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The FIP-1 like polyadenylation factor in trypanosomes and the structural basis for its interaction with CPSF30

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ABSTRACT

In trypanosomes transcription is polycistronic and individual mRNAs are generated by a *trans*-splicing/polyadenylation coupled reaction. We identified a divergent trypanosome FIP1-like, a factor required for mRNA 3′ end formation from yeasts to human. Here we showed that it is a nuclear protein with a speckled distribution essential for trypanosome viability. A strong interaction was found between TcFIP1-like and TcCPSF30, a component of the polyadenylation complex. We determined the specific amino acids in each protein involved in the interaction. Significant differences were found between the trypanosome interaction surface and its human counterpart. Although CPSF30/FIP1 interaction is known in other organisms, this is the first report mapping the interaction surface at the amino acid level.

Introduction

Nuclear pre-mRNA 3′ end formation involves a two-step reaction in eukaryotes: an endonucleolytic cleavage at the poly(A) site, followed by the polyadenylation of the newly generated 3′ end. This reaction requires *cis*-acting signal sequences on the pre-mRNA and *trans*-acting protein components [1].

mRNA maturation in trypanosomes, a group of unicellular eukaryotic parasites causative agents of Chagas disease and Sleeping sickness in humans, clearly deviates from the same process in most eukaryotes primarily because protein-coding genes are transcribed into polycistronic RNAs separated by relatively short intergenic regions (IRs).

Precursor RNAs are processed into individual mRNAs by two coupled reactions, the addition of a 39-nucleotide capped RNA (spliced leader, SL), through a *trans*-splicing event [2,3], followed by cleavage/polyadenylation. Both reactions are governed by a common polypyrimidine rich sequence present in the IRs [4]. As a consequence, there is no recognizable polyadenylation signal, such as the AAUAAA sequence of mammalian pre-mRNAs [6]. Another outcome of this arrangement is that regulation of gene expression is not controlled by promoter activity but by mRNA processing and stability [5]. Little is known about the protein complex involved in the coupled process but it emerges as an interesting anti-parasitic drug target since the process diverges from its mammalian host [7–9].

In mammals, the *trans*-acting protein complexes required for the cleavage step are, the cleavage/polyadenylation specificity factor (CPSF), the cleavage-stimulatory factor (CstF), the cleavage factors Im and IIm (CF Im and CF IIm) and the RNA polymerase II (pol II). The poly(A) polymerase (PAP) and the poly(A)-binding protein II (PABP II) are involved in the polyadenylation reaction [10].

Previous *in silico* searches in the trypanosome genomes identified the members of CPSF complex and a few members of the CF but those of the CstF seemed to be absent [8].

In this report, we identified a new member of the trypanosome polyadenylation complex: a divergent FIP1-like factor.

The FIP1 protein (Factor Interacting with PAP) was first identified in yeast and it was shown to have a crucial regulatory function in the polyadenylation reaction by controlling the activity of poly(A) tail synthesis through multiple interactions with the CPSF30 ortholog (Yth1) and PAP within the polyadenylation complex [11,12]. The same interactions were reported for the identified human FIP1 [13]. The sequence of FIP1 orthologues from different eukaryotes is not conserved except for a short domain of 57 amino acids that is a signature of this protein [13].

Here, we showed that the identified FIP1-like interacts with the trypanosome CPSF30 subunit and is essential for cell viability. In addition, we analyzed in detail the contact-binding surface of the

Abbreviations: Y2H, yeast two-hybrid; ONPG, ortho-nitrophenyl-β-galactoside; SOEing PCR, splice overlap extension PCR; CPSF, cleavage and polyadenylation specificity factor; FIP, factor interacting with PAP.

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trypanosomatid CPSF30/FIP1 in comparison with its human counterpart showing significant differences that could lead to the postulation of this interacting pair as a putative drug target. Our results also described the first contact surface of a CPSF30/FIP1 at the amino acid level.

Materials and methods

Data mining. Human polyadenylation factors were used as query sequences to screen the *Trypanosoma cruzi* and *Trypanosoma brucei* genome databases using TBLASTN (http://www.genedb.org/genedb/tcruzi/blast.jsp). Motifs and domains were searched in SMART (http://smart.embl-heidelberg.de/) databases.

Trypanosoma brucei growth, transfection and RNAi experiments. T. brucei procyclic cells (strain 1313) were grown at 27 °C in MEMpros media supplemented with 10% fetal bovine serum, and the appropriate antibiotics. Transfection and RNA knock-down analysis of *T. brucei* FIP1-like was done as previously described [14]. Total RNA samples from the induced (or uninduced) RNA knocked down cells was isolated by using the peqGold Trifast reagent (peqLab, Germany) according to the manufacturer's instructions and analyzed by Northern blot.

Cloning of human and T. cruzi coding sequences into the gateway system. Both TcCPSF30 and TcFIP1-like sequences were PCR amplified from 100 ng of total genomic DNA of *T. cruzi* CL-B clone using specific primers. *T. brucei* FIP1-like fragment for the RNAi experiment was PCR amplified from 100 ng of total genomic DNA of *T. brucei* 1313 strain using proper primers. The human orthologues were PCR amplify from human lymphocytes cDNA and cloned using specific primers (Supplementary file S1). All sequences were cloned into the gateway system as described [15].

Yeast two-hybrid assays and mutational analyses of CPSF30 and FIP1-like. All interaction experiments were performed exactly as previously described in [15] measuring the activation of two independent reporter genes (*His3* and *LacZ*). All quantitative ONPG assays were done as indicated in the Proquest™ kit manual (Invitrogen) and they are the result of two independent experiments performed in duplicate. Variants were generated by SOEing PCR as described previously [16] using specific primers. Alanine scan mutations were also generated by SOEing PCR (Supplementary file S1). All amplifications were performed using the proofreading AccuTaq enzyme (Sigma–Aldrich) and sequenced.

Production of polyclonal antibodies against TcFIP1-like and indirect immunoflorescence assay (IFA) on T. cruzi cells. T. cruzi epimastigotes were grown as described previously [17]. Recombinant FIP1-like His tagged protein was produced in *Escherichia* coli and purified using Ni-NTA column following the manufacturer's instruction (QIAGEN). Antibodies were produced in mice as previously described [18]. The IFA was done as previously described [9] with the following modifications: nuclear staining was done using propidium iodide (PI) and confocal images obtained in an Olympus FV300 microscope.

Results and discussion

Trypanosome FIP1-like diverged from the rest of its eukaryotic orthologues

By data mining within the trypanosome genome database, we found a previously unidentified subunit of the CPSF complex, a FIP1-like factor (Fig. 1A and Supplemental Fig. S1).

The FIP1 protein in *Saccharomyces cerevisiae* and human, shares a similar domain organization that consists of an acidic N-terminal region and a highly conserved central region followed by a Proline-rich domain (Fig. 1A). However, trypanosome FIP1-like shows a different architecture and it only conserves the fip1 domain region showing a 48% of identity with the human domain (Fig. 1B and Supplemental Fig. S1). In contrast with other FIP1 proteins, trypanosome FIP1-like is shorter (280 amino acids in *T. brucei* and 287 in *T. cruzi*) and it is the only FIP1 ortholog containing a CCCH zinc finger motif followed by a Q-rich stretch in the C-terminal region (Fig. 1A).

Trypanosome CPSF30 presents a similar domain organization compared with other organisms

The CPSF30 present in all eukaryotes is a poly-zinc finger protein with a highly conserved architecture from yeast to human. It contains five CCCH-type zinc finger motifs in *S. cerevisiae*, and it accommodates additional zinc finger motifs of the CCHC-type in human (one motif) and in *Drosophila melanogaster* (two motifs).

Trypanosome CPSF30, a 271 (*T. cruzi*) or 277 (*T. brucei*) amino acids protein, presents a high degree of similarity and the same domain organization compared with their orthologues (Supplemental Fig. S1). It has five CCCH-type zinc fingers and it also includes two CCHC-type zinc fingers in its C-terminal. The *T. brucei* protein was previously characterized as a *bona-fide* CPSF30 based on functional analyses [8].

FIP1-like is a nuclear protein essential for the parasite viability

Immunoflorescence assays using a specific polyclonal serum showed FIP1-like localized exclusively in the nucleus of the trypanosome cells with a speckled distribution pattern (Fig. 2A), compartmentalization that is consistent with its predicted function.

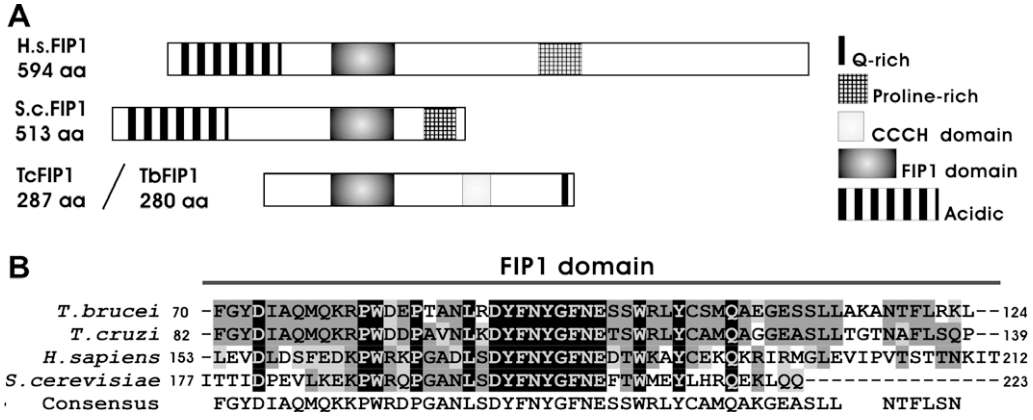

Fig. 1. Domain organization of trypanosome FIP1-like protein. (A) Schematic representation of FIP1 from human (H.s.FIP1.) and yeast (S.c.FIP1) compared with trypanosomes (Tc/TbFIP1). (B) Sequence alignment of the human and yeast fip1 domains compared with that of trypanosomes. Black box indicates 100% identical residues; white box corresponds to non-similar residues, light gray box indicates conservative changes; dark gray box shows blocks of similarity.

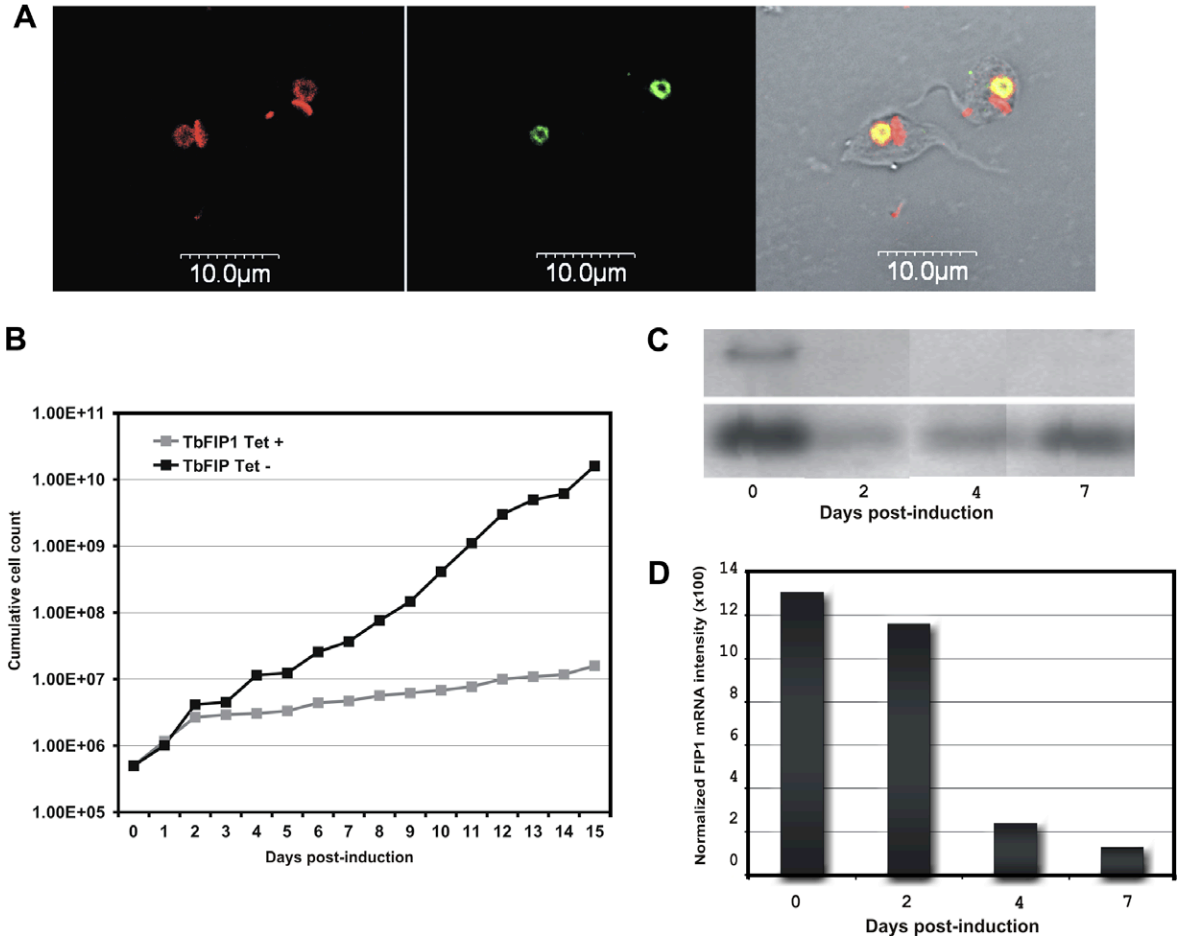

Fig. 2. Depletion of FIP1-like, a nuclear protein, caused growth defects on trypanosomes. (A) Immunolocalization of *Trypanosoma cruzi* FIP1-like. Left panel, DNA staining with propidium iodide (PI). Central panel, anti-FIP1 polyclonal serum incubated with a FITC conjugated α-mouse antibody. Right panel, merged image with DIC (Differential Interface Contrast). The oval shaped structure in PI staining corresponds to the kinetoplast DNA (the mitochondria of trypanosomes). Images were obtained using confocal microscopy. (B) Cumulative growth of *T. brucei* TbFIP1 knock-down cell line, measured during 15 days after tetracycline induction (gray) compared to uninduced cells (black). (C) Northern blot from the *T. brucei* TbFIP1-like knock-down cell line RNA extractions, measured 0, 2, 4 and 7 days post-induction, probed with FIP1 (upper panel) and with SRP as loading control (lower panel). (D) Intensity quantification of the northern blot bands showed in (C) normalized to the background.

RNA knock-down of its mRNA by RNA interference in *T. brucei* showed that a strong growth arrest occurred after day four compared to the uninduced control (Fig. 2B). The growth defects correlated with the decrease in the levels of FIP1-like mRNA in the RNAi induced parasites as shown by northern blots (Fig. 2C and D), indicating that the protein is essential for cell viability

In agreement, similar results were reported in *T. brucei* cells depleted of the CPSF30 protein [8].

Trypanosomes CPSF30/FIP1 are engaged in strong protein–protein interaction

Interactions between FIP1-like and CPSF30 were evaluated by a colorimetric quantitative Yeast-Two Hybrid (Y2H) assay. Our results showed a positive and strong interaction for the trypanosome pair (Fig. 3A). The human interaction was used as control. Interestingly, we also detected the hybrid interactions between human and *trypanosome* proteins in both configurations. However, interactions with the human proteins produced only half of the activation of LacZ reporter in the ONPG assay respect to the trypanosome pair (Fig. 3A).

Deletion analyses of the FIP1-like protein tracked down the minimum region of interaction to the fip1 domain since a deletion of nine amino acids from the core of fip1 domain completely abolished the binding to CPSF30 and a deletion of the C-terminal region (Δ140–287) had no effect (Fig. 3B).

Deletion analyses of the CPSF30 protein tracked down the minimum region of interaction to a 54 amino acids sequence that included zinc fingers (ZnF) 4 and 5 (Fig. 3C). Since deletion of ZnF-5 reduced the interaction by 60% of the original ONPG value and deletion of ZnF-4 abolished it, we concluded that ZnF-5 is probably modulating the interaction strength. Thus, we postulate that the complete information needed for binding and modulation is concentrated in the 54 amino acids region.

Mapping of the critical amino acids involved in the contact surface between T. cruzi CPSF30/FIP1

The specific amino acids responsible for CPSF30/FIP1 interaction are unknown although the interaction was reported in yeasts, humans and plants [13,19,20]. Thus, we performed an exhaustive Alanine scan analyses over the minimum contact regions.

Alanine substitutions were done for 11 selected positions within the fip1 domain. Substitutions were chosen based on the strong conservation in the alignment of Fig. 1B. We mutated every residue from the perfect conserved core $_{102}$DYFNYGFNE$_{112}$ plus residues R_{91} and W_{114} (Fig. 4A). To our surprise, the majority of amino acids from the core have a mild impact on the interaction (from none to 60%),

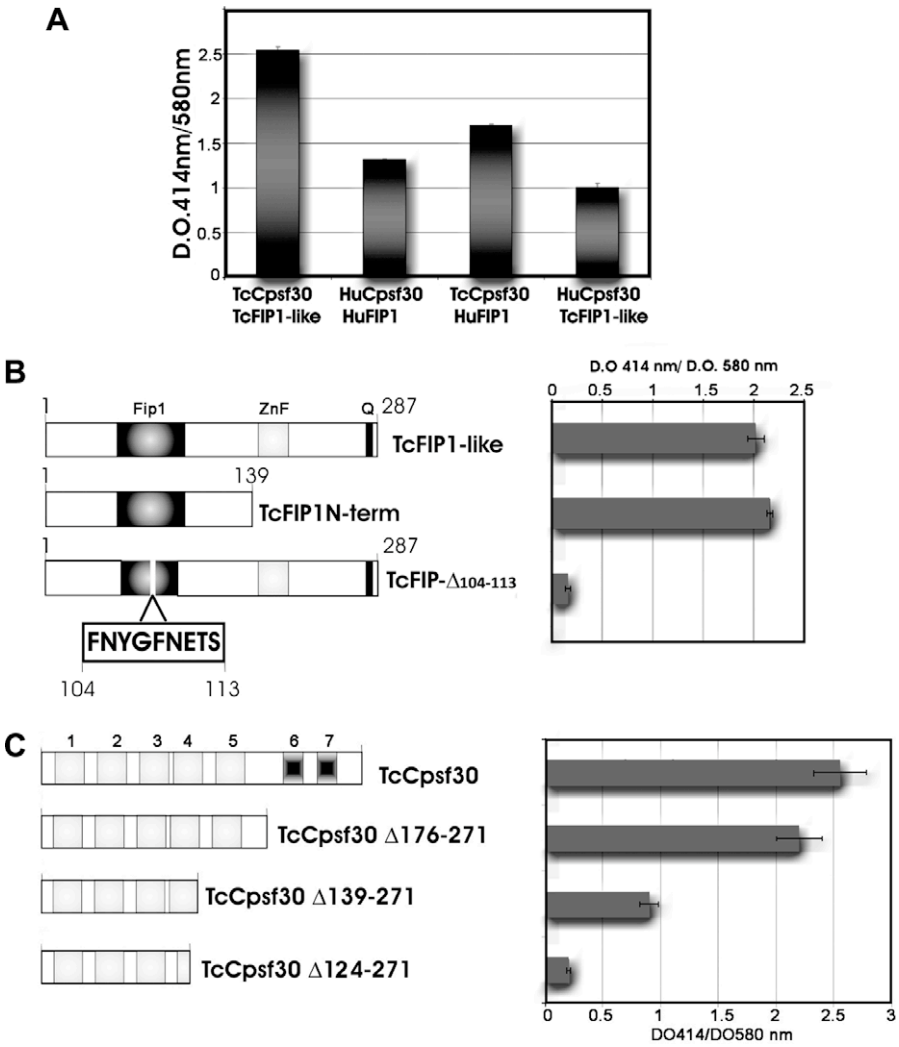

Fig. 3. Mapping the minimal interaction region between TcCPSF30 and TcFIP1-like. (A) Yeast two-hybrid interactions between *T. cruzi* proteins (TcCPSF30/TcFIP1-like), Human proteins (HuCPSF30/HuFIP1) and hybrid interactions (TcCPSF30/HFIP1 and HuCPSF30/TcFIP1-like), were tested by activation of LacZ reporter gene in liquid ONPG assays (see Materials and methods). (B) Left, deletion constructs of *T. cruzi* FIP1-like, N-term, first 139 residues from the N-terminal region of the protein; Δ104–113, deletion of 9 amino acids from the fip1 domain. Right, Y2H ONPG assay against DB-TcCPSF30. (C) Left, schematic representation of four different constructions of the *T. cruzi* CPSF30 protein, with sequential C-Terminal deletions. From 1 to 5, CCCH zinc fingers; 6–7, CCHC zinc fingers. Right, Y2H ONPG assay against AD-TcFIP1-like.

with the exception of E_{111} that abolished the interaction similar to the conserved residue W_{114} (Fig. 4A). More dramatic effects were seen when the scanned residues were assayed against the human CPSF30 (Supplemental Fig. S2A), where 7 out of the 9 mutated residues from the fip1 core almost abolished interaction. We established a comparative interaction rate between trypanosome/trypanosome and trypanosome/human protein interactions that allowed us to visualize the effects easily (Supplemental Figs. S3A and S3C). From this analysis, it is clear that replacements in the fip1 core have a more dramatic effect when FIP1-like is in contact with the human protein surface than the trypanosome surface. This is an interesting result since the scanned amino acids are 100% conserved compared with the human protein (Figs. 1B and 4A).

Alanine substitutions were also done for the minimum contact region in the trypanosome CPSF30. We performed 16 consecutive and independent mutations every three amino acids, replacing each triad by alanines in a sequential manner from residue 124 to 177 (Fig. 4B). Then, we analyzed the interactions against trypanosome FIP1-like. As shown in Fig. 4B, mutated positions GFC (124–126), CHL (132–134), RHV (135–137) corresponding to ZnF-4 abolished interaction while SCP (141–143), FYMA (144–147), GFC (148–150) and HPV (160–162) corresponding to ZnF-5 also prevented the interaction significantly. Other substitutions such as QEL (163–165) and YNR (166–168) in ZnF-5 have a mild effect. Interestingly, some substitutions such as NSV (169–171) have the opposite effect, increasing the interaction by 40% (Fig. 4B).

When the same set of substitutions was confronted with human FIP1 (Supplemental Fig. S2B), we found several significant differences. We used the comparative interaction rate to visualize them easily (Supplemental Figs. S3B and S3C). Mutations such as GFC (124–126), CHL (132–134), SCP (141–143) and GFC (148–150) have a more dramatic effect on the trypanosome interacting pair. In contrast, mutations such as PLG (151–153), PKC (154–156) or LRQ (175–177) almost abolished interaction with the human FIP1 (Supplemental Figs. S2B and S3B) while they have no effect on the trypanosome FIP1-like (Fig. 4B). Interestingly, two of the three residues were identical in these triads when compared to the human CPSF30 sequence (data not shown).

Taken the FIP1-like and CPSF30 mutation datasets together, they reveal that, while trypanosomes and humans share a common surface of interaction with conserved residues (Fig. 1B and Supplemental Fig. S1), the contribution of several of these conserved ami-

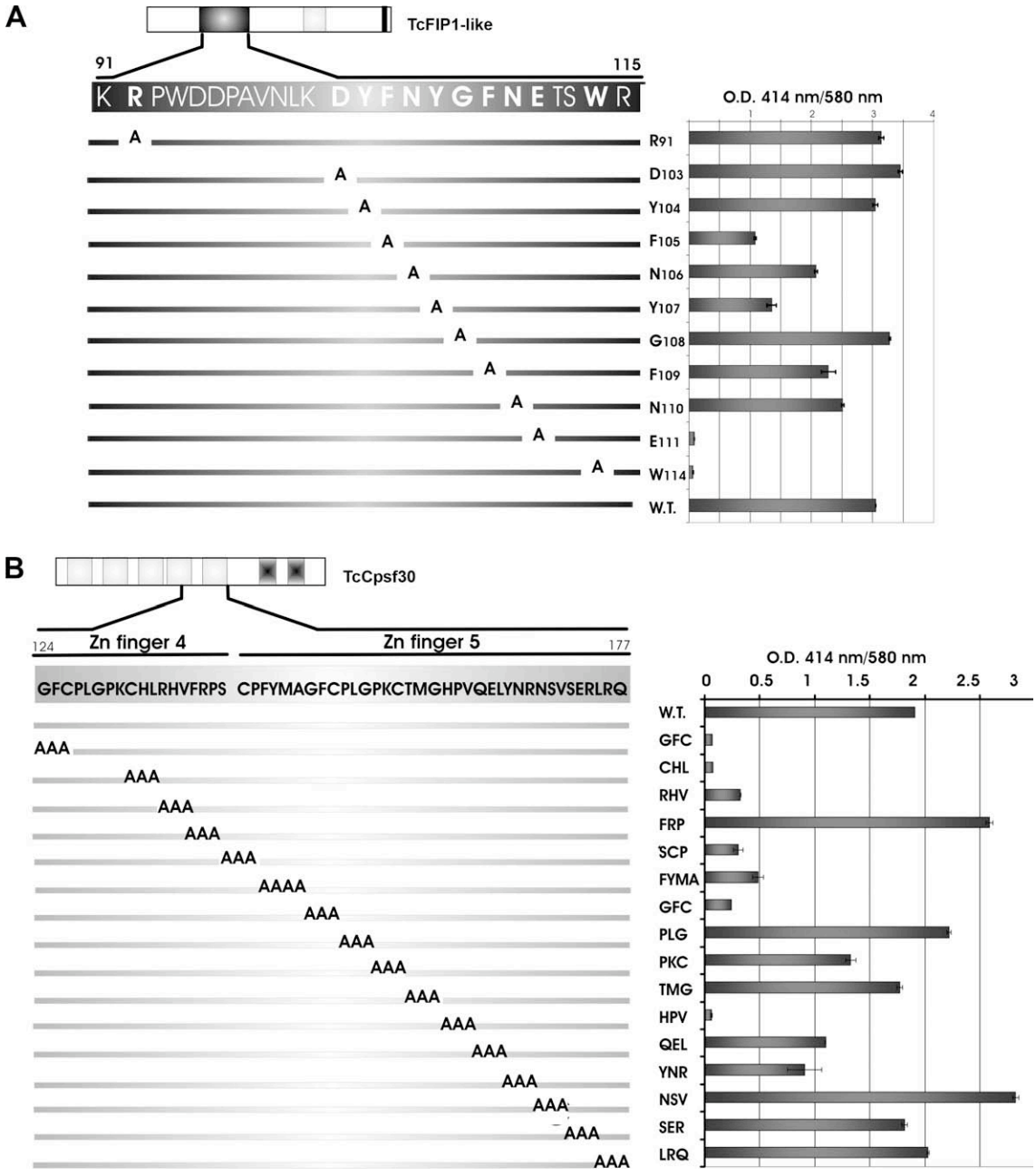

Fig. 4. Alanine scan analysis of the TcFIP1-like contact region. (A) Left, schematic representation of the 11 mutation constructions carried out on TcFIP1-like, encompassing the critical region for the interaction with TcCPSF30 protein. Right, Y2H ONPG assay for each AD-TcFIP1-like construct against DB-TcCPSF30. W.T., wild-type TcFIP1-like. (B) Left, schematic representation of the 16 mutation constructions carried out on TcCPSF30, encompassing the contact region with TcFIP1-like protein. Five light boxes, CCCH zinc fingers; two dark boxes, CCHC zinc fingers. Right, Y2H ONPG assay for each DB-TcCPSF30 construct against AD-TcFip1-like. W.T., wild-type TcCPSF30.

no acids to the contact interface seems to be quite different between them.

Our analysis also provides the basis to postulate the FIP1-like/CPSF30 interaction pair as a putative drug target against these human parasitic diseases. Since both proteins were shown to be essential for parasite viability (this work and [8]) and CPSF30 was also shown to block polycistronic mRNA processing leading to parasites death [8], then this putative drug target could be taken into a serious consideration for drug screenings.

Acknowledgments

The authors thank Prof. Dr. Christine Clayton for providing *T. brucei* parasite strains, plasmids and expertise for RNA interference assays. This work was supported by grants from FONCYT – PICT REDES 2003-00300, UBACyT X-153 (University of Buenos Aires) and PIP-CONICET 5492 to MV. MV is a member of the career of scientific investigator of CONICET, Argentina.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.01.182.

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	Orthologue accession number (geneDB)		length (aa)		% identity compared to human	
Protein	T.cruzi	T.brucei	T.cruzi	T.brucei	T.cruzi	T.brucei
Cpsf30	Tc00.1047053510219.30		271	277	47	33
Cpsf73	Tc00.1047053508693.10	Tb927.4.1340	625	770	45	48
Cpsf100	Tc00.1047053504109.110		802	818	23	26
Cpsf160	Tc00.1047053506871.140	Tb11.01.6170	1436	1452	26	25
PAP1	Tc00.1047053508153.440	Tb927.3.3160	485	487	29	27
	Tc00.1047053506795.50	Tb927.7.3780	645	687	35	34
PABP	Tc00.1047053506885.70	Tb09.211.0930	570	566	45	37
Cstf50	Tc00.1047053511365.10	Tb10.61.0570	522	517	26	24
Cstf64	n.i.	n.i.	-	-	-	-
Cstf77	n.i.	n.i.	-	-	-	-
CF1-25	Tc00.1047053509509.40	Tb927.7.1620	292	300	34	31
FIP1	Tc00.1047053510351.80	Tb927.5.4320	287	280	48*	51*

*** restricted to fip1 domain**

Figure S2

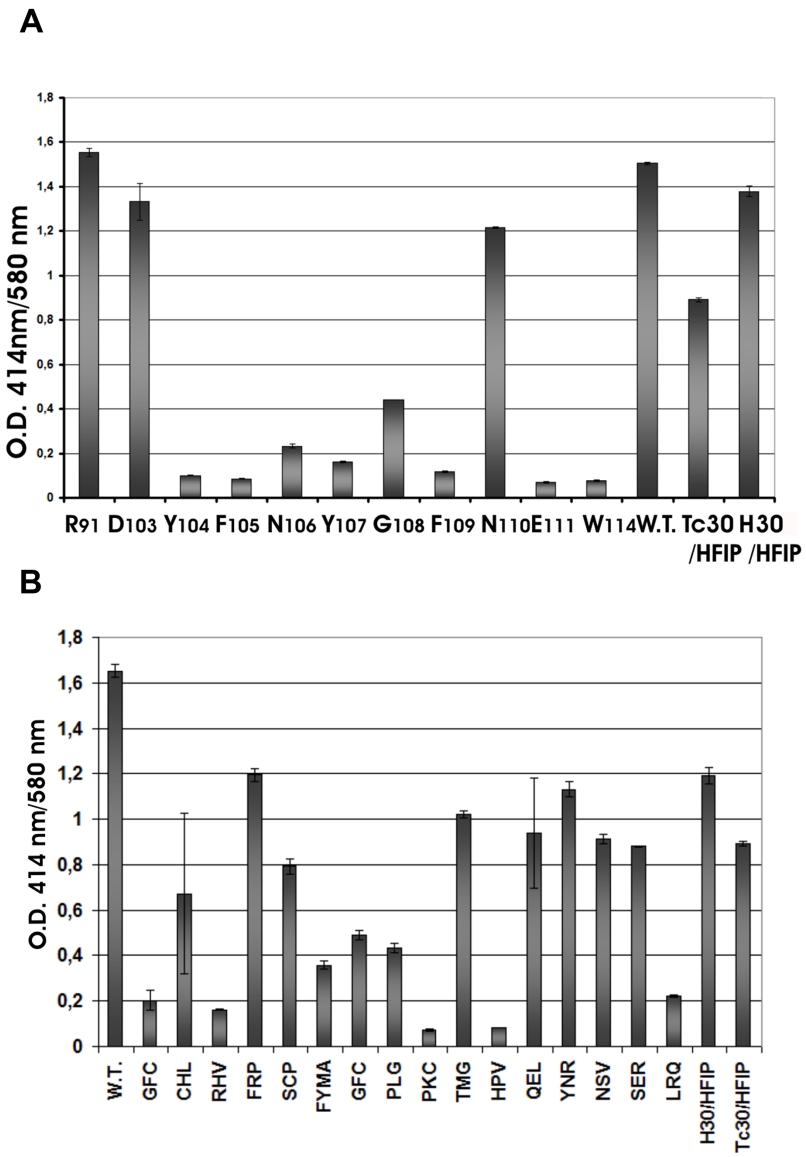

Figure S2. **A)** Y2H ONPG assay as in Figure 4A but performed against AD-human CPSF30. H30, human CPSF30; HFIP, human FIP1; Tc30, *T. cruzi* CPSF30. **B)** Y2H ONPG assay as in Figure 4B but performed against AD-human FIP1. H30, human CPSF30; HFIP, human FIP1; Tc30, *T. cruzi* CPSF30.

Figure S3

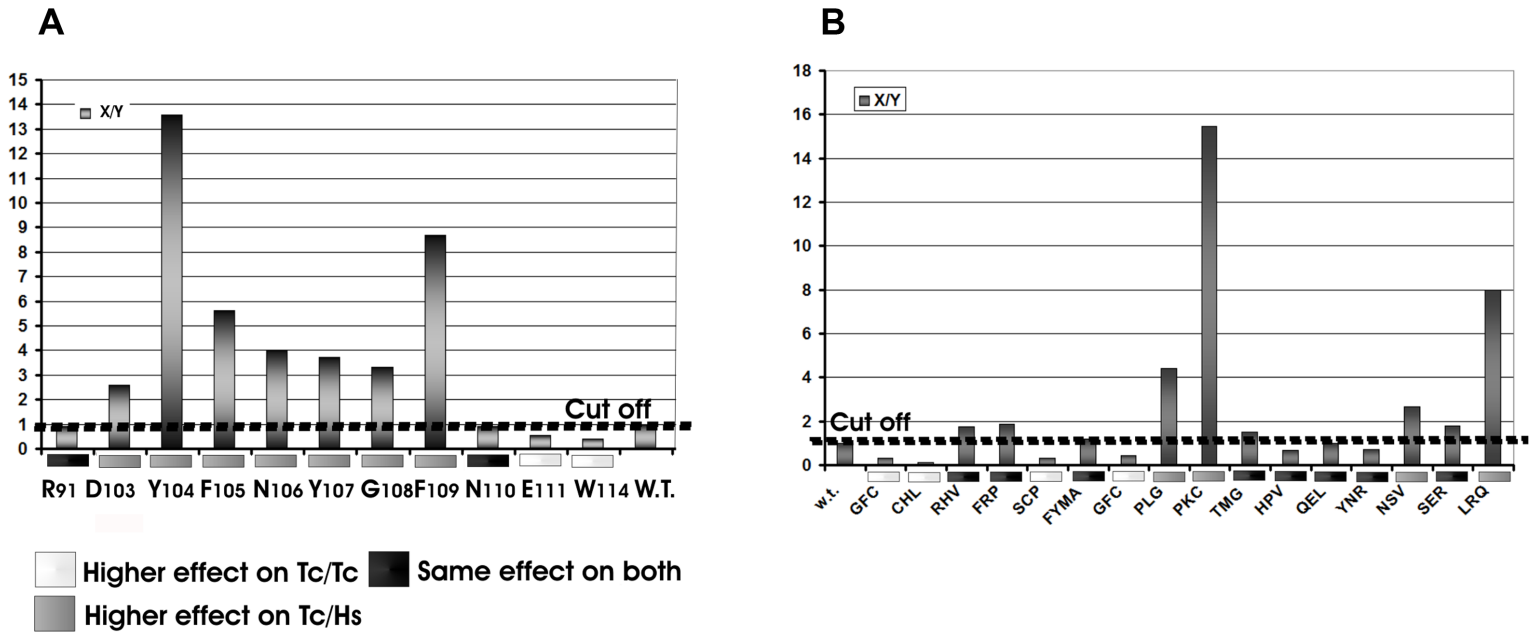

Figure S3. **A)** Comparative interaction rate between the trypanosome (Tc/Tc; TcCFIP1$_{scan}$ vs. TcCPSF30) and the hybrid interactions (Tc/Hs; TcFIP-like$_{scan}$ vs HuCPSF30). Grey scale below the graph shows the different mutation effects on the interactions. **B)** Comparative interaction rate between the trypanosome (Tc/Tc; TcCPSF30$_{scan}$ vs. TcFIP1-like) and the hybrid interactions (Tc/Hs; TcCPSF30$_{scan}$ vs HuFIP1). Grey scale below the graph shows the different mutation effects on the interactions. See comparative rate details below

Fig. S3 C. Comparative Rate

The rate (R) consists of two relative values which in turn, includes the ONPG value of each interaction normalized to the wild type. The first value (X) refers to the trypanosomal interaction value, and the second (Y) to the hybrid interaction value. The rate is calculated as follows:

X=(TcFIP1/ mutN_Tc30) / (TcFIP1/Tc30 wt);

Y= (HsFIP1/ mutN_Tc30) / (HsFIP1/Tc30 wt)

Where mutN represents every mutation construct

And then:

R= X/Y

This rate is an index of every mutation effect.

If X=Y, then the mutation did not affect the interaction. In contrast, if X or Y < 1 The mutation reduced the interaction.

In sum, if

R = 1, the mutation exerts the same effect on TcFIP/Tc30 and HsFIP/Tc30

R < 1, the mutation exerts a larger effect on TcFIP/Tc30 than HsFIP/Tc30

R > 1, the mutation exerts a lower effect on TcFIP/Tc30 than HsFIP/Tc30

Finally, we established an average line of cut off to compare the effects.