 520 ± 20 nm filters for blue and green fluorescence, respectively. Photographs were processed computationally to determine the overlap of DAPI (pseudocolor red) and GFP (green).

PROLIFERATION ASSAYS OF THE TRANSFECTED *T. cruzi* EPIMASTIGOTES

Proliferation of non-transfected wild-type epimastigotes and *T. cruzi* epimastigotes transfected with pTREX-*his-tcfen1-gfp*, pTREX-*tcfen1-gfp-his*, or empty vectors was determined by adjusting the parasites in the exponential growth phase, at a concentration of 20×10^6 parasites/ml in supplemented LIT medium as described above. Subsequently, aliquots of the cultures were daily obtained, diluted in PBS/10% formaldehyde, and the number of parasites was counted in a Neubauer chamber.

GENERATION OF A SUSTAINED H₂O₂ CONCENTRATION AND VIABILITY ASSAY OF TRANSFECTED EPIMASTIGOTES EXPOSED TO SUSTAINED OXIDATIVE STRESS

To generate sustained oxidative conditions, 13×10^6 parasites were incubated for 2 or 4 h in the presence of 5 mM glucose plus 10, 25, 50, and 100 mU glucose oxidase from *Aspergillus niger* (Sigma) at 28°C in LIT culture medium. These conditions generate a 45–65 µM H₂O₂ concentration as measured by the Amplex Red Hydrogen/Peroxide/Peroxidase kit (Invitrogen, [Sepúlveda et al., 2014]). Viability of *T. cruzi* epimastigotes overexpressing HIS-TcFEN1-GFP or TcFEN1-GFP-HIS exposed to H₂O₂ was evaluated by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay [Sepúlveda et al., 2014]. For this purpose, 100 µl of parasites in 96 wells plates were incubated with 10 µl of 5 mg/ml MTT reagent plus 0.22 mg/ml phenazine metosulfate. After additional incubation for 4 h, the generated water insoluble formazan dye was dissolved in 100 µl of 10% w/v SDS/0.01M HCl. The plates were further incubated 30 min at 37°C and optical density (OD) in each well was determined using a microplate reader (Multiskan FC, Thermo Scientific) at 570 nm.

BIOINFORMATICS AND MOLECULAR MODELING

Alignment and analysis of the nucleotide sequences was performed using the T-Coffee online program (<http://www.ebi.ac.uk/Tools/msa/tcoffee/>). The deduced amino acid sequence of TcFEN1 was used for molecular modeling, using the web portal Phyre2, available at www.sbg.bio.ic.ac.uk/~phyre2. For visualization of the models obtained from the server, the VMD version 1.9.1 program was used [Humphrey et al., 1996].

STATISTIC ANALYSIS

Proliferation and viability assays were measured in duplicate, and each experiment was conducted in triplicate. Data were analyzed using GraphPad Prism program (version 5.0.3) corresponding to mean \pm SEM. Significant differences in data were analyzed by two-way ANOVA with Bonferroni post-test.

RESULTS

A DNA FLAP ENZYMATIC ACTIVITY IS PRESENT IN *T. cruzi* HOMOGENATES

In order to evaluate whether an enzymatic activity showing flap catalytic properties was present in *T. cruzi*, protein extracts from the three cellular forms of this parasite (epimastigote, EPI; tripomastigote, TRYPO; amastigote, AMA) were incubated at 30°C for 10 and 30 min in the presence of the indicated flap substrate. A 7 mer fragment was generated by all homogenates tested, indicating the presence of a flap endonuclease activity in all parasite cellular forms (Fig. 1A). The enzymatic activity showed higher activity around pH 8.0 (Fig. 1B) and at 35–40°C (Fig. 1C), as seen in mammalian FEN1 [Balakrishnan and Bambara, 2013]. An epimastigote *T. cruzi* homogenate not only cut the flap substrate (Fig. 1A) but was also able to ligate the produced DNA fragments (the processed 35 mer oligo plus the P³² labeled 26 mer oligo), generating a labeled 54 mer

oligonucleotide (Fig. 1D, lane 2, arrow). This DNA repair activity was increased when an exogenous T4 DNA ligase was added to the reaction (Fig. 1D, lane 3, arrow). Therefore, *T. cruzi* presents a canonical flap activity and is able to ligate the DNA fragments processed by that flap activity.

A GENE CODING FOR A flap ENZYME IS PRESENT AND CONSERVED IN *T. cruzi*

The gene coding for an enzyme showing DNA flap activity in *T. cruzi* was searched in the Kinetoplastid Genomic Resource database (TriTrypDB, [Aslett et al., 2010]). An orthologous DNA coding sequence for a FEN1 endonuclease protein (1182 bp, GeneBank accession number XM_809865.1, gene ID TriTrypDB TcCLB.511867.110) was detected in the *T. cruzi* genome (strain CL Brener) corresponding to *Homo sapiens* FEN1 (GeneBank accession number NM_004111). The deduced amino acid sequence codes for 393 residues with a predicted protein of 44.2 kDa and an isoelectric

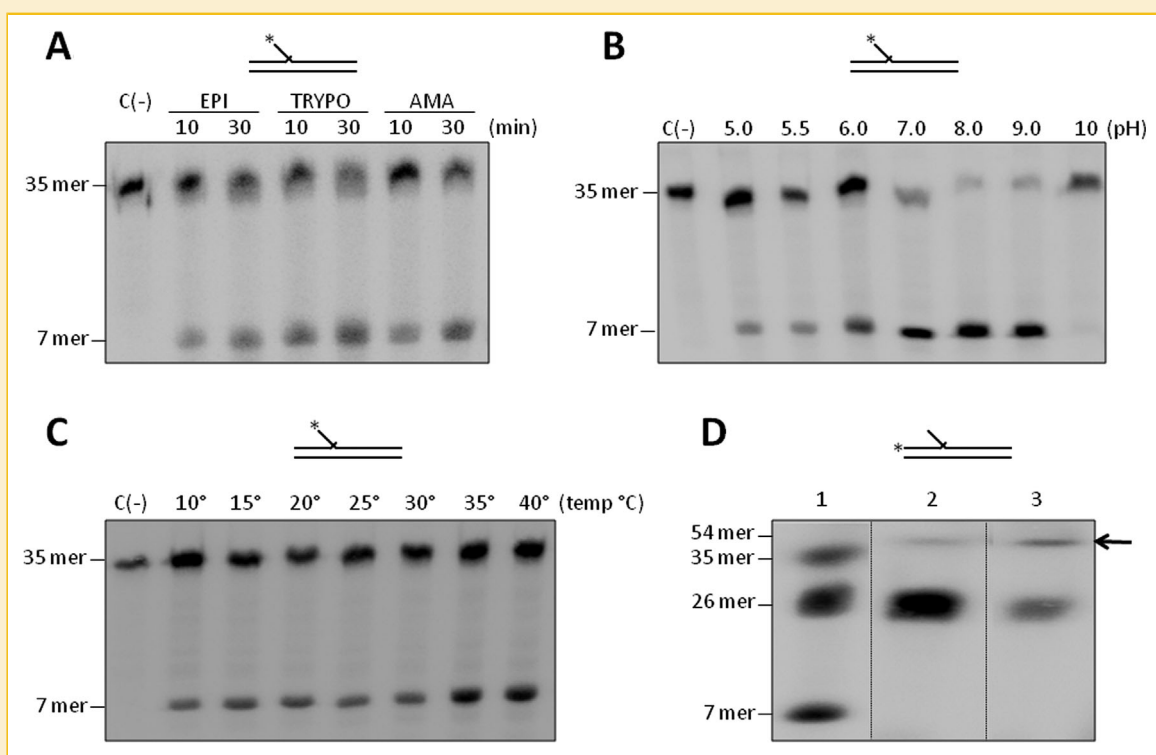


Fig. 1. DNA flap endonuclease activity in *Trypanosoma cruzi*. (A) *T. cruzi* homogenates present a DNA flap enzymatic activity. Protein extracts from *T. cruzi* epimastigotes (EPI), tripomastigotes (TRYPO), and amastigotes (AMA), were incubated in 50 mM HEPES pH 8.0 at 30°C for 10 or 30 min in the presence of the indicated flap substrate. A labeled 7 mer fragment was generated indicating the presence of a flap activity in all cellular forms of the parasite. C-, negative control, without protein extracts. (B) The effect of pH on the *T. cruzi* DNA flap activity. Parasite epimastigote protein extracts were incubated at different pHs, for 30 min at 30°C, in the presence of the indicated flap substrate. A labeled 7 mer fragment was generated showing the presence of a flap activity at all pHs assayed. C-, negative control, without protein extract. (C) The effect of temperature on the *T. cruzi* DNA flap activity. Parasite epimastigote protein extracts were incubated at different temperatures for 30 min, in the presence of the indicated flap substrate. A labeled 7 mer fragment was generated showing the presence of a flap activity. C-, negative control, without protein extract. In (A), (B), and (C), each assay was performed using 18 µg protein extract, 0.5 pmol substrate, 50 mM HEPES pH 8, and 50 mM MgCl₂, except for (B) in which different buffers were used, as indicated in Material and Methods. (D) DNA repair activity of *T. cruzi* epimastigote homogenates on an oligo processed by a parasite DNA flap endonuclease. A total of 18 µg of parasite epimastigote protein extracts were incubated for 1 h at 30°C, in 50 mM Tris pH 8.0, 50 mM MgCl₂, 10 mM ATP, and 0.5 mM of the indicated oligo substrate. The 35 mer oligonucleotide with a flap at 5' was cleaved by the *T. cruzi* flap endonuclease activity generating a 7 and a 28 mer oligo. The 28 mer oligo was ligated with the P³² labeled 26 mer oligo by a parasite ligase activity resulting in a labeled 54 mer oligo that represents the ligation of those DNA fragments. Lane 1: Oligonucleotide markers; lanes 2 and 3: The generated 54 oligonucleotide (arrow) in the absence (2) or presence (3) of 10 U of exogenous T4 DNA ligase.

point of 7.67. Figure 2 shows a multiple deduced amino acid sequences alignment of *T. cruzi* TcFEN1 with *Drosophila melanogaster* (GeneBank accession number 17647423), *Danio rerio* (GeneBank accession number 1036551479), *Homo sapiens* (GeneBank accession number 4758356), *Leishmania major* (GeneBank accession number 321438333), and *Sacharomyses cerevisiae* (GeneBank accession number 190409721) FEN1. TcFEN1 amino acid identity was 48.60% with *H. sapiens* FEN1, 48.85% with *D. melanogaster* FEN1, 50.76% with *D. rerio* FEN1, 46.23% with *S. cerevisiae* RAD27 (yeast FEN1 homolog), and 72.47% with *L. major* putative FEN1. Key catalytic amino acids specific of all FEN endonucleases [Tsutakawa et al., 2011] are fully conserved in the amino acid sequences of all enzymes depicted in Fig. 2, including TcFEN1. Therefore, TcFEN1 present sequence

features that are expected for a canonic flap DNA endonuclease. A cladogram constructed considering the derived amino acid sequences of all FEN1 enzymes depicted in Fig. 2A shows a close evolutionary relationship between TcFEN1 and *L. major* putative FEN1 (Fig. 2B).

The gene coding for TcFEN1 was amplified from genomic *T. cruzi* strain Dm28c DNA by PCR and inserted in a pGEM-T easy plasmid. The cloned gene was sequenced and compared with the sequence found in the database. We detected 12 nucleotides of difference between the TcFEN1 sequences derived from the annotated CL Brener strain and the Dm28c strain sequenced by us as well as the one published during the realization of this work [Grisard et al., 2014] (gene ID TriTrypDB TCDM_06623) (not shown). Those nucleotide differences generated three changed amino acids in the

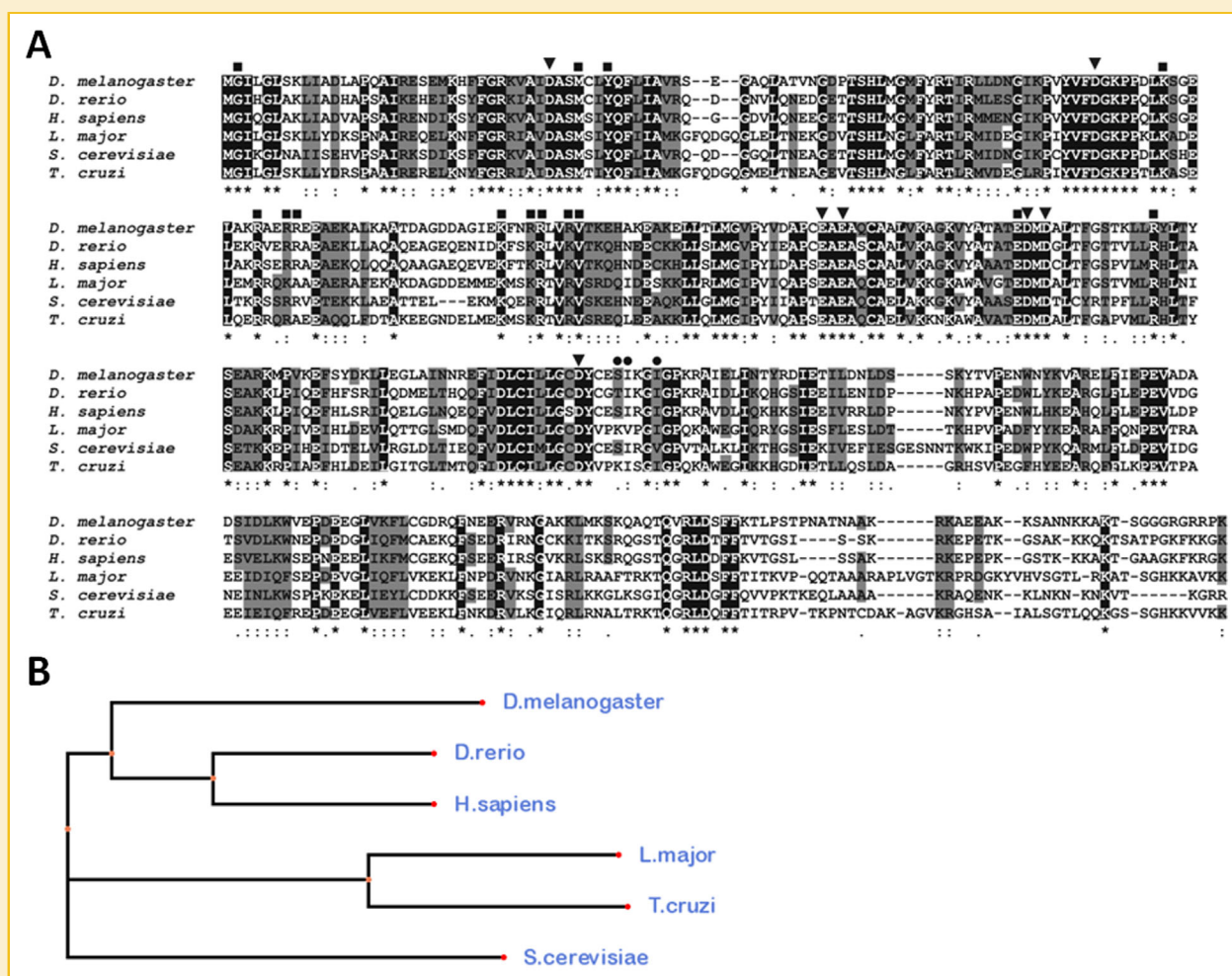


Fig. 2. Multiple amino acid sequences alignment (A) and deduced cladogram (B) of *T. cruzi* TcFEN1 with FEN1 from *Drosophila melanogaster*, *Danio rerio*, *Homo sapiens*, *Leishmania major*, and *Sacharomyses cerevisiae*. The multiple amino acid sequence alignment was performed using the T-Coffee online program (<http://www.ebi.ac.uk/Tools/msa/tcoffee/>). Black highlighted residues are identical match; gray highlighted are chemically similar residues. Arrow-heads (◄) are the seven conserved active site carboxylates characteristic of the FEN superfamily. In circles (○) are the residues that coordinate K+ ions, in human Ile238, Ile 241, and Ser237; while both Ile are conserved (except in *Sacharomyses*), the Ser 237 is changed to Lys in *L. major* and *T. cruzi*, both genus of the same family. It should be noted that Lys is also able to effectively coordinate K+ ions. Squares (◻) are the amino acids necessary for DNA contacts in the active site; all these amino acids are conserved except for an Ala instead of Arg at position 108 of the TcFEN1 protein. Again both Trypanosomatids show the same amino acid substitution. The cladogram (Fig. 2B) was constructed considering the derived amino acid sequences of all FEN1 enzymes analyzed and applying the PHY-FI program available in <http://cgi-www.daimi.au.dk/cgi-chili/phyfi/go>.

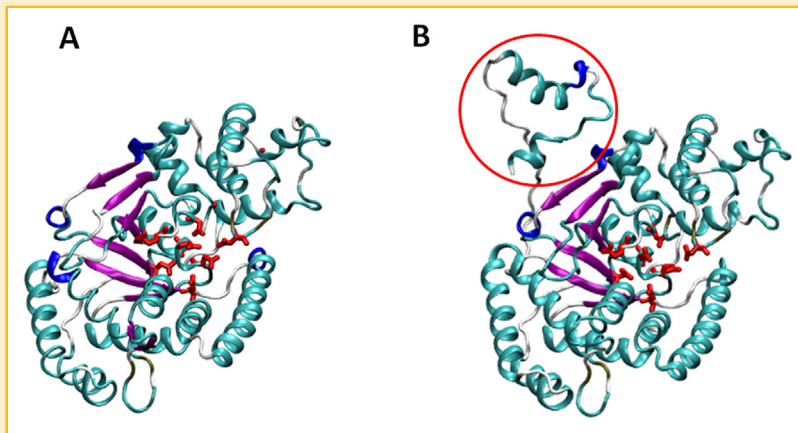


Fig. 3. Molecular modeling and structure of TcFEN1. (A) Modeling of the human FEN1 obtained from crystallographic data; (B) In silico modeling of TcFEN1. In red, amino acids that participate in the enzymatic catalysis; the red circle indicates the C-terminal end of TcFEN1.

protein; Lys147 (Brener) by Asn (Dm28c), Pro243 (Brener) by Ser (Dm28c), and Lys312 (Brener) by Glu (Dm28c); none of these amino acids corresponds to essential residues for the activity of FEN1 [Tsutakawa et al., 2011].

The deduced amino acid sequence of TcFEN1 (strain Dm28c) was used for molecular modeling, using the web portal Phyre2, available at www.sbg.bio.ic.ac.uk/~phyre2. When compared with the structure corresponding to human FEN1 (hFEN1), the predicted TcFEN1 protein showed a high structural similarity with their human counterpart, as expected from the sequence identity observed in the alignment (Fig. 2A). However, a notorious difference between these proteins, evidenced as an extended C-terminal region in TcFEN1, is clearly observed (Fig. 3, circle).

EXPRESSION AND ACTIVITY OF PURIFIED RECOMBINANT HIS-TcFEN1-GFP AND TcFEN1-GFP-HIS PROTEINS

To evaluate the enzymatic activity of purified recombinant parasite TcFEN1, *T. cruzi* epimastigotes were transfected with the expression constructs pTREX-*his-tcfn1-gfp* or pTREX-*tcfn1-gfp-his* to generate the recombinant proteins HIS-TcFEN1-GFP and TcFEN1-GFP-HIS, respectively. Those different versions of the TcFEN1-GFP recombinant protein were designed for the possibility that an N-terminal tag could be detrimental for the flap endonuclease activity as described for other recombinant FEN enzymes [Finger et al., 2012].

The HIS-TcFEN1-GFP recombinant protein is expressed in *T. cruzi* epimastigotes and when detected by Western blot using an anti GFP antibody it shows as a double band (Fig. 4A, lane 2, arrow). A

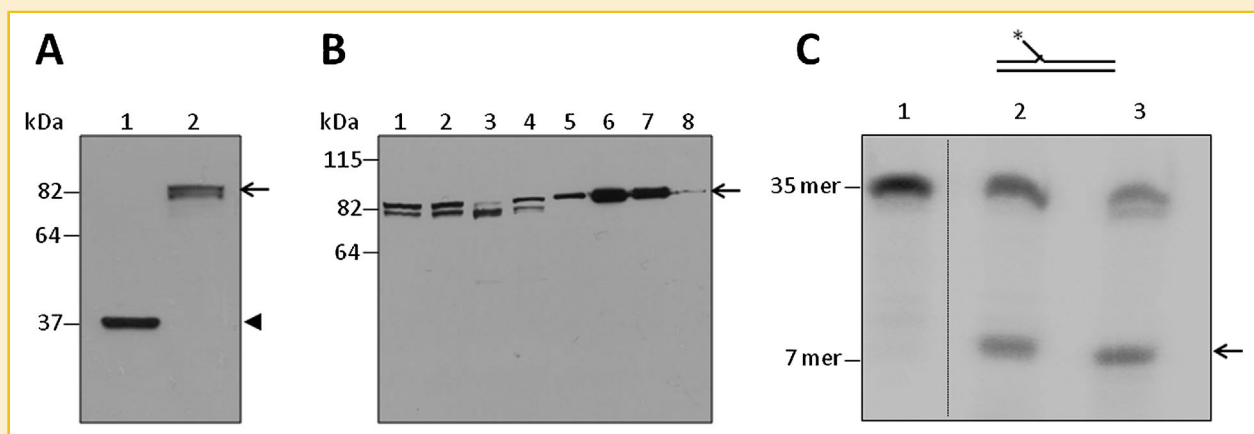


Fig. 4. Expression, purification, and activity of a recombinant TcFEN1-GFP protein. (A) A HIS-TcFEN1-GFP fusion protein was detected in homogenates from transfected epimastigotes by Western blot assay using an anti-GFP antibody. Lane 1, GFP control protein, arrowhead. Lane 2, HIS-TcFEN1-GFP fusion protein, arrow. (B) HIS-TcFEN1-GFP protein was purified under native conditions on a Ni-agarose column and detected by Western blot using an anti-GFP antibody. Lanes 1: HIS-TcFEN1-GFP transfected epimastigote homogenate; 2: 20,000g homogenate supernatant; 3: Protein not retained by the column; 4 and 5: Column washings; 6, 7, and 8: Column eluates. (C) A labeled 35 mer oligonucleotide containing a non-complementary region (flap) was incubated without enzyme (negative control, lane 1) or with *T. cruzi* trypomastigote homogenate (positive control, lane 2) or with the purified native HIS-TcFEN1-GFP protein (lane 3). A labeled 7 mer oligonucleotide is indicative of a flap endonuclease activity.

pTREX-*his-gfp* vector used as control expresses the GFP protein (Fig. 4A, lane 1, arrowhead). When further purified under native conditions, the HIS-TcFEN1-GFP enzyme corresponds to the upper band (Fig. 4B, arrow). This purified protein showed a DNA flap endonuclease activity evidenced by the appearance of a 7 mer oligo when incubated with the appropriate flap substrate (Fig. 4C, lane 3, arrow). Lane 1 shows the non-cleaved oligo substrate while in lane 2, the activity of the trypomastigote homogenate is observed (arrow). Similarly, the TcFEN1-GFP-HIS purified recombinant protein presents a flap endonuclease activity, clearly showing that the localization of the histidine tag has no effect on the enzyme endonuclease activity (not shown).

A HIS-TcFEN1-GFP FUSION PROTEIN EXPRESSED IN *T. cruzi* EPIMASTIGOTES IS LOCATED IN THE PARASITE NUCLEUS

Figure 5A shows a HIS-TcFEN1-GFP expressing epimastigote (phase contrast). DAPI DNA staining in the epimastigote nucleus (5B, arrow) co-localize with the HIS-TcFEN1-GFP fusion protein (Fig. 5C). DAPI/HIS-TcFEN1-GFP merge shows the absence of this enzyme in the *T. cruzi* kinetoplast (Fig. 5D). TcFEN1 is found in aggregates in the nucleoplasm. In previous work, we have shown that using pTREX-*gfp* control vector, the expression of the green fluorescent protein remains in the cytoplasm [Sepúlveda et al., 2014; Ormeño et al., 2016]. The subcellular localization of TcFEN1 was maintained when the parasites were treated with H₂O₂ (not shown). Similar results were obtained when the TcFEN1-GFP-HIS protein was assayed in epimastigotes.

TcFEN1 OVER-EXPRESSION INCREASES PARASITE PROLIFERATION

Transfected epimastigotes over-expressing HIS-TcFEN1-GFP and TcFEN1-GFP-HIS proteins were enriched by FACS (more than 90% parasites were over-expressing the enzymes) and the effect of both constructs on parasite proliferation was assayed. HIS-TcFEN1-GFP (Fig. 6A, triangles) and TcFEN1-GFP-HIS (Fig. 6B, triangles) over-expression induced a significant increase in cell proliferation as compared with non-transfected epimastigotes (6A and 6B, diamond) or with parasites transfected with GFP only (6A and 6B, squares).

TcFEN1 OVER-EXPRESSION INCREASES SURVIVAL WHEN PARASITES ARE EXPOSED TO SUSTAINED OXIDATIVE STRESS

Transfected epimastigotes over-expressing HIS-TcFEN1-GFP maintained in the exponential phase of growth were exposed to a sustained H₂O₂ production using the glucose/glucose oxidase system [Sepúlveda et al., 2014; Ormeño et al., 2016] for 2 (Fig. 7A) and 4 (Fig. 7B) hours. Afterwards, parasites viability was measured by the MTT assay. Clearly, over-expression of the TcFEN1 enzyme increases parasite survival.

DISCUSSION

T. cruzi proliferation and its survival to oxidative stress through DNA damage repair are essential for parasite persistence in its mammal and insect hosts. Considering that DNA flap enzymatic activity plays a key role both in DNA replication and in DNA repair, it

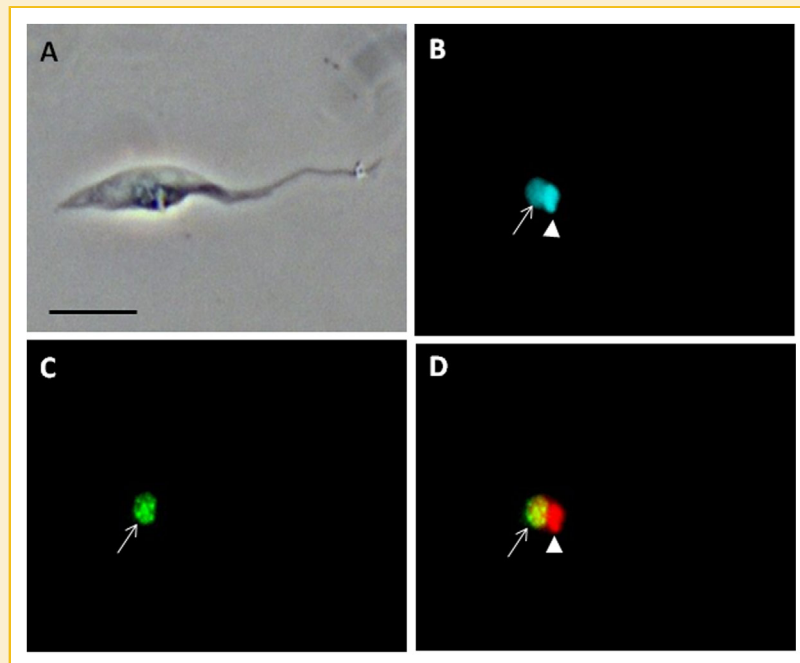


Fig. 5. The TcFEN1-GFP fusion protein is located in the nucleus of *T. cruzi* epimastigotes. pTREX-*his-tcfen1-gfp* transfected epimastigotes were fixed in 70% cold methanol for 30 min. (A) Phase contrast; (B) DNA DAPI staining; (C) HIS-TcFEN1-GFP direct fluorescence microscopy; (D) B and C merged. For merge DAPI was applied in red pseudo color. Arrows: nucleus; Arrowheads: kinetoplast. Bar 10 μ m.

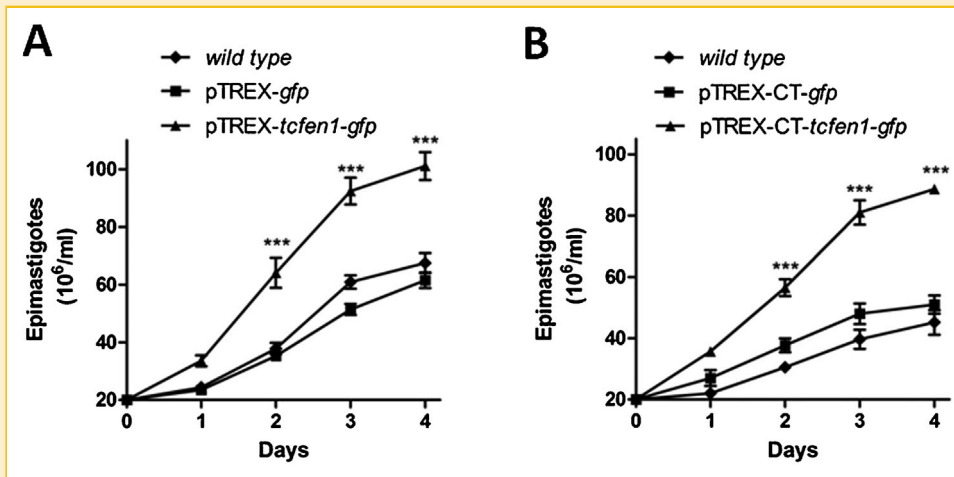


Fig. 6. HIS-TcFEN1-GFP and TcFEN1-GFP-HIS overexpression increase *T. cruzi* epimastigote proliferation. (A) pTREX-his-tcfen1-gfp transfected epimastigotes. (B) pTREX-tcfen1-gfp-his transfected epimastigotes. Parasites were maintained at the exponential phase of growth. Initial cell concentration was 20×10^6 parasites/ml and the number of cells was determined in a Neubauer chamber every 24 h. Results correspond to means \pm SEM from three independent experiments, *P*-value: * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001.

is of crucial importance to study the presence and activity of a DNA flap enzyme in that parasite.

It has been proposed that FEN1 is an ancient enzyme maintained during evolution to coordinate essential functions necessary to preserve cellular genome and viability. Its activity is present in prokaryotes as a domain associated with DNA polymerase I while is a separate enzyme in eukarya and archae [Kao et al., 2002; Zheng et al., 2007; Finger et al., 2012; Balakrishnan and Bambara, 2013]. The importance of FEN1 in the metabolism of DNA, the detection of other enzymes of the BER DNA repair pathway in *T. cruzi* [Cabrera et al., 2011; Sepúlveda et al., 2014; Ormeño et al., 2016], and the characterization of flap endonucleases in other protozoa, such as *P. falciparum* [Casta et al., 2008] strongly suggested us the existence of a flap endonuclease in *T. cruzi*.

FEN enzymes present a high degree of sequence homology between the various species studied, particularly in the amino-terminal and central regions of the protein, where the main substrate binding and catalytic domains are located [Tomlinson et al., 2010; Balakrishnan and Bambara, 2013]. The carboxy-terminal region, although is also involved in substrate binding [Stucki et al., 2001], presents a higher sequence diversity. It has been described the presence of post-translational modifications in this region, such as acetylations and phosphorylations that affect the enzyme activity [Friedrich-Heineken et al., 2003; Henneke et al., 2003]. This area is also of importance as a binding domain for other proteins that can regulate the activity of FEN1, such as PCNA [Chapados et al., 2004]. Trypanosome PCNA shows a conservation of its amino acid sequence compared to recent eukaryotes that is around or over

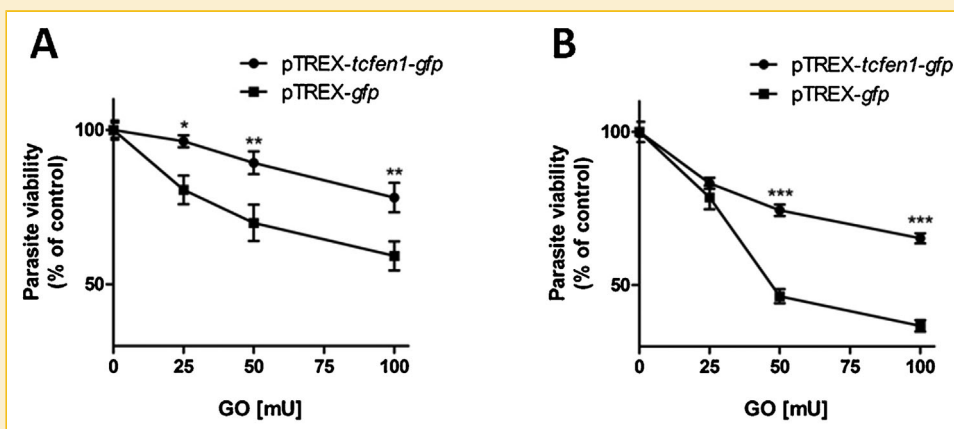


Fig. 7. Viability of TcFEN1 over-expressing epimastigotes submitted to sustained oxidative stress. HISTcFEN1-GFP over-expressing epimastigotes (circles) and parasites transfected with empty vector (squares) were treated for 2 (A) or 4 (B) h with a glucose-glucose oxidase system producing sustained H_2O_2 concentrations. Viability was determined by MTT assays. *P*-value: * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001.

50% (unpublished result), a figure similar to that we found for TcFEN1. These two proteins are evolutionarily related and their coordinated function is central for the maintenance of eukaryotic genomes. Therefore, it is not surprising that they are particularly conserved in *T. cruzi*. Other proteins related to DNA replication, such as Orc, or involved in DNA repair, as TcAPE1, TcAPE2, and Nth1, present a lower degree of conservation. This is probably due to the fact that those enzymes do not play a central role in the coordination of DNA replication and repair, as PCNA and TcFEN1.

It is remarkable that the TcFEN1 C-terminal region is longer than the human FEN1 counterpart, a characteristic shared by the *L. major* DNA flap endonuclease (Fig. 2A) and in a more extreme degree by *P. falciparum* PffFEN1 [Casta et al., 2008]. Since this region presents a higher sequence variability than other regions in FEN1s and it is involved in substrate binding and regulation of FEN1 [Stucki et al., 2001], it may represent differences in the functionality and regulation of these enzymes respect to their mammalian homologs, which may reflect an ancient bifurcation in the FEN1 enzyme group. More phylogenetic and biochemical studies are required to account for that difference.

Compartmentalization of nuclear processes is a particular feature of eukaryotes. Considering the importance of FEN1 enzyme activity in DNA replication and repair, among others, it would be expected to find this enzyme in the nucleus at specific areas. Indeed, when a HIS-TcFEN1-GFP fusion protein was expressed in *T. cruzi* epimastigotes in the exponential phase of growth, the enzyme was found as clusters in the parasite nucleus while GFP was in the cytoplasm. Previous work have shown that proteins related to DNA replication, such as TcOrc1/Cdc6 and TcPCNA are located in the parasite nuclear periphery when cells are in the S-phase of the cell cycle while they remain dispersed in the nucleus in G1 and G2/M phases [Calderano et al., 2011]. Considering the association of FEN1 to PCNA during DNA replication [Dovrat et al., 2014; MacNeill, 2016], we expected that TcFEN1 would be concentrated in the nuclear periphery in log-phase transfected epimastigotes. Indeed, TcFEN1 was found in aggregates similar to those described for PCNA and ORC proteins [Calderano et al., 2011] though we were not able to locate this enzyme at the nuclear periphery only. This finding may be explained considering that we are using asynchronous parasites over-expressing TcFEN1 or that we are generating a fusion HIS-TcFEN1-GFP protein or both. In any case, the aggregates may be a result of its interaction with PCNA and/or ORC1/Cdc6 proteins [Godoy et al., 2009]. Interestingly enough, TcAPE1 and TcAPE2 [Sepúlveda et al., 2014] as well as TcNth1 [Ormeño et al., 2016] DNA repair enzymes are also found in similar aggregates in the nucleus of *T. cruzi* suggesting that enzymes involved in DNA replication and repair behave as a related mechanism, as already proposed [Godoy et al., 2009]. Consequently, aggregates may be the result of loading of enzymes involved in DNA replication, such as ORC, PCNA, and TcFEN1, as well as those enzymes related to DNA repair, as TcAPE1, TcAPE2, TcNth1, and again TcFEN1, in the region of DNA replication in dividing trypanosomes.

Although FEN1 presents a sequence of nuclear destination [Finger et al., 2012; Balakrishnan and Bambara, 2013], flap endonuclease activity was also found in mitochondria of human cell lines [Kalifa et al., 2009]. We were not able to find the fusion TcFEN1-GFP

enzyme in the parasite kinetoplast; this is not an unexpected result taking in consideration that TcOrc1/Cdc and TcPCNA proteins are restrained to the nucleus [Godoy et al., 2009; Calderano et al., 2011]. Similarly, DNA base excision repair enzymes TcAPE1, TcAPE2, and TcNth1 are also located in the parasite nucleus only [Sepúlveda et al., 2014; Ormeño et al., 2016] while TcOGG1 (another DNA BER enzyme) is present in both compartments [Machado-Silva et al., 2016].

Another enzyme able of processing DNA flaps is DNA2, a helicase/nuclease that also participates in DNA replication and repair [Bae et al., 2001; Zheng et al., 2008; Duxin et al., 2009]. This enzyme processes a DNA flap generating a product that cannot be ligated. Instead, it cuts inside the single strand region of a DNA flap, requiring further processing for DNA ligation, as shown in *S. cerevisiae* [Bae et al., 2001]. In human cell lines, DNA2 was identified as a mitochondrial and a nuclear enzyme [Duxin et al., 2009], or only mitochondrial [Zheng et al., 2008]. DNA2 has been suggested as a candidate for FEN1 replacement and it has been shown that the activity of DNA2 and FEN1 stimulates each other [Zheng et al., 2008]. Since the mitochondrial DNA is an important target for oxidative damage, and we have determined that TcFEN1 is located in the *T. cruzi* nucleus only, it would be of interest to study whether a DNA2 homolog or similar protein is located in the parasite mitochondrion replacing TcFEN1 in the processing of DNA flaps.

In most eukaryotes, FEN1 plays important roles in the processing of DNA intermediaries presenting a nucleotide flap during DNA replication. Indeed, over-expression of TcFEN1 in replicative epimastigotes causes an important increase in parasite proliferation. This effect is not affected by the position of the enzyme amino acid sequence regarding that of the histidine tail marker. These results strongly suggest the participation of TcFEN1 in the correct excision of DNA flap structures during parasite DNA replication and processing.

Regarding to BER DNA repair, two sub-pathways have been identified in mammals. One is the short-patch DNA base repair (SP-BER), that results in DNA repair by replacement of a single nucleotide. The other subpathway, termed long-patch DNA repair (LP-BER), consists in the removal of several (2–10) nucleotides previous to the correct nucleotide insertion and ligation of the repaired DNA product [Robertson et al., 2009]. In mammals, though SP-BER is proposed as the major repair pathway, LP-BER still accounts for some DNA repair [Dianov et al., 1998]. In bacteria, LP-BER is a minor back-up pathway [Dianov and Lindahl, 1994]. In *S. cerevisiae* [Wang et al., 1993] and *P. falciparum* [Haltiwanger et al., 2000], SP-BER is absent, and LP-BER is the major DNA repair pathway. To date, there are no reports regarding the presence of the LP-BER in *T. cruzi*. Considering that over-expression of TcFEN1 augments epimastigote viability when submitted to sustained oxidative DNA damage, that this DNA repair enzyme is found in the parasite nucleus and that in other eukaryotes FEN1 is associated to the long-pathway DNA BER repair [Finger et al., 2012; Balakrishnan and Bambara, 2013], we propose that this sub-pathway should be functioning as a DNA repair mechanism in the *T. cruzi* nuclear genome.

Our results point to the presence and activity of a TcFEN1 enzyme in *T. cruzi*, involved in parasite proliferation and DNA repair. These

results are of importance to understand biochemical mechanisms of those basic processes that are fundamental for genome stability during evolution.

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