The RNA-associated proteins MKT1 and MKT1L form alternative PBP1-containing complexes in *Trypanosoma brucei*

Larissa Melo do Nascimento, Monica Terrao, Kevin Kamanyi Marucha, Bin Liu, Franziska Egler, and Christine Clayton*

From the Heidelberg University Centre for Molecular Biology (ZMBH), Heidelberg, Germany

Control of gene expression in kinetoplastids such as trypanosomes depends heavily on RNA-binding proteins that influence mRNA decay and translation. We previously showed that the trypanosome protein MKT1 forms a multicomponent protein complex: MKT1 interacts with PBP1, which in turn recruits LSM12 and poly(A)-binding protein. MKT1 is recruited to mRNAs by sequence-specific RNA-binding proteins, resulting in stabilization of the bound mRNA. We here show that PBP1, LSM12, and a 117-residue protein, XAC1 (Tb927.7.2780), are present in complexes that contain either MKT1 or an MKT1-like protein, MKT1L (Tb927.10.1490). All five proteins are present predominantly in the complexes, and we found evidence for a minor subset of complexes containing both MKT1 and MKT1L. XAC1-containing complexes reproducibly contained RNA-binding proteins that were previously found associated with MKT1. Moreover, XAC1- or MKT1-containing complexes specifically recruited one of the two poly(A)-binding proteins, PABP2, and one of the six cap-binding translation initiation complexes, EIF4E6–EIF4G5. Yeast two-hybrid assay results indicated that MKT1 directly interacts with EIF4G5. MKT1–PBP1 complexes can therefore interact with mRNAs via their poly(A) tails and caps, as well as through sequence-specific RNA-binding proteins. Correspondingly, MKT1 is associated with many mRNAs, although not with those encoding ribosomal proteins. Meanwhile, MKT1L resembles MKT1 at the C terminus but additionally features an N-terminal extension with low-complexity regions. Although MKT1L depletion inhibited cell proliferation, we found no evidence that it specifically interacts with RNA-binding proteins or mRNA. We speculate that MKT1L may compete with MKT1 for PBP1 binding and thereby modulate the function of MKT1-containing complexes.

Transcription initiation is a crucial point of gene expression regulation in most eukaryotes. However, in kinetoplastid protists, such as trypanosomes and leishmanias, transcription by RNA polymerase II is polycistronic (1), and mRNA precursors are co-transcriptionally processed to mature mRNAs by splicing and polyadenylation (2). To overcome the limitations imposed by such a system, the most abundant mRNAs are usually encoded by multicopy genes but may exceptionally be transcribed by RNA polymerase I, as exemplified by trypanosome major surface proteins (3). Translation and decay of mRNAs are also at least partially linked: optimal codon usage correlates positively with mRNA stability, enabling the correct steady-state levels of mRNAs and proteins from constitutively expressed genes to be maintained (4, 5). However, many genes also require more flexible controls, allowing expression to be modified during differentiation and in response to environmental stimuli. For example, the African trypanosome *Trypanosoma brucei* alternates between two hosts during its life cycle, living in the blood and tissue fluids of mammals and the digestive system of tsetse flies. Adaptation to various niches within the two hosts is accompanied by changes in levels and translation of several hundred mRNAs (6–12). Regulated control of mRNA processing, translation and mRNA decay relies mainly on RNA-binding proteins, which often (although not always) bind to the 3′-UTRs of mRNAs (reviewed in Ref. 13). miRNAs, however, appear to be absent (14).

Many RNA-binding proteins have been reported to control specific aspects of trypanosome gene expression (13). However, the only ones for which the molecular mechanism is well-understood are the zinc finger proteins ZC3H11 and ZC3H20. ZC3H11 binds to the 3′-UTRs of mRNAs that are required for survival after heat shock (15), whereas ZC3H20 binds to a subset of mRNAs that is preferentially expressed in procyclic forms (16, 17). Stabilization of mRNAs by ZC3H11 and ZC3H20 requires binding to MKT1, which in turn recruits PBP1, LSM12, and poly(A)-binding proteins (PABPs), especially PABP2 (16, 18). Trypanosome MKT1, PBP1, and LSM12 proteins were named after their previously characterized *Saccharomyces cerevisiae* homologues Mkt1, Pbp1, and Lsm12 (19). In both organisms, Pbp1/PBP1 recruits all of the other subunits as well as PABP. The metazoan Pbp1 homologue, ataxin-2, also interacts with both poly(A)-binding protein and Lsm12 (20, 21), but metazoans lack Mkt1. Pbp1 and ataxin-2 are incorporated into RNA-protein stress granules (22), as is *T. brucei* PBP1 (18), and polyglutamine tract expansions in ataxin-2 are implicated in neurodegenerative disease (20, 21). A point mutation in Mkt1 in the standard *S. cerevisiae* strain S288C has been linked to numerous minor defects (23–33); but the *T. brucei* MKT1 protein has the conserved WT residue at this position.
To assess the effects of proteins on bound RNAs in *T. brucei*, we use a tethering assay (34). The purpose of this assay is to find out whether any particular protein can affect mRNA behavior when it is bound to that mRNA in vivo, even when an investigator does not know which mRNAs (if any) the protein normally binds to. For this purpose, the protein of interest is tagged with a highly specific RNA-binding domain, such as the N peptide from bacteriophage λ, and co-expressed with a reporter mRNA that includes the cognate binding sequences (for the λN peptide, a sequence called "boxB"). This reliably results in binding or "tethering" of the experimental protein fusion to the reporter mRNA. Effects on reporter expression can then be measured and interpreted with some caveats. First, the activity may be spurious if the protein that is tested normally never binds to mRNAs. Second, for a genuine RNA-binding protein, the activity may be affected by the tag or might be different when the protein interacts with mRNA via its own RNA-binding domain rather than via the artificial tether. For our tethering assays, we use a trypanosome line that constitutively expresses a reporter mRNA bearing five copies of the boxB sequence in the 3'UTR, and AN-tagged proteins are expressed from a tetracycline-inducible promoter. Expression of λN-PABP, AN-PBP1, λN-LSM12, λN-MKT1, or AN-ZC3H111 all caused increases in reporter expression (18). Attachment of PABPs to mRNA 3'-UTRs increases mRNA levels (35–37), and this probably at least partially explains the mRNA-stabilizing effects. Pulldown followed by MS, as well as yeast two-hybrid analyses, showed that *T. brucei* MKT1 interacts with numerous RNA-binding proteins, often (though not always) via the motif (Y/W/V/I)(R/T/Q)H(N/D)P(Y) (18). Such RNA-binding proteins can therefore stabilize their target mRNAs and/or promote their translation by recruiting the MKT1–PBP1–LSM12–PABP complex (16–18).

We adapted the tethering assay to conduct a genome-wide screen for proteins that could influence mRNA fate (38). The protein encoded by Tb927.7.2780, eXpression ACtivator 1 (XAC1), was one of the strongest activators of gene expression in this screen (38, 39). In this paper, we show that XAC1 is present in two alternative complexes, containing PBP1, LSM12, and either MKT1 or an MKT1-like protein, MKT1L (Tb927.10.1490). We also find that both MKT1 and MKT1L are present mostly in complexes and that MKT1, but not MKT1L, is associated with many mRNAs and with a specific eIF4F-like translation initiation complex.

Results

**XAC1 is essential for trypanosome survival**

*T. brucei* XAC1 is 117 residues long and has no annotated functional domains (Fig. 1A). Syntenic homologues are present in all kinetoplastid parasite genomes examined, but not in the free-living *Bodo saltans* or evolutionarily more distant organisms. A 17-residue region is conserved in all sequences examined (Fig. 1B and black box in Fig. 1A). The sequences from *Trypanosoma* species have two regions rich in proline, alanine, glutamine, and serine further toward the N terminus (marked in red in Fig. 1).

The results of a high-throughput RNAi screen suggested that XAC1 is essential for trypanosome growth (40). To be able to monitor XAC1 protein levels, we integrated a sequence encoding a V5 tag immediately upstream of, and in frame with, an XAC1 ORF. Stem-loop RNAi induction (Table S1) resulted in a 75% reduction of V5-XAC1 protein within 16 h (Fig. 1C). At this point, protein synthesis was still normal (Fig. S1A), but no subsequent growth was seen (Fig. 1D).

**Composition of XAC1-containing complexes**

To investigate XAC1 interactions, we first made bloodstream-form trypanosomes in which one XAC1 gene included a C-terminal tag for tandem affinity purification (41) (XAC1-TAP), and the other XAC1 gene had been deleted (Table S1). Results from three tandem affinity purifications of XAC1-TAP are shown in Fig. 2A and Table S2. Results for GFP-TAP served as negative controls; some of these have been published previously (42, 43). The most strongly enriched proteins were MKT1, LSM12, PBP1, PABP2, and a protein related to MKT1, which we named MKT1L (Tb927.10.1490). Immunoprecipitation of YFP-tagged XAC1 via Myc-tagged LSM12 was confirmed and RNase-insensitive (Fig. S2A).

Previously, we had identified proteins associated with MKT1 in bloodstream forms after a single purification of C-terminally TAP-tagged MKT1, followed by nonquantitative MS. We had also found MKT1 interaction partners in a yeast two-hybrid screen (18). In addition to PBP1, LSM12, MKT1L, and MKT1, 24 proteins were significantly enriched with XAC1-TAP. Of these, 10 had previously been identified as direct or indirect partners of MKT1 (18) (Table 1). Most of the additional XAC1-associated proteins can also directly bind to mRNA, as judged by their presence in the mRNP proteome (39). (The mRNP proteome is a catalogue of all proteins that can be UV-cross-linked to poly(A)+ RNA.) Seven of the associated proteins have canonical RNA-binding domains, and nine of the additional associated proteins were shown to activate expression when tethered (38, 39) (Table 1), consistent with an ability to recruit the MKT1–PBP1 complex. XAC1 itself was not in the mRNP proteome (39), suggesting that it does not directly bind to mRNA.

**Structure and protein interactions of MKT1L**

The C-terminal part of MKT1L shares 20% identity with MKT1 but includes “Mkt1-like” PIN, N-terminal, and C-terminal domains (E-values 1e−22, 1e−15, and 2e−14, respectively) (Fig. 3A and Fig. S3). MKT1L is, however, distinguished from MKT1 by an extended N terminus; at the beginning, there are 35 residues consisting almost exclusively of glutamine and histidine, which are predicted to be disordered by mobibd_LITE. Similar low-complexity regions are found in the *Trypanosoma cruzi* and *Trypanosoma congolense* homologues, and the region is expanded to 130 residues in *Trypanosoma vivax* (Fig. S3).

In our previous studies of MKT1 interactions, we had used MKT1 with a C-terminal TAP tag. To confirm the interaction of MKT1L with XAC1, we therefore similarly inducibly expressed MKT1L-TAP and purified associated proteins. To our surprise, the only proteins that co-purified were known components of the MKT1 complex: LSM12 and XAC1 were significantly present, and PBP1 was enriched but below the significance cut-off (Fig. 2B and Table S2). No RNA-binding proteins were associated. These results, combined with
**PBP1-containing complexes in T. brucei**

### A.

previous data, suggest that the many additional proteins that were found to be associated with XAC1 were probably attached via MKT1, not MKT1L.

We next examined the proteins associated with MKT1 and MKT1L in procyclic forms. To avoid any possible effects of the large C-terminal tag or of inducible (over)expression, we used cell lines in which one of the relevant genes contained a sequence encoding a V5 tag at the 5′-end of the ORF (in situ V5 tag). V5-GFP expressed constitutively from the tubulin locus served as a negative control. V5-MKT1 from procyclic forms gave insufficient enrichment: this is tested later in the paper. We suspect that this is because the single round of purification any RNA-binding proteins with immunoprecipitated V5-MKT1.

### B.

results confirmed that the complexes are present in procyclic forms. To our surprise, there was no significant enrichment of any RNA-binding proteins with immunoprecipitated V5-MKT1. We suspect that this is because the single round of purification gave insufficient enrichment: this is tested later in the paper.

**MKT1 and MKT1L are in different complexes**

Our MS results showed the existence of two complexes: MKT1–XAC1–PBP1–LSM12 and MKT1L–XAC1–PBP1–LSM12. LSM12 and MKT1L are known to interact with PBP1 (18). The quantitative MS results suggested that each complex contained roughly equimolar amounts of XAC1, LSM12, PBP1, and either MKT1 or MKT1L. MKT1 and MKT1L were mostly
Figure 2. Proteins that co-purify with XAC1, MKT1, and MKT1L. A, volcano plot of bloodstream-form XAC1 interactors. The XAC1 gene was tagged in situ with a tandem affinity purification tag (protein A–tobacco etch virus cleavage site–calmodulin-binding peptide), and the other gene was replaced by a selectable marker (NPT). After two cycles of purification, the preparation was analyzed by MS. GFP-TAP served as the control. Detailed analysis using MaxQuant and PERSEUS was as described under “Experimental procedures.” The identities of selected spots are indicated; all significantly enriched proteins are listed in Table 1, and details are given in Table S2. The color code is as in Fig. 3; blue indicates a protein that is not part of the core complex. Normalized peak intensities of significantly enriched RNA-binding proteins were 40–1,000 times lower than that of MKT1, suggesting that each of these proteins is associated with only a small minority of XAC1-containing complexes. For details of all results in this figure, see Table S2. B, volcano plot of MKT1L interactors in bloodstream forms. MKT1L-TAP was inductively expressed in bloodstream forms and purified as in A. C, volcano plot of procyclic form MKT1L interactors. Procyclic forms with the MKT1L gene tagged in situ with a V5 tag were used. The V5-MKT1 protein was immunoprecipitated, and then the preparation was analyzed by MS. V5-GFP (pHD3141) served as the control. D, volcano plot of procyclic form MKT1L interactors. Procyclic forms with the MKT1L gene tagged in situ with a V5 tag (pHD2730) were used. The V5-MKT1L protein was immunoprecipitated, and then the preparation was analyzed by MS. V5-GFP served as the control. The apparent association of the 350-kDa protein intraflagellar transport protein Tb927.10.15700 could be an artifact: this protein contains 25 repeats of a lysine-containing peptide sequence, so it will be relatively easily detected if present as a contaminant.

present in different complexes, because MKT1 was not detected after purification of MKT1L-TAP from bloodstream forms, and MKT1L was not enriched after pulldown of V5-MKT1 from procyclic forms. However, MKT1L was detected after purification of MKT1 from bloodstream forms (18), and MKT1 was present after pulldown of V5-MKT1L from procyclic forms (Fig. 2), suggesting that there might be a small degree of association. To investigate the complexes in more detail, we used cell lines with in situ V5–tagged genes. By tagging at the N terminus, changes in regulatory 3′-UTR sequences are avoided; however, the change in the N terminus of the protein might affect protein stability.

We first analyzed protein abundance using denaturing polyacrylamide gels and quantitative Western blotting of the in situ tagged proteins. For bloodstream forms, quantitation of the signals suggested that both MKT1L and PBP1 are 2–4-fold less abundant than MKT1 (Fig. 3, B–D). Procyclic forms had less MKT1, with signals similar to those from MKT1L (Fig. 3, C and D). These results are consistent with published quantitative MS data (Fig. 3E) (46), which indicate that the total proteome of procyclic forms has roughly equal amounts of MKT1 and MKT1L, whereas in bloodstream forms, MKT1 is about 3 times more abundant than the MKT1L. These results are consistent with competition between MKT1 and MKT1L for PBP1.

A complex containing MKT1, PBP1, XAC1, and LSM12 has a predicted mass of 177 kDa, whereas a similar complex with MKT1L would be 204 kDa (Fig. 3F). Results from blue native gel electrophoresis are shown in Fig. 3G. In clear disagreement with results from denaturing gel electrophoresis, the signal from MKT1L for bloodstream-form extracts was much stronger than for the other proteins investigated. We speculate that most MKT1 is in complexes that are too large to enter the gel, such as polyosomes or aggregates, whereas most MKT1L complexes are free because they are not associated with mRNA, consistent with the lack of interaction with RNA-binding proteins.

We do not know whether the migration of the complexes in the gel reflects their molecular weights with any degree of
accuracy, and the migration of each could be influenced by the presence of additional proteins, such as PABP2, which were not assayed. The native gel resolved proteins down to about 60 kDa, but there were no MKT1L, PBP1, or XAC1 signals below 250 kDa. This suggests that MKT1L is present only within complexes. We cannot be certain about PBP1, but because it is substoichiometric, and a degradation product was visible in the denaturing gels (Fig. 3B), it seems likely that it is unstable as a monomer. The fastest-migrating MKT1 bands were at 140 and 180 kDa (Fig. 3G); these might be MKT1 multimers, or they might represent MKT1 association with other proteins that were not examined. The predominant band for XAC1, PBP1, and MKT1 (a in Fig. 3G) migrated at 400 kDa and might be an MKT1–XAC1–PBP1–LSM12 dimer. The 450 kDa band (b in Fig. 3G) might consist mostly of MKT1–XAC1–PBP1–LSM12 dimers, with a few mixed complexes; the latter could explain occasional co-purification of MKT1 with MKT1L, and vice versa (Fig. 2D). Equivalent monomeric complexes might be represented by the bands that migrate at 250 and 300 kDa, respectively (c in Fig. 3G). Complexes containing MKT1 and PBP1 but no detectable XAC1 migrated at 340 kDa. We concluded that MKT1 and MKT1L form separate complexes with PBP1 and XAC1 but that complexes containing MKT1 and MKT1L might also rarely associate with each other.

**Interactions of MKT1 and MKT1L with PBP1 and EIF4E6–EIF4G5 complexes**

To investigate interactions within MKT1 and MKT1L complexes, we used yeast two-hybrid assays (Fig. 4 (A and B) and Figs. S4 and S5). It was already known that PBP1 interacts with both LSM12 and MKT1, as shown in Fig. 3F (18). PBP1 (18) XAC1 and MKT1L fused with the DNA-binding domain (bait) activated by themselves, so mutual interactions of these proteins could not be tested (Fig. S5, A and B). Deletion of the repetitive N terminus of MKT1L, however, eliminated the self-activation (MKT1LΔN), and this protein, like MKT1, interacted with PBP1 (Fig. 4 (A and B) and Fig. S5C). The means by which XAC1 is associated with the complex is unresolved; we speculate that it too interacts with PBP1. CFB1, CFB2, and ZC3H11 all interact with MKT1 via C-terminal HNPY-like motifs, but MKT1LΔN was unable to interact with any of these proteins, showing that MKT1L does not recognize the motif (Fig. 4, A and B).

Intriguingly, the translation initiation complex EIF4E6–EIF4G5 (47) was present in the XAC1 affinity purification (Fig. 2A and Table S2). The enrichment was below the significance cut-off, and the intensities were about 100-fold lower than for MKT1 complex components. However, neither EIF4E6 nor EIF4G5 was detected in the GFP-TAP control, and the known abundant initiation complexes EIF4E3–G4 and EIF4E4–G3 (48) were also undetectable in the XAC1 preparations. G5-IP, which interacts with EIF4G5 (47), was also detected in two of three XAC1 preparations, but not the control. The translation helicase eIF4A1 was also in the XAC1 purification and was not detected with either GFP or MKT1L. A pulldown of *in situ* PPT-tagged EIF4E6, followed by Western blotting, indeed revealed association with V5-MKT1 but not V5-MKT1L (Fig. 4C and Fig. S2B). EIF4G5 also interacted with MKT1 in the two-hybrid assay (Fig. 4B and Figs. S4A and S5). The interactions between EIF4E6 and EIF4G5, and between EIF4G5 with

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**Table 1**

Proteins from bloodstream-form trypanosomes that co-purify with XAC1

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above shown were assessed using ImageQuant, and the resulting graph is shown below. The abundances were then measured by Western blotting, with overnight transfer to allow detection of high-molecular weight proteins. Signal intensities labeled are the two-hybrid assay, but MKT1L did not interact with EIF4G5 or EIF4E6 in the two-hybrid assay, but MKT1L did not interact with EIF4G5 or EIF4E6. These results suggest that EIF4E6 interacts with itself. These results are positive controls; there was also a novel interaction of EIF4G5 with itself. These results are positive controls; there was also a novel interaction of EIF4E6 with itself. These results are positive controls; there was also a novel interaction of EIF4E6 with itself. These results are positive controls; there was also a novel interaction of EIF4E6 with itself. These results are positive controls; there was also a novel interaction of EIF4E6 with itself. 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These results are positive controls; there was also a novel interaction of EIF4E6 with itself. These results are positive controls; there was also a novel interaction of(EIF4G5 with itself. These results suggest that EIF4E6–EIF4G5 complexes may be recruited to some mRNAs by MKT1. MKT1L did not interact with EIF4G5 or EIF4E6 in the two-hybrid assay, but MKT1LPBP1 interacted with both (Fig. 3A and Fig. S4). This could be an artifact caused by protein truncation; alternatively, the MKT1L N terminus might have a regulatory function, preventing the interaction in growing trypanosomes.

Do MKT1 and MKT1L interact with RNA-binding proteins in procyclic forms?

No associations of MKT1 with RNA-binding proteins were detected in procyclic form extracts after a single purification step. These proteins are strongly substoichiometric even after tandem affinity purification (Table S2), so after immunoprecipitation, they might have been insufficiently enriched to enable detection. We tested this for two proteins: ZC3H20 is more abundant in procyclic forms than in bloodstream forms,

Figure 3. Quantitation and complex composition. A, comparison of MKT1 and MKT1L, to scale. The different domains and their positions in the sequence are labeled. B–D, trypanosomes with in situ tagged MKT1 (pHD1973), MKT1L (pHD 3071), or PBP1 (pHD2166) were pelleted and boiled in SDS sample buffer. The abundances were then measured by Western blotting, with overnight transfer to allow detection of high-molecular weight proteins. Signal intensities were assessed using ImageQuant, and the resulting graph is shown below. ("Signal" is in arbitrary grayscale units.) B, Western blotting data. Experiment 2 is shown above and experiment 1 below. BS, bloodstream forms; PC, procyclic forms. The numbers of cells used per lane, multiplied by 10–5, are shown. C, amounts of V5-PBP1 and V5-MKT1L relative to V5-MKT1 in bloodstream forms. Individual results for different dilutions are shown. Cyan bars, arithmetic mean; magenta bars, median. D, results for cells in which both MKT1 and MKT1L were tagged. E, levels of PBP1, LSM12, and MKT1L relative to MKT1 in total proteomes of bloodstream and procyclic forms. The label-free quantification values from four replicate published proteomes for each stage (44) were used to calculate means and S.D. of the ratios. XAC1 was not reproducibly detected in bloodstream forms, perhaps because of its small size. These values are not reliable by themselves because some peptides are more readily detected than others, but the increased relative amount of MKT1L in procyclic forms is clear. F, diagrams of two possible complexes. Molecular weights of individual components and of the complexes are indicated; the numbers in parentheses are the complexes with a single molecule of PABP2. G, blue native gel electrophoresis of extracts from bloodstream and procyclic forms expressing V5-tagged XAC1, PBP1, MKT1, or MKT1L was followed by Western blotting and detection of the V5 tag. The numbers of loaded cell equivalents are indicated below the blot. There was no signal at all below the lowest band shown; the lowest molecular weight marker was at 66 kDa. Proteins detected in individual bands, with their approximate molecular weights, are indicated on the right using the color code in F. The presence of LSM12 in the complexes is assumed but was not tested in these experiments.

G5-IP, are positive controls; there was also a novel interaction of EIF4G5 with itself. These results suggest that EIF4E6–EIF4G5 complexes may be recruited to some mRNAs by MKT1. MKT1L did not interact with EIF4G5 or EIF4E6 in the two-hybrid assay, but MKT1LPBP1 interacted with both (Fig. 3A and Fig. S4). This could be an artifact caused by protein truncation; alternatively, the MKT1L N terminus might have a regulatory function, preventing the interaction in growing trypanosomes.
whereas ZC3H21 is procyclic-specific (16, 46). ZC3H20 and ZC3H21 have H(D/N)PY MKT1 interaction motifs, and the ability of ZC3H20 to activate when tethered in procyclic forms depends on this motif (16). Moreover, ZC3H20 was reproducibly detected in the bloodstream-form XAC1-TAP purification (Table S2 and Fig. 2A). To test for interactions, we used procytic trypanosomes in which both MKT1 and MKT1L had in situ N-terminal V5 tags (Fig. 4D). In these cells, we inducibly expressed GFP, ZC3H20, or ZC3H21 with C-terminal Myc tags. Myc-tagged ZC3H20 pulled down V5-MKT1 and a little V5-MKT1L, whereas GFP, or a mutant version of ZC3H20 lacking the MKT1 interaction motif, did not (Fig. 4D). Only V5-MKT1 was detected weakly in the ZC3H21-Myc pull-down. These results suggest that ZC3H20 and perhaps ZC3H21 associate with MKT1. Only a very small proportion of the MKT1, about one-thirtieth, co-precipitated with ZC3H20, which is expected if several different RNA-binding proteins with H(D/N)PY motifs compete for the same binding site on MKT1.

**MKT1L is essential in bloodstream forms and procyclic forms**

We had previously found that MKT1 depletion was rapidly lethal in bloodstream forms but had no effect in procyclic forms (18). We therefore attempted to delete both MKT1 genes in procyclic forms, using constructs bearing two different selectable markers (Fig. S6A). Although doubly resistant trypanosomes were obtained, the MKT1 ORF persisted in all clones (Fig. S6B), suggesting that duplication of the locus was necessary to allow cell survival. These results suggest that loss of
MKT1 in procyclic forms is at least severely deleterious and that it might be essential.

Results of an RNAi screen (40) suggested that MKT1L is an essential protein. Specific RNAi targeting MKT1L indeed resulted in strong growth inhibition in both bloodstream forms (Fig. 5, A and B) and procyclic forms (Fig. 5, C and D) within 1–2 days. Protein synthesis was uniformly decreased 24 h after MKT1L RNAi induction in bloodstream forms (Fig. S1B). This also happens after depletion of MKT1 (18), but it is not possible to tell whether the effect is direct or indirect. The results of additional experiments suggested that C-terminally TAP-tagged MKT1L is at least partially functional (Fig. S6C). Total protein synthesis was not affected after 24 h XAC1 RNAi (Fig. S1C), but this is uninterpretable because the amount of residual XAC1 was unknown.

Locations of XAC1 and MKT1L

To check the subcellular locations of XAC1 and MKT1L we used cell lines expressing tagged versions. Both V5- and YFP-tagged XAC1 were in the cytoplasm (Fig. 6, A and B). In the cell line expressing in situ YFP-tagged XAC1, the second allele of XAC1 was replaced by the NPT gene, demonstrating the functionality of the YFP-tagged version. Inducibly expressed MKT1L with a C-terminal TAP tag (Fig. 6C) and MKT1L tagged in situ with an N-terminal V5 tag (Fig. 6D) were also in the cytoplasm and almost completely excluded from the nucleus of bloodstream forms, as suggested by results from the Tryp-tag project (44).

Effects of XAC1 and MKT1L in the tethering assay

Because MKT1L interacts with PBP1, we expected that it would increase expression when tethered. Indeed, MKT1L tethering increased both CAT activity and mRNA (Fig. 7A). Because this was not seen in our initial tethering screen of protein fragments (38), the whole protein (or the whole Mkt-like domain) may be required, as is true for MKT1 (18). Tethering of XAC1 was known to increase expression of a CAT-boxB reporter (38). We now found that here too, the entire protein was required for the full increase in CAT activity and mRNA (Fig. 7B and Fig. S7). Tethering of XAC1 did not significantly affect ribosome loading on the CAT mRNA, suggesting that the major effect was on mRNA abundance (Fig. 7, C and D). Two proteins associated with XAC1 in bloodstream forms, EIF4E6 and a protein of unknown function, Tb927.10.15310, had not previously been tested at full length: both activated when tethered (Fig. S8).
MKT1-TAP binds many mRNAs

MKT1 and MKT1L can only enhance expression in vivo if they are associated with mRNA. In bloodstream forms, both MKT1L and MKT1 were found to be bound directly to poly(A)^+ mRNA, although the result for MKT1 (false discovery rate 0.003) was more convincing than for MKT1L (false discovery rate 0.014) (39). We expect MKT1 to bind to mRNA via its interactions with RNA-binding proteins, but evidence that MKT1L has similar interactions was absent. To identify bound mRNAs, we therefore pulled down tandem affinity-tagged proteins from lysates, released them by tobacco etch virus protease cleavage, and sequenced the associated RNAs. Reads were mapped to the T. brucei Lister 427 and TREU927 genomes.

Yields of mRNA in the MKT1L pulldowns were very low, and there was no evidence for specific mRNA binding (Table S3). In contrast, plenty of mRNA co-purified with MKT1. Although principal component analysis did not clearly separate the bound and unbound fractions (Fig. S9A) and there was no overall enrichment of any functional class (Fig. S9B and Table S3), there was clearly some specificity, because mRNAs encoding ribosomal proteins were significantly less MKT1-bound than other mRNAs (Fig. 8A and Fig. S9B). This was not an artifact due to their short mRNA length (Fig. 8B). Overall, these results suggest that MKT1 is associated with a large number of mRNAs, consistent with its interactions with many different RNA-binding proteins. Rather to our surprise, there was no correlation between MKT1 association and mRNA half-life (10) or ribosome density (9) (Fig. S8C). There was also no correlation with codon optimality (5) (not shown).

Discussion

Our results show that trypanosome PBP1, XAC1, and LSM12 form at least two different complexes. One contains MKT1, and the other contains MKT1L. In bloodstream forms, the MKT1-containing complex is associated with numerous different RNA-binding proteins and mRNAs and with the
EIF4E6–EIF4G5 complex (Fig. 9A). MKT1L, in contrast, appeared to have no association with either RNA-binding proteins or mRNAs. Results of native gel electrophoresis suggested that MKT1L and free MKT1 complexes are present mostly as dimers of PBP1–LSM12–XAC–MKT1(L) tetramers.

We suggest two alternative scenarios for the function of MKT1L. The first possibility is that the failure of MKT1L-TAP to bind RNA-binding proteins, and therefore mRNAs, was an artifact caused by the presence of the C-terminal tag. If so, MKT1L-based complexes might bind a set of RNA-binding proteins and mRNAs that is different from those bound by MKT1. This hypothesis is contradicted by evidence that MKT1L-TAP is at least partially functional. The second possibility is that MKT1L forms a separate complex with PBP1, either competing with MKT1 or acting as a reservoir for LSM12–PBP1–XAC1 complexes (Fig. 9B). Limited association between the two different complexes (Fig. 3G) might account for occasional co-purification of MKT1 and MKT1L and perhaps also for the presence of MKT1L in the mRNP proteome.

Figure 7. Tethered XAC1 and MKT1L increase expression from a reporter mRNA. A, tethering assay using XAC1 full-length protein and fragments. XAC1 domains are as in Fig. 1A. Levels of CAT activity and CAT mRNA in cell lines with inducible fusion protein expression were measured in the presence and absence of tetracycline. The ratios are shown in different colors for three independent cell lines. Expression of the tagged proteins is shown in Fig. S7 (A and B). B, tethering assay with MKT1L. Cells expressing CAT mRNA with or without boxB were used. Levels of CAT activity in independent cell lines with inducible fusion protein expression were measured in the presence and absence of tetracycline. Lines without a box fusion were measured once with and once without tetracycline as a negative control. Expression of the tagged protein is shown in Fig. S7 (A and B). C, migration of CAT-boxB mRNA in a sucrose gradient in the absence of AN-XAC1-Myc. The membranes stained with methylene blue were used as a loading control. Quantification (bottom) was done for two independent experiments using ImageJ. D, same as C but after induction of Myc-XAC1-AN expression for 24 h.
Association of MKT1-containing complexes with mRNAs was relatively promiscuous. We suggest that this is determined by numerous cooperative interactions, not only with specific RNA-binding proteins but also (via PBP1) with poly(A)-tail-bound PABP2 and with the EIF4E6–EIF4G5 initiation complex (Fig. 9A). MKT1-bound mRNAs were not particularly stable or well-translated. However, the behavior of mRNAs is affected not only by the presence of numerous different bound proteins (49), but also by codon optimality (4, 5). The action of MKT1 complexes may well compete with destabilizing or repressive proteins that are bound to other parts of the mRNA. Interestingly, mRNAs encoding ribosomal proteins were relatively depleted in the MKT1-bound fraction. These mRNAs are notable for their high codon optimality relative to other mRNAs (5). They also have unusually short untranslated regions (13) and therefore may associate with fewer RNA-binding proteins.

Our affinity purification of XAC1 confirmed the in vivo association of MKT1 complexes with proteins that bind to mRNA and can activate in the tethering assay and/or are known to stabilize mRNAs or activate translation (Table 1) (38, 39). These include ZC3H20 (16, 17), PUF9 (45), ZC3H28, PUF6, and CFB2 (39, 50). Less significantly enriched RNA-binding proteins were SLBP1, ZC3H5, ZC3H34, UBP2, ALBA3, and the known MKT1 partner ZC3H11. DRBD2 and DRBD3/PTB1, both of which have a polyglutamine repeats, were pulled down with XAC1 but had not previously been shown to be MKT1-associated. Neither MKT1 nor PBP1 was found by MS of T. cruzi DRBD2 preparations (51), and it was repressed in the tethering screens. DRBD3/PTB1, however, can stabilize mRNA (52–55).

Although PBP1 interacts with both PABP1 and PABP2 in the yeast two-hybrid assay (18), only PABP2 co-purified with XAC1. This result is consistent with a published study: PBP1 was enriched in pulldowns of PABP2, but not PABP1 (56). Under low-stringency conditions, PABP2 also was associated with MKT1 and its partners ZC3H20, ZC3H28, ZC3H39, PUF6, and PUF9 (56). It was notable that PABP2 did not co-purify with the MKT1L–PBP1–LASM12–XAC1 complex. Although it is possible that the extended N terminus of MKT1L inhibits the PBP1–PABP2 interaction, we suggest that stability of the PABP2–PBP1 association in vivo is enhanced if PBP1 is also bound to mRNA via additional interactions (Fig. 9A).

One of the most interesting results was the preferential association of XAC1-containing complexes with just one of the five possible EIF4E–EIF4G translation initiation complexes, containing EIF4E6 and EIF4G5. Moreover, EIF4G5 specifically interacts with MKT1 in the two-hybrid assay, and the EIF4E6–G5 complex, like XAC1, preferentially associates with PABP2 (56, 57). Results published so far suggest that EIF4E3 and EIF4E4 are the major translation factors in T. brucei (48). In procyclic forms, EIF4E6 may have a specialized role because its depletion caused flagellar defects, but the role of this complex in bloodstream forms is not known. Both EIF4E6 (this paper) and EIF4G5 (39) activate when tethered, which suggests that they are active in translation.

In conclusion, our results suggest that MKT1 complexes can enhance mRNA stability and translation through multiple interactions that, in combination, are absolutely essential for control of trypanosome gene expression (Fig. 9A). Several RNA-binding proteins each bind independently to different subsets of mRNAs with various degrees of affinity and sequence specificity. After recruitment of MKT1 complexes, their association with mRNA is stabilized through interaction of PBP1 with PABP2, whereas the interaction of MKT1 with EIF4G5 results in preferential cap-binding by EIF4E6, preventing decapping and stimulating translation initiation. Mkt1 homologues are present in very diverse organisms, including Toxoplasma and various amoebae in addition to fungi, but even in S. cerevisiae, its function is unknown. Our results suggest that it would be worthwhile, in those species also, to investigate whether Mkt1 directly influences mRNA stability or translation.

**Experimental procedures**

**Cells and plasmids**

Details of all plasmids and oligonucleotides are provided in Table S1. The culture conditions were as described (58). All
experiments were done with Lister 427 monomorphic bloodstream parasites expressing the Tet repressor and with procyclic forms expressing the Tet repressor and T7 polymerase (59).

Stable cell lines expressing V5- or YFP-tagged proteins were generated by integrating the tag-encoding sequence in frame with the coding region at the original locus (60, 61). Myc- and TAP-tagged proteins were obtained by tetracycline-inducible expression of fusion proteins from constructs integrated in the rRNA locus (62). Expression was induced using 200 ng/ml tetracycline. RNase against XAC1 or MKT1L was used inducing stem-loop plasmids in cell lines with one of the copies of XAC1 or MKT1L tagged with V5.

For the tethering assays, cell lines constitutively expressing a CAT reporter with actin 3'-UTR or boxB actin 3'-UTR were co-transfected with plasmids for inducible expression of fusion proteins from constructs integrated in the AN-XAC1-Myc full-length or fragment fusion proteins (38).

For gene knockouts, deletion cassettes were constructed using the Blastidin S deaminase (Bsd), Puromycin acetyltransferase (PAC), or neomycin phosphotransferase (NPT) genes. After transfection and selection, each resistance marker replaced one allele of MKT1 or MKT1L.

### 35S pulse labeling

3 × 10^7 cells were pelleted at 800 × g for 10 min at RT, transferred to a 1.5-ml Eppendorf tube, and washed twice with 1× PBS, followed by centrifugation at 4,000 × g for 3 min. The cell pellet was resuspended in 400 μl of labeling medium and incubated at 37 °C for 15 min. 2 μl of L-[35S]methionine (about 20 μCi) was added. The cells were incubated for 1 h at 37 °C. The cells were pelleted and washed twice with 1× PBS (4,000 × g for 3 min at RT). The pellet was resuspended in 15 μl of Laemmli lysis buffer, and the proteins were separated in a 12% SDS gel. The labeling medium was Dulbecco's modified Eagle's medium (Gibco, high-glucose, containing pyridoxine hydrochloride, lacking l-glutamine, sodium pyruvate, l-methionine, and l-cysteine), supplemented with 25 mM HEPES, 2 mM glutamine, 0.1 mM hypoxanthine, 1.5 mM l-cysteine, 0.0028% β-mercaptoethanol, 0.05 mM bathocuproinsulfate, and 10% heat-inactivated fetal calf serum (previously dialyzed against 30 mM HEPES, pH 7.3, 150 mM NaCl).

### Targeted yeast two-hybrid analysis

The Matchmaker Yeast Two-Hybrid System (Clontech) was used for protein–protein interaction analysis following the manufacturer's protocol. The DNAAs of the protein ORFs were PCR-amplified and cloned into both pGBKKT7 and pGADT7. The plasmids were co-transformed pairwise into AH109 yeast strains (Matchmaker 3 System, Clontech) and selected initially on double drop-out medium (minimal SD medium lacking Trp and Leu). Positive interactions were indicated mainly by growth on quadruple dropout (QDO) medium (minimal SD medium lacking Trp, Leu, His, and Ade) and sometimes confirmed by a blue color change due to X-α-gal present in the medium. The interaction between murine p53 and SV40 large T-antigen served as positive control, with Lamin-C and SV40 large T-antigen as negative bait (DNA-binding domain) and prey controls, respectively.

### Affinity purification for MS

All MS results are from three completely independent protein purifications. For tandem affinity purification from bloodstream forms, ~1 × 10^10 trypanosomes expressing TAP-tagged protein were harvested and used for tandem affinity purification as described previously (63). The elute was run 2 cm into a 1.5-mm NuPAGE™ Novex™ 4–12% BisTris protein gel (Thermo Fisher Scientific), stained with Coomassie Blue and distained with distaining solution (10% acetic acid and 50% methanol). The protein-containing gel area was analyzed by MS. Data were analyzed quantitatively. Affinity purifications from procyclic forms were done using cells expressing V5 in situ tagged proteins. 1 × 10^7 cells were harvested by centrifugation at 2,300 × g for 13 min at 4 °C. The cell pellet was washed in 10 ml of ice-cold PBS supplemented with protease inhibitors, followed by centrifugation at 2,300 × g for 8 min at 4 °C. Cell lysis was done by adding 1 ml of IP lysis/wash buffer with low salt concentration (20 mM Tris, pH 7.5, 150 mM KCl, 5 mM MgCl₂, 0.05% IGEPAI, 0.5 mM DTT) supplemented with 10 μg/ml aprotinin and 10 μg/ml leupeptin. Cells were pipetted up and down, passed 20 times through a 21-gauge × ½ needle and 20 times through a 27-gauge × ⅜ needle. The complete cell lysis was checked under light microscope slides. The sample was centrifuged at 10,000 × g for 15 min at 4 °C, and the supernatant was incubated for 4 h at 4 °C with 50 μl of V5 magnetic beads (20 mg/ml Dynabeads® M-280 Tosoylactivated, Invitrogen, coupled with anti-V5 tag antibody, clone SV5-Pk1, Bio-Rad). The unbound fraction was discarded, and the beads were washed twice with 1 ml of IP buffer and additionally twice more with 1 ml of ice-cold PBS containing protease inhibitors. The bound proteins were neutrally eluted with 50 μl of V5 peptide (V7754-4MG, Sigma) at 1 mg/ml in PBS, at 37 °C on a rotator for 10 min. The eluted sample was submitted to MS as described for the bloodstream forms.

### Quantitative MS data analysis

NanoFlow LC-MS² analysis was performed at the ZMBH MS facility, with an Ultimate 3000 liquid chromatography system directly coupled to an Orbitrap Elite mass spectrometer (both from Thermo Fisher Scientific, Bremen, Germany). Samples were delivered to an in-house packed analytical column (inner diameter 75 μm × 20 cm; CS–Chromatographie Service GmbH, Langerwehe, Germany) filled with 1.9 μm of Reprosil-Pur-AQ 120 C18 material (Dr. Maisch, Ammerbuch-Entringen, Germany). The mass spectrometer was operated in data-dependent acquisition mode, automatically switching between MS and MS². Raw files were processed using MaxQuant (version 1.5.3.30) for peptide identification and quantification. MS² spectra were searched against the T. brucei 927 annotated protein database, release 9.0 from TrTryptDB, and contaminants of Maxquant were included. We defined carbamidomethylation of cysteine residues as a fixed modification and acetyl (protein N-terminal), oxidation of methionine and deamidation of aspartic and glutamic acid as variable modifications and
PBP1-containing complexes in T. brucei

trypsin/P as the proteolytic enzyme with up to two missed cleavages allowed. The maximum false discovery rate for proteins and peptides was 0.01, and a minimum of two peptides per protein and a minimum peptide length of 7 amino acids were required. Peptides shared between orthologues and paralogues were assigned to the same protein group. Analysis was done in LFQ mode. MaxQuant calculated individual mass tolerances for each peptide, with the initial mass tolerances of the precursor ion set to 20 and 4.5 ppm for the first and main searches, respectively. All other parameters are default parameters of MaxQuant.

Statistical analysis of the results was performed using the Perseus toolbox (64) (version 1.6.2.1). Ratios were calculated from label-free quantification intensities. Missing values were imputed using the Perseus default settings of imputation (mean downshift = 0.3, S.D. downshift = 1.8). A two-sample t test was performed comparing the natural logarithm of the intensities from the experiments and the control groups. To confirm the specificities of the purifications, data from TAP-GFP or V5-GFP purifications were used as controls for bloodstream- and procyclic-form purifications, respectively.

Affinity purification and RNA-Seq

For RNA-Seq analysis, we used cells expressing TAP-tagged protein but omitted the second step of purification. The bound and unbound RNAs were purified using peqGOLD Trifast reagent (Peqlab), and rRNAs were depleted using RNA-complementary oligonucleotides and RNase H (65). Library-building (Bioquant, Heidelberg) and sequencing (EMBL, Heidelberg) were done as described (42).

Data were trimmed and aligned using the tryprnaseq pipeline (66), which includes Bowtie2 for alignment (67). Each read was allowed to align once to both the Lister427_2018 (68) and TREU 927 (69) genomes. The Lister 427 genome correspond to the cells used for the experiments and includes strain-specific expression sites and variant surface glycoprotein genes. However, the TREU 927 genome is much better annotated in chromosome-internal regions. When genes are present more than once, which is common in trypanosomes, the reads are shared among the different copies.

For statistical analysis (e.g. to look at enriched gene categories), we considered only a set of “unique” genes, in which only one representative of each repeated gene is present (adapted from Ref. 6). The functional categories were manually annotated (see Table S3). The ploidy of each of the “unique” genes was assessed using two data sets, for which each read was allowed to align 20 times. To ensure that each sequence was weighted appropriately, before the calculations, each “unique gene” read count was multiplied by the ploidy. Data were analyzed using DeSeqUI (70), an adapted version of DeSeq2 (71).

Northern blotting and polysome analysis

For Northern blotting, total RNA was extracted using peqGOLD Trifast reagent (Peqlab). Isolated RNA (typically 20 μg of total RNA or RNA purified from the entire gradient fraction) was resolved on formaldehyde-agarose gel, blotted to nylon membranes, and detected by hybridization with radioactive probes for CAT and β-tubulin (TB927.12370) mRNAs. Quantification was done using ImageJ software.

Protein detection and manipulation

Immunoprecipitation assays were done as described previously. 1 × 10^6 bloodstream trypanosomes expressing the tagged protein were harvested by centrifugation (800 × g, 10 min, 20 °C), washed with 1 ml of cold PBS, and lysed in hypotonic buffer (10 mM NaCl, 25 mM Tris-Cl, pH 7.5, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.1% IGEPA) by passing 10-20 times through a 21-gauge × ½ needle; for EIF4E, this was followed by 20 passes through a 27-gauge needle. After pelleting insoluble debris by centrifugation (17,000 × g, 10 min, 4 °C) and adjusting to 150 mM NaCl or KCl, the clarified lysate was used for immunoprecipitation with 30 μl of anti-Myc or anti-V5 coupled beads (Bethyl Laboratories) or with 10 μl of IgG-coupled magnetic beads (20 mg/ml Dynabeads® M-280 tosyl-activated (Invitrogen) coupled with purified rabbit IgG (Bio-Rad)) for pulldowns of PTP-tagged EIF4E6. The YFP-XAC1 fusion protein was immunoprecipitated using GFP-binding protein coupled to NHS-Sepharose that was kindly provided by Dr. Georg Stoecklin (ZMBH, Heidelberg University, Germany). The beads were incubated for 2 h at 4 °C and then washed four times at 4 °C (5 min of incubation followed by centrifugation at 850 × g, 5 min) with IPPI50 (10 mM Tris, pH 7.5, 150 mM NaCl or KCl, 0.1% IGEPA). Samples for Western blots were taken during the procedure, and the beads were boiled in SDS sample buffer. Proteins were detected by Western blotting according to standard protocols. Chloramphenicol acetyltransferase activity was measured in a kinetic assay involving partition of [14C]butyl chloramphenicol from the aqueous to the organic phase of scintillation fluid (72). The total protein concentration was measured by the Bradford method.

Blue native gel

Samples for native gel analysis were prepared as follows. 1 × 10^8 bloodstream trypanosomes expressing the in situ N-terminal V5-tagged protein were harvested by centrifugation at 800 × g, 8 min at RT, washed twice with 1 ml of cold 1% PBS, resuspended in 50 μl of native extraction buffer (25 mM HEPES, 150 mM sucrose, 20 mM potassium glutamate, 3 mM MgCl₂, 0.5% Igepal, 150 mM KCl, 0.5 mM DTT, 10 μg/ml leupeptin, 10 μg/ml aprotinin), incubated on ice for 30 min, and spun down at 17,000 × g for 15 min at 4 °C. Supernatants were prepared and electrophoresed in a precast 4–16% native PAGE, Novex BisTris Mini Gel following the manufacturer’s protocol (Life Technologies, Inc.). The Native Mark Unstained Protein Ladder (Life Technologies, Inc.) was used as standard protein sizes. Proteins were transferred to 0.45 μM Immun-Blot polyvinylidene difluoride membranes (Thermo Scientific™ Pierce™) with 1 × NuPAGE® Transfer Buffer at 25 V, 4 °C, overnight. Membranes were fixed in 8% acetic acid for 15 min and rinsed with water, after which the proteins were detected using the standard Western blotting procedure.
Data availability

The MS data are available via ProteomeXchange with identifier PXD018576. ArrayExpress accession numbers for the RNA-Seq data are as follows: XAC1 RNAi, E-MTAB-6239; TAP-MKT1 pulldown, E-MTAB-6907; TAP-MKT1L pulldown, E-MTAB-6904. Any other relevant underlying data that are not in the paper are available on request from the corresponding author, Christine Clayton (cclayton@zmbh.uni-heidelberg.de).

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Abbreviations—The abbreviations used are: PABP, poly(A)-binding protein; RT, room temperature; QDO, quadruple dropout; SD medium, synthetic defined medium; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; IP, immunoprecipitation; YFP, yellow fluorescent protein; TAP, tandem affinity purification.

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