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* (TbIF3a and TbIF3f) were overexpressed and purified, and 11 subunits were identified, TbIF3a through I minus j, which form a tight complex. Both TbIF3a and TbIF3f 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. TbIF3f and TbIF3a RNAi cell lines were modified to express tagged-TbIF3a and -TbIF3f, respectively. RNAi in combination with affinity purification assays indicated that both subunits are variably required for TbIF3 stability and integrity. The relative abundance of other subunits in the TbIF3f-tag complex changed little upon TbIF3a depletion; while only subunits TbIF3b, i, and e copurified comparably with TbIF3a-tag upon TbIF3f depletion. A genome-wide UV-crosslinking assay showed that several TbIF3 subunits have direct RNA-binding activity, with TbIF3c 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 TbIF3 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 (Oppenheimer 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 nu-

cleotides 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

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Article is online at <http://www.rnajournal.org/cgi/doi/10.1261/rna.058651.116>.

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2012). 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–tRNA_i 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 spe-

cifically 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 *LeishIF3a* 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 (Methot 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 IRESs 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 lo-

calized 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 peptides number and the percent protein coverage of the 11 *TbIF3* subunits were comparable and much greater than those of other co-purified 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 1. Subunit determination of the *TbIF3*-TAP complex and the *TbIF3f*-TAP complex. (A) Schematic drawing of a *TbIF3* subunit gene in frame fusion with a MH-TAP tag at the C terminus. The MH-TAP is composed of c-myc, 6xHis tag, calmodulin binding peptide, TEV protease cleavage site, and protein A as described previously (Guo et al. 2012). (B) Immunofluorescence assay of the subcellular localization of the tagged *TbIF3a* and *TbIF3f* proteins. (C) Overview of the procedures purifying the tagged *TbIF3* subunit complexes and determining the protein composition. TAP, tandem affinity purification; SR staining, SYPRO Ruby staining. (D) SDS-PAGE followed by SR staining of the purified *TbIF3a*-TAP (left lane) and *TbIF3f*-TAP (right lane) complexes. Individual band was excised and used for MS determination. The names of the proteins corresponding to each band are labeled. (E) SDS-PAGE followed by silver staining of the proteins associated with the tagged *TbIF3a* before (–) and after RNase A (+) treatment through TAP purification.

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

<i>TbIF3</i> subunit	Gene ID	Tagged- <i>TbIF3a</i>		Tagged- <i>TbIF3f</i>	
		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
elF1	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.

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 non-induced (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-hsaIF3a TAP cell line with RNAi induced (NI) or noninduced (I). (H) Western analysis of hsaIF3a-TAP in the cell lysate from PF *TbIF3a*-RNAi cells or from PF *TbIF3a* RNAi-hsaIF3a 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.

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 35 S-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 35 S-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 35 S-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-monomosome 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

FIGURE 3. Depletion of either *TbIF3a* or *TbIF3f* results in translation inhibition. 35 S-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 $\pm$ 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.

FIGURE 4. Effects of *TbIF3a* or *TbIF3f* repression on tagged *TbIF3f* or *TbIF3a* complexes, respectively. (A) Western analysis of the whole-cell lysate of PF 3a RNAi-3f TAP or 3f RNAi-3a TAP in which RNAi was not induced or induced for 48 h with rPAP reagent (upper panel). Coomassie blue staining of the membrane showed proteins load (lower panel). (B) Sedimentation profiles of the tagged-*TbIF3a* or -*TbIF3f* in the sucrose-gradient fractions by Western blot. The fractions from top to bottom are indicated. SYPRO Ruby stained gels of the *TbIF3f*-tag complexes (C) or the *TbIF3a*-tag complexes (D) purified from PF 3a RNAi-3f TAP or PF 3f RNAi-3a TAP cells via TAP purification, respectively, in which RNAi was not induced (NI) or induced for 48 h (I-48 h). Portions of SYPRO Ruby stained 8% gel are enlarged (C, right panel) to show the depletion of *TbIF3a* after 48 h of tet addition.

these two subunits for *TbIF3* stability. Sucrose-gradient centrifugation followed by Western analysis showed that *TbIF3a* depletion did not change the distribution of *TbIF3f*-TAP in the gradient fractions (Fig. 4B, upper panels); while *TbIF3a*-TAP up-shifted upon *TbIF3f* depletion (Fig. 4B, lower panels), suggesting that *TbIF3f*, but not *TbIF3a*, should be critical for the integrity of *TbIF3*. This speculation was further supported by the composition change of *TbIF3* upon repression of the subunit expression. As shown in Figure 4C, almost all other subunits copurified with the tagged-*TbIF3f* protein upon depletion of *TbIF3a*, suggesting that *TbIF3a* may be located at the periphery of the *TbIF3* complex or not critical for the assembly of *TbIF3*. In contrast, only subunits *TbIF3b*, *e*, and *i* were clearly observed to copurify with the tagged-*TbIF3a* protein upon depletion of *TbIF3f*, 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 eIF3 structure models in which eIF3a, *b*, *i*, and *g* form a stable subcomplex (Dong et al. 2013; Wagner et al. 2014), implying a distinctive *TbIF3* in structural assembly. More importantly, this result revealed a critical scaffolding role of *TbIF3f*. Therefore, *TbIF3a* and *TbIF3f* function differently in *TbIF3* 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 *TbIF3a* and *TbIF3f*

To examine whether or not IRES could initiate translation in *T. brucei*, the expressions 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-harboring 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 eIFs involved in IRES-mediated translation between trypanosomes and mammals may be functionally different.

The effect of *TbIF3a* and *TbIF3f* 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 *TbIF3a*-RNAi and *Tb3f*-RNAi cells and resulted in 3a RNAi-CrPV IRES and 3f

FIGURE 5. CrPV IRES could mediate translation in the presence of *TbIF3a* and *TbIF3f*. (A) Schematic representation of the dual-reporter vector with different IRES between an upstream *Rluc* ORF and a downstream *Fluc* ORF. Blue line segments show the Northern blot probe specific to *Rluc* or *Fluc*. The numbers represent the location of the probe sequence within the coding region of the reporters. (B) Luciferase activity assay of PF EMCV IRES-luc, HCV IRES-luc, and CrPV IRES-luc cells. Three clones from each cell line are marked as 1, 2, and 3 randomly. The parent cell line PF29-13 was used as control. (C) Real-time RT-PCR analysis of the total RNAs from one random clone of each cell line in panel A for *Rluc* and *Fluc* mRNA's measurement. The relative reporter mRNA levels of the cell PF EMCV IRES-luc were set as one. (D) Northern blot analysis of the cell PF luc-CrPV IRES and the control cell PF 29-13, with the probe specific to *Rluc* (lanes 1 and 2) or *Fluc* (lanes 3 and 4), then stripped and rehybridized with the probe specific to α -tubulin. EtBr staining of the rRNAs is shown. (E) Luciferase activity assay of the cell lysates from *TbIF3a* RNAi-CrPV IRES and *TbIF3f* RNAi-CrPV IRES cells in which RNAi was not induced (NI) or induced for 48 h (I-48). Three clones from each cell line, marked as 1, 2, and 3, were tested and the relative *Rluc/Fluc* ratio was calculated, with that in RNAi noninduced cells set as one. (F) Real-time RT-QPCR analysis of the total RNA from one random clone of each cell line in panel D. The relative *Rluc* and *Fluc* mRNA levels from RNAi induced cells were shown relative to those of the same cell lines in which RNAi was not induced. β -Tubulin was used as the internal control for all of these real-time RT-PCR analyses. At least three independent experiments were carried out, and the results are shown as mean $\pm$ SD.

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* knock-down (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 CLiCAP approach, as de-

scribed 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 - 32 P]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.

FIGURE 6. Genome-wide UV crosslinking assay of the RNA-binding activity of *TbIF3*. (A) Strategy of CLiTAP. CLiTAP represents UV crosslinking and TAP purification. (B) RNA-binding activity assay of the purified *TbIF3a*-TAP complex through CLiTAP. The purified complexes were resolved on a 10% SDS-PAGE gel, followed by SYPRO Ruby staining (left panel) and autoradiograph (right panel). The proteins corresponding to each band are indicated.

DISCUSSION

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 *LeishIF3e* 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 BF (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 *TbIF3*.

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 subcomplex 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-tRNA_i 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 trypanosomatids. The underdetection 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 IIIabc 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'-CACGTCGACATGTTGCAAGCGGAAGTA-3' plus 5'-CACGGATCCCTTCCCTTGAGGCGCTC-3' and the primers 5'-CACCTCGAGATGCGAAATTCGCTGGT-3' plus 5'-CACGGATCCACGGGTATTATTTCTCT-3', respectively. After digestion with SalI or XhoI, and BamHI, 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'-CACAAGCTTTTGAACCTCTGCAGGACG-3' and 5'-CACCTCGAGTACGCTGCACTAAGAAG-3' or the primers 5'-CACAAGCTTGCCAGATCACAGTAACG-3' and 5'-CACCTCGAGATATCCTGCATCATTCC-3', respectively. The PCR products were digested with XhoI and HindIII and inserted into similarly digested pZJM (Wang et al. 2000) to create pZJM-3a and pZJM-3f. Human genomic DNA was extracted from 293T cells and *eIF3a* was amplified by using RT-PCR with the primers 5'-CACGTCGACATGCCGGCCTATTTTCAGAG-3' and 5'-CACAGATCTACGTCGTACTGTGGTCCATC-3'. The PCR products were digested with SalI and BglII and then cloned into similarly digested pHD1344-MHTAP to create pHD1344-*hsaIF3a* TAP. To create the IRES-containing dual-reporter plasmids capable of integration into *T. brucei* genome, a pair of primers 5'-CACGTCGACATGACTTCGAAAGTTTATG-3' and 5'-CACGGATCCCTTACAATTTGGACTTTCC-3' were used to

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 SalI 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 μ g/mL), the resulting stable cell lines were designated PF *TbIF3a*-TAP and *TbIF3f*-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*, PF 29-13 and BF-SM cell lines were transfected independently with 10 μ g of NotI-linearized pZJM-3a or pZJM-3f. The resulted phleomycin-resistant clones were named PF *TbIF3a*-RNAi, BF *TbIF3a*-RNAi, PF *TbIF3f*-RNAi, and BF *TbIF3f*-RNAi. The PF *TbIF3a* RNAi-*hsaIF3a* TAP cell line was generated by transfecting the NotI-linearized pHD1344-*hsaIF3a* TAP into the PF *TbIF3a*-RNAi cell line. The PF 3a RNAi-3f TAP and 3f RNAi-3a TAP cell lines were generated by transfecting pHD1344-3f-TAP and pHD1344-3a-TAP into the PF RNAi cell lines *TbIF3a*-RNAi and *TbIF3f*-RNAi, respectively. The expression of these tagged genes was confirmed by Western blot. Ten micrograms of NotI-linearized pHD1344-CrPV IRES-luc, pHD1344-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 pHD1344-CrPV IRES-luc was additionally transfected into PF *TbIF3a*-RNAi and *TbIF3a*-RNAi cells, the resulting resistant clones were named PF 3a RNAi-CrPV IRES and 3f RNAi-CrPV IRES, respectively.

Cell growth

DsRNA was induced by adding 1 μ g of tet/mL. Growth of the RNAi cells was monitored in the absence or in the presence of tet and counted daily with a hemocytometer. The PF cells were maintained between 2×10^6 and 2×10^7 cells/mL while the BF cells were maintained between 1×10^5 and 2×10^6 cells/mL.

Immunofluorescence assay (IFA)

Subcellular localization of the tagged *TbIF3a* or *TbIF3f* 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.

DNA was visualized by treatment with DAPI (Roche). The fluorescence and phase-contrast images of the cells were captured with a fluorescence microscope (Olympus BX63).

Tandem affinity purification (TAP) and mass spectrometry analysis (MS)

A total of 1×10^{10} cells expressing TAP-tagged *TbIF3a* or *TbIF3f* were harvested and lysed, and the tagged complexes were purified through TAP purification essentially as described previously (Rigaut et al. 1999; Panigrahi et al. 2003). Briefly, IgG affinity chromatography, TEV protease cleavage, and calmodulin affinity chromatography were carried out in a stepwise order to purify the TAP-tagged complexes from the cell lysate. The purified proteins were resolved on a 10% SDS-PAGE gel and stained with SYPRO Ruby (Life Technologies) according to the manufacturer's instructions. The protein bands were cut and digested in gel with trypsin. In parallel, the total proteins were digested in solution with trypsin. All the protein samples were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis using the service provided by Beijing Genomics Institute (BGI). The *T. brucei* protein database downloaded from TriTrypDB. All MS/MS samples were analyzed using Mascot 2.3.02 (Matrix Science), and peptides and protein were specified using PeptideProphet and ProteinProphet algorithms.

RNA isolation and Northern blot

Total RNA was isolated from the RNAi cells in which RNAi was not 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 *TbIF3a* or *TbIF3f*. The RNAs from PF 3a RNAi-CrPV IRES and PF 29-13 cells were hybridized with the labeled probe specific to *Rluc* 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 Primer 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 μ g 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'-ATAACTGGTCCGAGTGGTG-3' plus 5'-AGGCCGCGTTACCA TGTAAG-3' and 5'-TTGTTTGGAGCACGGAAGAC-3' plus 5'-AAGACCTTCGGTACTTCGTCC-3', respectively. The sequences for β -tubulin have been described previously (Carnes et al. 2005). Each PCR reaction contained 2 μ L of cDNA, 8 μ L of forward and reverse primers (each at 0.75 μ M), and 10 μ L 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 that 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 *TbIF3a*-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 μ Ci 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 μ g/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 proteins synthesized within 60 min were calculated for each sample as cpm/ μ g protein.

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 μ g/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-3f TAP 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 *TbIF3a* 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-*hsaIF3a* TAP were separated on 8% SDS-PAGE gel for Western blot of the protein A-tagged *hsaIF3a*.

Luciferase reporter assay

Cell lysate was 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 *TbIF3a*-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 *TbIF3a*-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 phosphorimager (GE, Typhoon FLA 7000 IP).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

ACKNOWLEDGMENTS

We thank Dr. Ken Stuart for providing the pZJM, pHD1344tub, and pHD1344-MHTAP plasmids and *T. brucei* PF 29-13 and BSF-SM strains. We also thank Dr. Guangxia Gao for providing the plasmids pNL4-3RL-CrPV-FL, pNL4-3RL-HCV-FL, and pNL4-3RL-EMCV-FL. This work was supported by the National Natural Science Foundation of China (grant no. 81171601) and the

Natural Science Foundation of Guangdong Province (grant no. 2014A030313216).

Received August 6, 2016; accepted November 24, 2016.

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RNA 2017 23: 333-345 originally published online December 8, 2016

Access the most recent version at doi:[10.1261/rna.058651.116](https://doi.org/10.1261/rna.058651.116)

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