The unique Leishmania EIF4E4 N-terminus is a target for multiple phosphorylation events and participates in critical interactions required for translation initiation

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The eukaryotic initiation factor 4E (eIF4E) recognizes the mRNA cap structure and, together with eIF4G and eIF4A, form the eIF4F complex that regulates translation initiation in eukaryotes. In trypanosomatids, 2 eIF4E homologues (EIF4E3 and EIF4E4) have been shown to be part of eIF4F-like complexes with presumed roles in translation initiation. Both proteins possess unique N-terminal extensions, which can be targeted for phosphorylation. Here, we provide novel insights on the *Leishmania infantum* EIF4E4 function and regulation. We show that EIF4E4 is constitutively expressed throughout the parasite development but is preferentially phosphorylated in exponentially grown promastigote and amastigote life stages, hence correlating with high levels of translation. Phosphorylation targets multiple serine-proline or threonine-proline residues within the N-terminal extension of EIF4E4 but does not require binding to the EIF4E4's partner, EIF4G3, or to the cap structure. We also report that EIF4E4 interacts with PABP1 through 3 conserved boxes at the EIF4E4 N-terminus and that this interaction is a prerequisite for efficient EIF4E4 phosphorylation. EIF4E4 is essential for *Leishmania* growth and an *EIF4E4* null mutant was only obtained in the presence of an ectopically provided wild type gene. Complementation for the loss of *EIF4E4* with several EIF4E4 mutant proteins affecting either phosphorylation or binding to mRNA or to EIF4E4 protein partners revealed that, in contrast to other eukaryotes, only the EIF4E4-PABP1 interaction but neither the binding to EIF4G3 nor phosphorylation is essential for translation. These studies also demonstrated that the lack of both EIF4E4 phosphorylation and EIF4G3 binding leads to a non-functional protein. Altogether, these findings further highlight the unique features of the translation initiation process in trypanosomatid protozoa.

Introduction

Leishmania and Trypanosoma species are flagellated protozoa belonging to the family Trypanosomatidae, order Kinetoplastida, that are responsible for a large spectrum of diseases of worldwide impact.[1] These organisms are also characterized by unique features associated with their gene expression and regulation, such as the constitutive transcription of their genes as part of long polycistronic precursors that are further processed into monocistronic messages through *trans*-splicing and polyadenylation.[2,3] During *trans*-splicing, a conserved ~40-nt long spliced-leader (SL) sequence, capped by a novel cap4 structure (7-methyl-GTP followed by the first 4 nucleotides of the SL sequence modified

through methylation), is added to the 5′ end of all mature mRNAs.[4-6] This unusual mode of regulation precludes control at the level of transcription initiation and gene expression is regulated by posttranscriptional mechanisms at the level of mRNA stability and translation.[7-10]

Most eukaryotic mRNAs are defined at their 5′ and 3′ ends by the presence of the cap nucleotide and the poly-A tail, respectively. These structures are involved in different aspects of mRNA metabolism and both are required for efficient mRNA recognition by the translation machinery. Specific RNA-binding proteins, the translation initiation factor eIF4E (the cap binding protein) and the poly-A binding protein (PABP), are responsible for recognizing these structures and mediating their function in translation and

other processes.[11,12] eIF4E is part of the heterotrimeric translation initiation complex eIF4F, which also includes the RNA helicase eIF4A and the large scaffolding protein eIF4G. Interactions of eIF4F, through its eIF4G subunit, with a second initiation complex, eIF3, and PABP facilitate the recruitment of the 40S ribosomal subunit to the mRNAs and the initiation of translation.[13-16]

eIF4E is a small conserved cytoplasmic protein, which folds into a unique tridimensional structure found only in eukaryotic cells. Binding to the cap nucleotide is mediated by 2 conserved tryptophan residues localized within a slot positioned on the protein's convex side. In contrast, interactions with binding partners, such as eIF4G and eIF4E-binding proteins (eIF4E-BPs), involve yet another conserved tryptophan residue mapped to eIF4E's dorsal side.[17,18] In metazoans, regulation of eIF4E activity seems to be mediated by phosphorylation, which targets a conserved serine residue (S209) localized to its C-terminus. This phosphorylation decreases the affinity of eIF4E for the cap and is mediated by a MAP-kinase related kinase, named Mnk. eIF4E phosphorylation only occurs when it is bound to its eIF4G partner, responsible for recruiting the Mnk kinase through a direct interaction mediated by its C-terminal W2 domain. The role of S209 phosphorylation is still debatable with different lines of evidence alternatively suggesting stimulatory or inhibitory roles in eIF4E function during translation.[19-21] A better-defined role for phosphorylation in regulating eIF4E function involves the eIF4E-BP proteins in mammals, which are also targeted by phosphorylation linked to the mTOR cascade of protein kinases. eIF4E-BPs only bind to eIF4E in the absence of phosphorylation, hence preventing the eIF4F complex formation and inhibiting translation.[22,23]

In trypanosomatids, 6 distinct eIF4E homologues have been described (EIF4E1 to 6), all conserved in the various species and all sharing a number of conserved aromatic and other residues, which generally define the true eIF4E homologues.[24,25] EIF4E3 and EIF4E4 share the ability to participate in eIF4F-like complexes with EIF4G4 and EIF4G3, respectively, and both have been implicated in protein synthesis. These are strictly cytoplasmic proteins containing long N-terminal extensions displaying limited homology and which are not seen in other eIF4E homologues.[26] Several lines of evidence attribute EIF4E4 major functions in translation initiation in both *Leishmania* and *Trypanosoma brucei*. These include the ability to bind the trypanosomatid cap4, its association to polysomes and the specific binding to EIF4G3, the most likely eIF4G homologue involved in translation.[26-31] Nevertheless, EIF4E4 has been proposed to be a stage-specific translation factor being expressed only in the promastigote stage of *Leishmania amazonensis* and degraded during amastigote differentiation.[29] Our previous study showed that the two sets of eIF4E and eIF4G subunits from the EIF4E3/EIF4G4 and EIF4E4/EIF4G3 complexes are targeted for phosphorylation in both *L. amazonensis* and *T. brucei*, with the 2 eIF4E homologues being subjected to multiple phosphorylation events. For both organisms, EIF4E4 phosphorylation was consistently found to be associated with exponentially growing cells while phosphorylation of EIF4E3, in *L. amazonensis* only, was associated with stationary phase cells.[32] Phosphorylation of EIF4E3 was independently seen

to be associated with nutritional stress but this had no impact on its binding to the cap structure in an *in vitro* assay.[30]

Here, we provide a detailed characterization of the phosphorylation events within EIF4E4 through mutational analysis, binding assays and complementation studies. Multiple phosphorylation sites were identified within the unique N-terminal region of the *Leishmania* EIF4E4 but these were not essential for parasite viability and function. We also show that the unique interaction between EIF4E4 and PABP1, described previously,[29] maps to 3 conserved boxes of 10 amino acids each within the EIF4E4's N-terminus and not only is required for efficient phosphorylation but is also critical for EIF4E4 function.

Results

EIF4E4 is constitutively expressed but differentially phosphorylated during the 2 major life stages of *Leishmania infantum*

To study EIF4E4 function in a *Leishmania* species more readily capable of differentiating in culture and reproducing the major stages of the parasite's life cycle, *L. infantum* was chosen since standard protocols are available for its *in vitro* differentiation into amastigote-like forms, which resemble intracellular amastigotes in many aspects.[33-35] To evaluate EIF4E4 expression in *L. infantum* promastigotes and to compare with previously described results from *L. amazonensis* and *T. brucei*, Western blot analysis on protein lysates from parasites selected at different phases of growth was carried out using specific antibodies recognizing EIF4E4. As shown in **Figure 1A**, at the start of the culture (0 h time point) EIF4E4 is detected mostly as a single band, which rapidly (2 hours) shifted to isoforms of a higher molecular weight. These larger isoforms are predominant during the exponential growth phase (up to 2 days) but disappear when the culture reaches stationary phase where only the faster migrating isoforms are seen again. This pattern of high molecular weight isoforms of EIF4E4 observed in exponentially growing cells is equivalent to those previously seen in *L. amazonensis* and *T. brucei*. In both species, EIF4E4 was found as a phosphoprotein which specifically bound to a phosphoprotein purification column and on a 2-dimensional gel analysis it migrated as multiple spots with differences in size and isoelectric points compatible with phosphorylation events.[32] Treatment with alkaline phosphatase to determine that this post-translational modification was indeed phosphorylation was also attempted with the *Leishmania* orthologue, but EIF4E4 was seen to be very sensitive to degradation, even under very mild conditions and during short periods of time (data not shown).

EIF4E4 expression was also evaluated during *L. infantum* differentiation to amastigote forms. A pattern similar to the one seen for promastigotes was observed both during the differentiation process as well as with fully differentiated amastigotes (**Fig. 1B**). In exponentially grown differentiated parasites, the putatively phosphorylated EIF4E4 band is detected but when reaching stationary phase in the MAA medium, only the lower molecular weight band is seen. As a control for amastigote

differentiation, we used the amastigote-specific protein A2 (**Fig. 1B**, lower panel). After passaging to new amastigote medium, phosphorylation appears again. We have obtained similar results in promastigotes with an ectopically expressed HA-tagged EIF4E4, produced after stable transfection of *L. infantum* (**Fig. 1C**). Overall, these results indicate that EIF4E4 is regulated during the *L. infantum* life cycle, possibly through phosphorylation events associated with parasite growth stages of increased protein synthesis. However, no changes in EIF4E4 abundance were seen between promastigotes, differentiating cells and amastigotes.

The unique N-terminal region of the *Leishmania* EIF4E4 harbors several putative phosphorylation sites and conserved motifs

In the mammalian eIF4E homologue, the single known phosphorylation site is localized at the extreme C-terminus of the protein (residue S209, from a protein which is 217 amino acid residues in length),[19] a region poorly conserved and for which no sequence similarity was seen with the *Leishmania* EIF4E4. A high throughput analysis of the *L. donovani* phosphoproteome, however, has previously identified 2 phosphorylation sites within the N-terminus of the *Leishmania* EIF4E4.[36] and our recent work showed that most phosphorylation events targeting the *T. brucei* EIF4E4 are localized to its N-terminus.[32] Indeed, 9 phosphorylation sites were recently mapped to the *T. brucei* EIF4E4 N-terminus, after a more extensive phosphoproteomic analysis of procyclic and bloodstream cells.[37] This N-terminus is rich in prolines, mostly hydrophilic, and consists of over half of the length of the complete EIF4E4. It is not found in any known eIF4E homologue described so far outside the family Trypanosomatidae where it is detected only in the EIF4E3 and EIF4E4 orthologues. The two phosphorylation sites found in the *L. donovani* EIF4E4 orthologue and 7 out of the nine sites found within its *T. brucei* counterpart consist of serine-proline or threonine-proline (SP/TP) motifs, known target sites of different eukaryotic protein kinases.[38] Here, we carried out a sequence alignment between the unique N-terminal region of EIFE4 orthologues from multiple trypanosomatid species to evaluate the position and conservation of SP/TP motifs. For this alignment, an extension of ~140 residues missing from the annotated N-terminal sequences of several *Leishmania* EIF4E4 orthologues
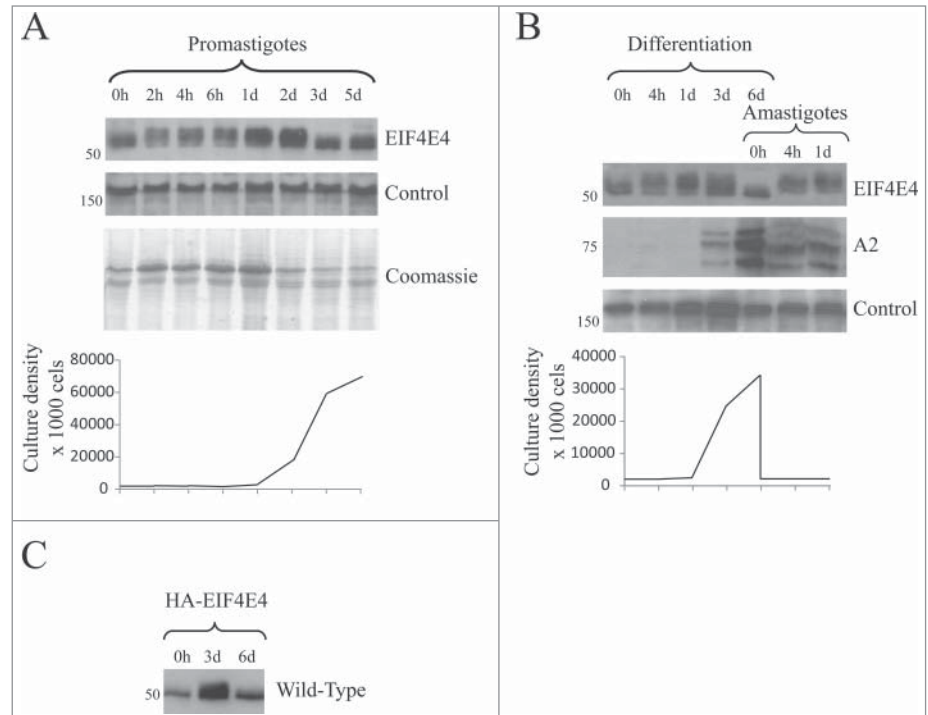

Figure 1. Expression analysis of *L. infantum* EIF4E4 during both promastigote and amastigote life stages. Western blotting showing the expression of EIF4E4 during distinct growth phases of both promastigote (**A**) and amastigote (**B**) life stages of *L. infantum*. For all lanes equal loads were run under denaturing conditions and blotted with a whole rabbit polyclonal serum directed against *Leishmania* EIF4E4. For both sets of experiments, the graphic representations of the cell counts from every aliquot are shown below the corresponding blots. In (**A**) the results from a single curve are shown with aliquots taken immediately after passaging (0h), at 2, 4 and 6 hour time points and daily after that (upper panel). As loading control, a cross-reacting band of high molecular weight non-specifically recognized by the EIF4E4 polyclonal serum is shown (middle panel). A Coomassie staining of equivalent amounts of the proteins loaded on the gel is presented in the lower panel. In (**B**) the results from 2 consecutive curves in amastigote medium (MAA) are shown (upper panel). The first curve (Differentiation) started with stationary-phase promastigotes passaged into MAA medium. Samples were taken immediately after passaging (0h), 4 hours later and at 1, 3 and 6 days to allow for differentiation into amastigote forms. The second curve (Amastigotes) was started with fully differentiated stationary phase amastigotes from the last day of the first curve. Expression of the amastigote-specific marker A2 was evaluated to confirm the amastigote differentiation pattern (lower panel). (**C**) Western blotting showing the ectopic expression of HA-tagged EIF4E4. After transfection in *L. infantum*, expression of the HA-EIF4E4 protein was evaluated at different growth phases (0h, 3d, 6d) by Western blot using an anti-HA monoclonal antibody. Numbers on the left of the panels indicate the position and size (in kDa) of selected molecular weight markers.

at TriTrypDB and other databases (including *L. major*, *L. infantum* and *L. mexicana*) was considered. This extension is encoded in the corresponding DNA sequences and has been found in other annotated *Leishmania* and *Trypanosoma* orthologues.[24,26] The alignment shown in **Figure 2A** confirms the presence of multiple SP/TP motifs within the N-terminus of the various EIF4E4 sequences. A minimum of 6 (in *T. cruzi*) and a maximum of 12 (in *T. brucei*) motifs are seen throughout the N-terminus of the various EIF4E4 orthologues. In the *L. infantum* orthologue, nine of these motifs are found and numbered 1 to 9 (**Fig. 2A, B**). The position of five of these motifs (1, 2, 4, 5, 6, 8 and 9) is conserved between the various *Leishmania* sequences, with 2 (5 and 9) strictly conserved in all trypanosomatid sequences analyzed, indicating a likely important role.
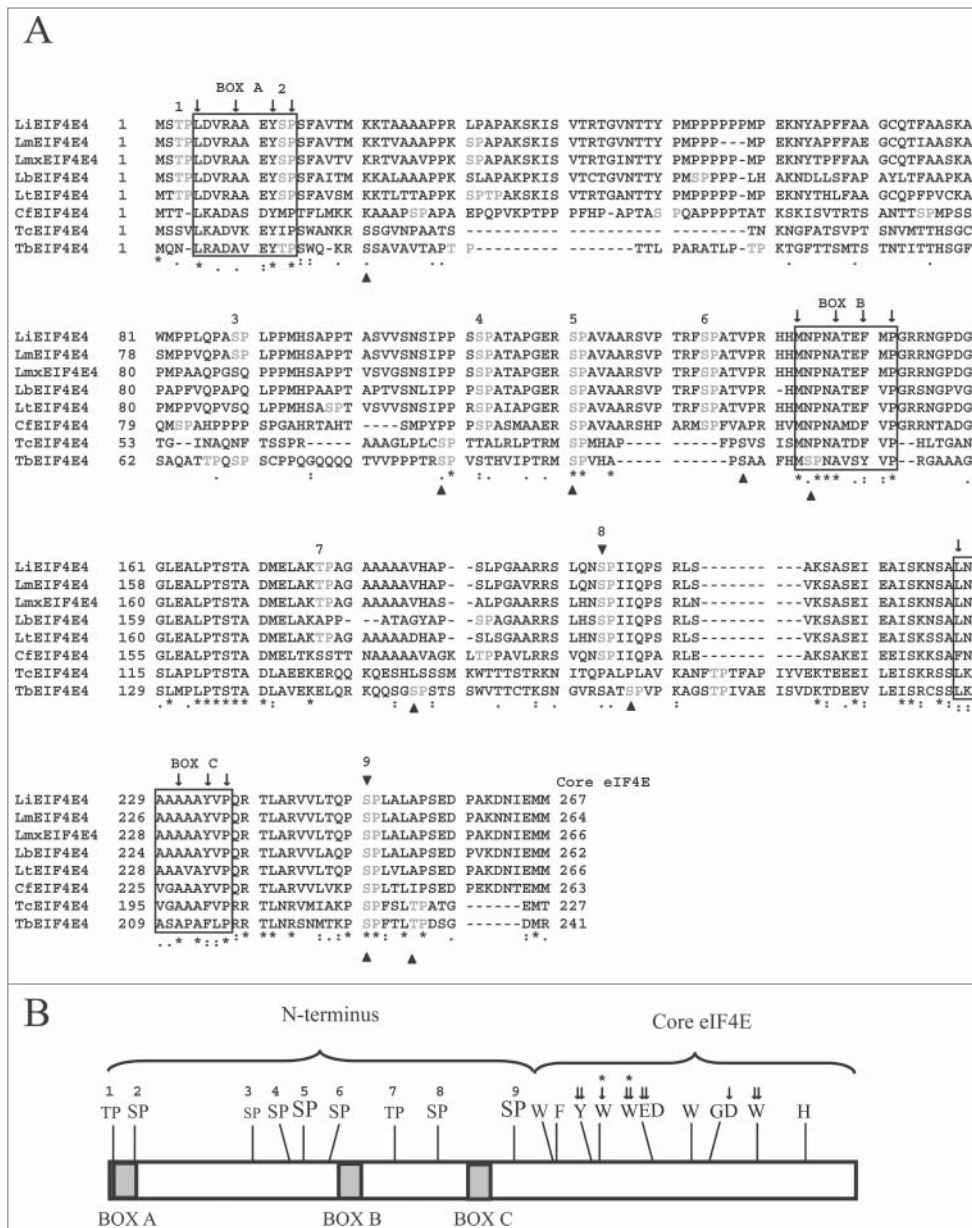
Functional evaluation of phosphorylation sites and conserved elements within the *L. infantum* EIF4E4 N-terminus

To confirm the role of individual SP/TP motifs as target sites for phosphorylation of *L. infantum* EIF4E4, site-directed mutagenesis was applied to eight of the identified motifs (with the exception of motif 3, not investigated here) by replacing serine or threonine residues with alanines. Sets of increasingly greater number of the SP/TP motifs were mutated and the mutant proteins were ectopically expressed in *L. infantum* cells. Western blot analysis of the resulting HA-tagged mutant proteins with the anti-HA antibody under conditions of exponential growth revealed that substitution of the S or T residues for alanines in motifs 1 and 2 did not lead to any detectable changes in the phosphorylation pattern while mutations in motifs 4, 5 and 6 induced a clear reduction in the number of isoforms (**Fig. 3A**). Mutations in motifs 7, 8 and 9 only also did not lead to a clear effect in isoform profile, however mutations in 8 motifs (1, 2, 4, 5, 6, 7, 8, 9) led to a major disappearance of the phosphorylated isoforms.
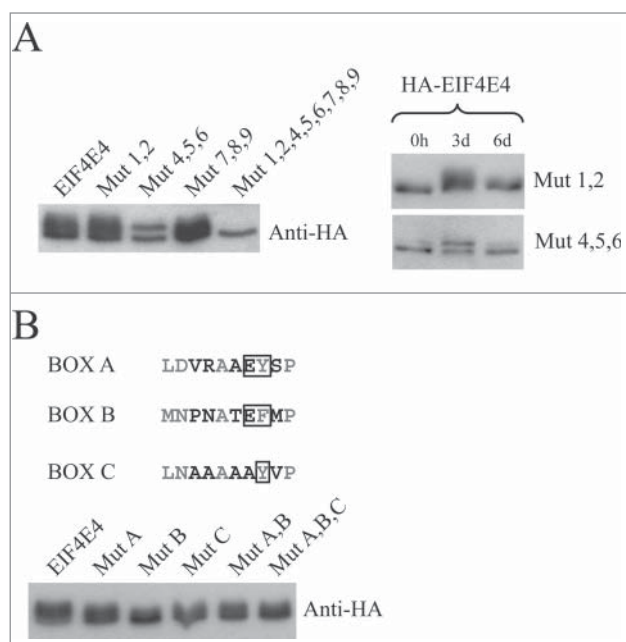
A

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                 BOX A   2
               1    ↓  ↓    ↓↓
LiEIF4E4    1  MSTPLDVRAA EYSPSFAVTM KKTAAAAPPR LPAPAKSKIS VTRTGVNTTY PMPPPPPPMP EKNYAPPFAA GCQTFAASKA
LmEIF4E4    1  MSTPLDVRAA EYSPSFAVTM KKTVAAAPPK SPAPAKSKIS VTRTGVNTTY PMPPP---MP EKNYAPPFAE GCQTIAASKA
LmxEIF4E4   1  MSTPLDVRAA EYSPSFAVTV KRTVAAVPPK SPAPAKSKIS VTRTGINTTY PMPPPPP-MP EKNYTPFFAA GCQTFAASKA
LbEIF4E4    1  MSTPLDVRAA EYSPSFAITM KKALAAAPPK SLAPAKPKIS VTCTGVNTTY PMSPPPP-LH AKNDLLSFAP AYLTFAAPKA
LtEIF4E4    1  MTTPLDVRAA EYSPSFAVSM KKTLTTAPPK SPTPAKSKIS VTRTGANTTY PMPPPPP-MP EKNYTHLFAA GCQPFPVCKA
CfEIF4E4    1  MTT-LKADAS DYMPTFLMKK KAAAPSPAPA EPQVPVKPTPP PFHP-APTAS PQAPPPPTAT KSKISVTRTS ANTTSPMPSS
TcEIF4E4    1  MSSVLKADVK EYIPSWANKR SSGVNPAATS ---------- ---------- --------TN KNGFATSVPT SNVMTTHSGC
TbEIF4E4    1  MQN-LRADAV EYTPSWQ-KR SSAVAVTAPT P--------- -------TTL PARATLP-TP KTGFTTSMTS TNTITTHSGF
               *.  *.  .  .:* * *::  .                                              ▲

                        3                                                    BOX B
                                                         4          5           6       ↓     ↓    ↓
LiEIF4E4   81  WMPPLQPASP LPPMHSAPPT ASVVSNSIPP SSPATAPGER SPAVAARSVP TRFSPATVPR HHMNPNATEF MPGRRNGPDG
LmEIF4E4   78  SMPPVQPASP LPPMHSAPPT ASVVSNSIPP SSPATAPGER SPAVAARSVP TRFSPATVPR HHMNPNATEF MPGRRNGPDG
LmxEIF4E4  80  PMPAAQPGSQ PPPMHSAPPT VSVGSNSIPP SSPATAPGER SPAVAARSVP TRFSPATVPR HHMNPNATEF MPGRRNGPDG
LbEIF4E4   80  PAPFVQPAPQ LPPMHPAAPT APTVSNLIPP PSPATAPGER SPAVAARSVP TRFSPATVPR -HMNPNATEF VPGRSNGPVG
LtEIF4E4   80  PMPPVQPVSQ LPPMHSASPT VSVVSNSIPP RSPAIAPGER SPAVAARSVP TRFSPATVPR HHMNPNATEF VPGRRNGPDG
CfEIF4E4   79  QMSPAHPPPP SPGAHRTAHT ----SMPYPP PSPASMAAER SPAVAARSHP ARMSPFVAPR HVMNPNAMDF VPGRRNTADG
TcEIF4E4   53  TG--INAQNF TSSPR----- AAAGLPLCSP TTALRLPTRM SPMHAP---- -----FPSVS ISMNPNATDF VP--HLTGAN
TbEIF4E4   62  SAQATTPQSP SCPPQGQQQQ TVVPPPTRSP VSTHVIPTRM SPVHA----- ------PSAA FHMSPNAVSY VP--RGAAAG
                 .                :        ▲     :.    ** *        ▲    *.*** .: :*           .

                        7                           8                                         ↓
LiEIF4E4  161  GLEALPTSTA DMELAKTPAG AAAAAVHAP- -SLPGAARRS LQNSPIIQPS RLS------- ---AKSASEI EAISKNSALN
LmEIF4E4  158  GLEALPTSTA DMELAKTPAG AAAAAVHAP- -SLPGAVRRS LQNSPIIQPS RLS------- ---VKSASEI EAISKNSALN
LmxEIF4E4 160  GLEALPTSTA DMELAKTPAG AAAAAVHAS- -ALPGAARRS LHNSPIIQPS RLN------- ---VKSASEI EAISKNSALN
LbEIF4E4  159  GLEALPTSTA DMELAKAPP- --AGTAYAP- -SPAGAARRS LHSSPIIQPS RLS------- ---VKSASEI EAISKNSALN
LtEIF4E4  160  GLEALPTSTA DMELAKTPAG AAAAADHAP- -SLSGAARRS LHNSPIIQPS RLS------- ---VKSASEI EAISKSSALN
CfEIF4E4  155  GLEALPTSTA DMELTKSSTT NAAAAAVAGK LTPPAVLRRS VQNSPIIQPA RLE------- ---AKSASEI EEISKKSAFN
TcEIF4E4  115  SLAPLPTSTA DLAEEKERQQ KQESHLSSSM KWTTTSTRKN ITQPALPLAV KANFTPTFAP IYVEKTEEEI LEISKRSSLK
TbEIF4E4  129  SLMPLPTSTA DLAVEKELQR KQQSGSPSTS SWVTTCTKSN GVRSATSPVP KAGSTPIVAE ISVDKTDEEV LEISRCSSLK
                .* .****** *:   *       :   ▲      :   .  :.    .. ▲    :        *: .*:   **: *:::

               BOX C            9
               ↓   ↓  ↓         ▼                   Core eIF4E
LiEIF4E4  229  AAAAAYVPQR TLARVVLTQP SPLALAPSED PAKDNIEMM 267
LmEIF4E4  226  AAAAAYVPQR TLARVVLTQP SPLALAPSED PAKNNIEMM 264
LmxEIF4E4 228  AAAAAYVPQR TLARVVLTQP SPLALAPSED PAKDNIEMM 266
LbEIF4E4  224  AAAAAYVPQR TLARVVLAQP SPLALAPSED PVKDNIEMM 262
LtEIF4E4  228  AAAVAYVPQR TLARVVLTQP SPLVLAPSED PAKDNIEMM 266
CfEIF4E4  225  VGAAAYVPQR TLARVVLVKP SPLTLIPSED PEKDNTEMM 263
TcEIF4E4  195  VGAAAFVPRR TLNRVMIAKP SPFSLTPATG ------EMT 227
TbEIF4E4  209  ASAPAFLPRR TLNRSNMTKP SPFTLTPDSG ------DMR 241
                ..* *::*:* ** *  ::* **: * *      :*.
                           ▲        ▲
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B

N-terminus — Core eIF4E

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  1  2       3  4 5  6      7     8        9  W F  Y W WED   W  GD  W     H
 TP  SP      SP SP SP SP    TP    SP       SP ↓↓ ↓ ↓↓ ↓↓↓    ↓  ↓↓  ↓
                                                 *   * *
 [BOX A]              [BOX B]        [BOX C]
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Figure 2. Amino acid sequence comparison of the EIF4E4 N-terminus from multiple trypanosomatid orthologues. (**A**) Clustal W alignment of the unique N-terminal part of EIF4E4 found in different trypanosomatid species. The position of the core eIF4E sequences, which characterize these proteins and are common to all known eukaryotic eIF4Es, is indicated and, when necessary, spaces were inserted within the various sequences (dashes) to allow better alignment. The three distinct elements found along this segment are indicated (boxes A to C - the arrows indicate the 4 conserved amino acid residues which led to their identification). Highlighted in gray are the putative serine (SP) or threonine (TP) phosphorylation sites (numbered from 1 to 9 for the *L. infantum* sites). Below the aligned sequences the residues conserved in all EIF4E4 orthologues analyzed are marked by an asterisk (*), with conservative (;) and semi-conservative (.) substitutions also indicated. The symbol ▼ above the sequences indicate the 2 serine residues previously confirmed to be targeted by phosphorylation in the *L. infantum* EIF4E4,[36] and which coincide with the SP/TP motifs 8 and 9. Below the *T. brucei* EIF4E4 sequence, the ▲ also highlights the serine/threonine residues found to be phosphorylated in this species.[37] Li: *L. infantum*; Lm: *L. major*; Lmx: *L. mexicana*; Lb: *L. braziliensis*; Lt: *L. tarentolae*; Cf: *Crithidia fasciculata*; Tc: *T. cruzi*; Tb: *T. brucei*. (**B**) Schematic representation of *L. infantum* EIF4E4. In the N-terminus all 3 boxes are represented in gray and the 9 putative phosphorylation sites are also indicated, with the sizes of the SP or TP letters representing their degree of conservation in different sequences based on the alignment shown in A. Within the core eIF4E region, unique aromatic and other residues which characterize the eIF4E sequences are indicated; with the one arrow (↓) are those which in other species or trypanosomatids have been implicated in eIF4G binding and with 2 arrows are those shown to be implicated in cap recognition.[24] The 2 tryptophan residues which have been mutated in this study (W305 and W333) are highlighted by an asterisk.

Figure 3. Identification of residues involved in EIF4E4 phosphorylation. (**A**) Expression analysis of HA-tagged EIF4E4 mutants containing multiple mutations in selected phosphorylation sites. Mutants were generated by replacing previously identified serine or threonine residues likely targeted for phosphorylation for alanines through site directed mutagenesis. In the left panel, the expression of the resulting mutant proteins in exponentially grown *L. infantum* (2 to 3 days growth) transfected with circular plasmids was evaluated. In the right panel, samples from 2 of the mutants whose gene were integrated into the BT1 locus were assayed during stationary and exponential growth conditions, as in **Figure 1C**. (**B**) Effect of mutations in boxes A to C on the expression of phosphorylated isoforms of EIF4E4. Mutations were introduced in each of the 3 boxes identified in *Leishmania* EIF4E4 (residues typical of these motifs are in gray) by replacing residues EY, EF, and Y in boxes A, B, and C, respectively for alanines. The expression of the resulting HA-tagged proteins was then evaluated in exponentially grown *L. infantum* cells by Western blot analysis using the anti-HA antibody. The results shown in both (**A**) and (**B**) were reproduced using a minimum of 2 batches of transfected cells using both episomal vectors and integrated genes. As much as possible, when comparing different mutants, the cultures were grown in parallel and assayed at the same stage of the growth curves and the same number of passages following transfection.

Since in some experiments some residual phosphorylation is still seen even in Mut 1, 2, 4, 5, 6, 7, 8, 9, it is possible that either motif 3 or a single other SP motif found within the core eIF4E region, but not investigated here, or even motifs deviating from the SP/TP consensus, could also be phosphorylated. Nevertheless, the bulk of the phosphorylation residues within the EIF4E4 N-terminus include those targeted by the mutagenesis approach.

Next, we investigated the contribution of the 3 boxes A-C in EIF4E4 phosphorylation. For this, mutations within conserved residues in boxes A through C were generated and additional constructs were made where two or three boxes were mutated. Western blot analysis of exponentially grown *L. infantum* recombinant strains expressing these mutant EIF4E4 proteins using an anti-HA antibody showed that individual mutations in all three boxes seem to interfere with EIF4E4 phosphorylation, leading to

a reduction in the number of isoforms, particularly those with higher molecular weights (**Fig. 3B**). The effect induced by mutation in box A was very subtle but a stronger effect was observed by mutations in boxes B or C. A clear effect was also observed when all three boxes were mutated, implying a role for these elements in phosphorylation. Since only two of the residues mutated within the boxes could be directly targeted by phosphorylation (tyrosine in boxes A and C), the results implicate these elements as possible docking sites for either protein kinases or protein partners, which would be required for phosphorylation to occur.

Interaction of EIF4E4 with EIF4G3 or efficient binding to the cap substrate is not required for EIF4E4 phosphorylation

In the best characterized example of eIF4E phosphorylation, that of mammalian eIF4E in S209, phosphorylation is dependent on the binding of eIF4E to eIF4G since the latter recruits the eIF4E kinase Mnk.[19-21] Within EIF4E4, the conserved W305 residue, highlighted in **Figure 2B**, has been shown previously to be required for the interaction with EIF4G3.[29] To determine whether binding of EIF4E4 to EIF4G3 is required for its phosphorylation, the W305 residue was replaced by an alanine (W305A). This mutation effectively abolished the ability of a.[35] S-labeled EIF4E4 protein to bind to a GST-tagged EIF4G3, as shown by *in vitro* pull-down assays (**Fig. 4A**) and in agreement with what has been described previously based on results with the yeast 2-hybrid system.[29] A second conserved tryptophan residue relevant for EIF4E4 function is W333 since the equivalent residue within the mammalian protein (W102) has been reported to be strictly required for cap binding and recognition.[39] The requirement of EIF4E4 phosphorylation for efficient mRNA binding was also investigated by substituting the W333 residue by alanine (W333A) in the wild type protein. The resulting mutant protein was first tested for its cap binding affinity by comparing its ability to bind to 7-methyl-GTP Sepharose beads with that of the wild type protein (**Fig. 4B**). Specific binding by the wild type EIF4E4 was observed, as previously reported,[26] compatible with the lower efficiency of binding by the trypanosomatid EIF4E4 to the classical cap analog, when compared with mammalian eIF4E.[24,26,27] However, no binding by EIF4E4 containing the W333A mutation was detected.

The two HA-EIF4E4 mutant proteins (W305A or W333A) were next expressed in *L. infantum* promastigotes and the presence of the phosphorylated isoforms was evaluated in exponentially grown parasites by Western blot analysis. No major changes in phosphorylation were observed for either mutant protein (**Fig. 4C**) when compared to the wild type EIF4E4 (see **Fig. 1C**), indicating that EIF4E4 phosphorylation does neither require the interaction with its eIF4G partner nor an efficient binding to the cap structure. These results are consistent with a mechanism associated with EIF4E4 phosphorylation that is distinct from the one described for the mammalian eIF4E.

Characterization of PABP binding motifs in EIF4E4 and requirement of PABP1 binding for EIF4E4 phosphorylation

The reduced phosphorylation of EIF4E4 observed when all three boxes were mutated (shown in **Fig. 3B**) indicates a possible

interference with the protein's ability to bind to protein partners or kinases. The *Leishmania* EIF4E4 N-terminus has been shown to interact directly with one of the three *Leishmania* PABP homologues (PABP1), a novel interaction not yet reported in other eukaryotes.[29] The EIF4E4 construct used by Zinoviev *et al.* lacks the first ~140 residues of EIF4E4 and the PABP1 binding region was mapped to the subsequent 86 residues of the protein, including most of boxes B and C. To investigate whether the effect of A-C box mutations on EIF4E4 phosphorylation might be associated with binding to PABP1, EIF4E4-PABP1 interactions were investigated *in vitro* through pull-down assays using a GST-tagged PABP1 and ^{35}S-labeled EIF4E4 mutant proteins. As shown in **Figure 5A**, GST-PABP1 binds very efficiently to wild type EIF4E4 and, although this experiment does not allow a proper evaluation of the affinities involved in the interaction, the binding between EIF4E4 and PABP1 seems to be as strong as or

even stronger than the binding between EIF4E4 and EIF4G3 (see **Fig. 4A**). The EIF4E4-PABP1 interaction was not altered when W305 was mutated to an alanine under conditions in which EIF4G3 binding was abolished (**Fig. 5A**). Regarding the A-C box mutations, no effect was seen when simultaneous mutations in both boxes A and B were present or when box C was individually mutated. However, mutations in all 3 boxes (Mut A, B, C) abolished the PABP1-EIF4E4 interaction. This effect was not due to a misfolding of the mutant protein since it was still able to efficiently bind to EIF4G3 (**Fig. 5A**).

To confirm these results *in vivo*, immunoprecipitations (IPs) were carried out from cellular extracts of recombinant *L. tarentolae* overexpressing either the wild type or selected mutants of the *L. infantum* HA-EIF4E4. We used the closely related *L. tarentolae* species [40] instead of *L. infantum* for these IP studies, as yield with *L. infantum* was systematically lower due to protein degradation. These assays were done using the commercially available monoclonal anti-HA antibody and the immunoprecipitated products were analyzed by Western blots with antibodies directed against *Leishmania* PABP1 and EIF4G3. As shown in **Figure 5B**, the IP with the W305A mutant specifically abolished the interaction with EIF4G3, but this interaction was not affected by mutations targeting most of the putative phosphorylation motifs or the three boxes A, B, and C. In the case of PABP1, interaction with EIF4E4 was specifically abolished only when the three boxes were mutated (**Fig. 5B**). A mutant with both the W305 residue as well as the three boxes mutated was also tested, and in this case neither PABP1 nor EIF4G3 were found in the immunoprecipitated material. Altogether, these data confirm the *in vitro* interactions (**Fig. 5A**).

To investigate the effect that the loss of both EIF4G3 and PABP1 binding might have on EIF4E4 phosphorylation, the
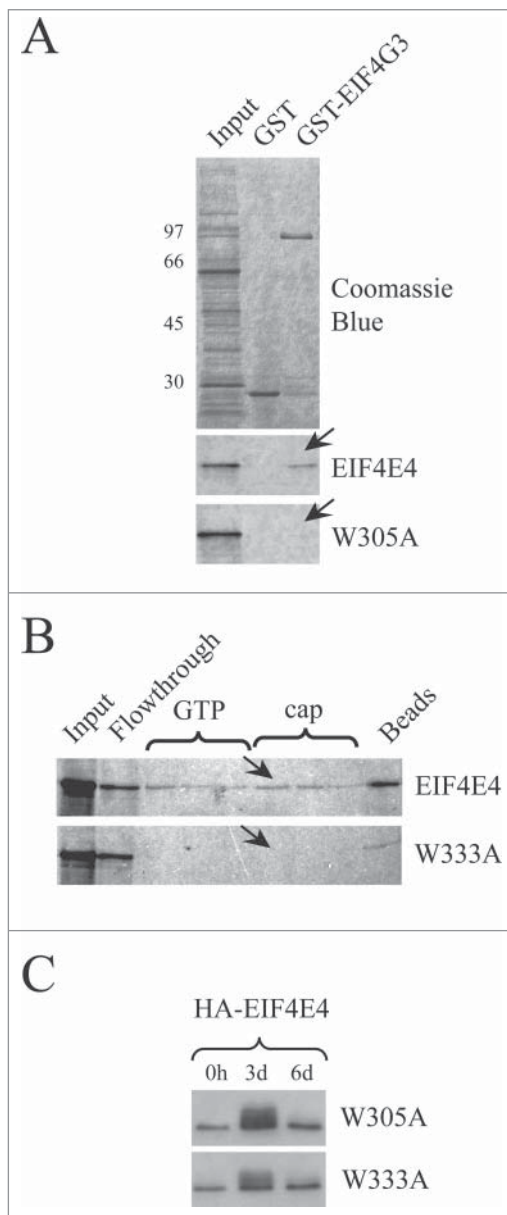

Figure 4. Investigation of EIF4E4 phosphorylation under conditions of impaired binding to EIF4G3 or to the cap structure. (**A**) Co-precipitation assay to confirm the effect of the W305A mutation in EIF4E4 binding to EIF4G3. A GST fusion of *L. major* EIF4G3 was used and assessed for its ability to bind to the *L. infantum* ^{35}S-labeled wild type EIF4E4 or to a mutant protein where the critical tryptophan residue (W305) implicated in binding to EIF4G3 was replaced by an alanine (both proteins were labeled with ^{35}S-methionine by *in vitro* translation). The upper panel shows the Coomassie-blue stained gel indicating the recombinant GST (negative control) or the GST-EIF4G3 fusion. The panel below shows the result from the assay comparing the wild type EIF4E4 with the W305A mutant protein. "Input" is equivalent to the same amount of labeled proteins (total translation reactions) used in the assay. The arrows indicate the bound protein fraction eluted with the SDS-PAGE sample buffer. (**B**) Cap binding assay to investigate the effect of the W333A mutation of EIF4E4 on its cap binding affinity. This assay evaluated the direct binding of the ^{35}S-labeled wild type EIF4E4 or the W333A mutant to a 7-methyl-GTP Sepharose resin. Non-specific binding was removed by washes with GTP, and specific elution was achieved with a cap analog ("cap" – see arrows). Aliquots of the various washes were run on SDS-PAGE and compared with samples from the original translation reaction ("Input") as well as the non-bound fraction ("Flow-through") and any protein remaining bound to the beads after the final washes, eluted in SDS-PAGE sample buffer ("Beads"). (**C**) Western blotting showing the ectopic expression of HA-EIF4E4 containing the W305A and W333A mutations at 0h and after 6 days of standard growth as well as during the exponential growth phase (3d).
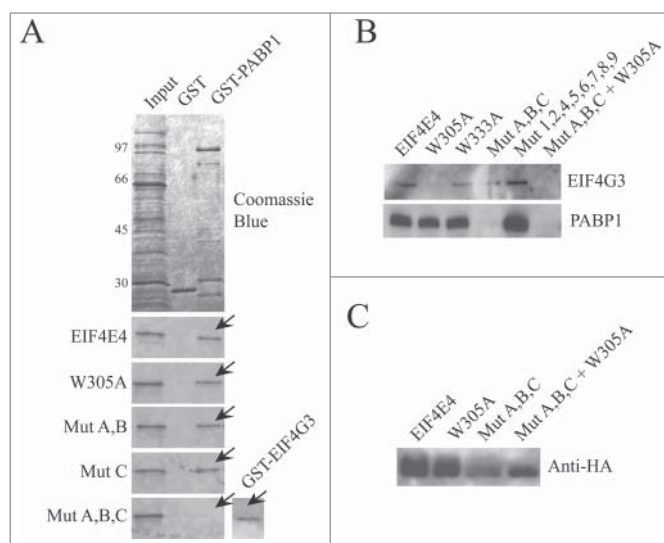
Figure 5. Defining the EIF4E4-PABP1 interaction and its role on EIF4E4 phosphorylation. (**A**) Co-precipitation assay to investigate the EIF4E4-PABP1 interaction. A *L. infantum* GST-PABP1 fusion was assessed for its ability to bind to the ^{35}S-labeled wild type EIF4E4 or mutants targeting the W305A residue or each of the 3 boxes. The upper panel shows the Coomassie-blue stained gel indicating the recombinant GST (negative control) or the GST-PABP1 fusion. The panels below show the results from the assays carried out comparing the wild type EIF4E4 with the various mutants. For EIF4E4 where all 3 boxes were mutated, the labeled protein was also incubated with the same GST-EIF4G3 described in **Figure 4A**. (**B**) Immunoprecipitation assays of cellular extracts from *L. tarentolae* promastigotes overexpressing the EIF4E4 wild type protein and selected mutants (*L. infantum* extracts were also essayed but, due to protein degradation, signals were hardly detectable; data not shown). Recombinant proteins were immunoprecipitated with magnetic beads bound to a monoclonal anti-HA antibody. The immunoprecipitated samples were then loaded on SDS-PAGE gels and blotted with antibodies specific to PABP1 and EIF4G3, as indicated. (**C**) Expression analysis to monitor phosphorylation of the EIF4E4 mutant lacking the PABP1 and EIF4G3 binding sites in exponentially grown *L. infantum*. The assay was carried out as described in **Figure 3**.

expression of the W305A + Mut A, B, C mutant was investigated in *L. infantum* cells and compared with mutants harboring only the W305A mutation or the mutations on the 3 boxes (**Fig. 5C**). As shown previously, loss of EIF4G3 binding did not alter EIF4E4 phosphorylation, which however was impaired when all 3 boxes were mutated and there was no PABP1 interaction. When both the PABP1 and EIF4G3 interactions were abolished, the decrease in EIF4E4 phosphorylation was even more significant and very little or no isoforms of high molecular weight associated with the phosphorylation events were detected (**Fig. 5C**). Overall, these results define the 3 boxes within the N-terminus of EIF4E4 as PABP1 binding sites and highlight the important role of the EIF4E4-PABP1 interaction on EIF4E4 phosphorylation.

EIF4E4 is essential for *Leishmania* growth

In *T. brucei,* RNAi mediated knockdown of EIF4E4 did not interfere with cell viability or growth in procyclic cells, although the protein was required for survival of the mammalian bloodstream form.[26] Here, attempts were made to generate haploid or diploid knockouts for the *EIF4E4* gene and evaluate its effect on *L. infantum* survival. **Figure 6A** highlights the gene replacement strategy used for generating the knockout, with the first gene copy being replaced by the hygromycin resistance gene (*HYG*) and the second allele with the puromycin resistance gene (*PURO*). No difficulties were observed for generating the haploid EIF4E4/EIF4E4::HYG mutant (HYG SKO). As shown in **Figure 6B**, hybridization of PstI-digested *L. infantum* total DNA with a probe consisting of the 500 bp of the intergenic region positioned immediately upstream of the *EIF4E4* gene (5′ IR probe) led to the appearance of the band corresponding to the targeted hygromycin allele (~0.9 kb), confirming the integration event. Attempts to generate a diploid knockout mutant by integrating the puromycin expression cassette into the EIF4E4/EIF4E4::HYG mutant failed (data not shown), however, most likely because EIF4E4 is essential for promastigote growth.

The essential requirement of EIF4E4 for *Leishmania* growth was confirmed by complementation studies where the second *EIF4E4* allele was successfully replaced by the puromycin expression cassette to generate a null mutant in the presence of an ectopically provided *EIF4E4* gene, as part of a neomycin resistance-encoded plasmid (**Fig. 6B, C**). In **Figure 6B**, in addition to the 0.9 kb *HYG*-targeted copy, a new band (~1.1 kb) corresponding to the integration of the *PUR* gene was detected in the blot hybridized with the 5′ IR probe, concomitant with the loss of the endogenous ~1.6 kb gene copy (DKO + EIF4E4 lane). Hybridization of the same blot with a probe corresponding to the *EIF4E4* coding sequence (CDS) revealed only the episomal copy of the gene at ~2.3 kb (**Fig. 6C**). This double inactivation of the *EIF4E4* gene, provided the complementation by the wild type allele, was also confirmed by Northern blot hybridization (not shown).

Binding of EIF4E4 to PABP1 is strictly required for EIF4E4 function *in vivo*

We used the above described complementation approach to investigate the contribution of key residues involved either in EIF4E4 phosphorylation or binding to cap or to EIF4G3 and/or to PABP1 on the essential function of EIF4E4. We therefore tested which of the EIF4E4 mutants was able to complement the EIF4E4::PUR/EIF4E4::HYG (DKO) null mutant and to maintain cell viability when provided ectopically. Results of ectopic complementation (or not complementation) with different EIF4E4 mutant proteins are summarized in **Figure 7A**. Unexpectedly, we have been able to generate an EIF4E4 null mutant by ectopically providing the W305A (loss of EIF4G3 binding) or the W333A (loss of cap binding) mutants as the sole source of EIF4E4 (**Fig. 6B, C**). These results are consistent with EIF4E being able to function even under conditions in which its cap recognition ability or its binding to EIF4G3 were impaired. Complementation with various phosphorylation mutants, including that with 8 of the 9 putative phosphorylation sites mutated (Mut 1,2,4,5,6,7,8,9) allowed inactivation of the endogenous gene, and these mutants were able to maintain cell viability and growth. As shown in the Western blot of **Figure 7B**, a single
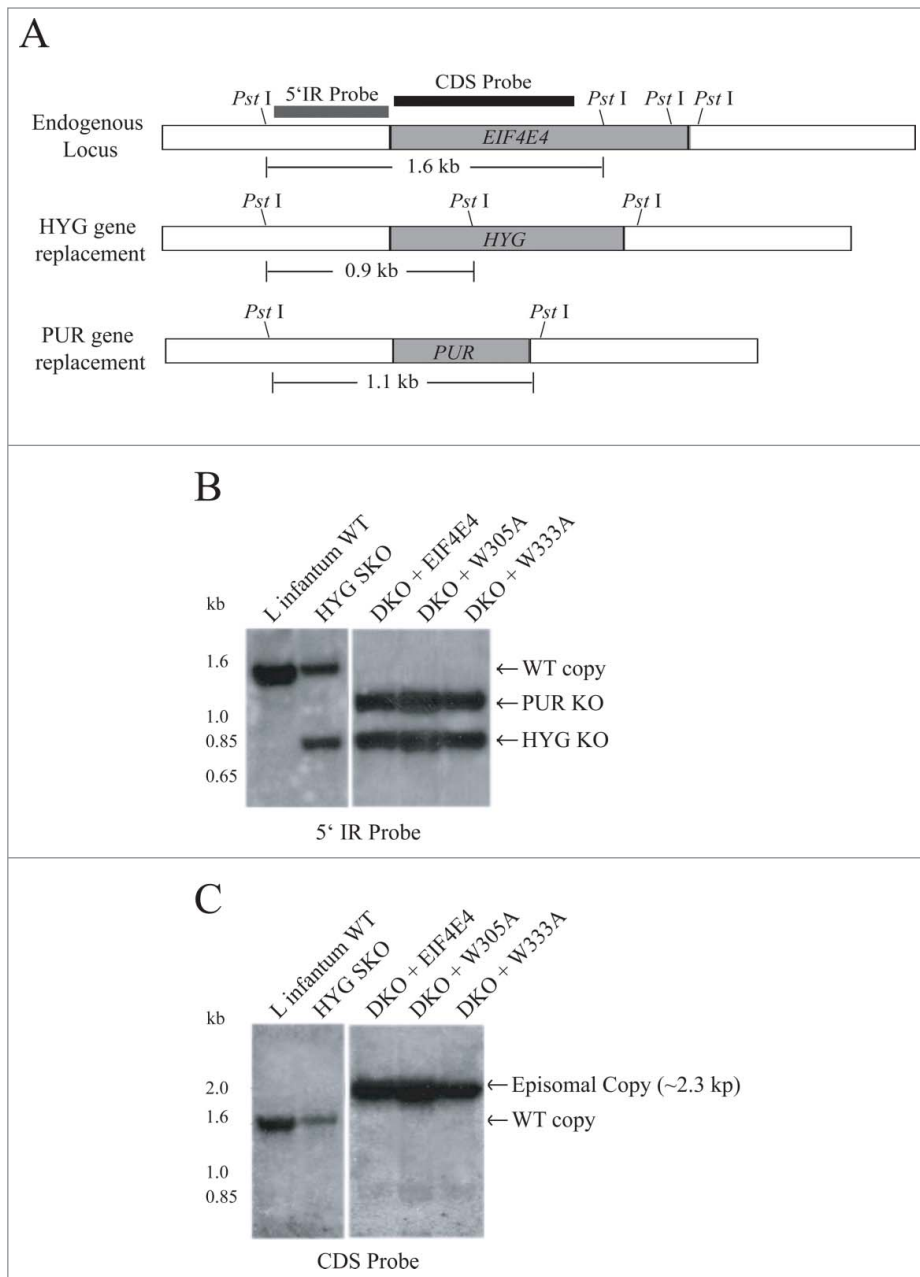
Figure 6. Generation of haploid and diploid *EIF4E4* gene knockout mutants in *L. infantum*. (**A**) Schematic representation of the endogenous *EIF4E4* gene as well as gene replacements following the single integration of the hygromycin (HYG) and/or puromycin (PUR) drug selectable markers. The probes used to confirm the integration events, corresponding to parts of the *EIF4E4* 5′ intergenic region (5′ IR) or its coding sequence (CDS), are also indicated. (**B and C**) Southern blots confirming the inactivation of the endogenous *EIF4E4* gene. Profiles of genomic DNAs from single (SKO; EIF4E4/EIF4E4::HYG) and double knockout (DKO; EIF4E4::PUR/EIF4E4::HYG) lineages digested with PstI and probed with the *EIF4E4* 5′ IR (**B**) or the *EIF4E4* CDS (**C**). The numbers on the left indicate the sizes in kb of molecular weight markers.

band representative of the dephosphorylated isoform of EIF4E4 was detected in Mut 1,2,4,5,6,7,8,9 with the antibodies directed against EIF4E4, confirming parasite viability in the absence of EIF4E4 phosphorylation. In contrast, EIF4E4 with both phosphorylation motifs and the EIF4G3 binding site mutated (Mut 2,4,5,6,7,8 + W305A) was unable to complement the lack of

the endogenous *EIF4E4* gene and therefore no *EIF4E4* null cell lineage was obtained. Mutations in boxes A and B or box C only, shown not to interfere with PABP1 binding or EIF4E4 phosphorylation, were able to complement the lack of the endogenous gene. In contrast, mutations in all three boxes (Mut A,B, C), which prevents PABP1 binding, effectively abolished the ability of the mutant protein to function in the complementation assay. Consequently, complementation with Mut A,B,C + W305A did not lead to the generation of an *EIF4E4* null cell lineage.

Next, we evaluated the growth of selected DKO lineages under conditions of promastigote and amastigote culture. Most of these cells showed no significant differences in growth rate, although a lag during the recovery phase from the second gene inactivation event was seen, when compared with the wild type EIF4E4 (data not shown). The only exception was observed with DKO lineages complemented with the W305A mutant lacking the ability to bind EIF4G3, which, despite their normal growth as promastigotes, were unable to differentiate and grow as amastigotes (**Fig. 7C**). Overall, these data support that interaction with PABP1 is strictly required for EIF4E4 function and cell viability while the EIF4G3 interaction is mostly required for *L. infantum* amastigote growth. Phosphorylation of EIF4E4 does not seem to be critical for its function, however, lack of phosphorylation coupled with loss of EIF4G3 binding (Mut 2,4,5,6,7,8+W305A, **Fig. 7A**) leads to a presumably inactive protein that is unable to complement the lack of the endogenous EIF4E4 protein.

To investigate more specifically the effect of EIF4E4 phosphorylation or its binding to EIF4G3 on promastigote and amastigote growth, we compared EIF4E4 haploid mutant cells (HYG SKO; EIF4E4/EIF4E4::HYG) overexpressing wild type EIF4E4 or mutants impaired in phosphorylation only (Mut 1,2,4,5,6,7,8,9) or in EIF4G3 binding (Mut W305A) or both. No differences in promastigote growth were observed. However, a significant delay in growth was observed in EIF4E4/EIF4E4::HYG amastigotes overexpression with Mut 2,4,5,6,7,8+W305A (**Fig. 8**). This suggests that lower levels (50% less) of EIF4E4 in the haploid mutant

impact more amastigote than pro-mastigote growth. It also suggests that overexpression of this non-functional mutant protein could have a dominant negative effect by sequestering some other factors, which might be required for efficient EIF4E4 function and translation in amastigotes. These data further indicate a possible redundancy between EIF4G3 binding and phosphorylation, which might be required for EIF4E4 activity during translation initiation.

Discussion

Previous published reports have attributed a major role of EIF4E4 in protein synthesis both in *Leishmania* and *Trypanosoma brucei*, although the lack of EIF4E4 detection in *L. amazonesis* amastigote-like forms and the non-essential phenotype after RNAi in *T. brucei* procyclic cells could suggest otherwise (reviewed in).[41] More recent data directly implicated EIF4G3, a key partner of EIF4E4, as an essential factor for efficient translation in *T. brucei*, which supports the importance of EIF4E4 for general protein synthesis.[31] Here, we describe several novel features of the *L. infantum* EIF4E4 with regards to its function and regulation. We show that EIF4E4 is constitutively expressed throughout the parasite life cycle but is specifically phosphorylated in exponentially grown promastigotes and amastigotes, hence suggesting a strong correlation between EIF4E4 phosphorylation and high levels of translation. Nine SP/TP phosphorylation motifs have been mapped within the unique N-terminal extension of EIF4E4 but these are not needed for binding to the cap structure or to EIF4G3. We also report that EIF4E4 interacts with PABP1 through 3 conserved boxes (A to C) at the EIF4E4 N-terminus, and that this interaction is important for phosphorylation and critical for EIF4E4 function *in vivo*. EIF4E4 is an essential gene in *L. infantum* as an *EIF4E4* null mutant was only generated in the presence of the wild type gene provided *in trans* as part of an episomal vector. Complementation studies demonstrated that only binding of EIF4E4 to PABP1, but not to EIF4G3, is essential for parasite viability. Constitutive expression of EIF4E4 suggests a key role of this factor in general translation during the different stages of *L. infantum* life cycle. However, it is possible that species-specific differences regarding EIF4E4 expression might occur, as in *L.*
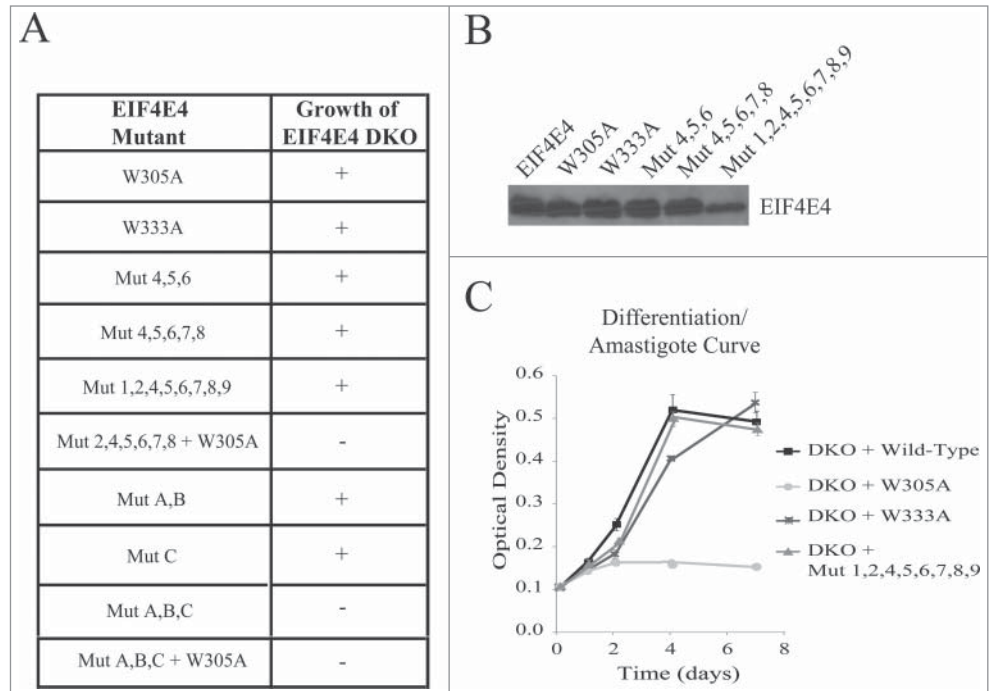

Figure 7. Evaluation of ectopically expressed EIF4E4 mutants in their ability to complement for the loss of the endogenous *EIF4E4* gene. (**A**) Complementation of EIF4E4 DKO with the different EIF4E4 mutants. A summary of the complementation experiments is shown in the table. The (+) stands for the ability of the mutant protein to complement for the loss of the endogenous *EIF4E4* gene through the generation of DKO lineages (EIF4E4::PUR/EIF4E4::HYG) and to maintain parasite viability (viable cells were recovered after the second knockout step with the puromycin selection marker, as shown in **Fig. 6**). The (-) defines those mutants which consistently failed to complement the loss of the *EIF4E4* gene. A minimum of three experiments was carried out with two distinct sets of SKO lineages. (**B**) Expression analysis of EIF4E4 wild type (EIF4E4) and selected mutant proteins in the DKO background, evaluated by a Western blot using the polyclonal serum directed against native EIF4E4. (**C**) Amastigote growth of the DKO lineages complemented with selected EIF4E4 mutants. Culture growth was monitored daily through optical density (OD) reading at 600 nm and observation under the microscope. The results are representative of a minimum of 3 experiments using two sets of independently transfected cells.

amazonensis EIF4E4 was not detected in amastigote-like forms, suggesting that another *Leishmania* eIF4E isoform, EIF4E1, may be regulating translation initiation in amastigotes.[29]

The identification of EIF4E4 phosphorylation sites as serine or threonine residues followed by a proline provides an indication of the nature of the kinases responsible for phosphorylation. Such sites are the preferred substrates of both mitogen-activated protein (MAP) kinases and cyclin-dependent kinases (CDK), enzymes associated with signal transduction pathways and cell proliferation.[38,42] Phosphorylation of EIF4E4 in multiple SP/TP sites occurs during periods of active cell growth and is rapidly induced upon passage of stationary cells to a fresh medium. This phosphorylation pattern is indeed compatible with a MAP kinase pattern of phosphorylation, which could also be maintained through CDK kinases. Mammalian eIF4E is specifically phosphorylated by the MNKs, another group of protein kinases which are known to be targeted by MAP kinases but recognize distinct phosphorylation sites.[20] MNKs are mainly found in metazoans and different organisms, such as yeasts and plants, seem to have evolved different mechanisms associated with the
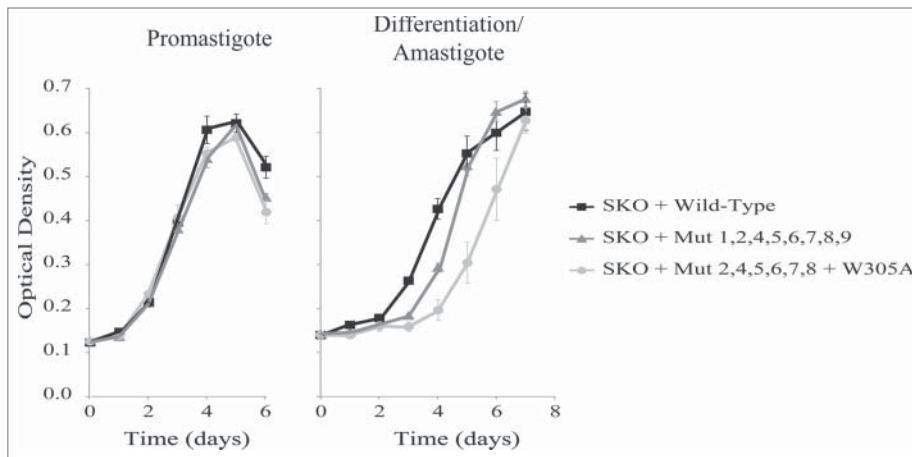
Figure 8. Effect of the overexpression of selected EIF4E4 mutants on amastigote growth. *L. infantum* cells lacking one of the *EIF4E4* gene copies through gene replacement using the hygromycin selectable marker (EIF4E4/EIF4E4::HYG) were transfected with episomal vectors expressing either the wild type HA-EIF4E4 copy or selected mutant proteins (Mut 1,2,4,5,6,7,8,9: a phosphorylation deficient mutant; W305A: impaired in its ability to interact with EIF4G3; Mut 2,4,5,6,7,8 + W305A: phosphorylation deficient and unable to interact with EIF4G3). Cells were grown as promastigotes (in SDM-79 medium) and axenic amastigotes (in MAA medium) as described in Materials and Methods. The results of the growth curves are representative of a minimum of three experiments using 2 sets of independently transfected cells. Standard deviations are derived from a single experiment done with 3 curves grown in parallel.

phosphorylation of eIF4E homologues.[21] Considering that multiple MAP and CDK kinases encoding sequences, many of unknown function, are found in trypanosomatid genomes,[43] it is likely that one or more of these kinases could be involved in EIF4E4 phosphorylation.

It is still unclear how phosphorylation of EIF4E4 would impact on its function. However, a similar pattern of phosphorylation was observed for PABP1 and it seems likely that there is an additive effect induced by the simultaneous phosphorylation of both protein partners, which should be involved in regulating their function (de Melo Neto et al, manuscript in preparation). In mammalian cells, the role of the single phosphorylation event targeting the mammalian eIF4E S209 residue, localized at the protein's C-terminal end, is still debatable and may be part of the normal cycle of events associated with cap-dependent translation initiation. This possibility has been reinforced recently by the demonstration that Mnk recruitment to eIF4F, and eIF4E phosphorylation, is influenced by the eIF3e subunit.[44] The pattern of phosphorylation of yeast eIF4E is more similar to that observed for the *Leishmania* EIF4E4 in that it targets two serine residues mapped to the protein's N-terminus,[45] but again the functional implications are unknown and the phosphorylation sites are distinct. A more clear mechanism linking regulation of protein activity through phosphorylation of an eIF4F subunit, which impacts on overall protein synthesis, has been recently proposed for the mammalian eIF4G1. Specific phosphorylation of eIF4G1 at a single serine residue by a CDK kinase active during mitosis has been shown to regulate eIF4F activity, and possibly protein synthesis, in a cell cycle dependent manner.[46,47]

A major novel finding here is the observation that the interaction between EIF4E4 and PABP1 is more critical for EIF4E4

function than its interaction with EIF4G3, and that it is essential and sufficient for the protein to function in translation initiation in *Leishmania*. Previous studies have predicted the PABP1 binding sites to the EIF4E4 N-terminal region,[29] but here we precisely mapped these binding motifs as the 3 boxes (A-C) within EIF4E4, which opens new avenues of investigation to better understand this interaction and its crucial role on EIF4E4 function. Further studies to define the PABP1 binding sites recognized by EIF4E4 would provide relevant information on how conserved is this type of interaction in other eukaryotic systems. The observation that the EIF4E4-EIF4G3 interaction, or EIF4E4 binding to the cap, is not critical for *Leishmania* promastigote viability, although the EIF4G3 interaction does seem to be important for amastigote growth, already highlights relevant differences not described elsewhere regarding the eIF4F mode of action. Considering the essentiality and relevance of EIF4G3 for translation initiation based both on the phenotype induced in *T. brucei* after its depletion through RNAi[31] and on the recent demonstration of its binding to the initiation complex eIF3 in *Leishmania*,[48] it is possible that interactions between EIF4E4-PABP1 and PABP1-EIF4G3 might compensate somehow for the loss of the direct EIF4E4-EIF4G3 interaction in promastigotes. Nevertheless, despite the fact that neither EIF4G3 binding alone nor EIF4E4 phosphorylation is strictly required for EIF4E4 function, impairing both activities leads to a non-functional protein with a possible dominant negative effect. A plausible explanation would be for the 2 activities, EIF4E4 phosphorylation and EIF4G3 binding, to be involved in recruiting the translation machinery to the mRNA. Aside from the cap binding activity required for recruiting the mRNA, 3 relevant interactions are then needed for a fully functional EIF4E4, mediated by the EIF4G3 and PABP1 binding motifs as well as phosphorylation. The last two, involving the EIF4E4 unique N-terminus, are not described in other eukaryotes and emerge as potential therapeutic targets for further investigation.

Materials and Methods

Parasite growth and expression analysis

Leishmania donovani infantum MHOM/MA/67/ITMAP-263 and *L. tarentolae* (strain Parrot-TarII) promastigotes were cultured in SDM-79 medium supplemented with 10% heat-inactivated fetal calf serum (Multicell, Wisent Inc.) and 5 μg/ml hemin (Sigma) at pH 7.0, 25°C. *L. infantum* axenic amastigotes were cultured in MAA/20 medium supplemented with 20% FCS (pH 5.5) at 37°C with 5% CO_2 for 4-5 days as described.[35]

For the expression analysis, growth curves were set up in SDM-79 medium starting with stationary phase *L. infantum* cells diluted to fresh medium at pre-established cell densities of 10^6 cells/ml. At selected time points after passaging, samples were harvested, resuspended directly into SDS-PAGE sample buffer and then submitted to denaturing SDS-PAGE and blotting, as previously described,[49] with rabbit polyclonal antibodies directed against *Leishmania* EIF4E4 (described previously.[32]) or mouse monoclonal antibodies directed against the A2 amastigote antigen (kindly provided by Dr Greg Matlashewski, McGill University) or the HA epitope tag (Anti-HA monoclonal antibody, 100 ηg/ml, Applied Biological Materials). For the differentiation/amastigote curves, late-stationary phase cells grown in SDM-79 (24 hours after the growth had stopped) were transferred to MAA medium to a density of 10^6 cells/ml and samples were taken at selected time points for processing, SDS-PAGE and blotting. Transfection procedures used for both circular plasmids (episomal expression) and linear DNA fragments (for integration and gene deletion) were carried out by electroporation as described previously.[50] Cells transfected with the EIF4E4 wild type and mutant constructs were selected with neomycin (G418, 20 μg/ml, Sigma) and those transfected with the linear DNA cassettes for *EIF4E4* gene deletion events with hygromycin B (40 μg/ml, Sigma) or puromycin (70 μg/ml, Sigma).

Sequence analysis

Sequence analysis and alignment were carried out using the ClustalW Multiple alignment tool available within the BioEdit Sequence Alignment Editor software (version 7.0.5.3) and the ClustalW2 website (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The sequences for the selected trypanosomatid EIF4E4 homologues used in the alignment are all available at the TriTrypDB Webpage (http://tritrypdb.org/). Occasional manual refinement of the alignment was performed and the identification of the serine-proline or threonine-proline (SP/TP) motifs was done through visual inspection of the sequences. TriTrypDB accessions: *Leishmania infantum* (Li)-LinJ.30.0460; *L. major* (Lm)-LmjF.30.0450; *L. mexicana* (Lmx)-LmxM.29.0450; *L. braziliensis* (Lb)-LbrM2903_30_0600; *L. tarentolae* (Lt)-LtaP30.0520; *Crithidia fasciculata* (Cf)-CfaC1_29_0520; *Trypanosoma cruzi* (Tc)-TcCLB.509037.40; *T. brucei* (Tb)-Tb927.6.1870.

Plasmid constructs DNA manipulations and Southern blot analysis

The *L. infantum* genomic DNA from the MHOM/MA/67/ITMAP-263 strain was isolated using DNAzol (Life Technologies) following the manufacturer's instructions. The full length wild type *EIF4E4* gene was amplified using primers flanked by sites for the restriction enzymes BamHI and HindIII and with the 3′ primer including a 27 nucleotide extension encoding a single copy of the HA epitope (YPYDVPDYA), immediately before the translation stop codon. Site-directed mutagenesis was carried out through two sets of PCR reactions using the full-length wild type *EIF4E4* gene as template. The first reaction generated a smaller mutagenized fragment, which was subsequently used as a

megaprimer for the second reaction to produce the full-length mutated *EIF4E4* genes. The various mutated genes were also flanked by sites for BamHI and HindIII and included the extension encoding the HA epitope at their 3′ end. Amplified fragments were first cloned into pGEM-T Easy vector (Promega), sequenced and subcloned into BamHI-HindIII sites of pSPBT1YNEOα [51] for expression in *L. infantum* (in this vector, the *EIF4E4* ORF was flanked by the 5′- and 3′-intergenic regions of the biopterin transporter gene 1 - *BT1*). The wild type gene and selected mutants were subsequently subcloned into the same sites of the pET21a vector (Novagen). To generate the constructs for gene deletion, a PCR fusion-based strategy was used as described previously,[52] where 500 bp fragments flanking the coding sequence from the *EIF4E4* gene were joined to the coding sequences of the hygromycin (*HYG*) or puromycin (*PUR*) resistance genes. The resulting amplified fragments were cloned into the pGEM-T Easy vector and sequenced. The vectors were digested with NotI (HYG vector) or EcoRI (PUR vector), and the linear fragments generated were gel purified and used for transfection. All oligonucleotides used for the various amplification and mutagenesis reactions are listed in Supplementary **Tables S1** and **S2**. All the amplified genes were sequenced multiple times after each subcloning step, and found to be identical to the *EIF4E4* gene sequence at the GenBank and TriTrypDB databases, with the exception of the targeted mutated residues, when expected. Southern blots were carried out using standard procedures with probes derived from PCR fragments generated using primers also listed in **Tables S1** and **S2**.

In vitro pull-down assays for measuring protein interactions

Co-precipitation/pull-down assays were essentially performed as described previously,[24] using Glutathione-Sepharose 4B beads (GE Healthcare) and affinity purified GST-tagged recombinant proteins. The production of *L. major* GST-tagged EIF4G3 has been previously described,[31] and since the identity in sequence between the *L. major* and *L. infantum* orthologues is 97%, the *L. major* protein was considered adequate to be used in these assays. GST-tagged *L. infantum* PABP1 was generated after amplification and cloning of its gene into pGEM-T Easy (using primers listed in **Table S3**), sequencing and subcloning into the BamHI and HindIII sites of a modified pGEX4T3 expression vector (GE Healthcare) having a HindIII restriction site inserted immediately before its NotI site. GST alone and both GST-tagged proteins were expressed in *Escherichia coli*, immobilized on the beads and assayed for their ability to bind to ^{35}S-labeled proteins. The labeled proteins, including the various EIF4E4 mutants, were obtained through the linearization of the corresponding pET21a derived plasmids with NotI, followed by transcription with T7 RNA polymerase in the presence of the cap analog and translation in the rabbit reticulocyte lysate (Promega or Ambion) supplemented with ^{35}S-methionine (Perkin Elmer).

Cap binding assay

Wild type EIF4E4 and the mutant W333A proteins were labeled with ^{35}S-methionine as described above after *in vitro* transcription and translation. The cap binding assays were

performed essentially as described previously,[24] using the 7-Methyl-GTP Sepharose 4B beads (GE Healthcare). Non-specific binding was removed by washes with 0.1 mM GTP and specific elution achieved with 50 μM cap analog.

Immunoprecipitation

The immunoprecipitation assays (IPs) were carried out using whole cytoplasmic extracts from *L. tarentolae* recombinant strains expressing selected HA-tagged EIF4E4 variants. These were produced as follows: late exponentially grown cells were harvested and washed once in PBS and once in lysis buffer (20 mM HEPES-KOH, pH7.4, 75 mM potassium acetate, 4 mM magnesium acetate, 2 mM DTT) supplemented with and 1x protease inhibitors (EDTA-free EASYpack Roche); the weight of the pelleted cells was measured and, after their ressuspension in lysis buffer to a concentration of 2-4x10^9 cells/ml, twice that weight of glass beads 150-200 μm in diameter (from Sigma) was added; lysis was carried through vortexing for 15 minutes at 4°C, followed by passaging 5 times through a 30 gauge needle and centrifugation at 13,000g for 15 minutes to remove cellular debris. The supernatants, the cytoplasmic extracts, were mixed with magnetic Dynabeads Protein G (Invitrogen) as per manufacturer's protocol. Briefly, 6 μl of the monoclonal anti-HA antibody was pre-bound to 60 μl of the beads for 1 h at 4°C followed by 2 washes with PBS and another 1 h incubation at 4°C with the soluble proteins (∼10 ODs at 260 nm of the cytoplasmic extracts for each IP). After 4-5 more washes with PBS, the resulting, specifically bound, immunoprecipitated antigen-antibody complexes were eluted in SDS-PAGE sample buffer and analyzed by SDS-PAGE and Western-blotting using antibodies against EIF4E4, EIF4G3 and PABP1 (previously described.[24,53]).

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