Supplementary Information for

Single p197 molecules of the mitochondrial genome segregation system of *T. brucei* determine the distance between basal body and outer membrane

Salome Aeschlimann1, 2, Ana Kalichava2, 3, Bernd Schimanski1, Bianca Manuela Berger2, 3, Clirim Jetishi2, 3, Philip Stettler1, 2, Torsten Ochsenreiter3* and André Schneider1*

*Corresponding authors

Email: torsten.ochsenreiter@unibe.ch, andre.schneider@unibe.ch

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Material and Methods

Transgenic cell lines

All procyclic cell lines are derivatives of *T. brucei* 427 grown at 27°C in SDM-79 supplemented with 5% (v/v) fetal calf serum (FCS). To generate a single marker inducible cell line, 427 cells were transfected with a modified pSmOx plasmid (1) where the puromycin resistance gene had been replaced with phleomycin resistance. The plasmid encodes both the T7 RNA polymerase (RNAP) and the Tet-repressor.

For Tet-inducible RNAi of p197 (Tb927.10.15750) cells were stably transfected with a NotI-linearized plasmid containing stem-loop sequences covering the 424nt long 3’ UTR of the p197 mRNA. RNAi plasmids targeting the ORFs of TAC65, TAC60 or TAC40 have been described previously (2, 3).

*In situ* C-terminal HA-tagging of p197 was done by stable transfection of PCR products using plasmids of the pMOtag series as templates (4) with primers defining the sites of homologous recombination leading to expression of tagged full length p197 or versions thereof lacking 334aa or 1383 aa of its C-tail. *In situ* N-terminal myc-tagging of p197 was also done using PCR products with primers defining the flanking region of the start codon of p197. The plasmid for amplification was a derivate of the pN-PURO-PTP (5) and was modified to have a triple myc tag instead of the PTP tag.

Plasmids for tetracycline-inducible expression of ΔC1383-HA, ΔN1228-HA, TAC65-myc and TAC60-myc were generated with a modified pLew100 expression vector (6) containing a puromycin or a blasticidin resistance cassette. For p197 truncations, PCR products corresponding to the ORF that either lacked the first 1228 or the last 1383 aa were amplified from genomic DNA and integrated into the vector allowing for C-terminal triple HA tagging. For TAC65 and TAC60 a PCR product corresponding to the whole ORF lacking the stop codon was amplified from genomic DNA and integrated into the vector allowing for C-terminal triple myc tagging.

To ensure functionality of double-tagged p197, a plasmid for the single knockout of p197 was generated by modification of a pMOtag43M vector (4). The 5’ flanking sequence of p197 (500 nucleotides before start codon) and the 3’ flanking sequence (495 nucleotides after stop codon) were amplified and ligated into Xhol/Nhel and BamH1/SacI restriction sites of the vector thereby flanking the hygromycin resistance gene. Because the 3’ flank of p197 has an internal Xhol restriction site (starting at nucleotide 226 after stop codon) the resistance cassette could be released with Xhol before electroporation of the single or double tagged p197 cell line. To generate the construct allowing inducible expression of the myc-Δrep-HA variant of p197, where the central repeat region was deleted, the region of the gene corresponding to the N-terminus (nt 1-1356) was amplified from genomic DNA and cloned into a vector allowing for N-terminal myc tagging. The region corresponding to the C-terminal part (nt 2965-5244) of p197 was amplified accordingly and integrated into a vector allowing for C-terminal HA-tagging. The construct encoding the myc-tagged N-terminus was then reamplified with primers containing flanking Ndel and a Xbal restriction sites, respectively. The resulting PCR product was then ligated into the construct encoding the C-terminal HA-tagged C-terminus using Ndel and Xbal to generate a fusion encoding myc-Δrep-HA. The Xbal site which marks the deletion of the repeat region could be used to insert the α-helical stretch of the *T. cruzi* p197 (TcBrA4_0086540, nt 1267-4698), to generate a construct encoding the myc-cruzi-HA version. For a schematic overview of p197 variants used in this study see SI Appendix, Fig. S9. An overview of all cell lines used in the study is shown in Table S1.
Immunofluorescence microscopy

One million cells were harvested, washed, resuspended in phosphate buffered saline (PBS) and allowed to settle on cover slides for 10 min. For whole cell IF analysis parasites were fixed using 4% paraformaldehyde (PFA) and permeabilized with 0.2% Triton X-100. For cytoskeletons cells were first permeabilized with 0.05% Triton X-100 for 10 sec, washed in PBS and then fixed with 4% PFA. Flagella were isolated as described (7). In brief, cells were harvested by centrifugation after EDTA (100 nM) was added to the culture, directly resuspended in extraction buffer (10 mM NaH₂PO₄, 150 mM NaCl, 1 mM MgCl₂, pH 7.2) containing 0.5% Triton X-100 and incubated for 10 min on ice. After centrifugation pellets were incubated on ice for 45 min with extraction buffer containing 1mM CaCl₂. Extracted flagella were then pelleted for 10 min at 10’000 g, resuspended in PBS, settled on a coverslip for 10min and fixed with 4% PFA.

Staining with antibodies has been done in a wet chamber, primary antibodies were diluted in PBS containing 2% bovine serum albumin (BSA), secondary fluorescent antibodies in PBS only. After incubation in cold methanol, dried coverslips were mounted onto Vectashield containing DAPI (Vectorlabs).

Z-stack images were acquired using a DMI6000B microscope equipped with a DFC360 FX monochrome camera and LAS X software (Leica Microsystems). Image figures were generated using Fiji software and the FigureJ Plugin. To quantify the images, z-projections were done, signals for the BB (bright YL1/2 signal), SAS6 and p197 determined by eye and counted. Number of counted cells or flagella are indicated in the corresponding figure legends. DIC images, DAPI signals and number of visible BBs per cells were used to determine the cell cycle stages.

Ultrastructure expansion microscopy (U-ExM)

10 million cells are centrifuged for 3 min at 2000 g and resuspended twice in 1 ml PBS. The resulting pellet is resuspended in 500 µl PBS and 50 µl cells are settled for 20 minutes on poly-D-lysine coated coverslips (12 mm, Menzel-Glaser). Coverslips are transferred into a 24-well plate filled with FAB solution (0.7% Formaldehyde (36.5-38%, Sigma) and 1% acrylamide (40%, Sigma) in PBS) and incubated for 5 hours at 37 °C. A plastic chamber is covered with wet tissue paper and parafilm and put on ice. One drop (35 µl) of monomer solution containing 19% sodium acrylate (AK Scientific 7446-81-3), 10% (wt/wt) acrylamide and 0.1% (wt/wt) N,N’-methylenebisacrylamide (Sigma) in PBS supplemented with 0.5% ammoniumpersulfate and 0.5 %tetramethylethylenediamine is placed on the parafilm. Coverslips are then carefully put on the drop with cells facing the gelling solution. Gelation is done for five minutes on ice, and subsequently samples are incubated at 37°C in the dark for one hour. Coverslips with gels are then transferred into a six well plate filled with 2 ml of denaturation buffer (200 mM SDS, 200 mM NaCl, and 50 mM Tris, pH 9 in ultrapure water) for 20 min at room temperature to allow the gel to detach from the coverslip. Gels are then moved into a 1.5 ml Eppendorf centrifuge tube filled with denaturation buffer and incubated at 95 °C for 1 hour. After denaturation, gels are placed in deionized water for a first round of expansions. Water is exchanged after 30 min, and gels are incubated overnight in deionized water. Subsequently, gels are washed twice for 30 min in PBS and incubated on a shaker with the primary antibody. All antibodies are diluted in PBS containing 2% BSA and gels are incubated for 3 hours at 37°C. Gels are then washed in 0.1% PBS-Tween 20 (PBS-T, Sigma) three times for 10 min while gently shaking and subsequently incubated with secondary antibodies. Gels are then washed in PBS-T three times for 10 min while gently shaking and finally placed in beakers filled with deionized water for expansion. Water is exchanged after 30 min before gels are incubated in deionized water overnight.

Mounting and image acquisition

The gel is cut into pieces that fit in a 36 mm metallic chamber for imaging. One piece is mounted on a 24 mm round poly-D-lysine functionalized coverslip already inserted in the metallic chamber and gently pressed with a brush to ensure adherence of the gel to the coverslip. Confocal
microscopy is performed on a Leica TCS SP8 using a 63 x 1.4 NA oil objective with the following parameters: z step size at 0.3 μm interval with a pixel size of 59 nm and a zoom factor of 8. LAS X software (Leica Microsystems), Huygens Professionals, ImageJ and Prism are used to analyse the images. The intramolecular distance and the distance between basal body and outer mitochondrial membrane is given by the average of three measurements (all three measurements performed at one kDNA). Statistics are performed using one-way ANOVA with post hoc Tukey's HSD (honestly significant difference) test.

**Calculation of expansion factors from U-ExM**

The expansion factor for single-tagged p197 (Fig. 1) is determined by measuring the lengths of the cell, the kDNA and the diameter of the nucleus before and after expansion. kDNA is measured by scoring the maximum length observed in each cell. The diameter of the nucleus is defined as the widest diameter observed in each cell. The length of the cell structure is determined by measuring their distance from the anterior to the posterior end of each cell. Twenty cells each were analyzed. The expansion factor relative to non-expanded cells is between 4.2 and 4.6.

The expansion factor for images from the p197 double-tagged cell lines (Fig. 5) are calculated based on basal body diameter (tubulin staining) in the expanded cells relative to the previously determined diameter of 250 nm in non-expanded cells (five basal bodies are measured per gel/experiment consisting of three measurements for each basal body (8)). The expansion factor is calculated to be between 4 and 4.7.

**Cell fractionation**

Two-step digitonin extractions were performed to analyze the subcellular localization of proteins. 1x10^7 cells were harvested, washed and resuspended in SoTE buffer (20 mM Tris HCl pH 7.5, 0.6 M sorbitol, 2 mM EDTA). An equal volume of SoTE containing 0.03% (w/v) digitonin was added for selective cell membrane permeabilization. After incubation on ice for 10min the sample was centrifuged for 5 min at 6’800 g. The supernatant (cytosolic fraction, SN1) was stored on ice and the pellet (P1) was then subjected to 1% digitonin extraction in SoTE, left on ice for 15 min and centrifuges at 21’000 g for another 15 min. Equal cell equivalents of the resulting supernatant (soluble organellar proteins, SN2), the pellet (insoluble proteins, P2), SN1 fraction and whole cell fractions (WC) were analyzed using SDS-PAGE. To analyze the solubility of ∆C1383 upon different RNAi inductions (Fig. 4E), only a one step digitonin fractionation (1%) has been done. Equal amount of WC, SN1 and P1 were analyzed using SDS-PAGE.

**Immunoprecipitations**

For immunoprecipitation of the ∆C1383 variant 2x10^8 cells were used to generate a mitochondria-enriched pellet using 0.015% of digitonin. The pellet was then solubilized by incubation in lysis buffer (20 mM Tris HCl pH 7.4, 100 mM NaCl, 0.1 mM EDTA, 10% glycerol) containing 1% digitonin and a protease inhibitor cocktail (Roche Complete, EDTA free). For immunoprecipitation with the ∆N1228 variant 2x10^8 whole cells were directly incubated in lysis buffer containing 1% digitonin (whole cell IP). After 15 min on ice, all samples were centrifuged for 15min at 21’000g. Half of the supernatant was either incubated with anti-c myc beads (Sigma) or with anti-HA beads (Roche) for 1 hour at 4°C. Afterwards, the flow-through was collected, beads were washed 3 times with lysis buffer containing 0.1% digitonin and proteins were eluted by boiling the beads in SDS-PAGE sample buffer lacking β-mercaptoethanol. Fractions of the input, the flow-through and the entire eluted sample were subjected to immunoblot analysis.
RNA extraction and RT PCR

To determine RNAi efficiency of the several cell lines total RNA was extracted using acid guanidinium thiocyanate-phenol-chloroform according to (9) and solubilized in 100% formamide. To validate TAC65 and TAC60 RNAi efficiency a Northern blot was performed using radioactively labeled DNA-probes that cover the whole ORF of TAC65 and TAC60 respectively. Ethidium bromide stain of the rRNAs has been used as a loading control. For p197 3'UTR RNAi efficiency validation total RNA was first treated with DNAse to ensure gDNA free RNA (DNA-free Kit, Ambion). First-Strand cDNA synthesis was performed using oligo(dT)$_{20}$ primers (SuperScript First Strand, Invitrogen). A control reaction without reverse transcriptase (-RT) has been done simultaneously. Same amount of each cDNA reaction (− and + RT) was taken to perform standard PCR using primers (forward: 5'AACTGAAGCCTGGATCCCAATCTGTGAAGAAGGGAG3'; reverse: 5'CCATCTCGAGTAATCCATAGGC3') for the 3'UTR of p197 (nt 1-236 after stop codon) with 28, 29 and 30 cycle rounds. PCR product of the α-tubulin ORF (nt 546-1249) has been used as loading control. Primers were, forward: 5'GGAGCCCTACAACTCTGTGC3'; reverse 5'CACCCTCTTCCATACCCTCA3'. PCR products were separated on a 1% agarose gel and stained with ethidium bromide.

Antibodies

The following non-commercial antibodies were used in the study. The dilution for immunoblots (IB) IF and U-ExM is indicated in parentheses. To produce a polyclonal rabbit antiserum against TAC40 (IB: 1:100, IF 1:100), the entire ORF of the protein containing an N-terminal His-tag, was recombinantly expressed in E. coli, purified via cobalt columns and used to immunize rabbits (Eurogentec). The polyclonal rabbit antiserum against ATOM40 (IB 1:10'000, IF 1:1,000, U-ExM 1:500) has previously been described (10). The polyclonal rabbit anti SAS6 antiserum (IF 1:400) and the monoclonal α-tubulin YL1/2 (IB 1:5'000, IF 1:1000) were kind gifts from Prof. Ziyin Li and Prof. Keith Gull, respectively.

The following commercially available monoclonal antibodies were used in the study: monoclonal mouse anti-myc antibody (Invitrogen, 132500; IB 1:2'000, IF 1:50), monoclonal mouse anti-myc (M4439, Sigma, U-ExM 1:500); monoclonal mouse anti-HA antibody (Enzo Life Sciences AG, CO-MMS-101 R-1000; IB 1:5'000, IF 1:1’000), rabbit anti-HA (H6908, Sigma, U-ExM 1:250), rat anti-HA high affinity (11867423001, Sigma, U-ExM 1:250), guinea pig anti α-tubulin (AA345, Geneva Antibody Facility, U-ExM 1:125), monoclonal mouse anti-EF1a antibody (Merck Millipore, product no. 05–235; WB 1:10'000).

Secondary antibodies for IB analyses were IRDye 680LT goat anti-mouse, IRDye 800CW goat anti-rabbit (LI-COR Biosciences; 1:20'000), and HRP-coupled goat anti-mouse (Sigma-Aldrich; 1:5'000). Secondary antibodies for the IF analysis were goat anti-mouse Alexa Fluor 596, goat anti-rat Alexa Fluor 488, goat anti-rabbit Alexa Fluor 488 (all from ThermoFisher Scientific; IF 1:1’000).

Secondary antibodies for U-ExM were Alexa Fluor 488 goat-anti-rabbit IgG (H+L) (Invitrogen 11038), Alexa Fluor 594 goat-anti-mouse IgG (H+L) (Invitrogen A11005), Alexa Fluor 488 goat-anti-rat IgG (H+L) (Life technologies A11006), anti-guinea pig 647 (Abcam 150187) and Alexa Fluor 647 goat-anti-rabbit IgG (H+L) (Life technologies 1620162). All secondary antibodies are diluted 1:500 except anti-guinea pig is diluted 1:200. DAPI (Sigma D9542, stock 5mg/ml) is diluted 1:1’000.

Miscellaneous

For growth curves cells were counted every day for 6 days with the Beckman Coulter (Z2 coulter Particle count and Size analyzer). All cultures have been diluted to 2x10$^6$ per ml and fresh
tetracycline was added in the induced cultures. Growth curves have all been done in triplicates. Immunoblots- and Northern blots were done as previously described (11).
Fig. S1. C-terminally HA tagged p197 is functional.

(A) Left panel, growth curve of the tetracycline (Tet) inducible p197 3'UTR RNAi cell line. SEM (n=3) are indicated. Inset, RT-PCR products of the 3'UTR of the p197 mRNA in uninduced or 2 days induced cells. Tubulin mRNA serves as loading control. Right panel, DAPI staining of whole cells of the p197 3'UTR RNAi cell line. Nuclei (orange), normal sized kDNAs (green) and overreplicated kDNAs (blue) are indicated with arrowheads. Scale bar 5µm. (B) Growth curve of the p197 3'UTR RNAi cell line expressing an RNAi-resistant in-situ C-terminally HA tagged p197 version schematically depicted at the top. Yellow, 175 aa repeats. Right panel as in (A). (C) Immunoblot of HA-tagged p197 from whole cell extracts separated by 4% SDS PAGE.
Fig. S2. p197 colocalizes with SAS6 throughout the cell cycle.
(A) IF analysis of cytoskeletons isolated from a p197-HA expressing cell stained with DAPI (blue), for the BB and pro-BB marker SAS6 (green) and for HA (red) as indicated. Representative images of the cell cycle stages with kDNA (K) nucleus (N) configurations of 1K1N, dK1N, 2K1N and 2K2N are shown. Scale bar 5µm. (B) Quantification (violin plot) of staining shown above. 436 cells in total from 3 different clones were analyzed for the number of SAS6 and p197-HA positive dots per cell, respectively.
Fig. S3. Quantification of IF analysis in main figure 2B and D.

(A) Percentage of 2 BB (bright YL1/2 spots) containing p197 3’UTR RNAi cells (3 days induced) with two, one or no ΔN1228-HA positive dots are indicated. In total 187 cells from 3 independent inductions analyzed. SEM are indicated. (B) As in (A) but a ΔC334-HA-expressing p197 3’UTR RNAi cell line induced for 0, 1 and 3 days was analyzed. In total 212 (day 0), 175 (day 1) or 228 (day 3) cells, respectively, from 3 independent inductions each were analyzed. SEM are indicated.
Fig. S4. Quantifications and replicates of main figure 4.

(A) Quantification of cell fractionation shown in figure 4A. Percentage of signal intensities in the SN1, SN2 and P2 fractions of the indicated proteins were measured in three independent experiments. SEM are indicated. (B, C and D) Replicates of the immunoprecipitations shown in figure 4B, C and D. (E) Left, quantification of the triplicate fractionations shown in figure 4E. The mean of the ratio between the signals of SN1 and P1 fractions are indicated. An ordinary one-way ANOVA t-test with Bonferroni correction was done (p<0.01: **). SEM are indicated. Right, Northern blots showing ablation of TAC60 and TAC65 mRNA in the corresponding RNAi cell lines, respectively. Ethidium bromide-stained rRNAs serve as loading control.
Fig. S5. Preparation of a cell lines exclusively expressing myc-p197-HA.

(A) Allelic situation of either wildtype cells (WT), cells containing double tagged p197 in the background of one wild-type allele of p197 (myc-p197-HA), or cells containing double tagged p197 in the background of a single knockout of p197 (myc-p197-HA x single KO) is schematically depicted (not to scale). Arrows and numbers indicate PCR primers that have been used for verification of the cell lines. (B and C) Genomic DNA of the three cells lines described in (A) was isolated and used as templates for the PCR reactions. A competition PCR with three primers (indicated with numbers) was used to verify the presence of the tag-containing constructs and the single knock outs of p197 on the genomic level. The presence of the N- and C-terminal tag encoding DNA sequences was verified by two independent PCR reactions. The myc-p197-HA x single KO cell line lacks a PCR product that in size corresponds to the one in WT cells, instead an additional PCR product for hygromycin is detected. In summary this shows successful knockout of the remaining p197 wild type allele.
Fig. S6. Growth of p197 central domain variants.

(A) Growth curve of wildtype cells (blue) and of cells exclusively expressing myc-p197-HA (red), schematically depicted at the top. SEM are indicated. (B) Growth curve of the p197 3’UTR RNAi cell line expressing RNAi resistant tet-inducible myc-cruzi-HA, schematically depicted at the top. SEM are indicated. Inset top, RT-PCR products of the 3’UTR of the p197 mRNA in uninduced or 2 days induced cells. Tubulin mRNA serves as loading control. Inset bottom, immunoblot showing inducible expression of myc-cruzi-HA. (C) Growth curve of the p197 3’UTR RNAi cell line expressing RNAi resistant myc-Δrep-HA, schematically depicted at the top. SEM are indicated. Inset top, RT-PCR products of the 3’UTR of the p197 mRNA in uninduced or 2 days induced cells. Tubulin mRNA serves as loading control. Inset bottom, immunoblot showing inducible expression of myc-Δrep-HA. (D) Immunoblot of whole cell lysates of cells expressing either full length myc-p197-HA, myc-cruzi-HA or myc-Δrep-HA. Same cell equivalents have been separated on a 4% SDS gel.
**Fig. S7.** p197 orthologues have a largely α-helical structure.

(A) Secondary structure of one 175 aa repeat from *T. brucei* p197 has been predicted using the Phyre2 online tool. It has an α-helix content of 96%. (B) Comparison of p197 homologues from the indicated organisms. Calculated molecular weights (with length of the proteins in parentheses), α-helical content and length of the longest essentially uninterrupted α-helical stretch are indicated.
Fig. S8. The N- and C-termini of p197 are more conserved than the rest of the protein.

Full length p197 orthologous or indicated parts of them from *T. brucei*, *Trypanosoma cruzi*, *Leishmania major* and *Paratrypanosoma confusum* were pairwise compared using BLAST. Percentage of identity is indicated.
Fig. S9. Schematic representations of p197 versions

p197 variants used in this study are shown schematically. Yellow highlights the repeat region. The number of repeat domains (yellow) are taken from TriTrypDB. However, as discussed in the paper the actual number of repeat domains (yellow) is much higher. To reflect this the corresponding aa numbers have been put in parentheses. Green indicate the central α-helical region of the T. cruzi p197. Constructs complementing TAC function are indicated. All p197 variants localized to the TAC region.
Table S1

| RNAi target | in situ tagging | inducible tagging | additional info | figures |
|-------------|-----------------|-------------------|-----------------|---------|
| p197 3'UTR | p197-HA         |                   |                 | S1A     |
| p197 3'UTR | ΔN1228-HA       |                   |                 | 2AB, S3A|
| p197 3'UTR | ΔC334-HA        |                   |                 | 2CD, S3B|
| p197 3'UTR | ΔC1383-HA       |                   |                 | 3AB, 4AE, S4AE|
| TAC40 ORF  | ΔC1383-HA       |                   |                 | 3C      |
| TAC40 ORF  | p197-HA         |                   |                 | 3D      |
| TAC60 ORF  | ΔC1383-HA       |                   |                 | 4B, S4B |
| TAC65 ORF  | ΔC1383-HA       |                   |                 | 4C, S4C |
| TAC60 ORF  | HA-ΔN1228, TAC65-myc |   |                 | 4AD, S4AD|
| p197 3'UTR | myc-p197-HA     |                   | single knockout background | 5ABCDE, S5ABC, S6AD|
| p197 3'UTR | myc-Δrep-HA     |                   |                 | 5ABCDE, 6BD|
| p197 3'UTR | myc-cruzi-HA    |                   |                 | 5ABCDE, 6CD|

Table S1: Cell lines used in this study
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