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CREditing: a tool for gene tuning in *Trypanosoma cruzi*

Lisandro A. Pacheco-Lugo [a,b], José L. Sáenz-García [a], Yirys Díaz-Olmos [c,d], Rodrigo Netto-Costa [c], Rodrigo S.C. Brant [a], Wanderson D. DaRocha [a,*]

[a] Laboratório de Genômica Funcional de Parasitos (GFP), Universidade Federal de Paraná, Paraná, Brazil
[b] Facultad de Ciencias Básicas Biomédicas, Universidad Simón Bolívar, Barranquilla, Colombia
[c] Instituto Carlos Chagas, Fiocruz-Paraná, Paraná, Brazil
[d] Facultad de Ciencias de la Salud, Universidad del Norte, Barranquilla, Colombia

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ABSTRACT

The genetic manipulation of *Trypanosoma cruzi* continues to be a challenge, mainly due to the lack of available and efficient molecular tools. The CRE-*lox* recombination system is a site-specific recombinase technology, widely used method of achieving conditional targeted deletions, inversions, insertions, gene activation, translocation, and other modifications in chromosomal or episomal DNA. In the present study, the CRE-*lox* system was adapted to expand the current genetic toolbox for this hard-to-manipulate parasite. For this, evaluations of whether direct protein delivery of CRE recombinase through electroporation could improve CRE-mediated recombination in *T. cruzi* were performed. CRE recombinase was fused to the C-terminus of *T. cruzi* histone H2B, which carries the nuclear localization signal and is expressed in the prokaryotic system. The fusion protein was affinity purified and directly introduced into epimastigotes and tissue culture-derived trypomastigotes. This enabled the control of gene expression as demonstrated by turning on a tandem dimer fluorescent protein reporter gene that had been previously transfected into parasites, achieving CRE-mediated recombination in up to 85% of parasites. This system was further tested for its ability to turn off gene expression, remove selectable markers integrated into the genome, and conditionally knock down the nitroreductase gene, which is involved in drug resistance. Additionally, CREditing also enabled the control of gene expression in tissue culture trypomastigotes, which are more difficult to transfect than epimastigotes. The considerable advances in genomic manipulation of *T. cruzi* shown in this study can be used by others to aid in the greater understanding of this parasite through gain- or loss-of-function approaches.

1. Introduction

The etiological agent of human Chagas disease, *Trypanosoma cruzi*, is a kinetoplastid protozoan parasite with a complex lifecycle involving both vertebrate and invertebrate hosts. Unlike other Trypanosomatid protozoa such as *T. brucei* and *Leishmania* spp., functional genomics studies in *T. cruzi* have been limited by the lack of accessible molecular tools. However, in recent years, clustered regularly interspaced short palindromic repeats with Cas9 (CRISPR-Cas9), a genome editing approach, has been successfully applied to *T. cruzi* (Peng et al., 2015; Lander et al., 2015, 2016; Lander et al., 2017; Chiurillo et al., 2017; Soares Medeiros et al., 2017; Burle-Caldas et al., 2018; Cruz-Bustos et al., 2018; Romagnoli et al., 2018). Two principal methods of this approach have been used to edit the genome of this protozoan: (i) endogenous expression of the Cas9 nuclease and single-guide RNA (sgRNA), and (ii) direct transfection of ribonucleoprotein complexes (Soares Medeiros et al., 2017; Burle-Caldas et al., 2018). Although CRISPR-Cas9 in *T. cruzi* has been shown to be a relatively efficient method in the ablation of endogenous genes (Lander et al., 2015; Peng et al., 2015; Chiurillo et al., 2017; Soares Medeiros et al., 2017; Cruz-Bustos et al., 2018) and tagging (Lander et al., 2016, 2017; Soares Medeiros et al., 2017), no published work exists that has demonstrated the efficiency of CRISPR-Cas9 in generating large genomic deletions in trypanosomatids. In other models, however, it has been shown that large genomic deletions by CRISPR-Cas9 can be relatively inefficient, with the consensus being that the larger the intended deleted fragment, the lower the efficiency. Thus, for the deletion of large fragments it is essential to use multiple sgRNAs to improve efficiency (Song et al., 2016), but this results in higher costs and increases the risk of off-target effects. Hence, the development of new strategies for *T. cruzi* that allow gene manipulation on a large scale is imperative.

* Corresponding author.
E-mail address: darocha@ufpr.br (W.D. DaRocha).

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In *T. cruzi*, inducible expression systems for recombinant proteins are not efficient (Burle-Caldas et al., 2015). However, fine-tuned gene expression systems that permit regulated expression of exogenous genes are an important tool for investigating the functional roles of encoded proteins. Furthermore, an efficient regulatory system in which gene expression can be tightly controlled, i.e. blocked or promoted under particular conditions, is essential to study the effects of genes that are expressed at very low levels, or for those genes whose products might be toxic to the cell.

The CRE-*lox* (Causes Recombination-locus of crossing (x) over) recombination system of the bacteriophage P1 is a widely used method of achieving conditional, targeted deletions, inversions, insertions, gene activation, translocation, and other modifications in chromosomal or episomal DNA (Bergemann et al., 1995; Sauer, 1998; Testa and Stewart, 2000). In the CRE-*lox* system, site-specific recombinases either excise or invert DNA between short target sequences (34 bp) consisting of an 8 bp core sequence, where recombination takes place, and two flanking 13 bp inverted repeats (Tronche et al., 2002). Depending on the *loxP* orientation, three results are possible: inversion, deletion or translocation.

One of the main limitations of the CRE-*lox* system is the enzyme activation inside the cell at the desired time and in the specific cellular compartment (Lewandoski, 2001). The regulated expression of CRE recombinase is essential as prolonged exposure to the enzyme can be lethal to cells (Chen and Behringer, 2001; Silver and Livingston, 2001). However, direct protein transduction or transfection of CRE recombinase within the cells has been shown to decrease the chance of insertional mutagenesis and the genomic instability induced by continuous CRE expression (Schmidt et al., 2000; Loonstra et al., 2001). In addition, the activity of CRE may be induced under the control of a regulatable promoter or the use of a fusion protein to modulate CRE activity with steroids (Jullien et al., 2007).

Previously, we showed that a conditional knockout using the Dimerizable CRE recombinase (DiCRE) system, which is ligand-dependent, resulted in very low recombination efficiency in *T. cruzi* (Kangussu-Marcolino et al., 2014). In the present work, we utilise direct protein delivery of CRE recombinase to improve the efficiency of recombination in this protozoan parasite.

2. Materials and methods

2.1. Parasites and culture conditions

Axenic cultures of G strain and Dm28c clone *T. cruzi* epimastigotes were maintained at 28 °C in liver infusion tryptose (LIT) medium containing 10% foetal bovine serum (FBS), streptomycin sulphate (0.2 g/l), and penicillin (200,000 U/l) (Thermo Fisher Scientific, USA). Epimastigotes in the exponential growth phase were used in all the following experiments.

2.2. In vitro metacyclogenesis and infection

To obtain metacyclic trypomastigotes (MTs), epimastigotes were pelleted by centrifugation and resuspended in Triatomine Artificial Urine (TAU) medium (190 mM NaCl, 17 mM KCl, 2 mM $CaCl_2$, 8 mM phosphate buffer, 2 mM $MgCl_2$, pH 6.8) at a density of 5×10^8 cells/mL. Parasites were then incubated at 28 °C for 2 h, and transferred to TAU3AAG medium (TAU medium plus 10 mM glucose, 2 mM L-aspartic acid, 50 mM L-glutamic acid and 10 mM L-proline – SIGMA, USA) and incubated at 28 °C for 72 h (Contreras et al., 1985). LLC-MK2 cells (ATCC, CCL-7) were maintained in complete RPMI 1640 medium (GIBCO, USA) supplemented with 10% FBS and infected with MTs at a concentration of 100 parasites/host cell. After 24 h of infection, the medium was removed and the cell monolayer washed with 1× PBS. RPMI medium was added and the infection was allowed to proceed for a further 96 h, then tissue culture-derived trypomastigotes (TCTs) were recovered from the supernatant for electroporation with 20 μg of purified *Tc*NLS::CRE (CRE recombinase fused to a *T. cruzi* nuclear localization signal) (see Section 2.4).

2.3. Construction of a plasmid for expression of full-length CRE recombinase in Escherichia coli

The nuclear localization signal (NLS) on *T. cruzi* histone H2B (Marchetti et al., 2000) was amplified by PCR using the primers TcH2B_FOR_XbaI (GCATCTAGAATGGCCACCCCCAAGAGCTCGTC) and TcH2B_REV_BamHI (CATGGATCCATGGTTGTTGATCGACTTG AG). The PCR fragments were digested with *Xba*I and *Bam*HI, and cloned into the *Nhe*I and *Bam*HI sites of a pET28a(+) vector (Novagen, USA), generating pET28a-*Tc*NLSH2B.

CRE recombinase was amplified by PCR with the primers CRErec_FOR-BglII (ACCAAGATCTAGAATGTCCAATTTACTGACC) and CRErec_REV-HindIII (TTTTAAGCTTAATGGCTAATCGCCATCTTCCA-GC) using pLEW100-CRE (Barrett et al., 2004), gifted by Dr. John Donelson (University of Iowa, USA), as the template. The PCR product was digested with *Bgl*II and *Hind*III, and cloned into the similarly digested pET28a-*Tc*NLSH2B vector, generating pET28a-*Tc*NLSH2B-CRE.

2.4. HYPERLINK "SPS:id::Sec1" TcNLS::CRE expression and purification

The *Tc*NLS::CRE protein was expressed in *E. coli* strain BL21 (DE3) carrying the plasmid pET28-*Tc*NLSH2B-CRE, which encodes a hexa-histidine tag at the N-terminus followed by a *T. cruzi* H2B histone-derived nuclear localization signal and CRE recombinase (Fig. 1A). *Escherichia coli* was cultured at 37 °C with shaking until reaching an OD600 of 0.4, and protein expression was then induced using 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG – SIGMA, USA). After 16 h of *Tc*NLS::CRE induction, cells were resuspended in lysis buffer (50 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 100 mM PMSF, 20 μg of lysozyme), incubated on ice for 30 min and thereafter sonicated for 6 min (20 s ON, 10 s OFF). Next, bacteria were centrifugated at 12,000*g* at 4 °C and the supernatant was passed through a 1 mL His-Trap column (GE Healthcare, Life Sciences, USA). Then, the column was washed with 10 volumes of wash buffer (20 mM Tris-HCl, 0.5 M NaCl, 30 mM imidazole). Finally, protein elution was carried out in 10 volumes of elution buffer (20 mM Tris-HCl, 0.7 M NaCl, 300 mM imidazole) in a peristaltic pump using 1 mL/min flux. Buffer exchange was performed by passing the eluted protein through a desalting 5 mL column (GE Healthcare, Life sciences, USA) and the protein was maintained in desalting buffer (20 mM Tris-HCl, 0.5 M NaCl). The protein was quantified by absorbance at 280 nm and visualised on a 12% SDS–PAGE gel.

2.5. In vitro assay for CRE recombinase activity

To test the activity of *Tc*NLS::CRE in vitro, the 9 kb vector pROCK-FEKO-PUR-Neo digested with *Not*I to linearize, was used as a substrate. This plasmid contains a fusion of the puromycin resistance gene and the HSV-TK gene, flanked by two *lox*P sites in the head-to-head orientation (Kangussu-Marcolino et al., 2014). For each reaction, 18 μM of purified *Tc*NLS::CRE were incubated with 900 ng of the linearized plasmid. The reaction was carried out in a final volume of 30 μL using three different buffers: NEB buffer 2 (NB2, New England Biolabs, USA), NEB buffer 3 (NB3), and a 1× CRE Recombinase Reaction Buffer (1× CRE; 33 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5) (Fig. 1D).

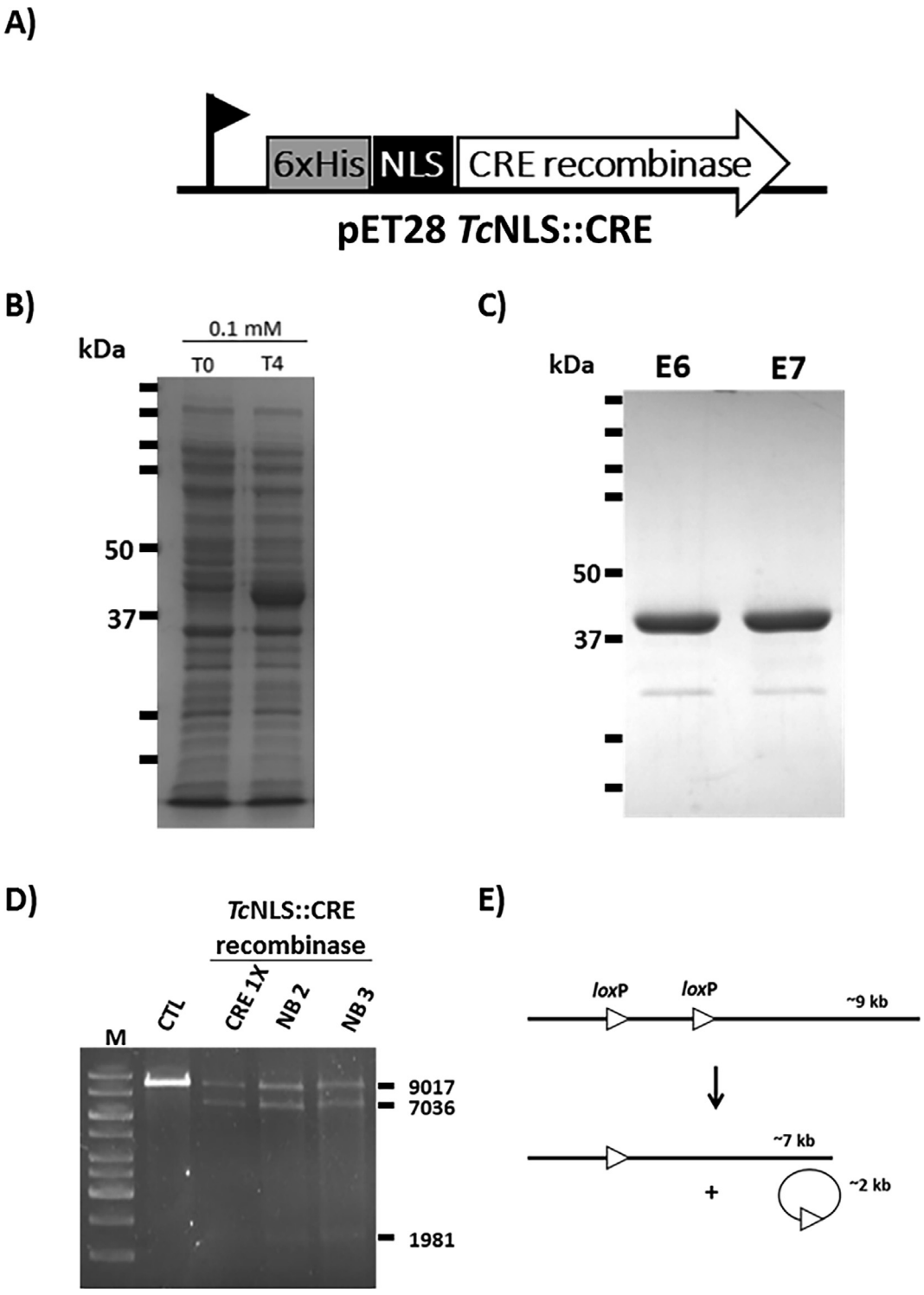

Fig. 1. Expression of CRE (**c**auses **re**combination) recombinase in *Escherichia coli*. (A) Schematic diagram of the CRE recombinase fused to a *Trypanosoma cruzi* nuclear localization signal (*Tc*NLS::CRE). (B and C) SDS–PAGE of expression and affinity purification of *Tc*NLS::CRE. T0 and T4 correspond to total extracts of BL21 star carrying pET28-*Tc*NLS-CRE at 0 h (uninduced) and 4 h after induction with isopropyl β-D-1-thiogalactopyranoside. E6 and E7 correspond to two elution fractions containing the recombinant protein (see Supplementary Fig. S3). Numbers on the left indicate the molecular weights (kDa) of the protein markers. (D and E) In vitro assay to test *Tc*NLS::CRE activity using different buffer compositions. The pROCK-FEKO-PUR-Neo plasmid (Kangussu-Marcolino et al., 2014) was linearized with *Not*I and used as a substrate for recombination. Control (CTL): 800 ng of linearized plasmid without *Tc*NLS::CRE. The substrate was incubated with *Tc*NLS::CRE in different buffers in lanes 1× CRE (33 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5), NB2, and NB3 (New England Biolabs buffers 2 and 3, respectively).

2.6. Construction of reporter plasmids to assess CRE recombinase activity in T. cruzi

The first reporter plasmid was constructed based on pAAV-FLEX-tdTomato, which was a gift from Dr. Edward Boyden (MIT Media Lab and McGovern Institute, USA) (Addgene plasmid #28306). The cassette, containing two pairs of *lox*P and *lox*2272 sites oriented head-to-head and flanking the inverted tdTomato coding sequence, was subcloned into pTREX-GFP-Neo (DaRocha et al., 2004b), generating pTREX-FLEX tdTomato-Neo (Fig. 2A).

The second reporter plasmid was constructed by cloning a fragment containing GFP flanked by *lox*66 and *lox*71, oriented head-to-head, from pGL2332 (Santos et al., 2017), a gift from Dr. Luiz R. O. Tosi (Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Brazil) into pTREX-GFP-Neo (DaRocha et al., 2004b), generating pTREX-Floxed GFP-Neo.

2.7. Construction of the floxed version of nitroreductase (NTR) and aldo–keto reductase (AKR)

First, NTR coding sequence (Dm28c ID: C4B63_56g60) was amplified by PCR using genomic DNA from *T. cruzi* Y strain with the primers NTR-For (5′-TACTTCCAATCCATGAGGAGAAATGACA TAAAAAGACGC-3′) and NTR-Rev (5′-TATCCACCTTTACTGTCAAA ACTTTCCCCACCGAACCAA-3′). The amplified NTR sequence was subcloned in the pCR2.1TOPO (Invitrogen, USA) vector and sequenced, then released from pCR2.1-*Tc*NTR through double digestion with *Spe*I and *Eco*RV. This sequence was finally cloned in a version of pTREX with a multiple cloning site flanked by *lox*2272 and *lox*P sites, generating the pTREX-FLEX *Tc*NTR-ON vector. In this context, ON refers to a sequence that is always expressed after transfection. To generate the OFF version of this vector, CRE recombinase was serially introduced as described below.

The AKR coding sequence (C4B63_175g10) was PCR amplified using the primers AKR_For (5′-TACTTCCAATCCATGAATTGCAATTA CAACTGTGTG-3′) and AKR-Rev (5′-TATCCACCTTTACTGTCACTC CTCTCCACCAGGGAA-3′). The amplified AKR was sequenced and cloned in the pTREX vector as described above for NTR, generating the pTREX-FLEX-*Tc*AKR-ON. The OFF version of AKR was generated through successive transfection steps with *Tc*NLS::CRE.

2.8. Transfection and drug selection

Trypanosoma cruzi G strain and Dm28c clone epimastigotes were transfected as previously reported (Pacheco-Lugo et al., 2017). In brief, 2×10^7 epimastigotes in the exponential growth phase were washed with Tb-BSF electroporation buffer (Schumann Burkard et al., 2011) and resuspended in 100 µL of the buffer. The parasites were electroporated in a Nucleofector 2b Device (Lonza, Switzerland) using the programme X-014. After transfection, the parasites were transferred to 5 mL of LIT medium and incubated at 28 °C. After 24 h post-transfection (pt) the cultures were diluted 1:5, and the antibiotics G418 (250 µg mL^{-1}) or hygromycin B (500 µg mL^{-1}), were added to select recombinant parasites.

For introduction of the CRE recombinase protein (*Tc*NLS::CRE) to the parasites, different molar concentrations of the CRE recombinase were electroporated as described above using 3×10^6 parasites and the U-033 programme. This electroporation procedure was repeated at day 2 or day 6 after the first electroporation.

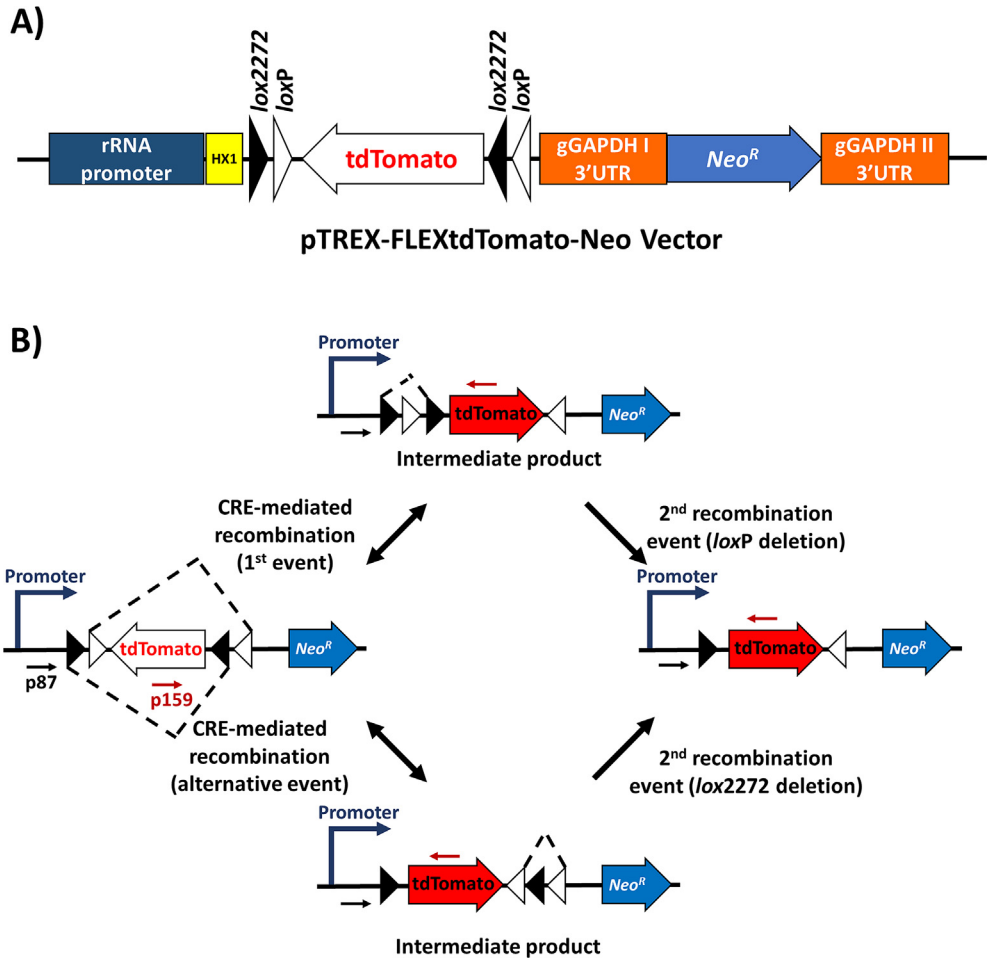

Fig. 2. Detection of CRE recombinase activity in vivo. (A) Scheme of the reporter plasmid for CRE activity. (B) Recombination events to revert tdTomato coding sequence in a construct containing two pairs of *lox* sites. p87 (red arrow) and p159 (black arrow) are primers used to estimate CRE-mediated recombination by PCR. Features are not to scale. The complete features characteristic of these plasmids (including regulatory sequences) can be seen in Supplementary Fig. S4.

2.9. Epimastigote sensitivity to benznidazole

The susceptibility of epimastigotes overexpressing NTR (NTR-ON) or recombined epimastigotes (NTR-OFF) to benznidazole (Bz) was evaluated. For this, Dm28c clone epimastigotes were stably transfected with pTREX-FLEX-*Tc*NTR-ON to generate the NTR-ON population; part of the G418-resistant population was then electroporated with *Tc*NLS::CRE to generate the NTR-OFF population. A total of 5×10^5 epimastigotes mL^{-1} were cultured in LIT + FBS medium with various concentrations of Bz (0–60 μM) in quintuplicate for 72 h at 28 °C in 96-well microtiter plates in a final volume of 100 μL. The plates were then incubated with 10 μL of alamarBlue™ Cell Viability Reagent (Thermo Fisher Scientific, USA) for 90 min and the reduction of resazurin to resorufin was measured at an excitation of 530 nm and an emission of 590 nm in a Tecan Safire Multimode Microplate Reader. The same procedure was performed using cultures overexpressing AKR (pTREX-FLEX-TcAKR-ON).

2.10. Detecting recombination through PCR analysis

To detect CRE-mediated recombination in cells carrying pTREX-FLEX tdTomato-Neo, PCRs were performed using the oligonucleotides p87 (TTTTAGATCTTTCTTCAAAATATGCAGCGG) and p159 (CATGTTGTTGTCCTCGGAGGAG). These primers amplify only the inverted version of tdTomato (tdTomato-ON) (see Fig. 2B). CRE-mediated excision of the cassette PurR-HSV_TK from cells carrying pROCK-FEKO-Pur Neo (FEKO-Epis reporter cells) was carried out as previously reported (Kangussu-Marcolino et al., 2014). All PCRs were performed using Platus Taq DNA polymerase (Sinapse Biotecnologia, Brazil) according to the manufacturer's protocol.

2.11. Flow cytometry analysis

For flow cytometry, 2.0×10^6 parasites electroporated with *Tc*NLS::CRE or 1× PBS (mock) were washed and resuspended in 1x PBS. Parasites were analysed in a FacsAria flow cytometer (Flow cytometry facility RPT08L/Carlos Chagas Institute — Fiocruz, Paraná, Brazil) at 24 h pt and 6 days pt. In each case, 20,000 events were acquired. The data were analysed using the FlowJo data analysis software package (V10) (TreeStar, USA).

2.12. Confocal microscopy analysis

Approximately 10^5 LLC-MK2 cells were left to adhere to glass coverslips in 24-well plates for 24 h then infected for 2 h with TCTs carrying pTREX-FLEX tdTomato-Neo (tdTomato-OFF) electroporated with or without *Tc*NLS::CRE. After infection, LLC-MK2 cultures were washed with PBS to remove non-internalised parasites and incubated with fresh media for 3 days before being fixed with methanol at −20°C. The slides were DAPI-stained and examined using a Nikon A1RSiMP confocal laser scanning microscope (Nikon, Tokyo, Japan). LLC-MK2 cells containing multiple parasites were imaged in three dimensions (3D z-stacking) to allow precise counting of amastigotes (using 20× or 60× objective lenses with an appropriate scan zoom for the particular cell and number of parasites). The imaging software Nis Elements 4.20 (Nikon, Tokyo, Japan) was used to visualise the images and produce animations in 3D using the z-stacks.

2.13. Determination of NTR mRNA expression by qPCR

Dm28c clone epimastigotes carrying pTREX-FLEX-*Tc*NTR-ON were electroporated with *Tc*NLS::CRE or not (mock control), and incubated in LIT medium until they reached 1×10^8 parasites/mL. Epimastigotes were harvested by centrifugation at 1500 *g* for 5 min, resuspended in TRIzol™, and the RNA was purified following the manufacturer's instructions. Purified RNA was treated with Turbo DNAse™ (Thermo Fisher Scientific, USA) at 37 °C for 30 min, and precipitated with ethanol/sodium acetate. One μg of RNA was used as template for cDNA synthesis using High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems, USA) following the manufacturer's instructions. Quantitative PCRs (qPCRs) were performed using primers qPCR-NTR645_FOR (TAGTGAAAG-CACTGGCAACG) and qPCR-NTR756_REV (AAATTGCCGTGT-CAAACCCT), and β-tubulin was used as an internal control. The qPCR assays were performed and analysed by GOgenetic (Curitiba, PR, Brazil).

3. Results

3.1. Crediting using recombinant TcNLS::CRE recombinase enables efficient recombination

Since a previous report using the DiCRE system showed poor recombination efficiency (Kangussu-Marcolino et al., 2014), one of the major aims of this study was to improve CRE-mediated recombination in order to provide a better molecular tool for genetic manipulation of *T. cruzi*. To achieve this, biologically active CRE recombinase was directly introduced to epimastigotes of *T. cruzi* through protein electroporation. This approach was based on previous work regarding the delivery of recombinant proteins, including CRE recombinase, by electroporation in other cell types (Deora et al., 2007; Furuhata et al., 2019), and Cas9 in *T. cruzi* (Soares Medeiros et al., 2017; Burle-Caldas et al., 2018). To this end, a recombinant fusion protein was designed, referred to as *Tc*NLS::CRE, consisting of an N-terminal histidine tag for efficient purification from *E. coli*, an NLS derived from histone H2B (Marchetti et al., 2000), and CRE recombinase from pLEW100-CRE (Barrett et al., 2004) (Fig. 1A). Then, *Tc*NLS::CRE was expressed in *E. coli* (Fig. 1B) and affinity purified to homogeneity (Fig. 1C). Purified fractions of *Tc*NLS::CRE were tested for their in vitro recombination ability using ~~a~~ linearized plasmid DNA of 9 kb as a substrate, which contained two *lox*P sites in the same orientation flanking a 2 kb fragment (Kangussu-Marcolino et al., 2014). When CRE-mediated recombination occurs the 2 kb fragment is removed, meaning the substrate is reduced to ~7 kb, which can be observed by agarose gel electrophoresis (Fig. 1D, E) of the in vitro assay. The *Tc*NLS::CRE was able to recombine ~50% of the substrate after incubation at 37 °C for 1 h (Fig. 1D, E). This efficiency of recombination was independent of the buffer composition used in the assay (NB2, NB3 or 1× CRE Recombinase Reaction Buffer. These results indicate that the recombinant CRE enzyme containing an N-terminal extension (His-tag, and *T. cruzi* H2B NLS) is active.

To track the activity of CRE recombinase in *T. cruzi* epimastigotes, pTREX-FLEX-tdTomato-Neo (Fig. 2A) was generated and introduced by transfection into the epimastigotes (Flex-tdOFF-EPIs). The pTREX-FLEX-tdTomato-Neo construct contains a ribosomal promoter that drives transcription of an inverted tdTomato sequence flanked by *lox*P and *lox*2272 in head-to-head orientation to allow tdTomato reversion (Fig. 2B). Purified *Tc*NLS::CRE was then introduced into Flex-tdOFF-EPIs using the electroporation conditions previously reported (Pacheco-Lugo et al., 2017). Thus, tdTomato expression can be directly detected by fluorescence microscopy or flow cytometry.

Flow cytometry analyses (Fig. 3A) and confocal microscopy images (Fig. 3B) were used to precisely determine the efficiency of CREditing in the *T. cruzi* epimastigotes. After transfecting Flex-tdOFF-EPIs with different molar concentrations of *Tc*NLS::CRE, a high proportion of parasites (up to 50%–60%) was observed to be expressing tdTomato as early as 24 h pt (Fig. 3A, B, Supplementary

Table S1). Interestingly, CRE-mediated recombination increased to ~88% when Flex-tdOFF-EPIs were re-transfected with purified protein 6 days after the first electroporation (Fig. 3D). However, there was a reduction in the percentages of tdTomato-expressing cells when parasites were re-transfected with just a 2 day interval after the first transfection, compared with those that had a 6 day interval. This reduction can be explained by toxicity (Supplementary Fig. S1), since a probable excess of CRE recombinase favours recombination (tdTomato ON) and causes cell death, as shown in *T. brucei* (Barrett et al., 2004; Scahill et al., 2008).

The recombination event was also confirmed at the DNA level by performing PCR using primers designed to amplify only the reverted version of tdTomato (Fig. 3C). As shown in Fig. 2B, two sequential recombination events depending on CRE activity are necessary to remove additional *lox* sites and make the recombination irreversible. PCR products revealed only one band compatible with 1028 bp, and the intermediate product was not detected, which is compatible with the occurrence of these two recombination events. Taken together, the recombinant *Tc*NLS::CRE-dependent recombination is highly efficient, allowing the expression of tdTomato in *T. cruzi* epimastigote forms after transient transfection of the protein.

3.2. Recombinant TcNLS::CRE turned off GFP expression with high efficiency

The efficiency of CREditing in turning off gene expression in a single recombination event was then assessed using different combinations of two *lox* sites: *lox*66 and *lox*71. When head-to-head *lox*66 and *lox*71 recombine, a wild-type *lox*P site and a double mutant *lox*P (*lox*72) site are generated (Fig. 4A). CRE-mediated inversion using *lox*66 and *lox*71 tends to happen only in the forward direction since the *lox*72 site exhibits reduced binding affinity for CRE recombinase (Albert et al., 1995). As shown in Fig. 4A, a plasmid containing GFP floxed by *lox*66 and *lox*71, known as pTREX-Floxed-GFP-Neo, was generated. In parasites carrying this construct, homogeneous populations of epimastigotes stably expressing GFP (GFP ON) were obtained, until *Tc*NLS::CRE was delivered by electroporation (Fig. 4B). After introduction of *Tc*NLS::CRE, there was a reduction close to 65% of GFP-OFF parasites at the later time points after electroporation (48 h pt and 6 days pt) compared with 24% of GFP-OFF parasites at 24 h pt (Fig. 4C). Despite this modest reduction in the percentage of GFP-positive parasites at 24 h pt, this culture showed a higher change in parasite fluorescence intensity (Fig. 4D). This complete loss of fluorescence by 6 days pt might be related to GFP stability.

3.3. TcNLS::CRE works independently of genomic context and can be used for selectable marker removal

As high efficiencies of recombination were observed in parasites carrying two different episomal fluorescent reporter plasmids, tdTomato (that was turned on) and GFP (that was turned off), the functionality of the CREditing in an endogenous locus of the parasite was assessed. Using the integrative vector pROCK-FEKO-PUR-Neo (Kangussu-Marcolino et al., 2014), a population of parasites was obtained that carried the puromycin-HSV-TK cassette flanked by two *lox*P sites in the same orientation in the beta-tubulin locus (Fig. 5A). After CRE-mediated recombination, a 1981 bp deletion could be detected by PCR amplification of the CRE "scar", giving a 295 bp PCR product (Kangussu-Marcolino

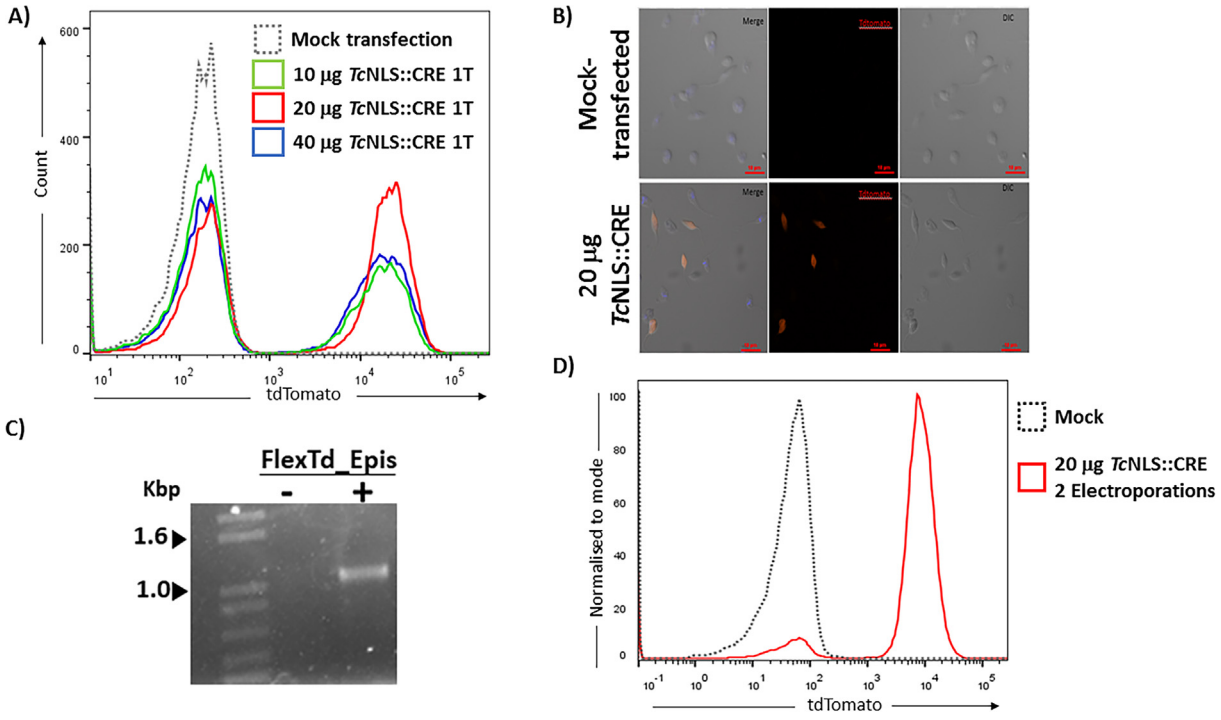

Fig. 3. CRE recombinase-mediated recombination using recombinant *Tc*NLS::CRE (CRE recombinase fused to a *Trypanosoma cruzi* nuclear localization signal) in *T. cruzi*. (A) Epimastigotes from *T. cruzi* G strain carrying an inverted sequence of tandem dimer Tomato (tdTomato) reporter gene (Flex-tdOFF-EPIs) were electroporated with different concentrations of *Tc*NLS::CRE and the efficiency of recombination was evaluated by flow cytometry at 24 h post-transfection and at 6 days post-transfection. A representative experiment is shown. Twenty thousand events were acquired for each sample and the gates were determined based on the wild-type histogram profile. (B) Confocal microscopy images of Flex-tdOFF-EPIs transfected with 20 µg of *Tc*NLS::CRE analysed at 6 days post-transfection. Scale bar = 50 µM. (C) Genomic DNA was extracted from Flex-tdOFF-EPI mock- and CRE-transfected epimastigotes and PCR was carried out to detect the reverted version of tdTomato. (D) Flex-tdOFF-EPIs were electroporated twice with a 6 day interval and the resulting culture was analysed by flow cytometry for tdTomato expression.

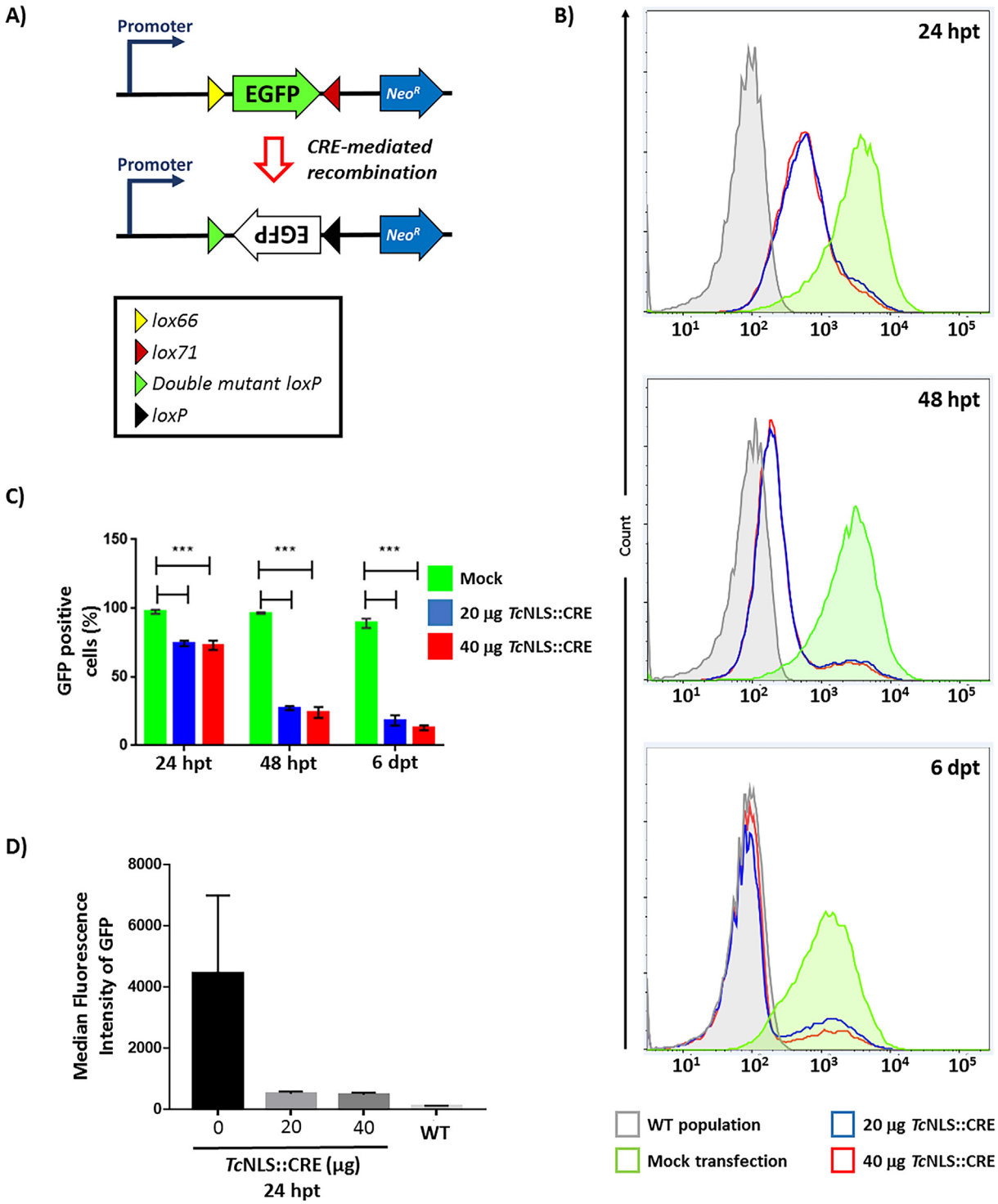

Fig. 4. High efficiency of CREditing in turning off GFP expression. (A) Epimastigote forms of *Trypanosoma cruzi* were stably transfected with a pTREX-Floxed-GFP-Neo plasmid, in which GFP expression is ON. (B) *Tc*NLS::CRE (CRE recombinase fused to a *T. cruzi* nuclear localization signal) was electroporated in these populations and GFP decay was checked by flow cytometry 24 and 48 h post-transfection (hpt) and at 6 days post-transfection (dpt). Twenty thousand events were acquired for each sample and the gates were determined based on the wild-type histogram profile. Data shown are representative of three independent experiments after transfection with 0 (green line; mock), 20 (red line), and 40 µg (blue line) of *Tc*NLS::CRE. The wild-type population is shown in grey. (C) The percentage of GFP-positive cells after introducing *Tc*NLS::CRE is shown. (D) The median fluorescence intensity (arbitrary unit) of GFP at 24 h post-transfection.

et al., 2014). To estimate the recombination efficiency, we carried out single-cell cloning, with 12 out of 15 clones positive for CRE scar amplification, indicating an efficiency of 80% (Fig. 5B). Taken together, these results demonstrate the utility of protein delivery by transfection to edit an endogenous locus in the parasite.

3.4. Conditional overexpression of NTR and aldo–keto reductase using CREditing

Bz and nifurtimox are nitroheterocyclic derivatives that need to be activated by intracellular nitroreductases to become toxic. In *T.*

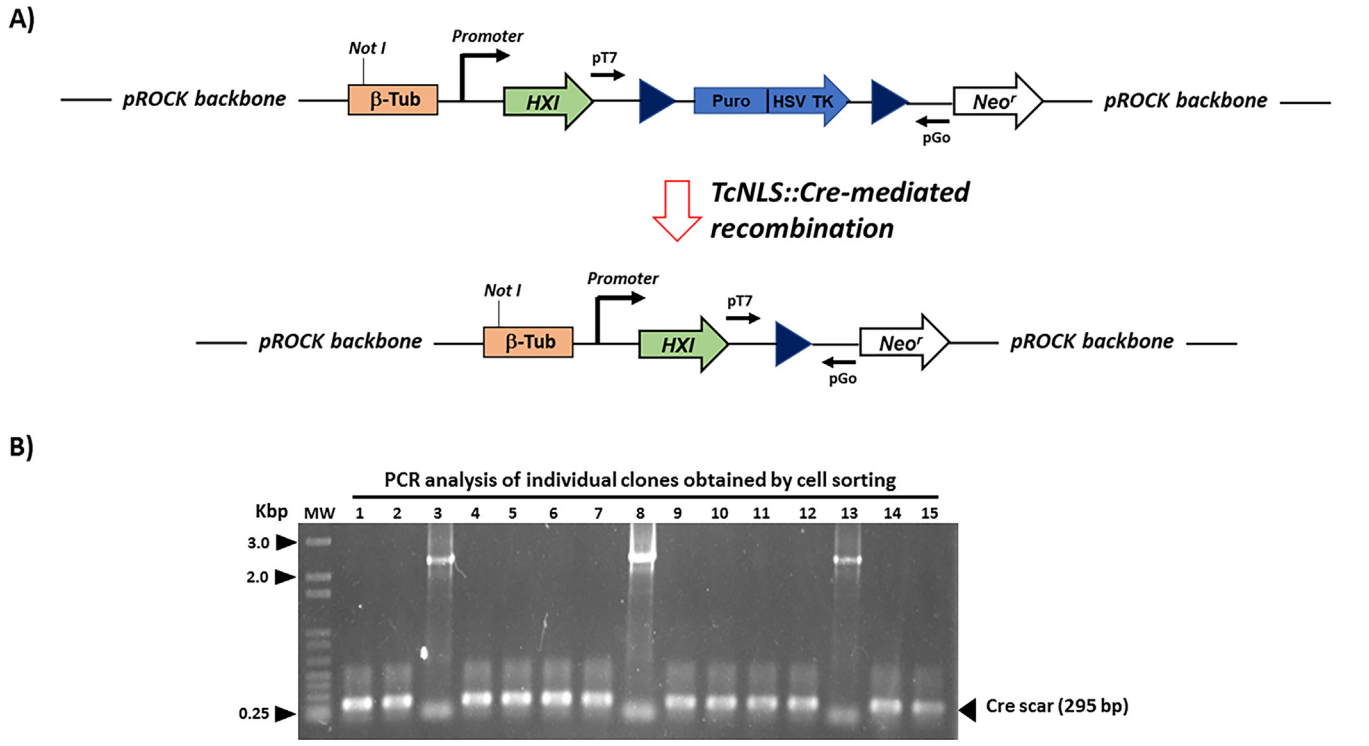

Fig. 5. *Tc*NLS-CRE (CRE recombinase fused to a *Trypanosoma cruzi* nuclear localization signal) is functional in an endogenous locus. (A) Epimastigote forms of *T. cruzi* were stably transfected with pROCK-FEKO-PUR, an integrative vector for the beta-tubulin locus carrying a puromycin-HSV-TK cassette flanked by *lox*P sites (blue arrowheads). After transfection with *Tc*NLS::CRE (+) or mock transfection with electroporation buffer (−), recombination was evaluated by PCR using pT7 and pGo primers (black arrows). (B) PCR analysis of individual single-cell sorted clones. The recombination events can be detected by PCR of a 295 bp fragment known as the CRE scar.

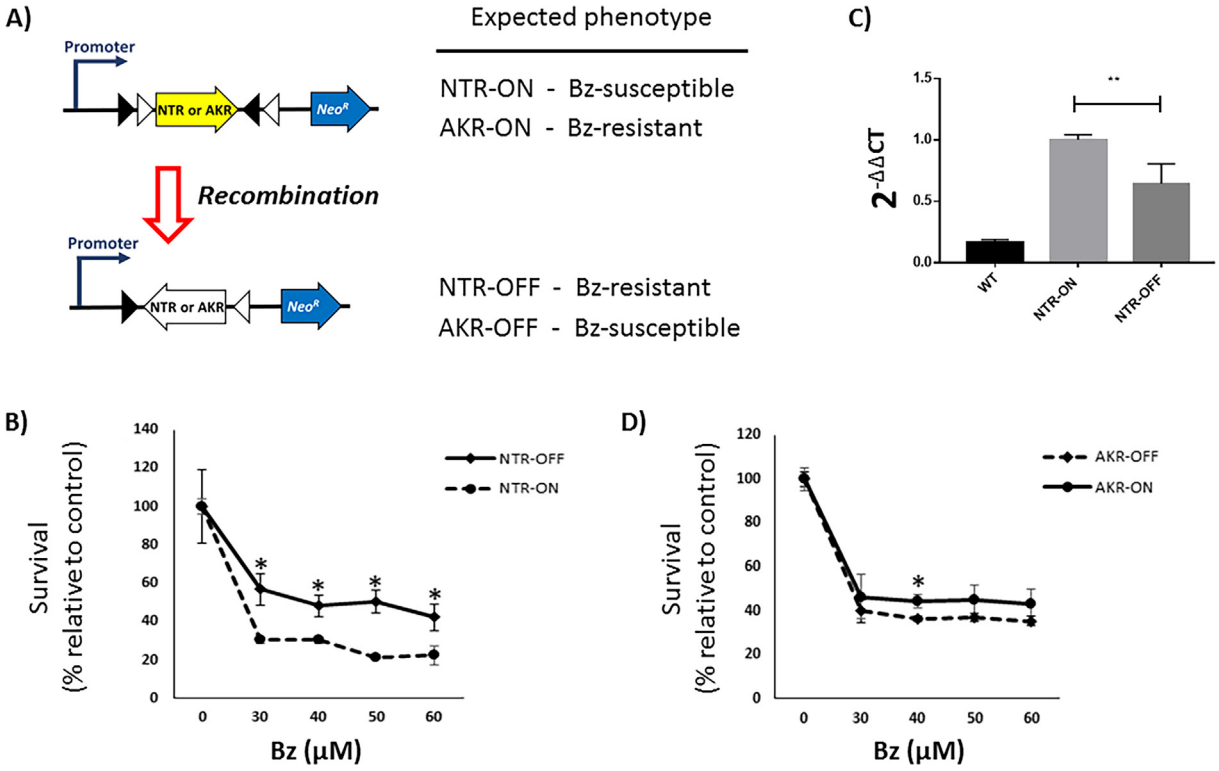

Fig. 6. Conditional nitroreductase and aldo–keto reductase overexpression using CREditing. (A) Schematic representation of the floxed-nitroreductase and AKR overexpressing cassettes in epimastigote forms of *Trypanosoma cruzi* Dm28c clone (NTR-ON and AKR-ON). CREdited populations were induced by delivering *Tc*NLS::CRE (CRE recombinase fused to a *T. cruzi* nuclear localization signal) generating parasite populations in which nitroreductase and aldo–keto reductase were turned off (NTR-OFF and AKR-OFF). All populations, NTR-ON, NTR-OFF, AKR-ON, and AKR-OFF (B and D) were incubated with different concentrations of benznidazole for 72 h and cell viability was evaluated using the alamarBlue test. Experiments were performed in triplicate with data presented as the mean ± S.D. $P < 0.05$. (C) NTR mRNA expression by quantitative PCR analysis. The data shows relative expression of the NTR gene after CRE recombinase electroporation. β-Tubulin gene was used as an internal control. ANOVA one-way test, $P < 0.005$.

brucei (Hall et al., 2011) and *Leishmania donovani* (Wyllie et al., 2013), NTR overexpression in parasites generates populations that are more sensitive to these pro-drugs. In the present study, the conditional expression of genes related to drug resistance/susceptibility in *T. cruzi*, was tested. For this, parasites overexpressing the *Tc*NTR gene (NTR-ON) were generated using pTREX-FLEX-*Tc*NTR ON. These parasites were then electroporated twice, with a 6 day interval, with *Tc*NLS::CRE to generate populations in which NTR expression was turned off (NTR-OFF) (Fig. 6A). Both populations (NTR-ON and NTR-OFF) were incubated with increasing concentrations of Bz for 72 h and cell viability was assessed by alamarBlue staining. As shown in Fig. 6B, the NTR-ON population was shown to be more sensitive to Bz than its floxed counterpart. As expected, the NTR mRNA expression dropped when NTR-ON parasites were treated with *Tc*NLS::CRE (Fig. 6C). The expression level of NTR mRNA in NTR-OFF cultures was higher than that of wild-type parasites, which has *Tc*NTR as a single copy gene per haploid genome. This difference in NTR expression between NTR-OFF and wild-type parasites suggests that the recombination could still be improved. In summary, the phenotypic change observed is compatible with the *Tc*NTR mRNA levels. This result demonstrates the use of this CREditing system in the investigation of gene function in the context of drug resistance.

Unlike NTR, it has been shown that overexpression of AKR improves Bz resistance in *T. cruzi* (González et al., 2017). AKR-ON and AKR-OFF populations were generated in a similar way as the NTR-ON and NTR-OFF populations. Parasites overexpressing floxed AKR (AKR-ON) were generated through transfection with pTREX-FLEX-AKR-ON, and a population of these parasites were further electroporated with *Tc*NLS::CRE (AKR-OFF) (Fig. 6D). These populations were then treated with Bz and a tendency to resist drug treatment was detected in AKR-ON parasites, however, a statistically significant difference was only seen when 40 μM of Bz were used (Fig. 6E).

3.5. Gene expression can be modulated in tissue culture-derived trypomastigotes by CREditing

Genetic manipulation of infective forms of *T. cruzi* is particularly challenging since MTs and tissue TCTs are non-replicative. Once CREditing was shown to be functional in epimastigotes, it was decided to test whether *Tc*NLS-CRE could be efficiently transfected into TCTs. First, an infection protocol using LLC-MK2 cells was employed to obtain TCT forms derived from epimastigotes stably carrying pTREX-FLEX-tdTomato-Neo. The TCTs carrying the floxed tdTomato (Flex-tdOFF-TCTs) were electroporated with *Tc*NLS-:: CRE. After transfection, FlexTd-OFF-TCTs cells were used to infect LLC-MK2 cells and analysed by confocal microscopy at 24, 48, and 72 h p.i. As shown in Fig. 7, TCTs transfected with *Tc*NLS:: CRE were infective to LLC-MK2 with detection of Flex-tdOFF_TCTs becoming Flex-tdON-TCTs as early as 24 h p.i., with intracellular forms showing high levels of tdTomato expression (Supplementary Fig. S2). In summary, CREditing allowed manipulation of gene expression through recombination in both epimastigote (replicative and non-infective) and TCT (non-replicative and infective) forms of *T. cruzi.*

4. Discussion

Functional genomics approaches in *T. cruzi* are challenging and this is further hindered as the parasites enter the non-replicative stages of their lifecycle. Molecular approaches that allow tight regulation of gene expression are still lacking for this parasite. Currently, the best system for inducible expression is the T7 RNA polymerase/Tet Repressor system (Taylor and Kelly, 2006). However, this system is not widely used, probably due to the lengthy and costly process of generating a cell line expressing the necessary components of the system prior to experimental use. Furthermore, there is some leakiness of the system in the absence of the inducer (tetracycline) (Taylor and Kelly, 2006; Piacenza et al., 2007; Laverrière et al., 2012). It would therefore be advantageous to have a system that allows transgene expression in a very well-controlled manner. In the present work, we evaluated a CREditing system that could be used to survey gene function in different stages of the *T. cruzi* lifecycle.

Due to the relatively high efficiency of genome editing shown by electroporation of recombinant Cas9 in *T. cruzi* (Soares Medeiros et al., 2017; Burle-Caldas et al., 2018), we expected a similar result for CRE recombinase, which could also circumvent the toxicity issue caused by constitutive expression of CRE recombi-

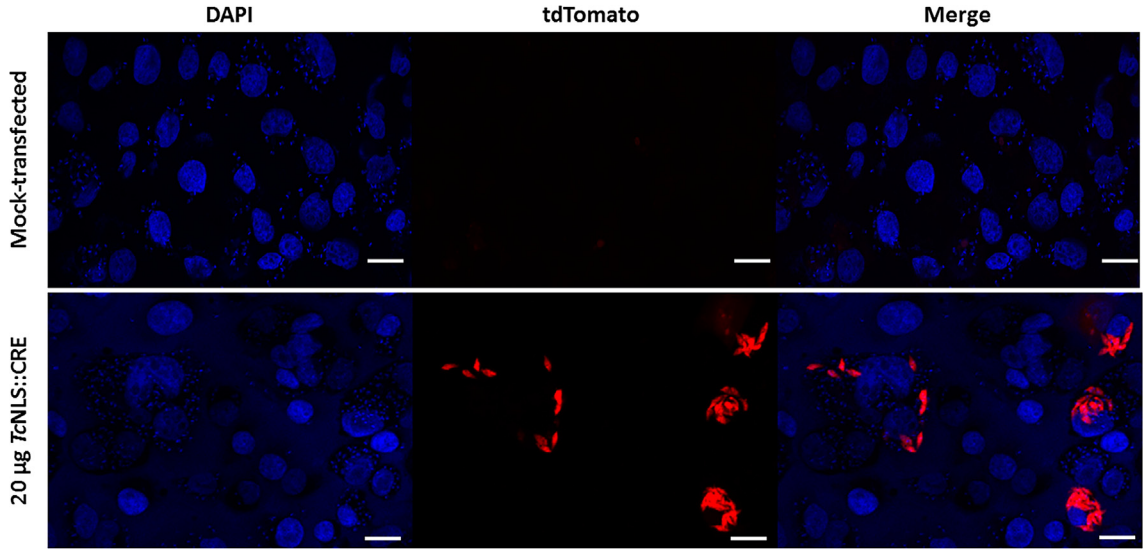

Fig. 7. Tissue culture-derived trypomastigote forms of *Trypanosoma cruzi* were efficiently transfected with *Tc*NLS::CRE (CRE recombinase fused to a *T. cruzi* nuclear localization signal). Floxed-tdTomato (a tandem dimer fluorescent protein) epimastigotes (tdTomato-OFF) were differentiated into metacyclic trypomastigote forms, which were used to infect a monkey kidney cell strain (LLC-MK2 cells) and trypomastigote forms were obtained from the supernatant after two rounds of infection. trypomastigote forms were washed once with electroporation buffer (Tb-BSF buffer), transfected with 20 μg of *Tc*NLS::CRE and immediately used to infect LLC-MK2 cells at a multiplicity of infection of 1:1. Cells were fixed and analysed by confocal microscopy at 40 h p.i. DNA was stained with DAPI (blue). Scale bars = 20 μm.

nase. Our results demonstrate that the delivery of a recombinant version of CRE recombinase fused to a *T. cruzi* nuclear localization signal (*Tc*NLS::CRE) by protein electroporation into epimastigote forms was successful, allowing the gene expression of fluorescent reporters to be turned on or off using different combinations of *lox* sites. In addition, we showed that this system could be useful for investigating gene function in the context of drug resistance mechanisms. This method resulted in high recombination efficiencies as early as 24 h after delivery of the *Tc*NLS::CRE in replicative forms of the parasite, reaching up to 60% of the cells. Previous reports have shown that transient transfections of plasmid DNA for reporter gene expression in epimastigotes result in between 15 and 35% positive cells (Pacheco-Lugo et al., 2017; Olmo et al., 2018), which is much lower than shown here using purified CRE recombinase. It is important to highlight that the constitutive expression of CRE recombinase has been shown to be toxic in several cell types, including in *T. brucei* (Schmidt et al., 2000; Loonstra et al., 2001; Silver and Livingston, 2001; Barrett et al., 2004). We found that using successive rounds of electroporation with the enzyme led to a reduction in fluorescence with the reporter tdTomato, which is turned on when recombination occurs. This result indicates that perhaps the elevated levels of CRE recombinase activity could be toxic (Supplementary Fig. S1).

Despite working efficiently in *T. brucei*, whereby there is at least four orders of magnitude greater gene expression compared with parasites without induction, the tetracycline-inducible system has been demonstrated to be much less efficient in *T. cruzi* (DaRocha et al., 2004a). More importantly, the system has shown a relatively high degree of leakage in the absence of tetracycline (Taylor and Kelly, 2006; Piacenza et al., 2007; Laverrière et al., 2012). Whereas the *Tc*NLS::CRE-mediated recombination can be more tightly regulated, as in the OFF mode the gene of interest (GOI) is encoded by the non-transcribed strand.

In general, when gene function is interrogated through overexpression or knockout of a GOI, it is carried out in replicative forms, the epimastigotes, due to the fact that they grow more easily and the protocol for their genetic manipulation is standard. In conventional approaches, epimastigotes are transfected and the constitutive expression of a GOI will cause biological changes/adaptations throughout the cell cycle that might affect the phenotype at the desired stage. However, CREditing allows deletion or overexpression without drug selection at almost any time in the extracellular stages. In the present work, we showed that gene manipulation is possible in both the epimastigote and tissue-derived trypomastigote forms by direct protein electroporation of the CRE recombinase. Confocal microscopy analysis (Fig. 7) showed between 10% and 20% of infected cells became tdTomato-positive 24 and 48 h p.i., respectively. Although the efficiency seems to be low, methods can be designed for enrichment of these populations through cell sorting, allowing more precise analysis as previously described (Padmanabhan et al., 2014). To our knowledge, there is only one work in the literature which has investigated the manipulation of TCTs, and that was done through the electroporation of plasmid DNA. In that work, efficiencies of 5% and 95% were obtained before and after cell sorting (Padmanabhan et al., 2014).

Gene knockout has been shown to be a powerful tool to explore gene function in *T. cruzi*. We asked if active CRE recombinase could remove a gene integrated in a locus of the parasite. We transfected epimastigote forms with the pROCK-FEKO-PUR-Neo plasmid, an integrative vector at the tubulin locus (Kangussu-Marcolino et al., 2014). Recombination led to the excision of 1981 bp, which results in a scar fragment that is easily detected by short-cycle PCR. This resulting fragment is 295 bp long, which corresponds to the length of a single *lox*P site as well as up- and downstream sequences. Interestingly, when we analysed individual clones of the floxed population we found a higher efficiency of recombination (80%, 12 out of 15 clones) (Fig. 6B). Several factors have been shown to affect the efficiency of CRE excision on the *lox* pair. One important factor is the nucleotide sequence identity in the spacer region of the *lox* site. Engineered *lox* variants which differ in the spacer region tend to have varied but generally lower recombination efficiencies compared with wild-type *lox*P, presumably through affecting the formation and resolution of recombination intermediates (Lee and Saito, 1998). In addition, the genetic location of the floxed sequences affects recombination efficiency, probably by influencing the availability of DNA by CRE recombinase (Liu et al., 2013). Therefore, the higher efficiency of recombination seen for the floxed cassette puromycin-HSV-TK can be explained by the flanking *lox*P sites (instead of the pairs *lox*2272/*lox*P and *lox*66/*lox*71, used for the reporters tdTomato and GFP, respectively) and the genomic locus (beta-tubulin in the last case).

Here, we applied the CRE-*lox* tool using constructs carrying the *lox* sites of interest at desirable positions. However, another approach is that one allele can be replaced by a selectable marker through homologous recombination and the other allele can be exchanged by the GOI flanked by *lox*P sites and a second selectable marker, as described by Scahill et al. (2008) in *T. brucei*. Alternatively, the GOI can be manipulated using CRISPR-Cas9 technology to allow the insertion of *lox*P sites flanking the GOI, so that recombination between *lox*P sites can be induced by transfecting active CRE recombinase. Recently, Damasceno et al. (2020) used the CRISPR-Cas9 system in *L. major* to replace the endogenous gene by the same GOI flanked by *lox*P sites plus a selectable marker. Later, they did conditional knock-out of RAD51 recombinase using the DiCRE system. This combined approach can help to evaluate the phenotype at specific stages of the lifecycle of the parasite by introducing the *lox* sites in epimastigotes using Cas9, followed by recombination at a particular stage.

In summary, application of the CRE-*lox* system tested here allowed gene expression to be turned on or off at specific lifecycle stages (epimastigotes and TCTs) and the deletion of a desired sequence integrated in the genome, without the need for previously modified parasites expressing CRE recombinase or its variants. Our results demonstrate that CREditing is a valuable genetic tool for the functional genomics toolbox of *T. cruzi*, and perhaps for related parasites. The CRE-*lox* technology described here provides a versatile tool for deletion or inversion of sequences that can be applied in order to remove engineered cassettes containing drug-selection cassettes floxed by *lox* sites, so that after successful drug selection, the selectable marker can be removed and reused for another round of stable transfection.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2020.06.010.

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