The eIF3 complex of *Trypanosoma brucei*: composition conservation does not imply the conservation of structural assembly and subunits function

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ABSTRACT

The multisubunit eukaryotic initiation factor 3 (eIF3) plays multiple roles in translation but is poorly understood in trypanosomes. The putative subunits eIF3a and eIF3f of *Trypanosoma brucei* (*Tb*IF3a and *Tb*IF3f) were overexpressed and purified, and 11 subunits were identified, *Tb*IF3a through l minus j, which form a tight complex. Both *Tb*IF3a and *Tb*IF3f are essential for the viability of *T. brucei*. RNAi knockdown of either of them severely reduced total translation and the ratio of the polysome/80S peak area. *Tb*IF3f and *Tb*IF3a RNAi cell lines were modified to express tagged-*Tb*IF3a and -*Tb*IF3f, respectively. RNAi in combination with affinity purification assays indicated that both subunits are variably required for *Tb*IF3 stability and integrity. The relative abundance of other subunits in the *Tb*IF3f-tag complex changed little upon *Tb*IF3a depletion; while only subunits *Tb*IF3b, i, and e copurified comparably with *Tb*IF3a-tag upon *Tb*IF3f depletion. A genome-wide UV-crosslinking assay showed that several *Tb*IF3 subunits have direct RNA-binding activity, with *Tb*IF3c showing the strongest signal. In addition, CrPV IRES, but neither EMCV IRES nor HCV IRES, was found to mediate translation in *T. brucei*. These results together imply that the structure of *Tb*IF3 and the subunits function have trypanosome-specific features, although the composition is evolutionarily conserved.

Keywords: translation; eukaryotic initiation factor 3; trypanosome

INTRODUCTION

*Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania major* can cause African sleeping sickness, American trypanosomiasis, and Leishmaniasis, respectively. They are unicellular protozoans and cycle between mammals and blood sucking insects. The proliferating forms of *T. brucei* in mammal blood and in tsetse fly intestine are designated bloodstream form (BF) and procyclic form (PF), respectively.

As anciently diverged organisms, trypanosomes possess many unique biological and metabolism features, such as tandem genes arrangement and polycistronic transcription (Opperdoes 1994; Martinez-Calvillo et al. 2004; Siegel et al. 2009). The production of mature mRNAs are through the coupled trans-splicing and polyadenylation (Matthews et al. 1994; Siegel et al. 2010). The resulted cap structure, named cap 4, is unusual and highly methylated in the first four nucleotides following the methylated guanosine (Bangs et al. 1992; Mair et al. 2000), whose full methylation is essential for maximized translation (Zamudio et al. 2009). Accordingly, the cap-binding translation initiation factor of trypanosomes, i.e., eIF4F, has unusual aspects as well, shown by an expanding number of subunit variants and a different combination (Dhalia et al. 2006; Freire et al. 2014; Moura et al. 2015). These unique features imply some trypanosome-specific translation patterns; however, little is known about the translation apparatus of trypanosomes, especially which one initiates translation.

Translation initiation is a complicated and highly ordered process and has been extensively studied in yeast and mammals. The largest translation initiation factor 3 (eIF3) plays multiple roles as a scaffold and a coordinator through the whole process (Hinnebusch 2006, 2014; Aitken and Lorsch 2017). This article is distributed exclusively by the RNA Society for the first 12 months after the full-issue publication date (see http://rnajournal.cshlp.org/site/misc/terms.xhtml). After 12 months, it is available under a Creative Commons License (Attribution-NonCommercial 4.0 International), as described at http://creativecommons.org/licenses/by-nc/4.0/.
Briefly, eIF3 mediates a multifactor complex eIF1–eIF1A–eIF3–eIF5 binding to a 40S ribosomal subunit and then recruits a ternary complex eIF2–GTP-Met-tRNAi to the 40S subunit (Sun et al. 2011; Sokabe et al. 2012). The resulting 43S preinitiation complex (PIC) attaches to the 5′-proximal region of an mRNA through eIF3–eIF4F interaction, and then scans downstream along the mRNA until the initiation codon, where it stops and results in 48S PIC. Finally, eIF5B promotes the joining of a 60S subunit into an 80S initiation complex and starts the translation. Beyond translation initiation, eIF3 has also been implicated to function at termination stage and is required for ribosome dissociation and recycling (Pisarev et al. 2007; Beznoskova et al. 2013).

Mammalian eIF3 is composed of 13 subunits, eIF3a through m. Based on a series of biochemical and cryo-EM reconstruction assays (Pisarev et al. 2008; Zhou et al. 2008; Elantak et al. 2010; Sun et al. 2011; Querol-Audi et al. 2013; des Georges et al. 2015), the structural assembly of human eIF3 and its interaction with the 40S ribosomal subunit have been elucidated clearly. Six PCI domain-containing subunits (eIF3a, c, e, k, l, and m) and two MPN domain-containing subunits, eIF3f and h, are arranged into a stable octamer; eIF3b, i, and g form a separate module, which adheres to the octamer through interacting with eIF3a; eIF3d attaches to the octamer through binding to eIF3e (Zhou et al. 2008; Karaskova et al. 2012; Querol-Audi et al. 2013; Aylett et al. 2015); while eIF3j is just loosely attached to the octamer by potentially interacting with eIF3a and b (Elantak et al. 2010). The PCI/MPN octamer resides on the solvent side of the 40S ribosomal subunit in a five-lobed shape with eIF3a and eIF3c establishing two contact points; the eIF3b–i–g module resides at the mRNA entrance with eIF3b interacting directly with the 40S subunit; eIF3d is located near the mRNA exit (Pisarev et al. 2008; des Georges et al. 2015). eIF3c, e, and d associate into a module and are involved in the eIF4G-binding and the subsequent mRNA recruitment to the ribosome (Villa et al. 2013).

The functions of individual eIF3 subunits have been underscored. Although not fully characterized, many of them appear to have additional functions beyond the scope of their general scaffolding roles in eIF3 and PIC assembly by showing essentiality for normal growth, development, and differentiation (Dong et al. 2004; Liu et al. 2007; Dong et al. 2009; Choudhuri et al. 2013) or over-expression in some disease conditions (Zhang et al. 2007). The underlying mechanisms were proposed to involve the specific RNA-binding activity and the selective translation control shown by some eIF3 subunits, particularly those not essential for protein synthesis and eIF3 activity, such as eIF3d, g, h, i, k, and l, etc. (Masutani et al. 2007; Choudhuri et al. 2013; Yin et al. 2013). RNA-binding assay and target mRNA determination are helpful to elucidate the function of eIF3 subunits. Accordingly, a recent genome-wide UV crosslinking assay showed that four human eIF3 subunits, eIF3a, b, d, and g, could bind specifically to some cell growth control-related mRNAs at the 5′-untranslated regions (5′-UTRs) and thus potentially endow eIF3 with positive or negative translation control on these genes’ expression (Lee et al. 2015). eIF3a has been suggested to regulate translation of a subset of messenger RNAs important for tumorigenesis, metastasis, cell cycle progression, drug response, and DNA repair (Dong et al. 2009).

The composition of trypanosomatid eIF3 was investigated, whereas the structure and function have not been characterized yet. Twelve eIF3 subunits, eIF3a through l, were predicted in T. brucei eIF3 and L. major eIF3 (termed as TbIF3 and LeishIF3, respectively) by deep informatics analysis, and further confirmed by affinity purification and mass spectrometry (MS) assay of the LeishIF3 complex (Rezende et al. 2014; Meleppattu et al. 2015). The gene encoding eIF3m was proposed to be absent in trypanosomatids. Although evolutionarily conserved in complex composition and in some characteristic motifs/domains within various subunits, such as PCI and MPN domains, each LeishIF3 or TbIF3 subunit displays a very low level of sequence identity when compared with their homologs from human and some other lower eukaryotes (Rezende et al. 2014). Among all the subunits, TbIF3f shows the lowest sequence identity at 9% in comparison with human eIF3f, while LeishIF3f shows 29% (Rezende et al. 2014). Meanwhile, it is worth noting that LeishIF3f and TbIF3a proteins lack a large fragment corresponding to ~620-amino acid length of the C-terminal region of human eIF3a, which was supposed to interact with eIF4B (Method et al. 1996) and 18S rRNA (Valasek et al. 2003) directly. These variations imply that eIF3 and the related translation regulation should have trypanosome-specific features, which sets a basis for finding effective targets against trypanosomatids.

Most translations occur in a cap-dependent manner in eukaryotes, while some viral mRNAs and a small number of mammalian cellular mRNAs use an internal ribosomal site (IRES) to initiate translation in a cap-independent manner. Based on the structure and the need for eIFs, IRESs are usually divided into three groups, with the IRESes of cricket paralysis virus (CrPV), hepatitis C virus (HCV), and encephalomyocarditis virus (EMCV) as the representatives of Group I, Group II, and Group III, respectively (Kieft 2008). The CrPV IRES-dependent translation is very simple and the 40S ribosomal subunit is enough to sustain it. In contrast, the translations mediated by HCV or EMCV IRES are complicated. They require not only the 40S subunit but also eIF2 and eIF3. Previous study has shown that human eIF3a and eIF3c use their highly conserved RNA-binding motif to bind to the HCV IRES and thereby promote HCV translation (Sun et al. 2013). Moreover, two more initiation factors, eIF1 and eIF5, are essential for EMCV IRES-initiated translation. It is not clear yet whether IRES could mediate translation initiation in trypanosomes or not; however, any exploration on it will provide more or less clues for
understanding the translation apparatus in these ancient organisms.

In the present study, we experimentally identified the composition of the tagged-TbIF3a and -TbIF3f complexes and estimated the roles of TbIF3a and TbIF3f in cell growth, total translation, IRES-mediated translation, and eIF3 structure assembly. Meanwhile, the subunits possessing RNA-binding activity were also examined using a genome-wide UV cross-linking assay.

RESULTS

Composition of the tagged-TbIF3a or -TbIF3f complexes

The composition of T. brucei eIF3 was identified through affinity purification and MS determination of the tagged TbIF3 subunits (Fig. 1). A MH-TAP tag was fused to the ORF of the putative TbIF3a or TbIF3f at the C terminus (Fig. 1A). After transfection into PF 29–13 cells and selection, the tagged TbIF3 subunit expressed constitutively from the β-tubulin locus driven by an endogenous promoter. IFA assay showed that the tagged TbIF3a and TbIF3f proteins were mainly localized in the cytoplasm (Fig. 1B), consistent with their potential function in translation. The proteins associated with the tagged TbIF3a or TbIF3f were purified and detected according to the procedures shown in Figure 1C. SDS-PAGE and SYPRO Ruby staining showed that these two protein samples shared a very similar band pattern (Fig. 1D). MS detection of each band revealed that these bands corresponded to the putative TbIF3a, b, c, d, e, f, g, h, i, k, and l proteins (Fig. 1D). MS analysis of the purified protein samples with in-solution digestion showed that the unique peptide number and the percent protein coverage of the 11 TbIF3 subunits were comparable and much greater than those of other copurified proteins, whether associated specifically or contaminated (Table 1, Supplemental Tables S1, S2). The putative TbIF3j was not detected, nor was any potential eIF3m-like counterparts. The putative TbIF1 was the only initiation factor detected in the purified TbIF3a- or TbIF3f-TAP complexes (Table 1), suggesting a strong interaction of TbIF1 with TbIF3. Further RNase A treatment did not alter the band pattern of the purified TbIF3a-tag complex and the relative abundance of each subunit (Fig. 1E), confirming that the assembly of TbIF3 is RNA-independent. Altogether, these results experimentally verified the composition of TbIF3, with each identified subunit exactly matching the bioinformatics prediction. Meanwhile, our results suggest that the identified 11 TbIF3 subunits associate tightly into a stable complex.

TbIF3a and TbIF3f are essential for viability

The essentiality of TbIF3a and TbIF3f for growth was assessed in PF and BF stages of T. brucei (Fig. 2). Four tet-inducible RNAi cell lines, including PF TbIF3a-RNAi, BF TbIF3a-RNAi, PF TbIF3f-RNAi, and BF TbIF3f-RNAi, were generated. The presence of tet induces the synthesis of dsRNAs targeting TbIF3a or TbIF3f (Fig. 2A). The growth of all the RNAi-induced cells, whether in PF or BF life stages, was severely inhibited; in contrast, the RNAi noninduced cells grew normally (Fig. 2B–E). Northern blot analysis revealed that the mRNA levels of TbIF3a or TbIF3f reduced significantly after RNAi induction for 48 h (Fig. 2F). Overall, significant growth inhibition of PFs and BFs upon repression of either TbIF3a or TbIF3f expression indicates that these two TbIF3 subunits are essential for the viability of both life stages of T. brucei.
FIGURE 2. *TbIF3a* and *TbIF3f* are necessary for the growth of PF and BF *T. brucei*. (A) Schematic representation of the dsRNA-expressing vectors. The number represents the location of the dsRNA sequence within the coding region of *TbIF3a* or *TbIF3f*. Growth curves of PF *TbIF3a-RNAi* (B), BF *TbIF3a-RNAi* (C), PF *TbIF3f-RNAi* (D), and BF *TbIF3f-RNAi* (E) cell lines in which RNAi knockdown *TbIF3a* or *TbIF3f* was induced (I) or noninduced (NI). (F) Northern blot analysis of the RNAi knockdown efficiency with the probe against the 5′-proximal coding region of *TbIF3a* or *TbIF3f*. NI and I-48 represent RNAi noninduced and induced for 48 h, respectively. EtBr-stained rRNA was used to show the loading. The targeted mRNAs are indicated on the right. (G) Growth of PF *TbIF3a-RNAi-hsalF3a TAP* cell line with RNAi induced (NI) or noninduced (I). (H) Western analysis of hsalF3a-TAP in the cell lysate from PF *TbIF3a-RNAi* cells or from PF *TbIF3a-RNAi-hsalF3a TAP* cells in which RNAi was noninduced (NI) or induced for 48 h (I-48 h) with rPAP reagent. Cumulative cell number represents the cell density multiplied by total dilution.

**TABLE 1.** Composition of the tagged-*TbIF3a* and -*TbIF3f* complexes

| *TbIF3* subunit | Gene ID | Tagged-*TbIF3a* | Tagged-*TbIF3f* |
|-----------------|---------|----------------|----------------|
|                 |         | Uni pep # | Coverage (%) | Uni pep # | Coverage (%) |
| a               | Tb927.7.6090 | 34        | 41.6         | 21        | 29.4         |
| b               | Tb927.5.2570 | 31        | 53.16        | 20        | 29.74        |
| c               | Tb927.10.8270/8290 | 27        | 43.11        | 13        | 18.78        |
| d               | Tb927.6.4370 | 25        | 43.28        | 15        | 31.72        |
| e               | Tb927.11.11590 | 13    | 53.51        | 6         | 24.94        |
| f               | Tb927.3.1680 | 17        | 60.38        | 11        | 50           |
| g               | Tb927.4.1930 | 17        | 48.53        | 10        | 37.13        |
| h               | Tb927.8.1170/1190 | 8    | 25.38        | 6         | 22.36        |
| i               | Tb927.11.9610 | 10        | 42.98        | 7         | 28.95        |
| k               | Tb927.11.15420 | 12       | 43.9         | 6         | 28.78        |
| l               | Tb927.10.4640 | 17       | 39.34        | 7         | 17.42        |
| j               | Tb927.3.2220 | ND        | ND           | ND        | ND           |
| k               | Tb927.11.5840 | 4        | 42.2         | 1         | 9.17         |

Uni pep # represents the number of the unique peptides; coverage (%) represents the percent protein sequence coverage by the identified unique peptides.
The effect of human eIF3a (hsaIF3a) expression on the growth deficiency upon TbIF3a depletion was estimated. hsaIF3a-TAP was introduced into the β-tubulin locus of PF TbIF3a-RNAi, resulting in the cell line PF TbIF3a RNAi-hsaIF3a TAP. Its growth was normal in the absence of tet but inhibited severely in the presence of tet (Fig. 2G), very similar to that of PF TbIF3a-RNAi cells (Fig. 2B). Further Western analysis indicated that the expression of hsaIF3a was well and not affected by the expression of dsRNA against TbIF3a (Fig. 2H). Therefore, the growth deficiency that resulted from TbIF3a depletion could not be rescued by its human homolog.

**RNAi knockdown of TbIF3a or TbIF3f inhibits translation initiation**

The functions of the putative TbIF3a and TbIF3f in translation initiation were investigated by performing 35S-Met incorporation assay and polysome profile analysis (Fig. 3). PF TbIF3a-RNAi or TbIF3f-RNAi cells in which RNAi was not induced or induced for 24 or 48 h were pulse-labeled with 35S-Met for 60 min. The cell extracts from equal numbers of cells were examined by SDS-PAGE. Coomassie blue staining of the gel showed that the total protein level changed little upon depletion of either TbIF3a or TbIF3f (Fig. 3A, lower panel), while an autoradiograph of the same gel revealed that the newly synthesized proteins reduced significantly at 24 h after RNAi induction and much more at 48 h (Fig. 3A, upper panel). Further scintillation counting the TCA precipitates showed that the radioactivity of the cells with RNAi induced for 24 or 48 h reduced to less than 40% or 8%, respectively, compared to the value of the cells with RNAi noninduced (Fig. 3B). The big reduction in 35S-Met incorporation therefore indicates that depletion of either TbIF3a or TbIF3f blocks total protein synthesis.

The inhibiting effect of TbIF3 subunit depletion on protein synthesis was also readily seen when polysome profiles were determined via sucrose-gradient centrifugation. Following RNAi knockdown of either TbIF3a or TbIF3f for 48 h, polysomes were severely dropped and 80S ribosomes increased dramatically according to the change of UV absorbance at 254 nm and the rRNA distribution in the gradient fractions (Fig. 3C). Given that a reduction in the polysome-to-monosome ratio is a hallmark of impaired translation initiation rates, these results together indicate that the putative TbIF3a and TbIF3f are required for translation initiation and TbIF3 activity.

**TbIF3a or TbIF3f knockdown exhibits a different effect on the structure assembly of TbIF3**

The role of TbIF3a in the formation of TbIF3 was estimated by analyzing the composition of the tagged-TbIF3f complex upon depletion of TbIF3a by RNAi, and vice versa (Fig. 4). The PF RNAi cell line TbIF3a-RNAi or TbIF3f-RNAi was modified to result in the cell line 3a RNAi-3f TAP or 3f RNAi-3a TAP, which expresses TAP-tagged TbIF3f or TbIF3a constitutively from the β-tubulin locus, respectively. Equal numbers of each cell line with or without RNAi induced for 48 h were harvested and lysed individually. Western analysis of the cell lysates showed that the tagged-TbIF3f or TbIF3a reduced moderately upon TbIF3a or TbIF3f depletion, respectively (Fig. 4A), suggesting an essentiality of

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**FIGURE 3.** Depletion of either TbIF3a or TbIF3f results in translation inhibition. 35S-Met was added to the PF TbIF3a-RNAi or TbIF3f-RNAi cells in which RNAi was not induced or induced for 24 or 48 h. After incubation for 60 min, the cells with equal numbers were collected and lysed. (A) The clear cell lysates were separated by SDS-PAGE followed by Coomassie blue staining (lower panel) and autoradiograph (upper panel). (B) The radioactivity of the newly synthesized proteins was measured by scintillation counting of the TCA-precipitated proteins. The assays were repeated three times and the results were shown as mean ± SD. (C) Polysome profile analysis of PF TbIF3a-RNAi and TbIF3f-RNAi cells in which RNAi was not induced (NI) or induced for 48 h (I-48 h) through sucrose-gradient centrifugation. Ninety-six fractions were taken and measured absorbance at 254 nm. Every eight fractions were combined, and RNAs were extracted and separated on agarose gel followed by EtBr staining to visualize rRNAs. The positions of 80S ribosome and polysomes are indicated.
These two subunits for TblF3 stability. Sucrose-gradient centrifugation followed by Western analysis showed that TblF3a depletion did not change the distribution of TblF3f-TAP in the gradient fractions (Fig. 4B, upper panel); while TblF3a-TAP up-shifted upon TblF3f depletion (Fig. 4B, lower panels), suggesting that TblF3f, but not TblF3a, should be critical for the integrity of TblF3. This speculation was further supported by the composition change of TblF3 upon repression of the subunit expression. As shown in Figure 4C, almost all other subunits copurified with the tagged-TblF3f protein upon depletion of TblF3a, suggesting that TblF3a may be located at the periphery of the TblF3 complex or not critical for the assembly of TblF3. In contrast, only subunits TblF3b, e, and i were clearly observed to copurify with the tagged-TblF3a protein upon depletion of TblF3f, while other subunits were almost indiscernible (Fig. 4D). This result suggested a stable subcomplex composed of subunits a, b, i, and e, which is somewhat different from other eukaryotic elf3 structure models in which elf3a, b, i, and g form a stable subcomplex (Dong et al. 2013; Wagner et al. 2014), implying a distinctive TblF3 in structural assembly. More importantly, this result revealed a critical scaffolding role of TblF3f. Therefore, TblF3a and TblF3f function differently in TblF3 structure assembly, and their respective roles appear to be somewhat distinct from their mammalian counterparts.

CrPV IRES could mediate translation in *T. brucei* in the presence of TblF3a and TblF3f

To examine whether or not IRES could initiate translation in *T. brucei*, the expression of different IRES-containing dual-reporters were detected in PF 29-13 cells. These dual-reporter vectors were pHD1344-based, with an upstream *Rluc* ORF and a downstream *Fluc* ORF linked by one out of EMCV IRES, HCV IRES, and CrPV IRES (Fig. 5A). Three cell lines, PF luc-EMCV IRES, PF luc-HCV IRES, and PF luc-CrPV IRES, were generated in which *Rluc* was expressed in a cap-dependent manner while *Fluc* in an IRES-dependent manner. Three clones from each cell line were examined for the reporters’ expression at mRNA and protein levels. As shown in Figure 5B, the Rluc activity values were similar among all these IRES-harborling clones and much higher than the background signal of the PF 29-13 cell; while the Fluc activity values in any of these EMCV IRES- or HCV IRES-containing clones were almost equal to the background signal. However, the Fluc activity values from CrPV IRES-containing cells were much greater. Total RNAs were isolated from one random clone of each cell line and used for reporters mRNA measurement, with 18S rRNA used as an internal control. The results showed that the relative mRNA levels of *Rluc* or *Fluc* were similar in all these tested clones (Fig. 5C), suggesting that the presence of IRES has no effect on transcription and mRNA stability. Northern blot analysis of the cell PF luc-CrPV IRES showed that the only hybridizing band of *Rluc* mRNAs and that of *Fluc* mRNAs were located at the same position with a size ranging from 3 to 4 kb (Fig. 5D), corresponding to the nucleotide length of *Rluc*-CrPV IRES-*Fluc* from the reporter plasmid. This result indicated that the mRNAs of *Rluc* and downstream *Fluc* are present as dicistronic *Rluc*-CrPV IRES-*Fluc* transcripts in the cell PF luc-CrPV IRES, and CrPV IRES should be responsible for the *Fluc* translation. Therefore, CrPV-IRES, but neither HCV-IRES nor EMCV-IRES, could initiate the translation in *T. brucei*, which suggests that elf3s involved in IRES-mediated translation between trypanosomes and mammals may be functionally different.

The effect of TblF3a and TblF3f repression on CrPV IRES-mediated translation was estimated. The CrPV IRES-containing dual-reporter vector was transfected and integrated into the β-tubulin locus of PF TblF3a-RNAi and TblF3f-RNAi cells and resulted in 3a RNAi-CrPV IRES and 3f RNAi-CrPV IRES.
RNAi-CrPV IRES cell lines, respectively. Similarly, three clones from each cell line were examined for the reporters’ expression by luciferase activity assay. As shown in Figure 5E, down-regulation of either TbIF3a or TbIF3f by RNAi did not alter the Rluc protein level but reduced the Fluc proteins to 50% compared to the RNAi noninduced cells. Real-time PCR analysis of the RNA samples from a random clone of either cell line showed that both Fluc and Rluc mRNA abundances were not affected upon TbIF3a or TbIF3f knockdown (Fig. 5F). Therefore, these results indicate that depletion of either TbIF3a or TbIF3f could result in repression of CrPV IRES-mediated translation.

**TbIF3 subunits TbIF3a, c, d, and h exhibit RNA-binding activity**

Whether and which TbIF3 subunit(s) could bind to RNA directly was detected by using the CLiTAP approach, as described previously (Yue et al. 2014). As shown in Figure 6A, the TbIF3a-TAP complex was purified from the PF TbIF3a-TAP cells after UV crosslinking. After RNase T1 treatment and TAP purification, the bound RNAs were labeled with [γ-32P]ATP. The purified complexes were resolved by SDS-PAGE. SYPRO Ruby staining was compared to the autoradiograph of the same gel, and the result showed that subunits TbIF3a, c, d, and h crosslinked directly to RNA with TbIF3c exhibiting the strongest signal; the overlapped TbIF3f and g proteins band corresponded to a very weak radioactive signal, suggesting a potential RNA-binding activity of f and/or g; while TbIF3e, i, k, and l did not show any visible radioactive signals, suggesting no direct RNA-binding activity. In addition, the RNA-binding activity of TbIF3b remained elusive because of the potential RNA signal overlapping between b and c (Fig. 6B). Overall, this genome-wide UV crosslinking assay indicates that at least four TbIF3 subunits, including TbIF3a, c, d, and h, have direct RNA-binding activity.
In the present study, we experimentally confirmed that the eIF3 complex of *T. brucei* is composed of 11 subunits, consistent with the previous bioinformatics prediction (Rezende et al. 2014), except that *TbIF3j* was not detected. Since the *TbIF3j* counterpart, *LeishIF3j*, was found in the *Leishmania* pull-down complex with a relatively low protein abundance factor, the missing detection of *TbIF3j* adds to the evidence that eIF3j may be loosely associated with the eIF3 complex in trypanosomes as well. Importantly, we demonstrated here that both *TbIF3a* and *TbIF3f* are structurally and functionally essential for *TbIF3*, although *TbIF3f* was not identified by deep informatics analysis due to an extremely low sequence identity to its homologs (Rezende et al. 2014). RNAi knockdown of either *TbIF3a* or *TbIF3f* could significantly inhibit growth of PFs and BFs (Fig. 2B–E) and shut down the translation initiation (Fig. 3). At translational termination stage, eIF3 is a principal factor that can promote both splitting of post-termination ribosomes into 40S and 60S subunits and initiation of a new round of translation (Kolupaeva et al. 2005; Pisarev et al. 2007; Beznoskova et al. 2013). Polysome profile analysis showed that depletion of either *TbIF3a* or *TbIF3f* resulted in significant accumulation of 80S ribosomes and dramatic reduction of polysomes, while no absorbance increase was observed in the potential 40S and 60S ribosome subunits area (Fig. 3C). Therefore, *TbIF3a* and *TbIF3f* subunits may be necessary for ribosome dissociation in *T. brucei*, and *TbIF3* should play a critical role in ribosome recycling.

Structural characterizations of the tagged *TbIF3* complex by reciprocally expressing *TbIF3a*-TAP and *TbIF3f*-TAP in *TbIF3f* RNAi and *TbIF3a* RNAi cells, respectively, reveal that these two subunits play different roles in the stability or integrity of *TbIF3* structure. *TbIF3a* knockdown caused a modest reduction in the tagged-*TbIF3f* protein level but had little effect on the composition of the residual *TbIF3f*-TAP complex (Fig. 4A–C), suggesting that *TbIF3a* is required for the stability of *TbIF3* but not indispensable for the integrity and the structure assembly. In stark contrast, mammalian eIF3a plays a critical scaffolding role in the octamer formation and is necessary for the entire eIF3 complex assembly (Masutani et al. 2007). Human eIF3a knockdown disrupted the entire eIF3 complex and severely reduced the octamer subunits (Wagner et al. 2014). The functional difference between *TbIF3a* and its mammalian homolog can be somewhat reflected by their sequence variations. Although the characteristic PCI domain, Spectrin repeats, and the putative RNA-binding motif are conserved (Wagner et al. 2014), the overall sequence identity is as low as 22%. Moreover, *TbIF3a* is just a little more than half the length of human eIF3a, while the function of this missing sequence has not been well characterized yet. We thus speculate that human eIF3a and *TbIF3a* are not functionally replaceable. As expected, expression of human eIF3a in PF *TbIF3a*-RNAi cells could not restore the growth defect of *T. brucei* upon *TbIF3a* repression by RNAi (Fig. 2G).

Distinct from the moderate impact of *TbIF3a* knockdown, *TbIF3f* knockdown caused the upshift of *TbIF3a* in the sucrose-gradient fractions and the disruption of the *TbIF3a*-TAP complex, and only *TbIF3b*, i, and e were clearly observed to copurify with *TbIF3a* (Fig. 4B,D). These results indicate that *TbIF3f* is essential for *TbIF3* stability and integrity and plays a principal role in *TbIF3* structure assembly. In contrast, mammalian eIF3f interacts with eIF3h directly, and seems not indispensable for other eIF3 subunits assembly, based on previous reports (Zhou et al. 2008; Pukala et al. 2009). In addition, *TbIF3f* knockdown resulted in polysome reduction and translation initiation inhibition (Fig. 3), while human eIF3f depletion promoted the translation initiation (Wen et al. 2012). In all, these observations indicate the functional divergence of *TbIF3a* and *TbIF3f* from their higher eukaryotic orthologs, implying a trypanosome-specific structural assembly of eIF3.

The absence of the eIF3m-encoding gene in *T. brucei* also highlights the specific structural features of *TbIF3*. As a PCI subunit, mammalian eIF3m forms a compact trigonal sub-complex with the MPN dimer eIF3f–eIF3h and mediates the association with the octamer mainly through eIF3f–eIF3m interaction (Zhou et al. 2005, 2008). eIF3m deficiency significantly down-regulated the subunits eIF3f, h, and c, and impaired the integrity of eIF3 (Zeng et al. 2013). Since eIF3m is essential for eIF3 structure and function in mammals, the absence of eIF3m (Rezende et al. 2014; Meleppattu et al. 2015) and the low sequence similarity of other eIF3 subunits suggest that the eIF3 of trypanosomatids should have unique structure and diverged subunits function. We speculate that eIF3m may have evolved for some specific functions in translation control under some particular conditions. However, more experiments are required to
decipher the roles of the individual subunit in TbIF3 structure and activity.

A previous study has shown that eIF3 can interact with eIF1, eIF1A, eIF2, and eIF5 directly to form a multifactor complex (MFC), which promotes the association of 40S ribosomal subunits and eIF2-GTP-Met-tRNAi into 43S PIC (Sokabe et al. 2012). The existence of this MFC has also been suggested in L. major based on the observation that LeishIF1, Leish1A, LeishIF2, and LeishIF5 could copurify with LeishIF3 (Meleppattu et al. 2015). Among them, the interaction between LeishIF1 and LeishIF3 is the strongest, with the relative protein abundance factor of LeishIF1 higher than other initiation factors. Consistently, we discerned a coprecipitation of the putative TbIF1 with the tagged-TbIF3a complex and the tagged-TbIF3f complex, but no other MFC components were identified, nor other eIFs interacting with eIF3 directly, such as eIF4G (Villa et al. 2013). The association of TbIF1 with TbIF3, however, adds to evidence that MFC is potentially present in trypansomatids. The under-detection of other eIFs in our purification may be due to the transient or weak interaction and/or stringent two-step affinity purification. This speculation was supported by the missing TbIF3j in the purified TbIF3 complex, while its L. major homolog was detected in the purified LeishIF3 complex (Meleppattu et al. 2015).

The dual-reporter system containing different types of IRES works well in mammalian cells (Zhu et al. 2012 and references therein). However, luciferase activity assay and RNA analysis showed that only CrPV IRES, but neither EMCV IRES nor HCV IRES, could initiate reporter translation in T. brucei (Fig. 5B–D). These results suggest that the eIFs involved in EMCV IRES- or HCV IRES-mediated translation, including eIF1, eIF2, and eIF3 (Kieft 2008), should be different from their mammalian counterparts in composition, subunits arrangement or function. Given that the presence of a 40S ribosomal subunit is enough to initiate CrPV-IRES-mediated translation, we propose that the mechanism of TbIF3a or TbIF3f knockdown inhibiting the CrPV-IRES-mediated translation can be attributed to the accumulation of 80S ribosomes and the potential decrease of 40S subunits. Although the T. brucei ribosome has an unusually large and unique arrangement of expansion segments revealed by a high-resolution cryo-EM (Hashem et al. 2013), this unusual structure did not interfere with the translation of CrPV IRES-mediated translation.

Direct RNA-binding activity is indispensable for eIF3 functioning in translation initiation and translation control. The genome-wide UV crosslinking experiment has shown that several TbIF3 subunits have direct RNA-binding activities with various efficiencies (Fig. 6B). The most notable is TbIF3c, which exhibits a much stronger RNA-binding signal than other RNA-binding subunits. In contrast, its human homolog did not show any RNA-binding activity in a genome-wide UV crosslinking experiment (Lee et al. 2015). However, a highly conserved helix-loop-helix (HLH) RNA-binding motif (RRM) was predicted in eIF3c, which has been verified to contribute to the binding to the IIabc domain within HCV IRES and direct HCV IRES-dependent translation together with TbIF3a (Sun et al. 2013). Sequence alignment between TbIF3c and human eIF3c showed that their overall sequence identity was as low as 20.4% and the HLH domain was not predicted in TbIF3c by Phyre2 (Supplemental Fig. S1), a protein structure prediction software (Kelley and Sternberg 2009). These results suggest that TbIF3c may use different RNA-binding motif(s) for cellular mRNA binding. Further determination and characterization of the RNAs bound to TbIF3a, c, d, and h will be helpful to uncover the mechanisms of TbIF3 underlying translation initiation and translation control in the species of Trypanosomatids.

In conclusion, our data experimentally highlight the trypanosome-specific structural assembly of TbIF3 and subunit function, although the composition is conserved evolutionarily from protozoan to mammals. These findings provide a basis for future investigation of translation in trypanosomes, and suggest that TbIF3a and TbIF3f can be potential targets for developing new drugs against trypanosomes.

MATERIALS AND METHODS

Plasmid construction

To create the vectors expressing C-terminally TAP-tagged proteins, T. brucei 427 genomic DNA was used as a template to amplify the full-length open reading frames (ORFs) of TbIF3a and TbIF3f by using the primers 5′-CACCTCGAGCATGTTGCAAGCGGAAGTA-3′ plus 5′-CACGGATCCCTTCCCTTGTAGGCGCTC-3′ and the primers 5′-CACCTGGAGATCGAAATTCGCTGTGT-3′ plus 5′-CACGGATCCACCGGATTATTCCTCCT-3′, respectively. After digestion with SalI and XhoI, the PCR products were cloned into the similarly digested pHD1344-MHTAP (Guo et al. 2012), which contains a MH-TAP-coding region following the cloning sites (Fig. 1A), to generate the constructs pHD1344-3a-TAP and pHD1344-3f-TAP. The plasmids expressing tetracycline (tet)-inducible RNAi for TbIF3a or TbIF3f were constructed as follows: A 600- or 616-bp fragment corresponding to the middle region of TbIF3a ORF or the 5′-terminal region of TbIF3f ORF (Fig. 2A) was amplified from the genomic DNA by using the primers 5′-CACCAAGCTTTTGAACCTCTGAGAGG-3′ and 5′-CACTCGAGTACGCTGCATTAGCG-3′ or the primers 5′-CACAGCTGGCCAGATCAAGCT-3′ and 5′-CACCTGGAGATCCTACTGCCTG-3′, respectively. The PCR products were digested with HindIII and HindIII and inserted into similarly digested pZ2M (Wang et al. 2000) to create pZ2M-3a and pZ2M-3f. Human genomic DNA was extracted from 293T cells and eIF3a was amplified by using RT-PCR with the primers 5′-CACCTGGACATGCACCGGCGCTT-3′ and 5′-CACTCGATCATCTAGGTCGTTCTGCAC-3′. The PCR products were digested with SalI and BglII and then cloned into similarly digested pHD1344-MHTAP to create pHDI344-hsIF3a TAP. The PCR product containing the 5′-UTR of TbIF3a and TbIF3f was amplified by using the primers 5′-CACAGCTGGACATGCACCGGCGCTT-3′ and 5′-CACTCGATCATCTAGGTCGTTCTGCAC-3′ and then cloned into similarly digested pHD1344-MHTAP to create pHDI344-hsIF3a TAP
amplify the Renilla luciferase (Rluc)-CrPV IRES-firefly luciferase (Fluc), Rluc-HCV IRES-Fluc, and Rluc-EMCV-IRES-Fluc from the plasmids pNL4-3RL-CrPV-FL, pNL4-3RL-HCV-FL, and pNL4-3RL-EMCV-FL (Zhu et al. 2012), respectively. After digestion with Sall and BamHI, these PCR products were inserted into the plasmid pHD1344tub (Carnes et al. 2005) digested with XhoI and BamHI to generate pHD1344-Dual-luc-CrPV IRES, pHD1344-Dual-luc-CrPV IRES, and pHD1344-Dual-luc-CrPV IRES. The restriction sites are underlined. The plasmids pHD1344-MHTAP (Guo et al. 2012) and pHD1344tub (Carnes et al. 2005) target the integration of the inserted genes into β-tubulin locus.

**Cell culture and cell line generation**

The starting cell lines *T. brucei* PF 29-13 and BF single-marker (BF-SM) were maintained in SDM-79 medium and HMI-9 medium, respectively, supplemented with 10% fetal bovine serum and selection marker(s) as described previously (Wirtz et al. 1999). The plasmids pHD1344-3a-TAP and pHD1344-3f-TAP were linearized by NotI and individually transfected into PF 29-13. After selection with puromycin (1 µg/mL), the resulting stable cell lines were designated PF THF3a-TAP and THF3f-TAP. Expression of the tagged genes was determined by Western blot. To generate tet-inducible RNAi cell lines in both life stages of *T. brucei* were transfected independently with 10 µg of NotI-linearized pZJM-3a or pZIM-3f. The resulted pheomycin-resistant clones were named PF THF3a-RNAi, BF THF3a-RNAi, PF THF3f-RNAi, and BF THF3f-RNAi. The PF THF3a RNAi-βtubulin TAP cell line was generated by transfecting the NotI-linearized pHD1344-βtubulin3a TAP into the PF THF3a-RNAi cell line. The PF 3a RNAi-βtubulin3aTAP and 3f RNAi-3aTAP cell lines were generated by transfecting pHD1344-3f-TAP and pHD1344-3a-TAP into the PF RNAi cell lines THF3a-RNAi and THF3f-RNAi, respectively. The expression of these tagged genes was confirmed by Western blot. Ten micrograms of NotI-linearized pHD1344-CrPV IRES-luc, pHDI344-HCV IRES-luc, or pHD1344-EMCV IRES-luc were transfected into PF 29-13, and the puromycin-resistant clones were designated PF luc-CrPV IRES, PF luc-HCV IRES, or PF luc-EMCV IRES, respectively. The linearized pHDI344-CrPV IRES-luc was additionally transfected into PF THF3a-RNAi and THF3f-RNAi cells, the resulting resistant clones were named PF 3a RNAi-CrPV IRES and 3f RNAi-CrPV IRES, respectively.

**Cell RNA isolation and Northern blot**

Total RNA was isolated from the RNAi cells in which RNAi was induced or induced for 48 h, or from PF 3a RNAi-CrPV IRES and PF 29-13 cells, using the TRIzol Reagent (Life Technologies) according to the manufacturer’s instructions. Twenty micrograms of RNA from each sample was resolved on a formaldehyde-containing 1.2% agarose gel and then transferred to a Hybond N+ membrane (GE Amersham Biosciences). The RNA samples from RNAi cells were hybridized with the DIG-labeled probe specific to THF3a or THF3f. The RNAs from PF 3a RNAi-CrPV IRES and PF 29-13 cells were hybridized with the labeled probe specific to Fluc or Fluc (Fig. 5A), and then stripped and rehybridized with the probe specific to α-tubulin, which was used as a control. Probe labeling and signal detection were conducted with DIG High Prime DNA Labeling and Detection Starter Kit II according to the manufacturer’s instructions (Roche Diagnostics). The rRNAs were observed through ethidium bromide (EtBr) staining.

**Real-time reverse transcriptase PCR (real-time RT-PCR)**

Real-time RT PCR was carried out to measure the mRNA levels of *Rluc* and *Fluc*. Total RNAs were isolated from the cells expressing different IRES-containing reporters in the absence or presence of tet for 48 h as described above. Five micrograms of RNA were treated with DNase I (Promega) and the integrity was confirmed by visualizing on an agarose gel. The cDNA templates for real-time PCR were reversely transcribed from 2 µg treated RNA by using random hexamers and PrimeScript RT reagents (TaKaRa). Control reactions without reverse transcriptase were performed to eliminate genomic DNA contamination. The cDNA reaction mixtures were diluted 1:5 in water as the template for PCR detection of *Rluc* and *Fluc*, and

**Immunofluorescence assay (IFA)**

Subcellular localization of the tagged THF3a or THF3f was determined by IFA as described previously (Lerch et al. 2012). The rabbit polyclonal antibody against c-myc (Sigma-Aldrich) and the FITC-labeled anti-rabbit IgG (Sigma-Aldrich) were used as primary and secondary antibody, respectively, to visualize the tagged proteins.

**Real-time reverse transcriptase PCR (real-time RT-PCR)**

Real-time RT PCR was carried out to measure the mRNA levels of *Rluc* and *Fluc*. Total RNAs were isolated from the cells expressing different IRES-containing reporters in the absence or presence of tet for 48 h as described above. Five micrograms of RNA were treated with DNase I (Promega) and the integrity was confirmed by visualizing on an agarose gel. The cDNA templates for real-time PCR were reversely transcribed from 2 µg treated RNA by using random hexamers and PrimeScript RT reagents (TaKaRa). Control reactions without reverse transcriptase were performed to eliminate genomic DNA contamination. The cDNA reaction mixtures were diluted 1:5 in water as the template for PCR detection of *Rluc* and *Fluc*, and
further diluted 1:50 for amplification of the internal control β-Tubulin. The sequences of the primers for Rluc and Fluc are 5′-ATACTGGCCAGCTGTTG-3′ plus 5′-GGCGGCGGTATTCCA TGAAA-3′ and 5′-TTGTTTTGAGCAAGGAAGAC-3′ plus 5′-AAAGACCTTCCGTACTCGTCC-3′, respectively. The sequences for β-Tubulin have been described previously (Carnes et al. 2005). Each PCR reaction contained 2 µL of cDNA, 8 µL of forward and reverse primers (each at 0.75 µM), and 10 µL of SRBR green PCR Supermix (Bio-Rad). The amplification condition was 95°C for 5 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec, using Bio-Rad CFX96 thermocycler. Each reaction was carried out in triplicate. Thermal dissociation curves confirmed the PCR generated a single amplicon. The target mRNA levels were normalized to β-Tubulin.

35S-Met pulse labeling assay

The pulse labeling and incorporation counting assay was carried out according to the previous description with some modifications (Dhalia et al. 2006). Briefly, PF TbIF3α-RNAi and TbIF3f-RNAi cells in which RNAi was not induced as a control, or induced for 24 or 48 h, were pulse-labeled with 100 µCi of 35S-Met/mL for 60 min at 27°C. Labeling was terminated by adding 3 volumes of stop solution (1.2 mg/mL methionine and 0.1 mg/mL cycloheximide [CHX] suspended in PBS). Some cells were lysed directly with 1× SDS-PAGE loading buffer and loaded onto SDS-PAGE gels at 1×106 cells/lane. Gels were stained, fixed and dried, and radiolabeled proteins were detected by autoradiography. Meanwhile, some cells were lysed with buffer IPP150 (Rigaut et al. 1999) plus 1% Triton X-100 followed by trichloroacetic acid (TCA) precipitation, and the incorporated radioactivity was counted in a Beckman LS 6000 IC scintillation counter. Parallel incubations in the presence of 50 µg/mL CHX and counting were performed to eliminate the incorporation of radiolabel by processes other than cytoplasmic protein synthesis. Protein concentration was determined using Protein Assay Dye Reagent Concentrate (Bio-Rad) following the manufacturer's instructions. The residual 9 mL cell lysates were used for TAP purification. Similarly, the whole-cell lysates of PF TbIF3a-RNAi or TbIF3a-RNAi-hsulIF3a TAP were separated on 8% SDS-PAGE gel for Western blot of the protein A-tagged hsulIF3a.

Polysome profile analysis

Of note, 2 × 109 of PF TbIF3a RNAi or TbIF3f RNAi cells in which RNAi was not induced or induced for 48 h were harvested immediately after addition of CHX to 0.1 mg/mL, and lysed with the buffer A (10 mM, pH 7.5, Tris–HCl, 10 mM MgCl2, 200 mM KCl, 100 µg/mL cycloheximide, 1 mM DTT) supplemented with 1% Triton X-100 and complete protease inhibitors (Roche). After centrifugation at 16,000g at 4°C for 15 min, the clear lysate was loaded onto 11 mL linear sucrose gradient (5% to 45% sucrose in the buffer A) and centrifuged at 38,000 rpm for 2.5 h at 4°C in a SW40 Ti rotor. Ninety-six gradient fractions were collected from the bottom to top by puncturing the centrifuge tube at the bottom, ~0.1 mL per fraction. Polysome profiles were obtained by monitoring the absorbance of each fraction at 254 nm. Every eight fractions were combined and total RNAs were isolated by using TRIzol LS reagent (Life Technologies) according to the manufacturer’s instructions. The sedimentation profiles of rRNA were monitored on an agarose gel by EtBr staining.

Sucrose-gradient centrifugation and Western blot

Linear sucrose-gradient was made in buffer IPP150 instead of buffer A as described above. A total of 1 × 1010 of PF 3aRNAi-3fTAP or 3f RNAi-3a TAP cells with RNAi noninduced or induced were harvested and lysed with 10 mL buffer IPP150 containing 1% Triton X-100. One milliliter of the clear cell lysate was loaded onto the sucrose gradient and centrifuged as described above. Twelve fractions were collected from top to bottom and loaded on a 10% SDS-PAGE gel and transferred onto a PVDF membrane. The membrane was blocked in 10% nonfat milk powder in PBST (10 mM phosphate buffer, pH 7.2, 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature, and then washed with PBST and probed with rPAP reagent (Sigma-Aldrich) against the protein A-tagged TbIF3α or TbIF3f (1:1000) for 1 h at room temperature. The membrane was washed with PBST and the proteins were visualized using the ECL system. The residual 9 mL cell lysates were used for TAP purification. Similarly, the whole-cell lysates of PF TbIF3a-RNAi or TbIF3a-RNAi-hsulIF3a TAP were separated on 8% SDS-PAGE gel for Western blot of the protein A-tagged hsulIF3a.

Luciferase reporter assay

Cell lysates were prepared using passive lysis buffer (Promega) and Rluc and Fluc luciferase activity was measured using the Dual Luciferase Assay kit (Promega) according to the manufacturer’s instructions.

UV cross-linking combination with tandem affinity purification (CLiTAP)

CLiTAP was carried out to assess whether and which TbIF3 subunit could bind to RNA directly as described previously (Yue et al. 2014). Briefly, 2 × 109 of PF TbIF3α-TAP cells were harvested and irradiated by UV light at 254 nm followed by lysis with buffer IPP150 plus 1% Triton X-100, RNase T1 treatment and clarification by centrifugation. Through TAP purification, the TbIF3α-TAP complexes attached on the calmodulin beads were treated again with RNase T1 followed by [γ-32P]ATP (PerkinElmer) labeling RNA. Then the beads were boiled in 1× SDS-PAGE loading buffer and the denatured protein sample was separated on 10% SDS-PAGE gel followed by SYPRO Ruby staining according to the manufacturer’s instructions (Life Technologies) and the radioactive signal was visualized by exposure to a phosphorimagery (GE, Typhoon FLA 7000 IP).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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