

3' adenylation determines mRNA abundance and monitors completion of RNA editing in *T. brucei* mitochondria

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Expression of the mitochondrial genome in protozoan parasite *Trypanosoma brucei* is controlled post-transcriptionally and requires extensive U-insertion/deletion mRNA editing. In mitochondrial extracts, 3' adenylation reportedly influences degradation kinetics of synthetic edited and pre-edited mRNAs. We have identified and characterized a mitochondrial poly(A) polymerase, termed KPAP1, and determined major polypeptides in the polyadenylation complex. Inhibition of KPAP1 expression abrogates short and long A-tails typically found in mitochondrial mRNAs, and decreases the abundance of never-edited and edited transcripts. Pre-edited mRNAs are not destabilized by the lack of 3' adenylation, whereas short A-tails are required and sufficient to maintain the steady-state levels of partially edited, fully edited, and never-edited mRNAs. The editing directed by a single guide RNA is sufficient to impose a requirement for the short A-tail in edited molecules. Upon completion of the editing process, the short A-tails are extended as (A/U) heteropolymers into structures previously thought to be long poly(A) tails. These data provide the first direct evidence of functional interactions between 3' processing and editing of mitochondrial mRNAs in trypanosomes.

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Introduction

RNA processing in the mitochondria of trypanosomatid protozoa begins with a nucleolytic partitioning of multi-cistronic transcripts into ribosomal RNAs and pre-mRNAs, which are then subjected to 3' polyadenylation and U-insertion/deletion RNA editing. In *Trypanosoma brucei*, editing is required for expression of 12 of the 18 protein-encoding genes. Multi-

protein complexes carry out editing reactions (20S editosome), guide RNA (gRNA) 3' uridylylation (RNA editing TUTase 1 (RET1)), and mRNA–gRNA annealing (mitochondrial RNA binding proteins 1 and 2 (MRP1/2)) (reviewed by Simpson *et al*, 2004; Stuart *et al*, 2005). In the steady-state RNA population, pre-edited, highly heterogeneous partially edited and fully edited forms have been distinguished (Abraham *et al*, 1988; Decker and Sollner-Webb, 1990; Sturm and Simpson, 1990).

Polyadenylation ubiquitously regulates RNA stability, even though the exact role of the poly(A) tail may differ among cellular organelles or within the same compartment (reviewed by Martin and Keller, 2007). The virtually universal nuclear polyadenylation of eukaryotic mRNAs is accomplished by macromolecular complexes bearing poly(A) polymerase (PAP) as a catalytic subunit and is coupled to transcription, splicing, and nuclear export (Proudfoot, 2004). Conversely, adenylation of intergenic transcripts and various aberrant RNAs by the non-canonical Trf4/5 PAPs leads to RNA degradation in the nucleus of budding yeast (reviewed by Houseley *et al*, 2006). Cytoplasmic polyadenylation generally stimulates translation and is often targeted to specific mRNAs through RNA binding proteins (Huang and Richter, 2004; Stevenson and Norbury, 2006). Despite the mitochondrion's monophyletic origin, the roles of polyadenylation in this organelle are diverse. For example, yeast mitochondrial mRNAs are not polyadenylated, whereas in plant mitochondria (Gagliardi *et al*, 2004) and bacteria (Kushner, 2004), the poly(A) tail is a signal for degradation. In human mitochondria, polyadenylation stabilizes mRNAs whereas de-adenylation by polynucleotide phosphorylase has the opposite effect (Nagaike *et al*, 2005).

Existing data suggest that polyadenylation has a key role in regulating *T. brucei* mitochondrial genome expression by altering mRNA stability. In addition to the sequence heterogeneity created by mRNA editing, mitochondrial transcripts have either short (~20–25 nucleotides (nt)) or long (~120–250 nt) A-tails (Bhat *et al*, 1992). The length of the poly(A) tail appears to correlate with the editing status of the mRNA: pre-edited forms possess only short A-tails whereas never-edited and edited mRNAs have both short and long A-tails (Bhat *et al*, 1992; Read *et al*, 1994; Militello and Read, 1999). *In vitro* experiments have demonstrated that synthetic pre-edited mRNAs with short A-tails degrade rapidly, whereas partially or fully edited messages were relatively stable in mitochondrial extracts (Ryan *et al*, 2003; Kao and Read, 2005). However, PAP activity has never been detected in mitochondrial extracts and the abundance of mitochondrial transcripts has not been linked to their polyadenylation status *in vivo*.

We have previously pointed out that the RNA editing TUTases, RET1 (Aphasizhev *et al*, 2002) and RET2 (Aphasizhev *et al*, 2003; Ernst *et al*, 2003), are homologous

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to several proteins encoded in trypanosomal genomes (Aphasizhev, 2005). Indeed, the non-mitochondrial TUT3 (Aphasizhev *et al*, 2004) and TUT4 (Stagno *et al*, 2007b) have been characterized as RNA uridylyl transferases. Beyond Kinetoplastida, however, data mining of eukaryotic genomes has identified 'non-canonical' PAPs, such as animal cytoplasmic Gld-2-type and yeast nuclear 'quality control' Trf4/5 PAPs, as the proteins most closely related to TUTases (not shown). This apparent ambiguity was partially resolved by examining the X-ray structures of TUT4 in complexes with UTP or ATP, and the demonstration that TUTase's active site may be hospitable to ATP binding (Stagno *et al*, 2007a). Therefore, we hypothesized that some TUTase-like proteins in trypanosomes are non-canonical PAPs. To search for the mitochondrial PAP, we set out to analyse the NTP substrate specificity and cellular function of a candidate TUTase-like protein with a putative mitochondrial importation signal, previously designated TUT5 (Aphasizhev, 2005).

Here we show that TUT5, re-named kinetoplast poly(A) polymerase 1 (KPAP1), is a mitochondrial PAP essential for parasite viability and mitochondrial function. This enzyme is directly involved in the synthesis of short A-tails that are required and sufficient for the steady-state abundance of never-, partially, and fully edited mitochondrial mRNAs. The pre-edited transcripts were either unaffected or increased because of the loss of KPAP1 and the ensuing lack of short A-tails. Editing directed by a single gRNA is sufficient to impose a requirement for the short A-tail in partially and fully edited molecules. Further, A/U heteropolymers, previously thought to be long poly(A) tails, are appended to the fully edited mRNAs upon completion of the editing process. Thus, the A/U-tail may represent a hallmark of translationally competent mRNAs. Proteomic analysis of the affinity-purified KPAP1 complex revealed a novel RNA processing particle composed of RNA binding and pentatricopeptide repeat (PPR) proteins, and polypeptides with no similarities beyond Kinetoplastida.

Results

KPAP1 is a mitochondrial PAP

Protein sequences of RET1, RET2, TUT3, and TUT4 were used to search Kinetoplastida genome databases (<http://www.genedb.org>), leading to the identification of the KPAP1 gene (Supplementary Figure S1). Trypanosomal nuclear PAP (Mair *et al*, 2000) was not detected in these searches. The recombinant KPAP1 (Figure 1A) showed a preference for ATP in a nucleotidyl transfer assay (Figure 1B). To verify the enzymatic identity, KPAP1 with a D97A mutation in the catalytic site was purified and found to be inactive (Figure 1B). Apparent molecular mass determined by gel filtration on Sepharose 12 column in 150 mM of KCl (not shown) closely matched the predicted value for the His-tagged protein (61 kDa), indicating that recombinant KPAP1 is a monomer. To assess the processivity of ATP polymerization by KPAP1, the reactions were carried out under competitor challenge conditions. Following pre-incubation of the RNA substrate with enzyme, extensions were initiated by addition of ATP plus increasing concentrations of unlabelled RNA. The product length was linearly reduced from 20–25 to 2–3 adenosines, illustrating a distributive nucleotidyl transferase activity (Figure 1C).

Western blotting of *T. brucei* subcellular fractions demonstrated that KPAP1 is localized in the mitochondrial matrix. As shown in Figure 1D, KPAP1 was enriched in the density gradient-purified mitochondrial fraction. A subunit of the 20S editosome, MP81 (Drozd *et al*, 2002), showed a similar distribution, indicating that KPAP1 is localized to the mitochondrial matrix. A truncated form of KPAP1 was also observed in the mitochondrial fraction. The cytoskeletal (β -tubulin) and cytosolic (HSP70.4) proteins were not detected, verifying the correct segregation of subcellular compartments. KPAP1 localization in live *T. brucei* cells was confirmed by expressing its C-terminal fusion with enhanced yellow fluorescence protein (eYFP). The KPAP1-eYFP colocalized with the membrane potential-dependent MitoTracker Red CMX-Ros dye (Figure 1E). Interestingly, the apparent concentration of KPAP1 was observed at two punctate antipodal regions adjacent to the kDNA disk.

KPAP1 is essential for cell viability and mitochondrial function

As seen from RNA interference (RNAi) analysis, the *KPAP1* gene is essential for the viability of insect (procyclic form (PF)) *T. brucei*. Upon RNAi induction with tetracycline, cumulative cell growth was monitored in comparison to mock-treated culture. The growth inhibition was followed by elimination of live cells from the culture (Figure 2A). The KPAP1 protein was undetectable after 50 h of RNAi induction (Figure 2B). To address the appearance of a second band in the mitochondrial extract (Figure 1D, KPAP1 panel), RNAi was induced for ~50 h and subcellular fractionation was carried out. Both bands disappeared in the mitochondrial fraction depleted of KPAP1 by RNAi (Figure 2C).

Maintenance of the mitochondrial membrane potential ($\Delta\Psi_m$) is essential for PF and BF (bloodstream) trypanosome viability (Schnauffer *et al*, 2005; Brown *et al*, 2006). The depolarization of the mitochondrial membrane over the course of KPAP1 RNAi was monitored in the PF *T. brucei* (Supplementary Figure S2). RNAi was induced at 24-h intervals and individual cultures were maintained in the presence of tetracycline. Cell staining with membrane potential-sensitive dye MitoTracker Red CMX-Ros and FACS analysis for all time points were performed at the same time. Treatment with CCCP (carbonylcyanide *m*-chlorophenylhydrazone) protonophore, which discharges the pH gradient across the inner membrane, was included as a control for the loss of membrane potential. In this experiment, cell division arrest was observed between 72 and 96 h, which coincided with the appearance of a predominant population of cells lacking a membrane potential. Therefore, KPAP1 is essential for cell viability and mitochondrial function.

KPAP1 interacts with RNA editing complexes, but its depletion does not affect editing activity

Previous work has suggested that polyadenylation exerts different effects on the stability of edited and pre-edited mRNAs in mitochondrial extracts (Kao and Read, 2005). RET1 was also implicated in the UTP-dependent mRNA degradation *in organello* (Militello and Read, 2000; Ryan and Read, 2005). To investigate KPAP1 interactions with RNA editing complexes, immunoprecipitations with anti-KPAP1 antibody were performed in the mitochondrial extract. The core components of the 20S editosome, RNA editing

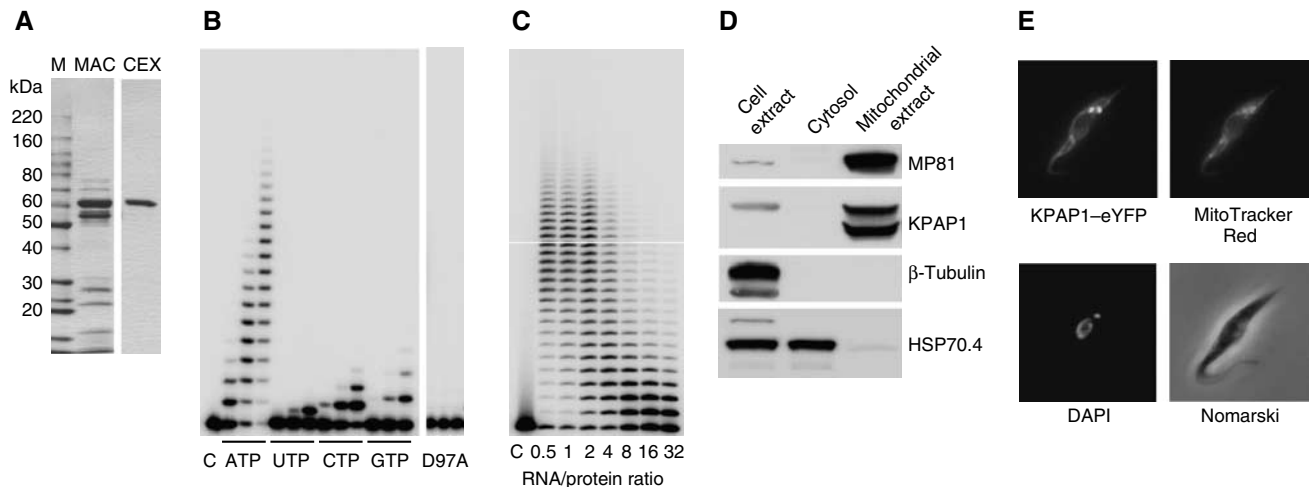


Figure 1 KPAP1 is a mitochondrial PAP. (A) Purification of the recombinant KPAP1 from *E. coli*. M: mol. mass marker; MAC: metal affinity chromatography; CEX: cation exchange column. Proteins were separated on 8–16% gradient SDS-PAGE. (B) NTP specificity of KPAP1. The 5'-labelled 24-mer 6[A] RNA was incubated with KPAP1 at increasing concentrations of NTPs (1, 10, and 100 μ M). The mutant D97A protein was tested in the presence of ATP. C: control RNA. The products were resolved on a 15% polyacrylamide/8 M urea gel. (C) Primer challenge assay. The 5'-labelled 6[A] RNA and KPAP1 were pre-incubated at final concentrations of 25 and 50 nM, respectively, for 10 min at 27°C. Reactions were started by simultaneous addition of ATP to 100 μ M and unlabelled 6[A] RNA to 0, 25, 50, 100, 200, 400, and 800 nM. Incubation was continued for 30 min. (D) Subcellular fractionation of procyclic *T. brucei*. Approximately 10 μ g of protein from hypotonic cell extract, cytosolic fraction, and purified mitochondria was separated on SDS-PAGE. Western blotting was performed with antibodies against KPAP1 and mitochondrial (MP81), cytoskeletal (β -tubulin), and cytosolic (HSP70.4) proteins. (E) Intracellular distribution of KPAP1. The C-terminal KPAP1-eYFP fusion was expressed in pLew79-based vector (Wirtz *et al*, 1999). Fluorescent images were captured in the presence of MitoTracker Red CMX ROS and DAPI stains.

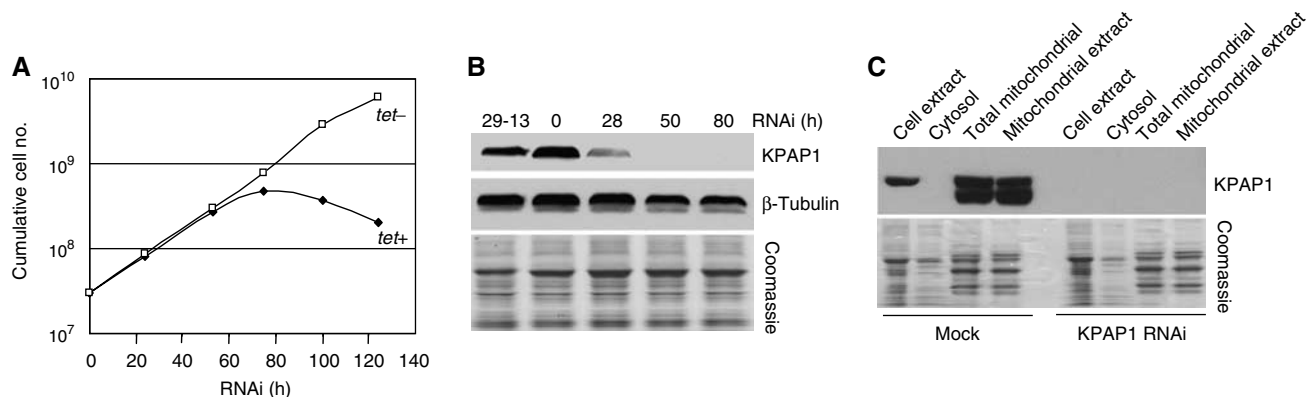


Figure 2 KPAP1 is an essential gene required for mitochondrial function. (A) Cumulative cell growth after KPAP1 RNAi induction in procyclic *T. brucei*. (B) Depletion of KPAP1 protein in PF *T. brucei*. Cell lysates from the parental cell line (29-13) and tet-induced cells were separated on SDS-PAGE and probed with anti-KPAP1 antibody. (C) Subcellular fractions obtained from KPAP1 RNAi cell line were probed with anti-KPAP1 antibody. The Coomassie blue staining was used as loading control.

ligase 1 (REL1) and MP81, and RET1 were detected in the co-immunoprecipitated (co-IP) material (Figure 3A). These interactions do not appear to be RNA-mediated, as RNase A and high-salt treatment had no effect on co-IP. Insignificant depletion of editing proteins was observed when all detectable KPAP1 was immunoprecipitated from the mitochondrial extract (not shown), suggesting that only minor fractions of the 20S editosome and RET1 are stably associated with KPAP1.

To assess the effects of KPAP1 RNAi on RNA editing complexes and editing activity *in vivo*, mitochondrial extracts were obtained from the *T. brucei* parental cell line (29-13), and cells were subjected to KPAP1 RNAi for ~50 h. The extracts were fractionated on 10–30% glycerol gradients and each fraction was tested for the 20S editosome (Figure 3B). This approach reportedly detects even minor alterations in

the editosome structure (Aphasizhev *et al*, 2003; Kang *et al*, 2006), yet no apparent differences were observed. The peak fraction 9 from both gradients, adjusted for REL1 adenylation signal and total protein amount, showed virtually identical U insertion (Figure 3C) and U-deletion (data not shown) editing activities on a pre-cleaved mRNA substrate (Igo *et al*, 2000).

Next we analysed gRNA abundance and size distribution. The 5' labelling of primary transcripts, such as gRNAs, with guanylyltransferase in the presence of [α -³²P]GTP (Blum *et al*, 1990) detected no appreciable difference caused by KPAP1 RNAi (Figure 3D). As a control for the loss of oligo[U] tail in gRNAs (Aphasizhev *et al*, 2003), RNA isolated from cells depleted of RET1 by RNAi was subjected to the same labelling procedure. These results show that KPAP1 interacts with RNA editing components, but its depletion does not

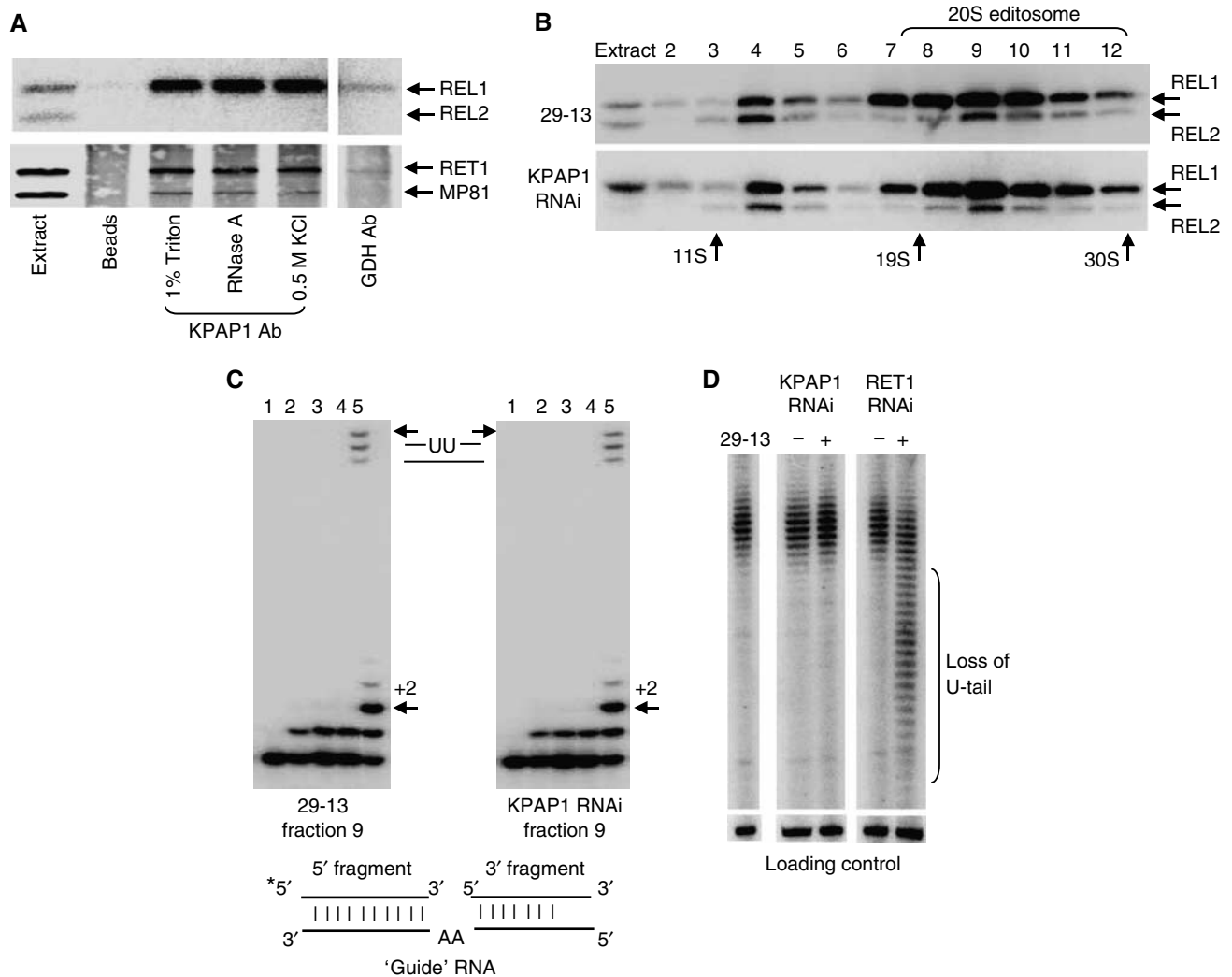


Figure 3 Inhibition of *KPAP1* expression does not affect RNA editing complexes. **(A)** Co-immunoprecipitation of *KPAP1* with 20S editosome and *RET1*. Mitochondrial extract (200 μ l, \sim 5 mg protein/ml) from *T. brucei* was incubated for 1 h with 10 μ l of magnetic beads pre-coated with antigen-purified anti-*KPAP1* antibodies. Additional 30 min washes with 1% Triton or RNase A (0.1 mg/ml) in PBS buffer, or with 0.5 M KCl, were performed to assess the stability of *KPAP1* interactions. Immunoprecipitated material was adenylated on beads, separated on SDS-PAGE, and probed for *RET1* and *MP81* on immunoblotting. Co-IP with antibodies against glutamate dehydrogenase (*GDH*) served as a negative control. **(B)** Sedimentation of the 20S editosome from *KPAP1*-depleted mitochondrial extracts. Mitochondrial extract was fractionated on a 10–30% glycerol gradient. Fractions were incubated with [α - 32 P]ATP to detect editing ligases *REL1* and *REL2* and separated on SDS-PAGE. Sedimentation standards (catalase (11 S), thyroglobulin (19 S), and *E. coli* 30S ribosome subunit) migrated as indicated by arrows. **(C)** U-insertion editing activity in the peak gradient fraction. RNA substrates for the pre-cleaved editing assay were assembled as follows: (1) 5' fragment, no proteins added; (2) 5' fragment; (3) 5' fragment + 'guide' RNA; (4) 5' fragment + 3' fragment; (5) fully assembled substrate for +2 addition, 5' fragment + 3' fragment + 'guide' RNA. Positions of +2 guided U-insertions and ligation of edited product are shown by arrows. **(D)** gRNA labelling. Total RNA was isolated from the parental cell line (29-13), *KPAP1*, and *RET1* RNAi cells. Guide RNAs were 5' labelled with [α - 32 P]GTP in the presence of vaccinia virus guanylyltransferase and separated on 10% polyacrylamide/urea gel. The unidentified cytosolic RNA labelled with [α - 32 P]GTP was used as a loading control.

affect the integrity of editing complexes, editing activity, or gRNA abundance.

Purification of mitochondrial polyadenylation complex

To analyse complexes associated with *KPAP1*, mitochondrial extract from *T. brucei* expressing a TAP-tagged (Puig *et al*, 2001) fusion protein was subjected to sedimentation in a 10–30% glycerol gradient. Fractions were pre-incubated with [α - 32 P]ATP to self-adenylate editing ligases, separated by gradient Tris-glycine PAGE in the presence or absence of SDS, and transferred to nitrocellulose membrane (Figure 4A). Western blotting with PAP reagent, which detects the protein A moiety in the TAP tag, and exposure to phosphor storage

screen were used to detect polyadenylation and editing complexes, respectively. SDS-PAGE analysis showed that *KPAP1* complexes sediment in the 15–30S range, whereas native gel separation revealed *KPAP1*-containing particles with apparent molecular weights ranging from \sim 0.4 to 1.2 MDa (Figure 4A). Unassociated *KPAP1*-TAP, which would be expected to migrate at \sim 80 kDa in the native gel and sediment at the top of the gradient, was also detected in the mitochondrial extract.

Tandem affinity purification of *KPAP1* complex from mitochondrial extract produced a set of \sim 15 polypeptides, which was further analysed by western blotting (Figure 4B) and mass spectrometry (Table I). Consistent with immuno-

Table I Proteins identified in TAP tag affinity-purified KPAP1 complex

Protein	Band analysis	Total complex digest		Motifs
		Untreated	RNase	
Tb11.02.5820 (KPAP1)	24 (122)	5 (10)	12 (39)	PAP core PAP assoc.
Tb927.6.2230 (TBRGG1)	4 (18)	6 (16)	8 (43)	RGG
Tb927.2.3180 (TBPPR1)	22 (61)	7 (30)	5 (43)	PPR
Tb09.211.3720	11 (42)	5 (22)	3 (32)	PPR
Tb927.8.3170	9 (32)	ND	ND	PPR
Tb11.02.3180	8 (22)	ND	ND	PPR
Tb11.01.5980	4 (16)	5 (16)	3 (21)	PPR
Tb10.389.0070 (EF-Tu)	11 (55)	2 (6)	8 (43)	EF-Tu
Tb11.01.7510	13 (34)	ND	ND	ND
Tb11.47.0024	9 (34)	ND	ND	ND
Tb927.7.2570	5 (19)	2 (10)	1 (7)	ND
Tb927.2.3800	3 (13)	2 (7)	1 (5)	ND
Tb927.4.4150	ND	3 (7)	2 (14)	ND
Tb927.1.3010	ND	3 (7)	ND	ND
Tb11.02.5390	ND	2 (11)	ND	ND

Number of unique peptides matches in Mascot searches with e-value below 0.05 is provided. The total number of matches is shown in parentheses. ND, none detected.

the same amount of purified material demonstrated that some of the put-MBP polypeptides were no longer detected while coverage decreased for others indicating RNA-based interactions (Table I). Finally, the predicted mitochondrial elongation factor Tu was abundant in all preparations.

Enzymatic activities of the polyadenylation complex

We next wished to see whether the purified KPAP1 complex would produce elongation patterns similar to those of the recombinant protein (Figure 1B and C) or long A-tails observed *in vivo*. To generate an RNA molecule that resembles an *in vivo* substrate for KPAP1, the 3' fragment of the pre-edited mRNA for small ribosomal protein subunit 12 (RPS12) was fused to the modified HDV ribozyme sequence (Supplementary data). The ribozyme self-cleavage produced a defined 3' end terminating with five encoded uracil residues. In the presence of ATP, activities of the recombinant enzyme and purified KPAP1 complex, adjusted for KPAP1 concentration, were virtually indistinguishable (Figure 4C, left panel). Addition of UTP into the reaction revealed the presence of a processive TUTase activity, which is in agreement with KPAP1-RET1 co-IP (Figure 3A) and immunoblotting of the purified KPAP1 complex (Figure 4B).

Reactions with KPAP1 complex did not produce the expected 20–25 nt A-tail of the pre-edited mRNAs, likely because of low enzyme concentration. Given the distributive nature of KPAP1 activity (Figure 1C), we tested whether increasing the concentration of the recombinant protein would lead to the formation of such a structure. Surprisingly, at concentrations above 50 nM, short A-tail was synthesized but the additions effectively stopped at 20–25 nt (Figure 4D). These findings indicate that KPAP1 does not require associated factors for synthesis of short A-tail but is unable to add longer moieties because of an intrinsic self-limiting mechanism.

Inhibition of KPAP1 expression reduces the abundance of never-edited and edited mRNAs

To assay the changes in mRNA abundance caused by KPAP1 RNAi, we carried out 'poisoned' primer extensions with oligonucleotides that hybridize downstream of editing do-

mains (Aphasizhev *et al*, 2002). The 3' regions of cytochrome oxidase subunit 3 (CO3) mRNA (Feagin *et al*, 1988) and ATP synthase subunit 6 (A6) (Bhat *et al*, 1990) were chosen as representative pan-edited mRNAs. Cytochrome oxidase subunit 2 (CO2) mRNA was analysed because editing is limited to the insertion of only four uridines (Benne *et al*, 1986) and is guided by a *cis*-acting RNA element (Golden and Hajduk, 2005 and references therein). The never-edited cytochrome oxidase subunit 1 (CO1) mRNA was tested with β -tubulin mRNA serving as a loading control (Figure 5A). The edited forms of all mRNAs, as well as the CO1 transcript, decreased in abundance. To further assess KPAP1 RNAi effects, the relative abundance of eight pre-edited, corresponding edited, and four never-edited mRNAs was determined by quantitative RT-PCR (qRT-PCR). In a uniform pattern, the rRNAs and pre-edited mRNAs either remained unaltered or increased, indicating lack of effects on transcription and nucleolytic processing of multi-cistronic transcripts. In contrast, all edited transcripts showed a 45–80% reduction (Figure 5B). Never-edited mRNAs also declined to various extents (Figure 5C).

To determine whether depletion of KPAP1 led to a loss of poly(A) tails, we used northern blotting to examine changes in transcript sizes (Figure 5D). In agreement with primer extension and qRT-PCR data, the abundance of both short A-tail (ST) and long A-tail (LT) forms of CO1 mRNA declined, whereas no changes were observed for β -tubulin mRNA and 12S rRNA. The CO2 probe, which hybridizes to both pre-edited and edited mRNAs (+4Us), detected a loss of the slower-migrating band, which would correspond to mRNA with a long A-tail. Probes that specifically bind to pre-edited and edited forms, and to both species, were used for the cytochrome *b* (Cyb) transcript. As expected, the edited mRNA was reduced by ~80%, but the pre-edited form increased by ~20% and became shortened. A similar pattern was observed for the pan-edited A6 mRNA. The accumulation of non-adenylated never-edited or edited transcripts in the steady-state mRNA population was not detected, likely because of rapid degradation. Furthermore, the loss of edited mRNAs apparently does not depend on the number of editing events or the positioning of editing blocks within an mRNA.

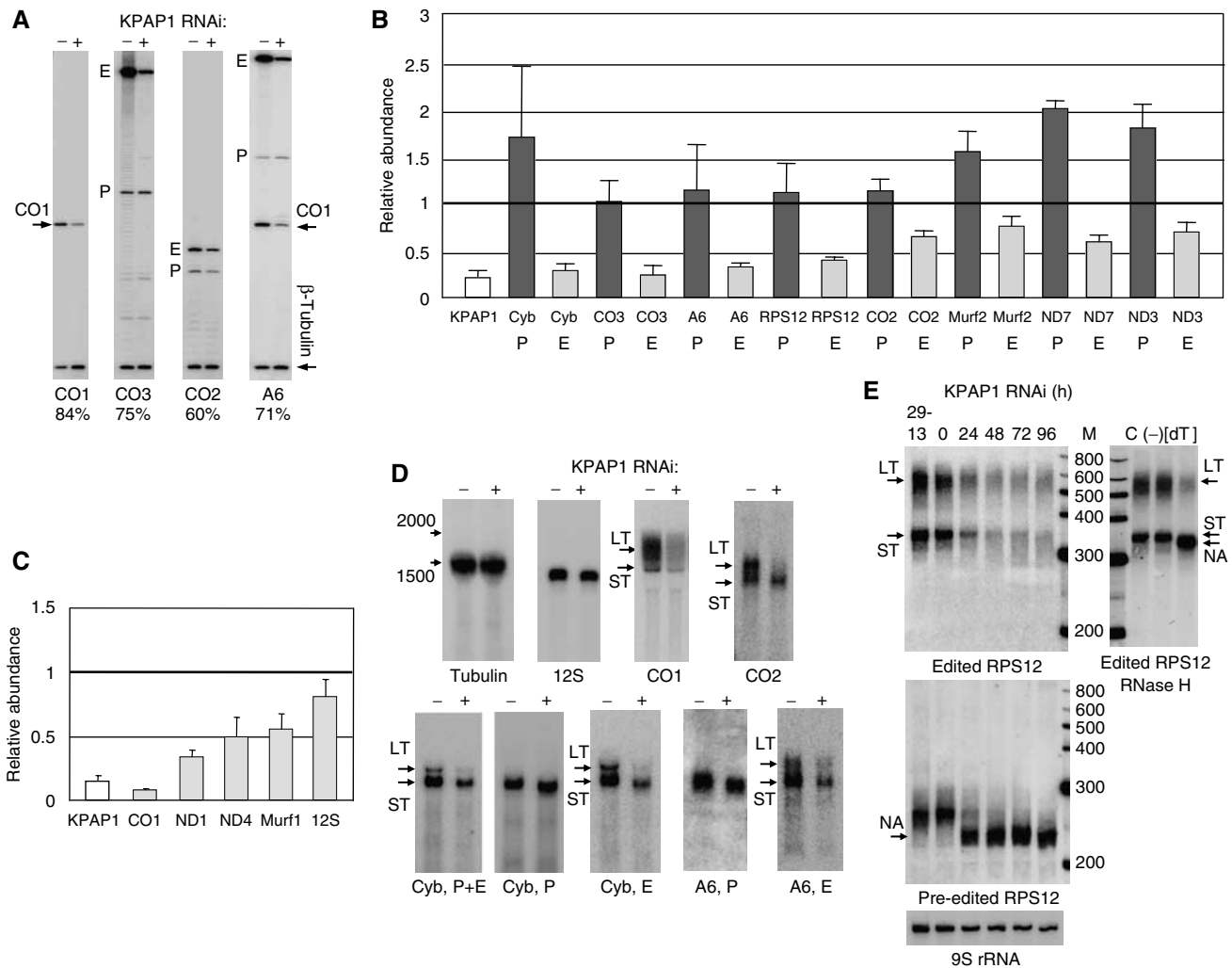


Figure 5 Polyadenylation determines the abundance of never-edited and edited mRNAs. (A) ‘Poisoned’ primer extension analysis. DNA oligonucleotides were hybridized with total RNA and reverse transcription was performed in the presence of ddGTP (Missel *et al*, 1997). Reaction products were separated on 10% polyacrylamide/urea gel. Single-extension products were observed with never-edited CO1 mRNA and β -tubulin. In the A6 panel, primers for β -tubulin, A6, and CO1 mRNA were used in a single reaction. Only extension products are shown, and 5'-labelled DNA primers are omitted. The relative decrease in abundance was calculated assuming the mitochondrial mRNA/ β -tubulin mRNA ratio in mock-induced cells as 100%. (B) Quantitative RT-PCR analysis of edited mRNAs. The RNA levels averaged from three replicates were normalized to β -tubulin mRNA. The thick line at 1 indicates no change in relative abundance. (C) Relative abundance of never-edited mRNAs and 12S ribosomal RNA. (D) Northern blotting of the never-edited (CO1), *cis*-edited (CO2), 5'-edited (Cyb), and pan-edited (A6) mRNAs. Gel bands corresponding to mRNAs with long-tail (LT) or short-tail (ST) adenylation patterns are shown by arrows. Total RNA (25 μ g) isolated from uninduced (-) and induced (+) KPAP1 RNAi cells was separated on 1.4% agarose-formaldehyde gel. (E) RPS12 mRNA decay in the course of KPAP1 RNAi. Total RNA was separated on 6% polyacrylamide/urea gel and sequentially probed for edited and pre-edited RPS12 transcripts, and 9S mitochondrial ribosomal RNA. Long-tail (LT), short-tail (ST), and non-adenylated (NA) forms are shown by arrows. C: untreated RNA; (-), RNase H digestion minus DNA oligo.

Collectively, these results demonstrate a uniform reduction of edited and never-edited transcripts in KPAP1 RNAi cells. The lack of apparent defects in transcription, nucleolytic processing of multi-cistronic transcripts (Figure 5), and RNA editing processes (Figure 3) implies that the increase in the decay rates of never-edited and edited transcripts is a most likely consequence of KPAP1 knockout.

Edited mRNAs with short and long A-tails are equally impacted by KPAP1 RNAi

The next step investigated whether the abundance of mRNAs distinguished by short and long A-tails changes synchronously with RNAi progression. Northern blotting of the never-edited CO1 revealed heterogeneous A-tails of more

than 200 nt, whereas edited mRNAs possessed more homogenous long A-tails of \sim 200 nt (Figure 5D). For a more precise length assertion of short and long A-tails, we analysed the mRNA encoding RPS12 (Maslov *et al*, 1992). Its smaller size made the transcript amenable to high-resolution separation on acrylamide/urea gels. As seen in Figure 5E (edited RPS12 panel), long tail (LT) and short tail (ST) forms of edited RPS12 mRNA showed a simultaneous decrease upon RNAi induction. The pre-edited form lost \sim 25 nucleosides (NA, Figure 5E, pre-edited RPS12 panel) but its abundance remained unaffected.

Because the non-adenylated edited mRNAs were undetectable by northern blotting, we set out to determine the length of poly(A) tail in both forms of edited RPS12. Total RNA was

incubated with 18-mer oligo[dT] and treated with RNase H (Figure 5E, RNase H panel). In the presence of oligo[dT], ST and LT edited forms collapsed into a single band corresponding to the predicted length (325 nt) of the fully edited RPS12 mRNA. The quantitation of the underexposed gels allowed us to estimate the length of the short and long A-tails as 20–25 and 240–250 nt, respectively. In summary, these results indicate that the presence of a short poly(A) tail is essential for the maintenance of edited mRNAs in mitochondria. Conversely, the steady-state level of pre-edited mRNAs does not depend on 3' adenylation.

KPAP1 adds short A-tails to pre-edited and never-edited mRNAs

The short A-tails apparently have distinct roles in maintaining the abundance of pre-edited and never-edited transcripts, although both types are thought to originate from the nucleolytic processing of the same precursor RNA. The non-adenylated pre-edited mRNAs are readily detectable by northern blotting whereas non-adenylated never-edited transcripts are not (Figure 5). Therefore, the question arose if KPAP1 is responsible for the short A-tail addition to never-edited mRNAs. To directly analyse poly(A) tails in pre-edited and never-edited mRNAs, total RNA was isolated from cells subjected to KPAP1 RNAi for various time periods, followed by circularization with T4 RNA ligase, transcript-specific cDNA synthesis, and PCR amplification in a procedure known as circular RT-PCR (cRT-PCR; Brogna, 1999). Primers were designed to allow sequencing analysis of poly(A) tails plus the 5' and 3' regions of respective mRNAs species (Supplementary Figure S3). The cRT-PCR produced products of expected sizes for adenylated and non-adenylated forms of pre-edited RPS12 mRNA (Figure 6A). Products obtained from the parental cell line and after 48 h of RNAi induction were cloned and sequenced. The statistical analysis of the short A-tail in pre-edited RPS12 mRNA showed an average length of ~25 nt (Figure 6B). In agreement with northern blotting data (Figure 5D and E), the A-tail was lost in all 30 clones obtained from KPAP1 RNAi cells. Several sequences also had heterogeneous 3' deletions (Supplementary Table S1) extending into the mRNA for up to 90 nt.

Applying cRT-PCR to never-edited CO1 mRNA generated products consistent with the presence of the short A-tails (Figure 6C). We were unable to detect PCR products corresponding to CO1 mRNA with a long A-tail. The analysis of A-tails in 47 CO1 clones from the parental cell line and 45 clones from KPAP1 RNAi demonstrated a statistically significant reduction in the number of adenosines per A-tail in KPAP1 RNAi (Figure 6D). Approximately 22% of all clones contained non-adenylated transcripts and RNAs missing 3–4 encoded nucleotides. In contrast to pre-edited RPS12 mRNA, the lack of more extensive 3' deletions in CO1 may indicate a processive degradation of non-adenylated never-edited mRNAs. Importantly, mRNA intermediates consistent with 5'–3' degradation pathway have not been detected among sequenced RPS12 or CO1 clones. Although the relative decay rates have not been measured, the structure of degradation intermediates suggests that the decrease in edited and never-edited mRNA abundance was caused by the 3'–5' degradation of non-adenylated transcripts.

In agreement with earlier reports (Decker and Sollner-Webb, 1990; Read *et al*, 1992), we have found that the short A-tails of both pre-edited RPS12 and never-edited CO1 mRNAs contain randomly incorporated U's, one or two at a time. Because of the low U content, the A/U ratio has not changed with any statistical significance in KPAP1 RNAi. To conclude, the short A-tails of pre-edited and never-edited mRNAs are synthesized by KPAP1 and appear to be slightly different in length but similar in nucleoside composition.

Editing of a single block converts the short A-tail into a stabilizing signal

As polyadenylation is likely to precede RNA editing (Koslowsky and Yahampath, 1997), we examined whether editing alters the function of the short poly(A) in the pre-edited transcripts from a neutral feature into a signal required for edited mRNA maintenance. Amplification of the pre-edited RPS12 mRNA did not discriminate transcripts that were unedited or edited in a region flanked by the primer binding site (A479) and the A-tail (positions 131–221; Supplementary Figure S3). Therefore, it was possible to observe a correlation between the editing and polyadenylation patterns within the same molecule. Analysis of 29 clones showed that in the parental cell line, six clones contained sequences that were fully edited in the seven editing sites adjacent to the mRNA's 3' end. In addition, 17 more clones were correctly edited in six sites. Further, extensive random editing upstream of this region was observed in 21 clones (Supplementary Figure S4 and Supplementary Table S1). Similar random editing patterns have been reported for Cyb and CO3 mRNAs (Decker and Sollner-Webb, 1990). Only five out of 29 clones carried unedited sequences. In contrast, among the 30 RPS12 clones obtained from the KPAP1 RNAi cells, only four sequences were edited in the first seven sites, and four more underwent random editing to a much lesser extent (Supplementary Figure S4).

Editing of these seven sites constitutes the first editing block, which is covered by a single gRNA, for example, E09 (KISS database; Ochsenreiter *et al*, 2007). The complete editing of this block most likely creates a binding site for the next gRNA, thus ensuring a 3'–5' polarity of the editing process (Maslov and Simpson, 1992). Because the 20S editosome and gRNA processing machineries remain intact in KPAP1 RNAi cells (Figure 3), and the editing took place in the first editing block (Supplementary Figure S4), it is unlikely that the loss of edited sequences was due to a compromised editing of non-adenylated mRNAs. Rather, a switch to poly(A)-tail-dependent stabilization of the edited mRNAs occurs upon editing of the first 3' block.

Long poly(A/U) tails are the hallmarks of fully edited mRNAs

The editing-dependent switch of function for the short A-tail must be followed, at some point, by addition of ~200 nt. This extended structure does not have an apparent effect on mRNA stability *in vitro* (Kao and Read, 2005) and its synthesis requires KPAP1 activity (Figure 5E). We next set out to analyse the structure of long tails and the temporal order of their appearance in edited mRNAs. The poly(A) tails are presumably difficult to amplify and clone because of the instability of homopolymeric DNA regions in *Escherichia coli*. Indeed, application of cRT-PCR to edited RPS12 mRNA

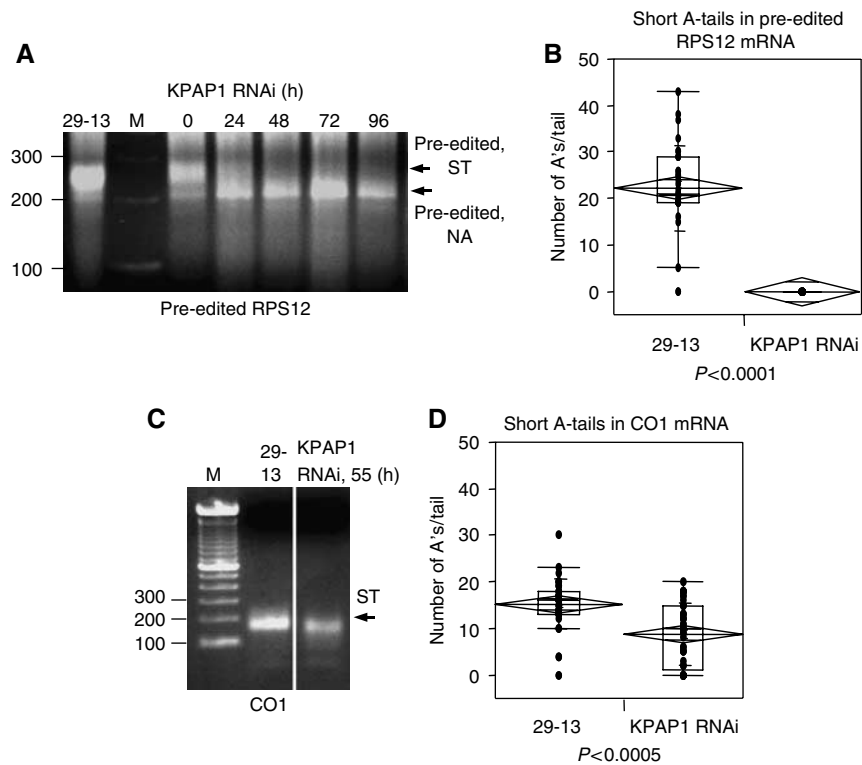


Figure 6 Short A-tails are synthesized by KPAP1. (A) cRT-PCR amplification of pre-edited RPS12 mRNA. PCR products were separated on 2% agarose gel. The pre-edited adenylated (ST) and non-adenylated (NA) forms are shown by arrows. (B) Distribution of A's per tail in pre-edited RPS12 mRNA. Box plot shows the distribution of A's per tail with possible outlying points in the 25–75 percentile range. The middle line identifies the median sample value. The upper and lower 'whiskers' highlight the range of non-outlying data points. The diamond illustrates the sample mean and 95% confidence interval. The line across each diamond represents the group mean. The vertical span of each diamond represents the 95% confidence interval for each group. (C) cRT-PCR amplification of the CO1 mRNA. (D) Distribution of A's per short A-tail in CO1 mRNA after KPAP1 RNAi.

produced two PCR products migrating with ~100 bases difference (Figure 7A). We were able to clone LT products only from the parental cells; four out of 100 clones contained sequences extending beyond the short A-tail (Figure 7B).

As expected, the region between the PCR primer binding site and the A-tail was fully edited in the ST and LT forms. In the ST form, a statistically significant reduction in the number of A's per short A-tail was observed as a consequence of KPAP1 RNAi (Figure 7B). We also noticed a 35% increase in the number of edited RPS12 transcripts missing the entire A-tail. However, heterogeneous deletions extending into the encoded region of the pre-edited form were not detected (Supplementary Figure S4). This indicates a processive decay of non-adenylated edited RPS12 mRNA similar to that observed for non-adenylated never-edited CO1 mRNA.

Remarkably, sequencing of four LT PCR products (Figure 7B) revealed that the homogenous 5' segment of ~20 adenosine nucleosides, or the short A-tail, was extended by an ~80-nt-long random addition of A (70%) and U (30%) residues (Figure 7C). The distinct U-rich structure of the long tail suggested that uridylyl transferases may be involved in the 3' processing of mitochondrial mRNAs, which is consistent with RET1 association with polyadenylation complex (Figure 4).

The long A/U-tails appear to be a characteristic of edited mRNAs (Figures 5 and 6), but the question remained whether these additions are present solely in the fully edited mRNAs, that is, in which editing of the 5' ends has been completed. Alternatively, the A/U extension may be added to the

partially edited mRNA, in which editing has proceeded beyond the first 3' editing block. To resolve the temporal order of the long A/U-tail addition with respect to 3'–5' polarity of editing (Maslov and Simpson, 1992), we hybridized RNA isolated from RET1 and KPAP1 RNAi cells with a 38-mer DNA probe for the 5' end of pre-edited RPS12 mRNA (A605, positions 33–70; Supplementary Figure S3). This region is edited by the insertion of 47 and deletion of 3 U's, which would prevent hybridization if editing took place (Figure 7D, right panel). In addition, the PCR-generated DNA probes (Supplementary Figure S3) against pre-edited RPS12 mRNA were hybridized with the same membrane (Figure 7D, left panel). Sequential hybridization with a probe for the fully edited form is shown in Figure 7E. In the parental and mock-induced cells, partially edited molecules were three-fold more abundant than pre-edited mRNAs, but long A/U-tails were not detected (Figure 7D, right panel). In RNA isolated from RET1-depleted cells, the partially and fully edited transcripts showed an ~90% decrease in abundance, as would be expected from impeded RNA editing due to loss of gRNA 3' uridylylation (Aphasizhev *et al*, 2003). The slower-migrating ~550- and 700-nt-long RPS12 species also appeared but their identities have not been investigated further. In RET1 RNAi cells, the abundance of pre-edited RPS12 transcripts increased while their A-tails remained intact (Figure 7D). Apparently, inhibition of RNA editing did not affect the stability of the pre-edited mRNAs. Most importantly, partially edited mRNA (Figure 7D, right panel) did not have the long A/U extension characteristic of the fully

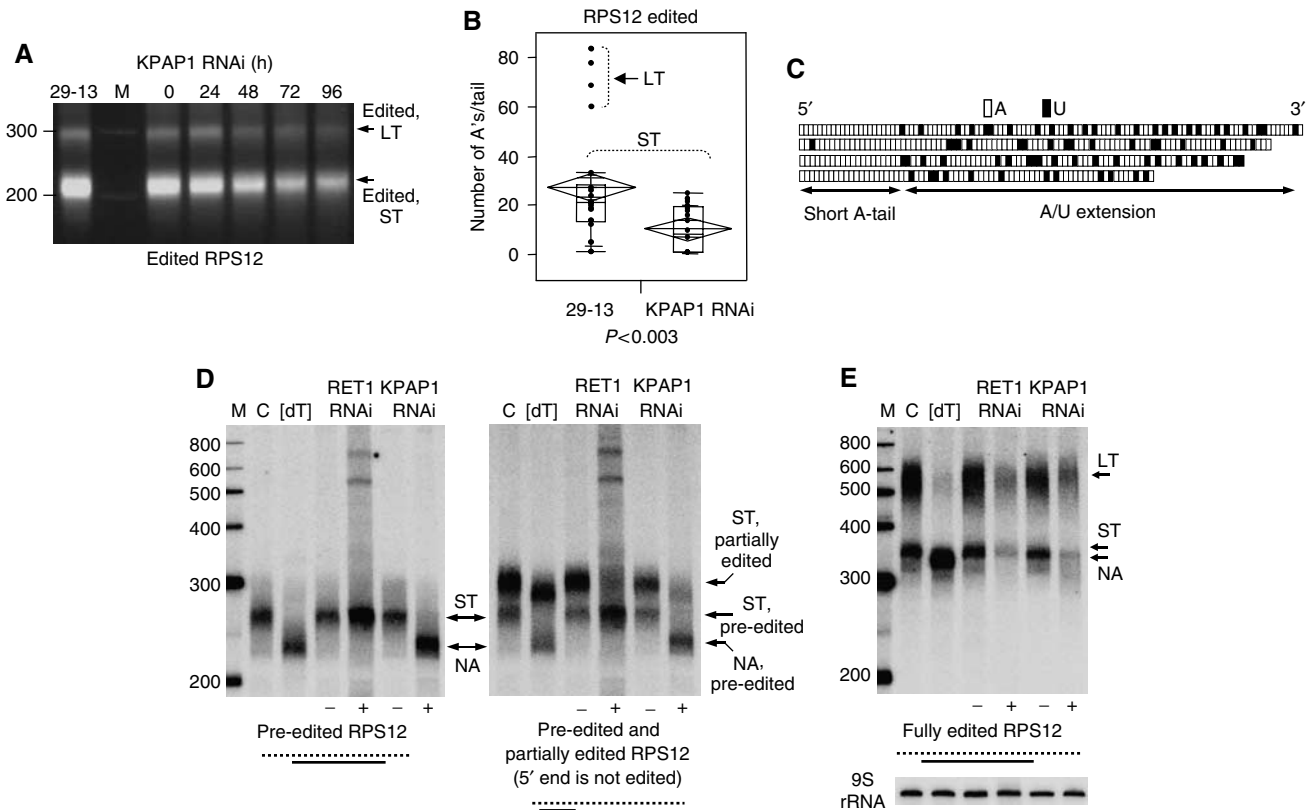


Figure 7 The A/U extension constitutes the ‘long A-tail’ of fully edited RPS12 mRNA. **(A)** cRT-PCR amplification of the long A-tail (LT) and short A-tail (ST) forms of the fully edited RPS12 mRNA. **(B)** Distribution of A’s per tail in edited RPS12 mRNA following RNAi knockout of KPAP1. **(C)** Schematic representation of the A/U extensions in fully edited RPS12 mRNA derived from LT PCR products. **(D)** Northern blotting of pre-edited and partially edited RPS12 mRNAs in RNA isolated from RET1 and KPAP1 RNAi cells. The schematic positioning of DNA probes (solid line) within mRNA (dotted line) is shown under each panel; the sequences are provided in Supplementary Figure S3. [dT]: RNase H treated; C: control RNA; 9S rRNA: mitochondrial small subunit rRNA. **(E)** Hybridization of the same membrane as in (D) with a probe for the fully edited RPS12 mRNA (Supplementary Figure S3).

edited form (Figure 7C and E). Consistent with the northern blotting results, analysis of the cRT-PCR clones confirmed that the 5’ ends of RPS12 mRNAs with long A/U extensions contained only fully edited sequences (not shown). Hence, we have concluded that the A/U extension is added to the short A-tail, which is required and sufficient for the maintenance of mRNA undergoing U-insertion/deletion, upon completion of the editing process.

Discussion

We report on the identification and functional characterization of the trypanosomal mitochondrial PAP termed KPAP1. Collectively, our results show that the short (20–25 nt) A-tails are essential for maintaining the steady-state level of never-edited and edited mRNAs. In the edited mRNAs, the long (100–200 nt) poly(A/U) extensions are added to the short A-tails upon completion of the editing process. It is possible that A/U structures are characteristic of translationally competent fully edited mRNAs. Although the nucleotide composition of ‘long A-tails’ in never-edited mRNA remains to be established, one may speculate that it should be similar to that of the fully edited transcripts. Our inference that the long ‘A-tail’ is a specific feature of never-edited and fully edited, but not partially edited, mRNAs disagrees with an earlier report (Militello and Read, 1999). We used northern hybridization

with a selective probe for the extensively edited 5’ end in RPS12 mRNA, and sequencing analysis of the 5’ region to verify this conclusion.

The degradation of mRNAs lacking poly(A) tails appears to be a processive 3’–5’ reaction that nevertheless does not affect non-adenylated pre-edited mRNAs. The rapid loss of non-adenylated edited mRNAs, and evidently functional RNA editing and gRNA processing machineries in KPAP1 RNAi cells prompted a search for the signal that converts the A-tail into a stabilizing feature of edited mRNA. We found that the U-insertion/deletion events beyond the first editing block impose a requirement for 3’ adenylation to maintain the transcript. It is conceivable that upon binding of the next gRNA, the editosome dissociates or moves to the upstream block, exposing the edited mRNA to 3’–5’ degradation. In cases of limited editing at the 5’ end of mRNA (Cyb) or editing guided by a *cis*-gRNA (CO2), the edited mRNAs still depend on 3’ adenylation. This may indicate cross-talk between the editing and polyadenylation complexes or deposition of editing factors on pre-existing short A-tails.

Purification of the polyadenylation complex supported the notion that RNA editing TUTase 1, an enzyme responsible for gRNA 3’ uridylylation (Aphasizhev *et al*, 2003), is involved in mRNA 3’-end processing (Ryan and Read, 2005). Analyses of KPAP1 and RET1 RNAi cell lines suggested that synthesis of a short A-tail by KPAP1 is an initial processing step for

pre-edited and never-edited mRNAs. The short tail's length is consistent with the extension patterns of recombinant KPAP1 (Figure 4D). It is appealing to speculate that after cleavage of the multi-cistronic precursor, the short A-tail is added to pre-edited and never-edited transcripts, which then become inefficient substrates for KPAP1. Quite possibly, the re-activation of such substrates for KPAP1 to synthesize longer extensions is achieved by uridylylation. Therefore, the A/U extensions may be synthesized by recycling of poly(A) and poly(U) polymerase activities, which are both present in the purified polyadenylation complex. The *in organello* phenomenon of UTP-stimulated, RET1-dependent mRNA degradation (Ryan and Read, 2005) may be rationalized assuming that an increase in the U content of A/U extensions leads to mRNA destabilization.

The critical question of signalling between completion of mRNA editing and 3'-end A/U-addition remains. In a possible scenario, the direct readout of RNA sequence generated *de novo* by RNA editing may be followed by the recruitment of an RET1-containing particle, in which KPAP1 and RET1 compete for the RNA substrate. Identification of five PPR proteins in the KPAP1 complex highlights these molecules as potential transcript-specific sensors. Indeed, PPR proteins ubiquitously function as sequence-recognition factors in post-transcriptional RNA processing in organelles (reviewed in Delannoy *et al*, 2007). Although the biological roles of all proteins reported in this study must be investigated further, it should be noted that PPR proteins may have general and transcript-specific roles. The results of PPR1 knockdown, for example, are consistent with its general role in the synthesis of 'long A-tails'. Upon induction of PPR1 RNAi, these structures were lost and mitochondrial function was compromised (Mingler *et al*, 2006; Pusnik *et al*, 2007). Predictably, the short A-tails and mRNA abundance were not affected, which leaves defects in translation of mRNAs missing A/U extensions as a plausible cause. Identification of the EF-Tu elongation factor as one of the major polypeptides in the KPAP1 complex also indicates a probable coupling of mitochondrial polyadenylation and translation processes.

Materials and methods

Expression, mutagenesis, and purification of KPAP1

The KPAP1 gene was PCR-amplified, re-sequenced, and inserted into pET28c vector (Novagen) to generate N-terminal 6xHis fusion protein. Enzymatic assays and RNA substrates are described in Supplementary data.

Trypanosome culture and RNAi

The RNAi expression plasmids were generated by cloning a fragment of the KPAP1 gene (24–600) into pT7-177 vector that allows for tetracycline-inducible, T7 RNA polymerase-driven

expression (Wickstead *et al*, 2002). The construct was transfected into procyclic 29-13 *T. brucei* strains (Wirtz *et al*, 1999) followed by clonal selection of phleomycin-resistant cell lines. RNAi was performed as described by Djikeng *et al* (2004).

Mitochondrial extract preparation and fractionation

Mitochondria purification, lysate preparation, glycerol gradient fractionation, and adenylation reactions were carried out according to Pelletier *et al* (2007). Tandem affinity purification and sample preparation for mass spectrometry were performed as described (Aphasizhev and Aphasizheva, 2007).

Western blotting and immunoprecipitation

Rabbit polyclonal antibodies against the recombinant KPAP1 were purified on antigen-affinity column. For western blotting, 2.5×10^6 cells were lysed in SDS loading buffer, separated on 8–16% gradient SDS-PAGE, and transferred to nitrocellulose membrane. For immunoprecipitation, purified mitochondria were resuspended at 0.3 g (wet weight)/ml in 50 mM HEPES (pH 8.0), 60 mM KCl, 10 mM MgCl₂, and 0.4% NP-40 and sonicated three times at 9 W for 5 s. The extract was spun at 18 000 g for 30 min to remove the debris. KPAP1 antibody (2.5 µg) was pre-bound to 10 µl of Dynabead-Protein A (Invitrogen) and incubated with 200 µl of mitochondrial extract for 1 h at 4°C followed by three washes for 30 min with 0.3% of Triton X-100 in PBS.

Fluorescent microscopy

A KPAP1-eYFP fusion gene was cloned into the pLew79 vector, which allows for tetracycline-inducible expression, and the construct was transfected into procyclic 29-13 *T. brucei* (Wirtz *et al*, 1999). Parasites were incubated with 100 nM MitoTracker Red CMX ROS.

RNA analysis

Total RNA was isolated by a modified procedure (Chomczynski and Sacchi, 1987). Detailed methods for primer extension, qRT-PCR, northern blotting, RNase H digestion, gRNA labelling, mRNA 3' end cloning and statistical analysis, and oligonucleotides used are described in Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Accession numbers

GenBank accession numbers for the sequences reported in the paper are EF650028 and EF650030 for KPAP1 from *T. brucei* and *L. major*, respectively.

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