

1 ***N*⁶-methyladenosine in poly(A) tails stabilize VSG 2 transcripts**

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19 **Keywords**

20 *N*⁶-methyladenosine, m⁶A, RNA stability, RNA modification,
21 trypanosomes, VSG, antigenic variation, poly(A) tail

23 **Summary**

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25 RNA modifications are important regulators of gene expression. In
26 *Trypanosoma brucei*, transcription is polycistronic and thus most regulation
27 happens post-transcriptionally. *N*⁶-methyladenosine (m⁶A) has been detected in
28 this parasite, but its function remains unknown. Here we show that ~50% of the
29 m⁶A is located in the poly(A) tail of the monoallelically expressed Variant Surface
30 Glycoprotein (VSG) transcript. m⁶A residues are removed from the VSG poly(A)
31 tail prior to deadenylation and mRNA degradation. Using genetic tools, we
32 identified a 16-mer motif in the 3'UTR of VSG that acts as a cis-acting motif
33 required for inclusion of m⁶A in the poly(A) tail. Removal of this motif from the
34 VSG 3' UTR results in poly(A) tails lacking m⁶A, rapid deadenylation and mRNA
35 degradation. To our knowledge this is the first identification of an RNA
36 modification in the poly(A) tail of any eukaryote, uncovering a novel post-
37 transcriptional mechanism of gene regulation.

40 Introduction

41 *Trypanosoma brucei* (*T. brucei*) is a protozoan unicellular parasite that
42 causes lethal diseases in sub-Saharan Africa: sleeping sickness in humans and
43 nagana in cattle¹. In humans, the infection can last several months or years
44 mostly because *T. brucei* escapes the immune system by periodically changing
45 its variant surface glycoprotein (VSG)². The *T. brucei* genome contains around
46 2000 antigenically distinct VSG genes, but only one VSG gene is actively
47 transcribed at a given time. Transcriptionally silent VSG genes are switched on
48 by homologous recombination into the BES or by transcriptional activation of a
49 new BES², resulting in parasites covered by ~10 million identical copies of the
50 VSG protein³.

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52 VSG is essential for the survival of bloodstream form parasites. VSG is not
53 only one of the most abundant proteins in *T. brucei*, but it is also the most
54 abundant mRNA in bloodstream forms (4-11% of total mRNA)^{4,5}. VSG mRNA
55 abundance is a consequence of its unusual transcription by RNA polymerase I
56 and its prolonged stability. The half-life of VSG mRNA has been estimated to
57 range from 90-270 min, contrasting with the 12 min, on average, for other
58 transcripts⁶. The basis for its unusually high stability is not known. It is thought to
59 derive from the VSG 3'UTR, which contains two conserved motifs, a 9-mer and
60 a 16-mer motif, found immediately upstream of the poly(A) tail. Mutational studies
61 have shown that the 16-mer conserved motif is essential for VSG mRNA high
62 abundance and stability⁷, even though its underlying mechanism is unknown.

63 VSG expression is highly regulated when the bloodstream form parasites
64 undergo differentiation to the procyclic forms that proliferate in the insect vector⁸.
65 The BES becomes transcriptionally silenced and VSG mRNA becomes
66 unstable⁹, which results in rapid loss of VSG mRNA and replacement of the VSG
67 coat protein by other surface proteins (reviewed in¹⁰). The mechanism by which
68 VSG mRNA becomes unstable during differentiation remain unknown. The
69 surface changes are accompanied by additional metabolic and morphological
70 adaptations, which allow procyclic forms to survive in a different environment in
71 the insect host¹⁰.

72 RNA modifications have been recently identified as important means of
73 regulating gene expression. The most abundant internal modified nucleotide in
74 eukaryotic mRNA is *N*⁶-methyladenosine (m⁶A)^{11,12}, which is widespread across
75 the human and mouse transcriptomes and is often found near stop codons and
76 the 3' untranslated regions of the mRNA encoded by multiple genes^{13,14}. m⁶A is
77 synthesized by a methyltransferase complex whose catalytic subunit, METTL3,
78 methylates adenosine in a specific consensus motif. Demethylases responsible
79 for removing m⁶A from mRNA have also been identified. m⁶A affects several
80 aspects of RNA biology, for instance contributing to mRNA stability, mRNA
81 translation, or affecting alternative polyadenylation site selection (reviewed in¹⁵).

82 Here we show that *N*⁶-methyladenosine is an RNA modification enriched
83 in *T. brucei* mRNA. Importantly, this study revealed that m⁶A is present in mRNA
84 poly(A) tails, and half of m⁶A is located in only one transcript (VSG mRNA). We
85 identified a cis-acting element required for inclusion of m⁶A at the VSG poly(A)
86 tail and, by genetically manipulating this motif, we showed that m⁶A blocks
87 poly(A) deadenylation, hence promoting VSG mRNA stability. We provide the first

88 evidence that poly(A) tails of mRNA can be methylated in eukaryotes, playing a
89 regulatory role in the control of gene expression.

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91 **Results**

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93 ***m⁶A is present in *T. brucei* messenger RNA***

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95 To investigate if *T. brucei* RNA harbours modified nucleosides that could
96 play a role in gene regulation, we used liquid chromatography-tandem mass
97 spectrometry (LC-MS/MS) to detect possible modifications of RNA nucleosides.
98 Thirty seven possible modified nucleosides were found in total RNA (Table S1),
99 most of which had been previously detected in *T. brucei* RNA including Am, which
100 is found in the mRNA cap structure, and m³C, m⁵C and Gm in tRNA and rRNA^{16–}
101 ¹⁸. Mass-spectrometry analysis of mRNA revealed a clear peak that
102 corresponded to m⁶A. Given the importance of this modification for RNA
103 metabolism in other eukaryotes, we focused on this specific modification in *T.*
104 *brucei*.

105 First, we tested if m⁶A was mainly present in mRNA, or if it was present in
106 other type of RNA molecules (rRNA, tRNA and other non-coding RNAs). We also
107 compared two different stages of the parasite life cycle, the mammalian
108 bloodstream and the insect procyclic forms. An m⁶A nucleoside standard was
109 used as a control. The chromatograms of the poly(A)-enriched fraction (mRNA)
110 revealed a single peak corresponding to the 282→150 mass transition and an
111 elution time of 10 min (Fig. 1A), confirming it represents m⁶A. The chromatograms
112 of total RNA and poly(A)-depleted samples also contained a peak with an
113 identical mass transition, but the elution time was much earlier (6.5 min), which
114 likely reflects N¹-methyladenosine (m¹A), a modification with the same mass and
115 earlier elution times, and is commonly found in rRNAs and tRNAs^{19,20}. The m⁶A
116 peak in poly(A)-depleted RNA was barely detectable, indicating that most (if not
117 all) m⁶A is present in mRNA and absent from rRNA and tRNAs. Similar results
118 were obtained in RNA fractions obtained from the procyclic form insect stage
119 (Extended Data Fig. S1A).

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121 The m⁶A standard allowed us to quantify the abundance of m⁶A in both
122 stages of the life cycle (Extended Data Fig. S1B). As expected, the levels are
123 very low in total and poly(A)-depleted fractions. In contrast, in both bloodstream
124 and procyclic forms mRNA fractions, m⁶A represents 0.06% of total adenines in
125 mRNA (Fig. 1B). In other words, 6 in 10,000 adenosines contain a methyl group
126 at position 6. This proportion is lower than in mammalian cells (0.1–0.4%,^{11,12}).

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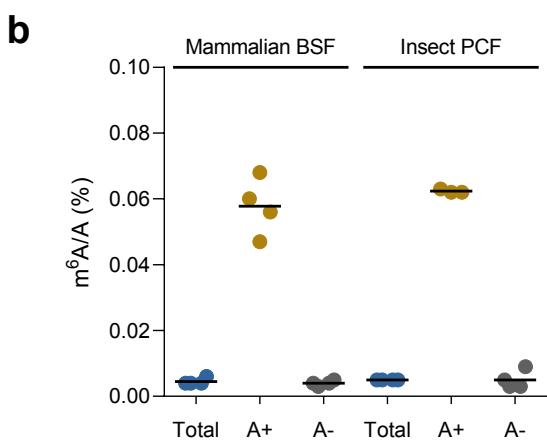
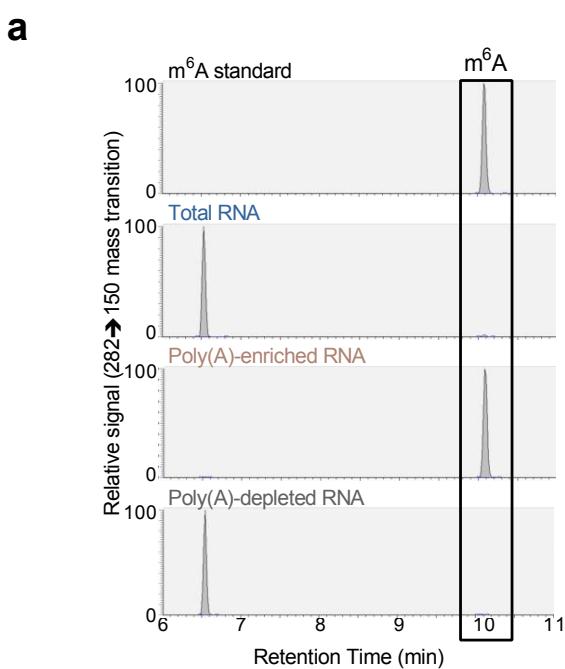


Figure 1. m⁶A is present in *T. brucei* mRNA in both insect and mammalian stages of the life cycle.

a. Chromatograms obtained based on an LC-MS/MS analysis of a m⁶A standard and three RNA samples of *T. brucei* mammalian stages (BSF): total RNA, RNA enriched with oligo(dT)-beads (i.e., poly(A)-enriched RNA) and RNA that did not bind poly(T)-beads (i.e., poly(A)-depleted RNA). RNA was hydrolyzed and dephosphorylated and individual nucleosides were resolved by HPLC. m⁶A was readily detected in the poly(A)-enriched RNA. Another peak eluting at ~6.6 min, corresponding to the tRNA and rRNA-enriched nucleotide m¹A, was detected in the poly(A)-depleted and total RNA samples. Identical analysis was performed in RNA from the insect life-cycle stage (PCF) – Fig. S1A. **b.** Levels of m⁶A detected by LC-MS/MS in the RNA samples indicated in panel (A) and quantified using the standard curve in Fig. S1B.

(see also Extended Data Fig. S1)

161 **m⁶A is enriched in the VSG poly(A) tail**

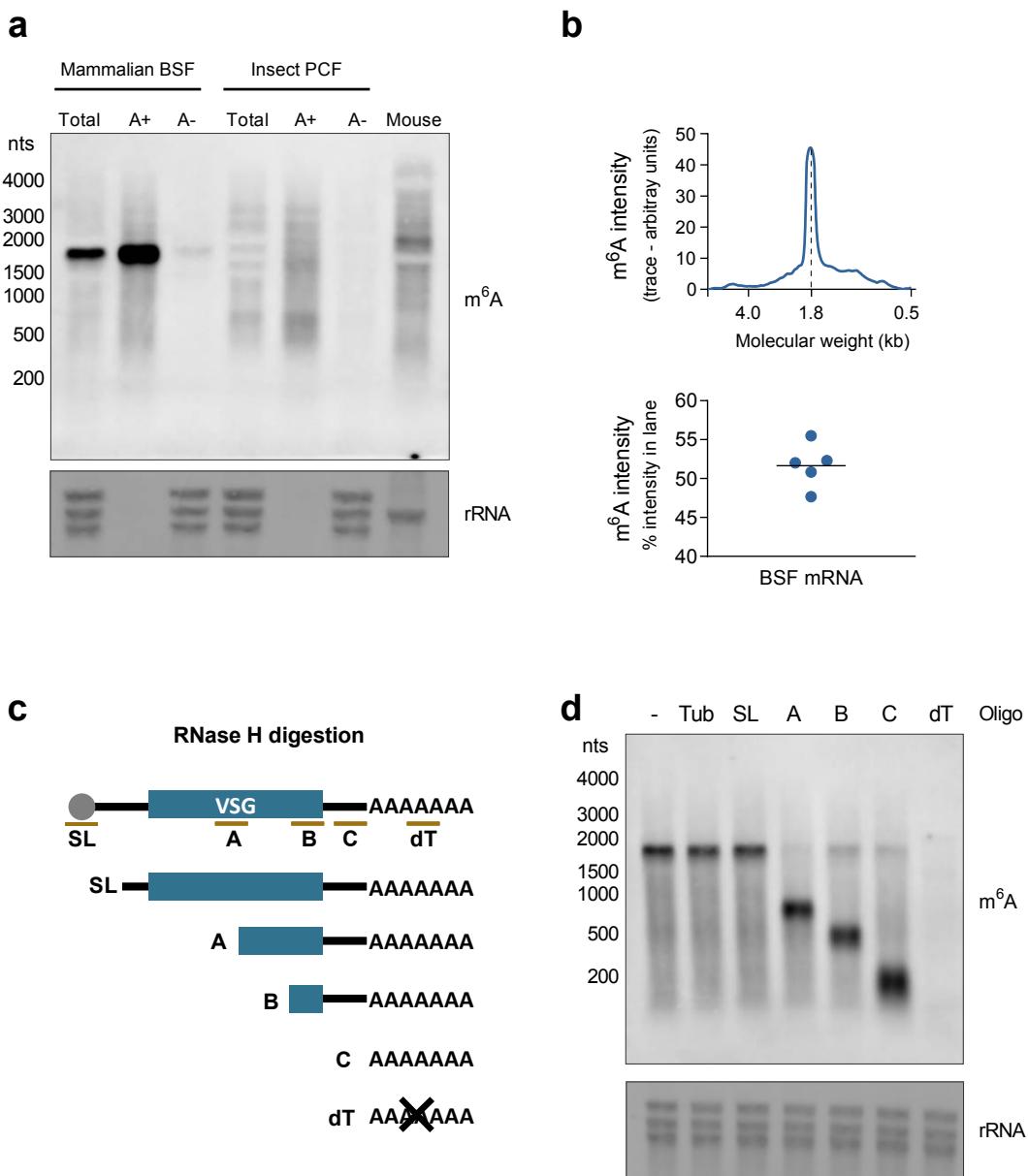
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163 During their life cycle, parasites need to adapt to living in different
164 environments. The most recent studies have shown that around 30% of
165 transcripts and around 33-40% of proteins are differentially expressed between
166 these two life cycle stages^{21,22}. It is possible that m⁶A is not equally distributed
167 qualitatively and quantitatively in the transcripts of the two stages of the life cycle.
168 To test this hypothesis, performed immunoblotting with an antibody that
169 recognizes m⁶A. This antibody specifically recognizes m⁶A with minimal cross-
170 reaction with unmodified adenosine or m¹A (Extended Data Fig. S2). Mouse liver
171 total RNA was used as a control. As expected¹³, the immunoblotting of liver total
172 RNA revealed a smear in the entire lane, indicating that multiple RNA molecules
173 of different sizes harbour m⁶A (Fig. 2A).

174 Next, we examined m⁶A in total RNA, poly(A)-enriched and poly(A)-
175 depleted RNAs derived from bloodstream and procyclic forms (Fig. 2A).
176 Consistent with the results obtained by LC-MS/MS, the poly(A)-depleted fraction
177 showed a weak m⁶A signal, revealing that m⁶A is absent or below detection levels
178 in rRNAs and tRNAs. In contrast, an m⁶A-positive smear was detected in poly(A)-
179 enriched fraction both in bloodstream and procyclic forms, confirming that m⁶A is
180 present in multiple mRNA molecules in both stages of the life cycle. Strikingly,
181 however, the sample of bloodstream forms revealed an intense band of around
182 1.8 kb. This band accounts for ~50% of the m⁶A signal intensity in the lane (Fig.
183 2B), suggesting that m⁶A in bloodstream forms is highly enriched in one type or
184 several similarly sized types of transcripts.

185 The most abundant transcript in bloodstream forms is the VSG, which
186 accounts for ~5% of the total mRNA⁵. To test if the specific band is VSG, we
187 repeated the immunoblot assay but site-selectively cleaved the VSG transcript
188 with Ribonuclease H (RNase H) and monitored the mobility of the m⁶A band.
189 Poly(A) RNA was incubated with DNA oligonucleotides that annealed at different
190 sites along the length of the VSG transcript. RNase H digestion of the RNA:DNA
191 hybrids result in fragments of predicted sizes (Fig. 2C-D). If the VSG transcript is
192 the band with the intense m⁶A signal, the band detected by immunoblotting would
193 “shift” to one or two of the fragments of smaller size.

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Figure 2. m⁶A is present in the poly(A) tail of VSG mRNA and other transcripts.

a. Immunoblotting with anti-m⁶A antibody. RNA samples (from left to right): total RNA (Total), Poly(A)-enriched (A+) RNA and Poly(A)-depleted (A-) RNA from two life cycle stages (BSF and PCF). The last lane contains total mouse liver RNA (Mouse). 2 µg of total RNA, 2 µg of poly(A)-depleted RNA and 100 ng of poly(A)-enriched RNA was loaded per lane. rRNA was detected by staining RNA with methylene blue to confirm equal loading between total and poly(A)-depleted fractions. As expected the rRNA are undetectable in the poly(A)-enriched fraction. b. Intensity of the m⁶A signal in immunoblot, measured by Image J, in the whole lane containing the poly(A)-enriched RNA of bloodstream forms. The intensity of the ~1.8 kb band was compared with the signal intensity of the entire lane, and averaged from 5 independent samples. c. Diagram displaying the location of the oligonucleotides used in RNase H digestion of VSG mRNA. The digestion products detected in the immunoblot (panel D) after incubation with each oligonucleotide (SL, A, B, C, dT) are also indicated. d. Immunoblotting with anti-m⁶A antibody of mammalian bloodstream forms total RNA pre-incubated with indicated oligonucleotides and digested with RNase H. 2 µg of total RNA were loaded per lane. Staining of rRNA with Methylene Blue confirmed equal loading. SL: spliced leader, dT: poly deoxy-thymidines. Tub: α-Tubulin. (see also Extended Data Fig. S3)

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215 We first performed RNase H digestion of RNA pre-incubated without any
216 oligonucleotide or with a control oligonucleotide that annealed with α -tubulin
217 (another abundant transcript in *T. brucei*) did not affect the mobility of the m⁶A
218 band (Fig. 2D). Next, we used an oligonucleotide that hybridized to the spliced
219 leader (SL) sequence, a 39nt sequence that contains the mRNA cap and that is
220 added to every mRNA by a trans-splicing reaction²³. RNase H digestion of RNA
221 pre-incubated with the SL oligonucleotide showed no effect on m⁶A
222 immunoblotting signal (Fig. 2D). This indicates that m⁶A is neither present in
223 spliced leader sequence, nor in the mRNA cap structure. In contrast, when we
224 used oligonucleotides VSG-A, VSG-B and VSG-C, which hybridized to three
225 different unique sites in the VSG sequence (Fig. 2C), we observed that the major
226 m⁶A band shifted, and in all three conditions, the 3' end fragment contained the
227 entire m⁶A signal. Importantly, VSG-C oligonucleotide is adjacent the beginning
228 of the poly(A) tail. Thus, the 3' fragment released upon RNase H digestion with
229 VSG-C corresponds to the poly(A) tail of VSG mRNA. This fragment, which
230 contains the entire m⁶A signal from the VSG transcript, is heterogeneous in length
231 and shorter than 200 nt (Fig. 2D).

232 To further confirm that the 3' fragment released after incubation with VSG-
233 C and RNase H corresponds to the VSG poly(A) tail, we performed RNase H
234 digestion in RNA pre-incubated with a poly(T) oligonucleotide. Consistent with
235 the results using VSG-C, the major band detected by m⁶A-antibody completely
236 disappears, further supporting the idea that in bloodstream forms most m⁶A is
237 present in the poly(A) tail of VSG mRNA. Interestingly, digestion of RNA
238 hybridized with poly(T), also abolished the smear detected by m⁶A-antibody,
239 indicating that m⁶A present in non-VSG transcripts is also most likely located in
240 their poly(A) tails. Notably, a similar approach to digest poly(A) tails does not
241 affect m⁶A levels in mammalian mRNA of HeLa cells¹³. Thus, this is the first
242 demonstration that a poly(A) tail can harbour a modified nucleotide.

243 Our results show that although VSG mRNA is only 4-11% of total mRNA,
244 it accounts for ~50% of cellular m⁶A, suggesting that VSG mRNA is preferentially
245 enriched in m⁶A compared to other mRNAs. Based on the m⁶A frequency in the
246 transcriptome and the enrichment in VSG, we estimate that there are nearly four
247 m⁶A molecules per VSG mRNA. In contrast, among the non-VSG mRNAs, only
248 one in five mRNAs are predicted to contain m⁶A.

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250 **Removing m⁶A precedes deadenylation and degradation of VSG transcript**

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252 The half-life of VSG transcript is 90-270 min, while the median mRNA half-
253 life in trypanosomes is 13 min⁶. Given that removal of the poly(A) tail often
254 precedes RNA degradation, we hypothesized that the presence of m⁶A in the
255 poly(A) tail could account for this exceptional VSG mRNA stability.

256 We tracked m⁶A levels in VSG mRNA as it undergoes degradation in three
257 independent conditions. We first inhibited transcription in bloodstream form
258 parasites with actinomycin D (ActD) and, for the next 6 hours, we quantified the
259 amount of VSG mRNA that remains (by qRT-PCR). We also measured the levels
260 of m⁶A in VSG (by immunoblotting) and the length of the VSG poly(A) tail using

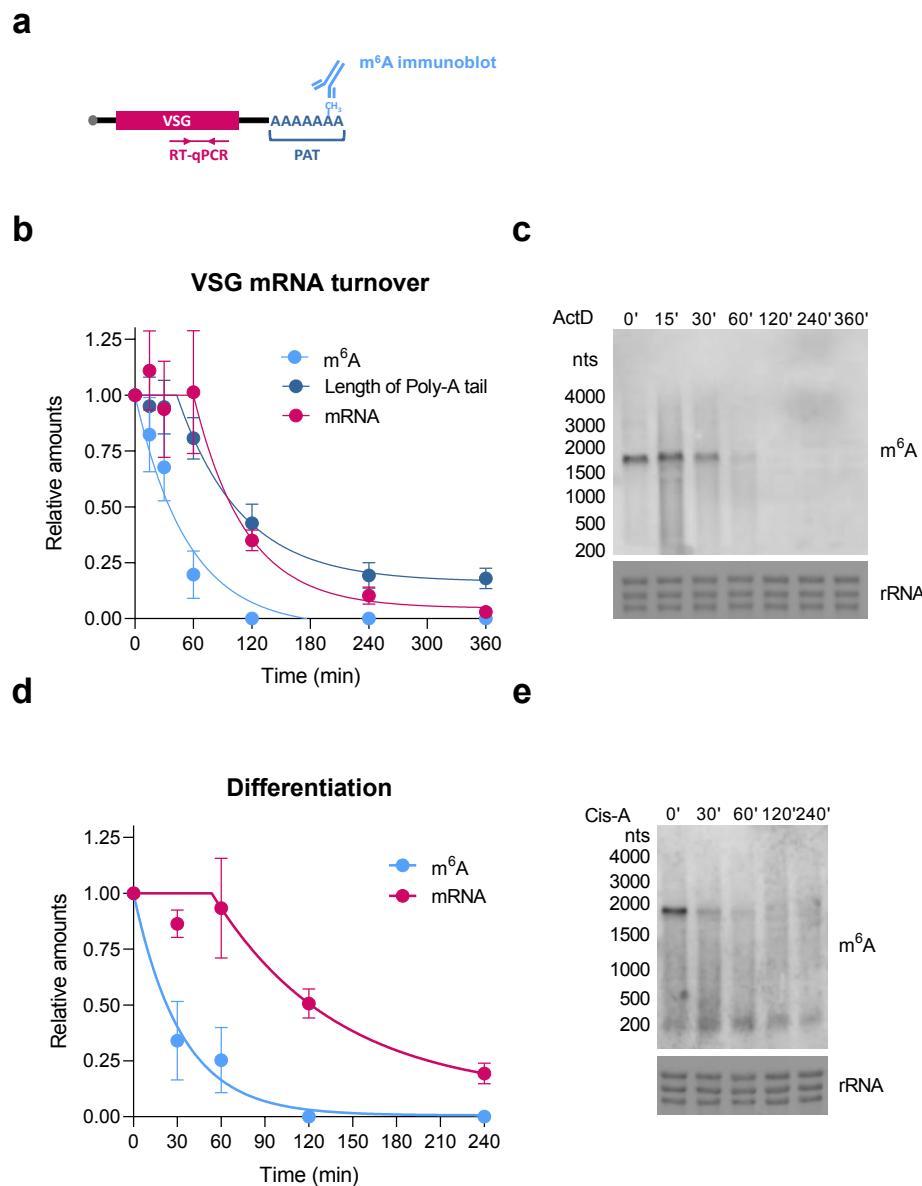
261 the Poly(A) Tail-Length Assay (PAT), which involves ligation of adaptors to the 3'
262 end of poly(A) tails and two consecutive PCRs using VSG-specific forward
263 primers (Fig. 3A). The amplified fragments contain part of the ORF, the 3'UTR of
264 VSG transcript and the downstream poly(A) tail, whose size is variable between
265 different transcript molecules.

266 VSG mRNA has previously been shown to exhibit biphasic decay: in the
267 first hour after transcription blocking VSG mRNA levels remain high and, only in
268 a second phase, do VSG mRNA levels decay exponentially^{9,24}. Consistent with
269 these earlier findings, we detected no major changes in mRNA abundance during
270 the first hour after actinomycin D treatment (lag phase, or first phase); however,
271 afterwards VSG exhibited exponential decay (second phase) (Fig. 3B). During
272 the one hour lag phase, the length of the VSG poly(A) tail was unchanged, but
273 then it rapidly shortened during the second phase. This indicates that there is a
274 specific time-dependent step that triggers the rapid shortening of the VSG poly(A)
275 tail and the subsequent degradation of the VSG transcript. Immunoblotting
276 revealed that the m⁶A levels also decreased, but strikingly the loss of m⁶A
277 preceded the shortening of the poly(A) tail and subsequent mRNA decay (Fig.
278 3C). In fact, m⁶A levels decrease exponentially during the first hour after
279 actinomycin D, taking around 35 min for total mRNA m⁶A levels to drop 50%,
280 while VSG mRNA only reached half of the steady-state levels around 2hr (Fig.
281 3B). These results indicate that m⁶A is removed from VSG mRNA prior to the
282 deadenylation of the poly(A) tail, which is quickly and immediately followed by
283 degradation of the transcript.

284 When bloodstream form parasites undergo cellular differentiation to
285 procyclic forms, VSG is downregulated as a consequence of decreased
286 transcription and decreased mRNA stability⁹. To test whether m⁶A is also rapidly
287 removed from VSG mRNA prior to its developmentally programmed degradation,
288 we induced differentiation *in vitro* by adding cis-aconitate to the medium and
289 changing the temperature to 27°C. Parasites were collected and total RNA was
290 extracted in different time points. Quantitative qRT-PCR showed that the levels
291 of VSG mRNA stayed stable for around one hour, which was followed by an
292 exponential decay (Fig. 3D). Importantly, immunoblotting analysis showed that
293 during parasite differentiation, m⁶A intensity in the VSG mRNA dropped faster
294 than the VSG-mRNA levels, reaching half of the steady-state levels in 23 min
295 (Fig. 3D-E). Thus, during parasite differentiation, we observed again that the
296 removal of m⁶A precedes the loss of VSG mRNA levels.

297 In addition to differentiation, another trigger for VSG mRNA degradation is
298 translation inhibition, which can be achieved by treating parasites with drugs such
299 as puromycin²⁵. We treated bloodstream form parasites with puromycin and
300 collected RNA samples for 4 hours. Consistent with previous studies, qRT-PCR
301 revealed that VSG mRNA is actively degraded and it takes around 39 min to
302 reach half of steady state levels (Extended Data Fig. S3). Immunoblotting
303 revealed, once again, that m⁶A is removed earlier from the VSG transcript with a
304 half-life of about 10 min (Extended Data Fig. S3).

305 Overall, these results show that in three independent conditions, m⁶A is
306 removed from the VSG transcript earlier than the VSG transcript is deadenylated
307 and degraded, suggesting that m⁶A may need to be removed from the VSG
308 transcript before it can be degraded.



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311 **Figure 3. m⁶A is removed from VSG mRNA prior to its degradation.**

312 a. Diagram of VSG2 mRNA transcript. Primers used for qRT-PCR are indicated in pink, primers
313 used for PAT assay are indicated in dark blue and methylated adenosines of the poly(A) tail are
314 indicated in light blue. b. Quantification of VSG2 transcript levels, m⁶A signal and the length of
315 poly(A) tail after transcription halt by actinomycinD (ActD). Total RNA of bloodstream parasites
316 was extracted after various time-points of ActD treatment. Values were normalized to 0 hr.
317 Transcript levels were measured by qRT-PCR (pink), m⁶A levels were measured by
318 immunoblotting signal (light blue) and the length of the poly(A) tail was measured by PAT assay
319 (dark blue). c. Immunoblotting with anti-m⁶A antibody of bloodstream form total RNA extracted
320 from parasites treated with actinomycin D for increasing times. 2 µg of total RNA were loaded per
321 lane. Staining of rRNA with Methylene Blue confirmed equal loading. d. Quantification of VSG2
322 transcript levels, m⁶A signal and the length of poly(A) tail during parasite differentiation from
323 bloodstream to procyclic forms. Total RNA was extracted at different time-points after inducing
324 differentiation with cis-aconitate. Values were normalized to 0 hr. Same color code as in Panel B.
325 e. Immunoblotting with anti-m⁶A antibody of parasites differentiating to procyclic forms. 2 µg of
326 total RNA were loaded per lane. Staining of rRNA with methylene blue confirmed equal loading.
327 (see also Extended Data Fig. S3)

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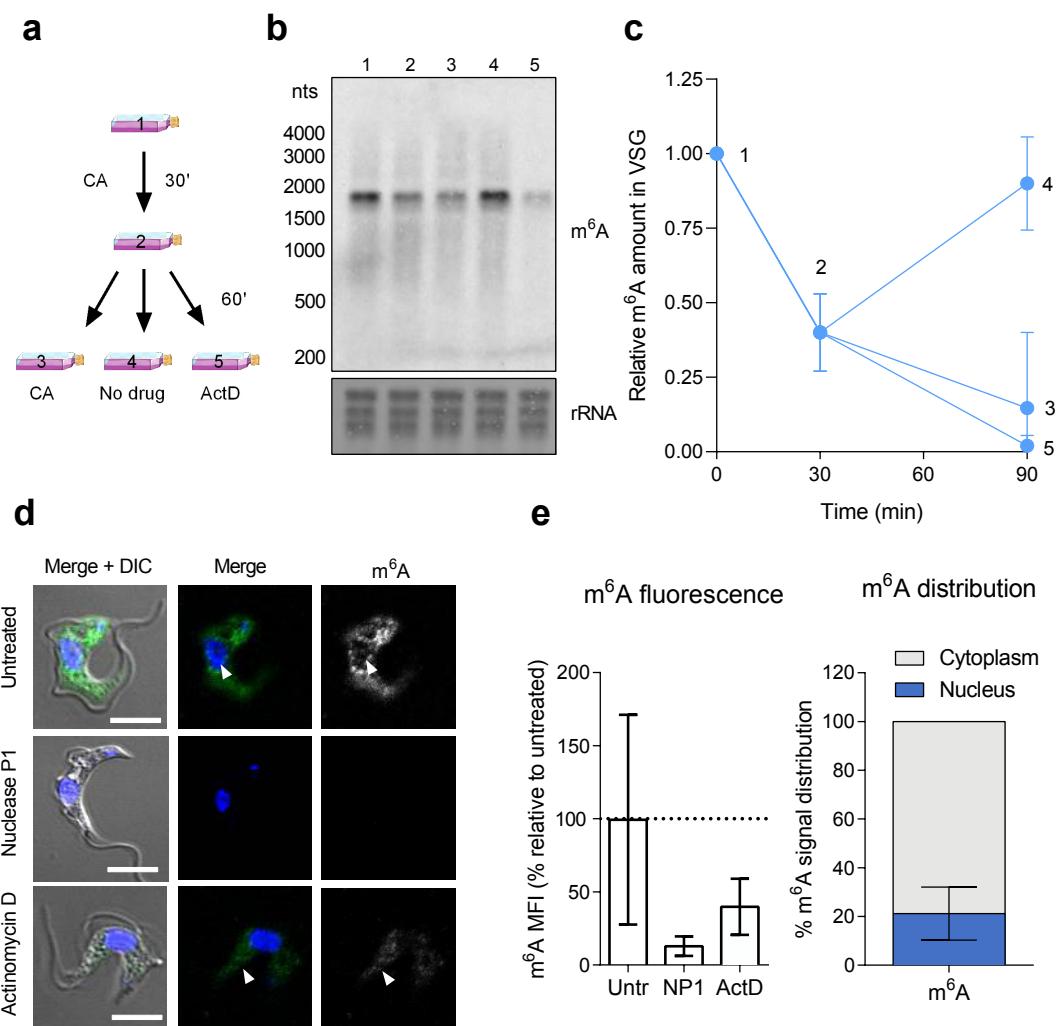
330 **Inclusion of m⁶A in VSG mRNA is dependent on *de novo* transcription**

331 In most organisms, m⁶A is generated by methylation of adenosine
332 residues within a specific consensus sequence by the METTL3
333 methyltransferase or its orthologs¹⁵. Given that in *T. brucei*, m⁶A is present in the
334 poly(A) tail, a different mechanism is likely used. Indeed, trypanosomes lack a
335 METTL3 ortholog²⁶, indicating that a different pathway would be required to
336 acquire m⁶A in the poly(A) tail. To understand how m⁶A accumulates in the VSG
337 mRNA, we used parasite differentiation as a natural inducible system of VSG
338 downregulation. This process is reversible in the first two hours²⁷. Parasite
339 differentiation was induced by adding cis-aconitate for 30 min (as described
340 above, Fig. 3D), and then was washed away. As shown above, immunoblotting
341 revealed that m⁶A was reduced after 30 min of cis-aconitate treatment (Fig. 4A-
342 B, Fig. 3E). In contrast, when cells were allowed to recover for 1 hr in the absence
343 of cis-aconitate or other drugs, we observed that the intensity of the m⁶A signal
344 returned to normal levels (Fig. 4A-B). These results indicate that, if differentiation
345 is halted and the levels of VSG mRNA are re-established (Extended Data Fig.
346 S4), VSG m⁶A is quickly recovered.

347 To test if the recovery of m⁶A levels after cis-aconitate removal was due
348 to *de novo* transcription, parasites were cultured in the presence of actinomycin
349 D. We observed that the intensity of m⁶A in the VSG transcript was not recovered.
350 Instead, the m⁶A levels decreased by ~20% (Fig. 4A-B). Overall, these results
351 indicate that *de novo* transcription is required to re-establish m⁶A levels in VSG
352 mRNA.

353 If m⁶A is included in VSG mRNA soon after transcription, and if it remains
354 in the poly(A) tail until it gets degraded, we should be able to detect m⁶A in the
355 nucleus and in the cytoplasm of the parasites. Immunofluorescence analysis with
356 an antibody against m⁶A showed a punctate pattern both in the nucleus (20%)
357 and cytoplasm (80%) (Fig. 4D-E). To confirm that this m⁶A signal originated from
358 RNA, we incubated the fixed cells with nuclease P1 prior to the antibody staining,
359 which specifically cleaves single-stranded RNA without any sequence-specific
360 requirement. This treatment caused a marked reduction in the intensity of the
361 m⁶A signal, indicating that the immunoreactivity of the m⁶A antibody derives from
362 RNA, and not non-specific interactions with cellular proteins (Fig. 4C-D). As an
363 additional control, we treated with actinomycin D for 2 hr prior to
364 immunofluorescence analysis. This treatment is expected to result in reduced
365 cellular mRNA levels. Immunostaining with the m⁶A antibody showed a drop in
366 the intensity of the m⁶A signal by around 40% (Fig. 4D-E), which is similar to the
367 reduction observed by immunoblotting (Fig. 3B-C). Overall, these data support
368 the idea that the m⁶A immunostaining results likely reflect m⁶A in mRNA and not
369 a non-specific binding of the antibody to the cells.

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372 **Figure 4. Inclusion of m⁶A in the VSG poly(A) tail depends of *de novo* transcription.**

373 **a.** Experimental setup. Parasites were treated with cis-aconitinate (CA) for 30 min, time at which
374 the compound was washed away. Parasites were placed back in culture in 3 different conditions
375 for an extra hour: in the presence of cis-aconitinate, without cis-aconitinate, without cis-aconitinate but
376 with transcription inhibitor actinomycin D (ActD). Labels 1-5 indicate the conditions at which
377 parasites were collected for Immunoblotting analysis (panel B). **b.** Immunoblot with anti-m⁶A
378 antibody of total RNA from bloodstream parasites collected at each of the 5 labelled conditions
379 (panel A). **c.** Quantification of Immunoblotting in panel A. All levels were normalized to time point
380 0 hr. **d.** Immunofluorescence analysis showing m⁶A localization in mammalian BSF. Parasites
381 were treated with Nuclease P1 (NP1) or actinomycin D (ActD) to confirm signal-specificity. Nuclei
382 were stained with Hoechst. Arrow in top row points to m⁶A signal in the nucleus. Arrow in bottom
383 panel points to weak m⁶A signal; scale bars, 4 μ m. **e.** Quantification of mean fluorescence intensity
384 (MFI) levels of m⁶A in untreated BSF, nuclease P1 (NP1)-treated BSF, and actinomycin D (ActD)-
385 treated BSF. Raw MFIs were obtained, the average of the untreated BSF equalled to 100%, and
386 all other values normalized to 100%. Error bars indicate SEM. Results shown are the mean of n
387 = 2 independent experiments, and n = 500 parasites per condition. **f.** Quantification of the
388 proportion of m⁶A signal in nucleus and cytoplasm across 500 parasites (untreated BSF). The
389 proportion was calculated by segmentation dividing the parasite body and nucleus, and
390 quantifying fluorescence intensity in each segment. Error bars indicate SEM. DIC, differential
391 interference contrast.

392 (see also Extended Data Fig. S4)

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394 **Inclusion of m⁶A in VSG poly(A) tail is dependent on neighboring cis-acting
395 motif**

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397 m⁶A is added to the VSG mRNA poly(A) tail soon after transcription,
398 probably still in the nucleus. m⁶A is not simply targeted to all poly(A) tails in the
399 cell since, if this were the case, all mRNAs would be methylated and VSG
400 methylation would only account for 4-11% of total cellular m⁶A. We therefore
401 asked how the VSG poly(A) tail is selected for preferential enrichment of m⁶A. It
402 has been previously shown that each VSG gene contains a conserved 16-mer
403 motif (5'-TGATATATTTAACAC-3') in the 3'UTR adjacent to the poly(A) tail that
404 is necessary for VSG mRNA stability⁷. It has been proposed that a currently
405 unknown RNA-binding protein may bind this motif and stabilize the transcript by
406 an unknown mechanism⁷. Here, we hypothesized that this 16-mer motif may act
407 *in cis* to promote inclusion of m⁶A of the adjacent poly(A) tail.

408 To test the function of the 16-mer motif, we could not simply mutagenize
409 it because this would lead to an irreversible loss of VSG protein, which is lethal
410 for the parasites⁷. Therefore, we created two reporter cell-lines, in which a second
411 VSG gene (*VSG117*) was introduced in the active BES, from where *VSG2* is
412 normally transcribed (Fig. 5A). As a result, these reporter cell-lines are VSG
413 double-expressors, i.e. they simultaneously express *VSG2* and *VSG117* at the
414 cell surface. In the cell line designated “VSG double expressor 1”, or “DE1”, the
415 *VSG117* gene contains the endogenous *VSG2* 3'UTR which harbors the 16-mer
416 motif. In the second cell-line, “VSG double expressor 2”, or “DE2”, *VSG117*
417 contains the same 3'UTR, except that the sequence of the 16-mer motif was
418 scrambled (5'-GTTATACAAAACCTTTT-3') (Fig. 5A). As has been previously
419 reported, the transcript levels of *VSG2* and *VSG117* are dependent on each other
420 and are dependent on the presence of the 16-mer motif⁷. Quantitative RT-PCR
421 analysis showed that the two VSGs have roughly the same levels in DE1 cell-
422 line. However, in DE2, *VSG117* transcript is about 7-fold less abundant than
423 *VSG2*. (Fig. 5B, 5D).

424 To test whether the 16-mer motif is required for inclusion of m⁶A in VSG
425 poly(A) tails, we performed m⁶A immunoblotting of cellular RNA obtained from
426 the two double-expressor cell lines. Given that *VSG2* and *VSG117* transcripts
427 have similar sizes (~1.8 kb), we used RNase H to selectively cleave *VSG2* before
428 resolving the RNA on gel. *VSG2* cleavage was performed by incubating the total
429 RNA sample with an oligonucleotide that hybridizes to the *VSG2* open reading
430 frame, followed by incubation with RNase H (as described in Fig. 2). As expected,
431 the *VSG2*-m⁶A-containing fragment is smaller and runs faster on gel (Fig. 5C).
432 An “m6A index” was calculated by dividing the relative intensity of m⁶A in each
433 VSG band (Fig. 5C) by the corresponding relative transcript levels measured by
434 qRT-PCR (Fig. 5B). A low m⁶A index indicates a given transcript has fewer
435 modified nucleotides (Fig. 5D).

436

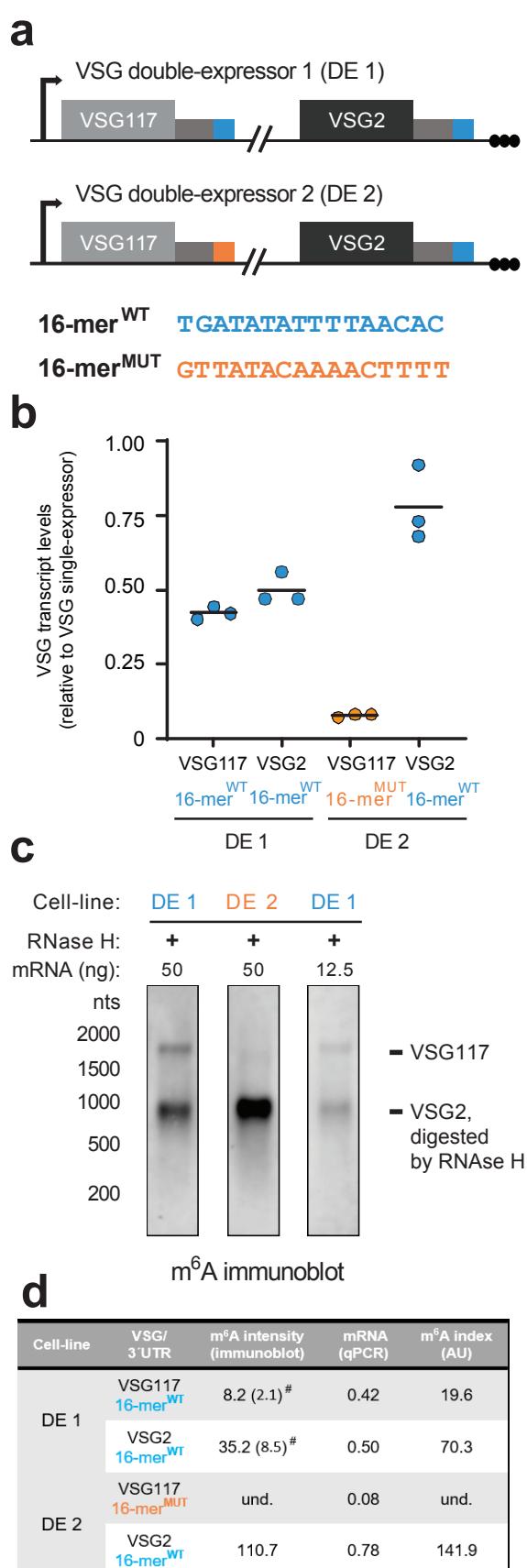
437

438

439 **Figure 5. Conserved VSG 16-mer
440 motif is required for inclusion of m6A
441 in adjacent poly(A) tail.**

442 a. Diagram of 16-mer motif VSG double-
443 expressor (DE) cell-lines. *VSG117* was
444 inserted immediately downstream of the
445 promoter of the active bloodstream
446 expression site, which naturally contains
447 *VSG2* at the telomeric end. In VSG double
448 expressor 16-mer^{WT} cell-line, *VSG117*
449 contains its endogenous 3'UTR with the
450 conserved 16-mer motif (sequence shown in
451 blue). In VSG double expressor 16-mer^{MUT}
452 cell-line, the 16-mer motif was scrambled
453 (sequence shown in orange). b. Transcript
454 levels of *VSG117* and *VSG2* transcripts
455 measured by qRT-PCR in both reporter cell-
456 lines. Levels were normalized to transcript
457 levels in cell-lines expressing only *VSG2* or
458 only *VSG117*. c. Immunoblot with anti-m⁶A
459 antibody of mRNA from VSG double-
460 expressor cell-lines. RNase H digestion of
461 *VSG2* mRNA was used to resolve *VSG2*
462 and *VSG117* transcripts. Different quantities
463 of the same VSG double expressor 16-mer^{WT}
464 cell-line was loaded in two separate lanes
465 (50ng and 12.5ng) to show that the *VSG117*
466 band is detectable in both conditions. d. m⁶A
467 index calculated as the ratio of m⁶A intensity
468 and mRNA levels, measured in panels c.
469 and b., respectively. und., undetectable. #
470 intensities measured in lane 3 of Figure 5C
471 (see also Extended Data Fig. S5)

472
473
474
475



476 Whenever the 3'UTR of *VSG* transcripts contain a 16-mer^{WT} (*VSG*s with a
477 blue box in Fig. 5A), *VSG* m⁶A bands are detectable by immunoblot and the m⁶A
478 index varies between 20-140 arbitrary units. In contrast, when the 16-mer motif
479 is mutagenized (*VSG117* with orange box in Fig. 5A), the *VSG* m⁶A is
480 undetectable (Fig. 5C), and the m⁶A index therefore is too low to calculate. These
481 results indicate that the motif is required for inclusion of m⁶A in the *VSG* transcript.

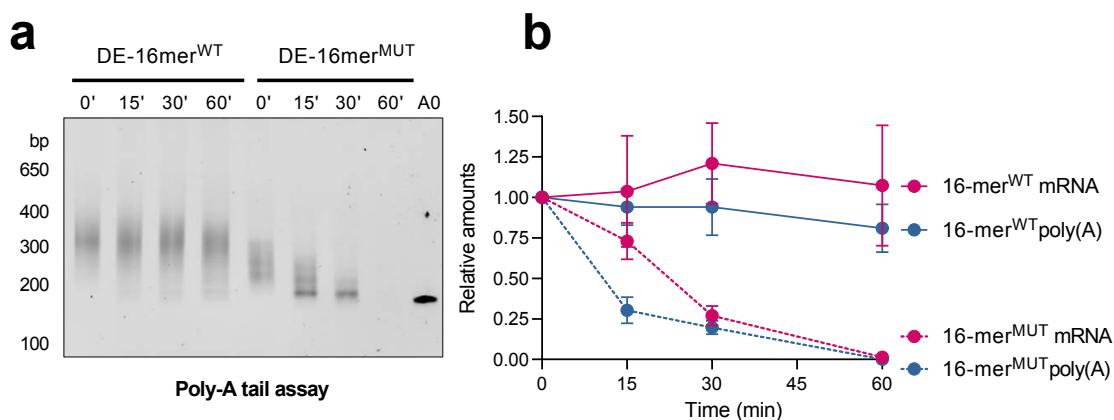
482 In contrast, if the 16-mer motif played no role in m⁶A inclusion, the *VSG117*
483 m⁶A index would be identical in both cell-lines (DE1 and DE2), i.e. around 20.
484 Given that the qRT-PCR quantifications showed the relative intensity of *VSG117*
485 16-mer^{MUT} is ~0.10 (Fig. 5B), the predicted intensity of the *VSG117* 16-mer^{MUT}
486 m⁶A band would have been 20 x 0.10 = 2.0 arbitrary units. To be sure that a band
487 with this level of m⁶A would be detected on an immunoblot, we ran a more diluted
488 DE1 RNA sample in lane 3 (Fig. 5C). The intensity of the *VSG117* 16-mer^{WT} band
489 is 2.1 arbitrary units (Fig. 5C and 5D), and it is easily detected in the immunoblot.
490 Given that we could not detect any band corresponding to a putative methylated
491 *VSG117* 16-mer^{MUT} in DE2 (even after over exposure of the immunoblot,
492 Extended Data Fig. S5), we conclude that the *VSG* conserved 16-mer motif is
493 necessary for inclusion of m⁶A in the *VSG* poly(A) tail.
494

495 **m⁶A is required for *VSG* mRNA stability**

496 The unusual localization of m⁶A in the poly(A) tail, and the lack of a
497 METTL3 ortholog, suggests that the underlying biochemistry of m⁶A formation in
498 trypanosomes is different from what has been described in other eukaryotes.
499 Given that, at this stage the mechanism of m⁶A formation in the poly(A) tail is
500 unknown and therefore cannot be directly blocked, we used the genetic mutants
501 of the 16-mer conserved motif to enquire about the function of m⁶A in *VSG*
502 mRNA.

503 To test the role of the 16-mer motif on poly(A) length on mRNA stability,
504 we measured *VSG* mRNA stability in 16-mer^{WT} and 16-mer^{MUT} cell-lines. *VSG*
505 mRNA half-life was measured by blocking transcription for 1 hr with actinomycin
506 D (the duration of the lag phase during *VSG* mRNA decay) and the levels of *VSG*
507 mRNA were followed by qRT-PCR. PAT assay clearly shows that, when the
508 *VSG117* transcript contains the 16-mer motif (16-mer^{WT} cell-line), the length of
509 the *VSG117* poly(A) tail is stable for 1 hr (Fig. 6A-B). In contrast, *VSG117*
510 transcripts containing a scrambled 16-mer motif exhibited very rapid shortening
511 of the poly(A) tail. In this case, there was no detectable lag phase—instead, the
512 length of the poly(A) tail was reduced to 25% of its original length after just 15
513 min, and was undetectable after 1 hr (Fig. 6A-B). Consistent with the fast kinetics
514 of poly(A) deadenylation, in the absence of an intact 16-mer motif, *VSG117*
515 transcript levels decayed very rapidly with a half-life of ~20 min (Fig. 6B).

516 Overall, these experiments show that when the *VSG* conserved 16-mer
517 motif is mutated and the m⁶A is lost, the *VSG* transcript is no longer stable and
518 exhibits rapid poly(A) deadenylation and a marked reduction of mRNA stability.
519



520

521 **Figure 6. VSG 16-mer motif delays poly(A) tail deadenylation.**

522 a. The length of the VSG poly(A) tail was measured using Poly(A) tailing (PAT) assay. Specific
523 primers confer specificity to the transcript being analysed. WT and Mut-16-mer cell-lines were
524 compared. A0 corresponds to the minimum length amplified by PAT and it comprises the end of
525 the VSG open reading frame and the whole 3'UTR. b. Quantification of *VSG117* transcript levels
526 and the length of poly(A) tail after transcription halt by actinomycin D. Total RNA of bloodstream
527 parasites was extracted after the indicate times. Values were normalized to 0 hr. Transcript levels
528 were measured by qRT-PCR (pink) and the length of the poly(A) tail was measured by PAT assay
529 (dark blue).

530

531 **Discussion**

532

533 In this work we report the identification and the function of the first RNA
534 epitranscriptomic modification in the poly(A) tail of mRNA. We show that the
535 stability and abundance of VSG transcripts stem from a novel mechanism in
536 which the presence of m⁶A in the poly(A) tail inhibits RNA degradation, probably
537 by blocking deadenylation. We also show that m⁶A is regulated by processes that
538 induce destabilization and degradation of VSG mRNA such as cellular
539 differentiation.

540 The classic function of a poly(A) tail is to keep mRNAs from being
541 degraded and to promote translation. Poly(A)-binding proteins (PABPs) bind the
542 poly(A) tail and stimulate mRNA translation by interaction with translation
543 initiation factors²⁸. Removal of the poly(A) tail by deadenylase complexes (e.g.
544 Ccr4-Not complex) allow the unprotected mRNAs to enter in the 5' → 3' or
545 3' → 5' degradation pathways^{29,30}. In this report, we identified a novel mechanism
546 by which a poly(A) tail contributes to mRNA stability. We found that the poly(A)
547 tail of VSG, a transcript that represents 4-11% of mRNAs from *T. brucei*
548 bloodstream forms, contains half of the m⁶A present in the cell. Our estimates
549 suggest that each VSG poly(A) tail has around 4 modified adenosines. When we
550 measured VSG mRNA decay rates by treating with transcription-blocking drugs,
551 we found that m⁶A is removed from the VSG poly(A) tail before the tail is
552 deadenylated and mRNA is degraded, raising the possibility that m⁶A normally
553 prevents these processes. A similar observation was made during parasite
554 differentiation. The removal of m⁶A may therefore be the step that initiates
555 deadenylation and mRNA decay. To test this idea, we removed a 16-mer motif in
556 the 3'UTR, which we find is necessary for the inclusion of m⁶A in the VSG poly(A)

557 tail. When m⁶A levels in VSG mRNA become undetectable, by mutating this
558 sequence element, the VSG mRNA exhibits rapid deadenylation and mRNA
559 decay. Overall these data support the idea that m⁶A acts as a stabilizer of the
560 VSG mRNA.

561 The presence of m⁶A in the poly(A) tail is so far unique to trypanosomes.
562 In other eukaryotes, m⁶A has been mainly detected by m⁶A mapping approaches
563 around the stop codon and 3' UTR, where it plays a role in mRNA stability and
564 translation¹⁵. A mapping study was recently published in *T. brucei* in which the
565 authors conclude that m⁶A localizes in internal regions of transcripts³¹. m⁶A was
566 not reported to be in the poly(A) tail in this previous study. However, m⁶A mapping
567 relies on aligning m⁶A-containing RNA fragments to genomic sequence. Since
568 the poly(A) sequence is not encoded in the genome, and m⁶A-containing
569 fragment from the poly(A) tail would not be mappable and therefore not detected
570 in this or any other previous m⁶A mapping study.

571 It remains unclear how m⁶A gets into the poly(A) tail. The presence of m⁶A
572 in the poly(A) tail suggests that an unusual RNA-methyltransferase will directly or
573 indirectly bind to the 16-mer motif and methylate adenosines that are either
574 adjacent to the 16-mer motif or become more proximal via a loop-like
575 conformation of the poly(A) tail. This would explain why orthologs of the canonical
576 METTL3 enzyme does not exist in the trypanosome genome²⁶. Unfortunately,
577 previous efforts from other labs have been unsuccessful at identifying proteins
578 that specifically bind to the 16-mer motif^{7,32}. An alternative mechanism would be
579 if m⁶A is incorporated into the poly(A) tail while polymerisation takes place. If this
580 was true, there should be m⁶ATP inside the cell. Unbiased metabolomics³³ did
581 not support the existence of m⁶ATP, although studies specifically focused on
582 detecting this nucleotide would be required to completely disprove this model.

583 Deadenylation is the first step in the main mRNA decay pathway in
584 eukaryotes³⁴. *T. brucei* is not an exception²³. In this study we showed that m⁶A
585 seems to protect the poly(A) tail from deadenylation. The molecular mechanism
586 behind this stabilizing effect is unknown. It is possible that the deadenylases are
587 inefficient on a methylated poly(A) tail. There is structural and biochemical
588 evidence that poly(A) tails adopt a tertiary structure that facilitates the recognition
589 by some deadenylases (CAF1 and Pan2)³⁵. When a poly(A) tail contains m⁶A,
590 just like when it contains guanosines, the tertiary structure may be not properly
591 assembled and deadenylase activity is inhibited. In this model, a putative
592 demethylase may be required to remove the methyl group and only then poly(A)
593 tail would be deadenylated. Alternatively, the stabilizing effect of m⁶A could result
594 from recruitment of a specific RNA-binding protein, that prevents the poly(A) tail
595 from being deadenylated.

596

597 *T. brucei* has around 2000 VSG genes, but only one is actively transcribed
598 at a given time². By a process of antigenic variation, parasites evade the host
599 immune response by changing an old VSG surface coat to a new VSG coat that
600 is antigenically distinct. m⁶A may be removed from VSG mRNA, facilitating the
601 switch from the old VSG mRNA, so that it can be more rapidly replaced by a new
602 VSG coat protein. Since m⁶A stabilizes the VSG mRNA, it will also be important
603 to determine whether inability to synthesize m⁶A in poly(A) tails results in more
604 frequent switching to new VSG coat proteins. The role of m⁶A in differentiation

605 remains to be established but it is possible that a delayed removal of VSG would
606 interfere with the interaction of the parasite with the midgut wall.

607 The Rudenko lab has proposed that the maximal amount of VSG mRNA
608 per cell is dependent on a post-transcriptional limiting factor dependent on the
609 presence of the 16-mer motif ⁷. We propose that this limiting factor is the rate of
610 inclusion of m⁶A in the poly(A) tails. When the 16-mer motif is present in both
611 VSG genes, both get partially methylated and their abundance is reduced to
612 about half of a single-VSG expressor; however, when the 16-mer motif is absent
613 from one of the VSGs, the second VSG is more methylated and the transcripts
614 become more abundant.

615 As far as we know, our work describes the first RNA modification in poly(A)
616 tails. We show that m⁶A is present in the poly(A) tail of *T. brucei* mRNAs, it is
617 enriched in the most abundant transcript (VSG), and that m⁶A acts as a protecting
618 factor stabilizing VSG transcripts. It will be important for future studies to identify
619 the enzymes and proteins involved in adding, reading, or removing this RNA
620 modification. Given the importance of VSG regulation for chronic infection and
621 parasite transmission, drug targeting such enzymes is expected to result in an
622 important loss of parasite virulence. Understanding these regulatory
623 epitranscriptomic processes may open up possibilities for developing therapeutic
624 strategies to treat sleepiness sickness.

625
626

627 **References**

628

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746 **Supplementary information**

747

748 Figures S1 to S5

749 Tables S1 to S3

750

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752

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774

775

776 **Author contributions**

777 I.J.V., J.P.M., M.D.N and J.A.R performed experiments.

778 I.J.V., J.P.M., M.D.N, J.A.R, F.A.B, S.R.J and L.M.F planned experiments &
779 analyzed data.

780 I.J.V., F.A.B., J.A.R., S.R.J and L.M.F conceived study.

781 I.J.V., S.R.J and L.M.F wrote manuscript, with contributions of all remaining
782 authors.

783

784 **Competing interests**

785 The authors do not have conflicts of interest.

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790

791 **Methods**

792

793 **Cell culture and cell-lines**

794 *Trypanosoma brucei* bloodstream form (BSF) parasites (EATRO 1125
795 AnTat1.1 90:13, and Lister 427 antigenic type MiTat 1.2, clone 221a, Single
796 Marker (SM) cell line³⁶ were cultured in HMI11, supplemented with 10% Fetal
797 Bovine Serum, at 37°C in 5% CO₂³⁷. Procyclic forms were cultured in DTM
798 supplemented with 10% Fetal Bovine Serum at 27°C^{38,39}. Parasites were
799 routinely grown in the presence of the selectable drugs: neomycin 2.5 µg·mL⁻¹,
800 hygromycin 5 µg·mL⁻¹ for EATRO 1125 AnTat1.1 90:13 and neomycin 2.5 µg·mL⁻¹
801 for SM. Transcription was inhibited by treating parasites with 5 µg·mL⁻¹ of
802 actinomycin D (Sigma A4262). Differentiation of slender into stumpy forms was
803 induced by adding 6 mM cis-aconitate (Sigma A3412) to the culture and dropping
804 temperature to 27°C. Protein translation was inhibited with 50 µg·mL⁻¹ of
805 puromycin (ant-pr-1, Invivogen).

806 Parasite cell lines were generated by transfection of *T. brucei* SM with
807 plasmid p221- purVSG117UTR (Addgene plasmid 59732) or with p221-
808 purVSG117UTRmut (Addgene plasmid 59732,7). The 16-mer mutagenized motif
809 was introduced in a primer that was used to PCR amplify p221-purVSG117UTR
810 plasmid. Amplification was performed with Phusion High-Fidelity DNA
811 polymerase (ThermoScientific). After elimination of original plasmid template by
812 digestion with *Dpn*1 (NEB R0176), amplification products were transformed into
813 *E. coli* JM109 (Promega L2005). Plasmids were isolated and purified from
814 bacteria, digested with *Not*1-HF (NEB R3189) and *Xba*1 (NEB R0146), ethanol
815 precipitated and transfected. Transfections were made with the AMAXA
816 nucleofector II (Lonza Bioscience), program X-001, using transfection buffer (90
817 mM sodium phosphate, 5 mM potassium chloride, 0.15 mM calcium chloride, 50
818 mM HEPES, pH 7.3). After overnight growth, transfected clones were selected
819 by adding 1 µg·mL⁻¹ puromycin.

820

821 **LC-MS/MS**

822 RNA samples were digested with nuclease P1 (sigma N8630, 1 U per 50
823 ng) for 2h at 37°C in buffer (2.5 mM ZnCl₂, 40 mM NH₄Ac, pH 5.3). After
824 dephosphorylation was done by addition of 10 U of Antarctic phosphatase (NEB
825 M0289) in the buffer provided for 2h at 37°C. Digested samples were diluted in
826 mili-Q water (10 ng/µL) and filtered with Microcon – 30 kDa. Chromatographic
827 separation was performed on a liquid chromatography systemUltimate 3000
828 RSLCnano (Thermo Fisher Scientific). Column – CORTECS® P3 (3 mm x 150
829 mm, 2.7 µm particle size, Waters Corporation). Mobile phase consisted of water
830 containing 0.1% formic acid (A) and methanol containing 0.1% formic acid (B).
831 The used elution gradient (A:B, v/v) was as follows: 100:0 for 2 min; 100:75 at 4
832 min; 75:30 at 9 min; 30:70 at 12 min; 0:100 at 15 min; 0:100 isocratic elution from
833 15.5 to 20 min. Samples were separated by liquid chromatography (column
834 Waters CORTECS T3 2.7µ 3.0 x 150 mm, Gradient with mobile phase A of water
835 0,1% formic acid and mobile phase B of methanol 0,1% formic acid.) and
836 analyzed in triple quadrupole Thermo Scientific TSQ Endura mass spectrometer

837 with electrospray ionization in positive mode, and followed by multiple reaction
838 monitoring. The ion mass transitions for m⁶A was 282,090>[150,059-150,061]
839 and for adenosine was 268,061>[136,110-136,112]. Calibration curves were
840 done with chemical standards and area under the curve (peak) integrated. To
841 calculate m⁶A to adenosine molar ratio, m6A and adenosine amounts were
842 calculated in the same sample injection. The ion mass transition of other modified
843 nucleosides are shown in Supplementary Table 1.

844

845 **RNA isolation and handling**

846 RNA was extracted from bloodstream and procyclic form parasites with
847 TRIzol (Invitrogen). 10-100 million parasites were lysed in 1 mL of TRIzol. RNA
848 was isolated according to the instructions of the manufacturer. RNA was treated
849 with DNase I (NEB M0303) (1 U per 2.5 µg of RNA) for 20 min. Reaction was
850 inactivated by adding 5 mM EDTA and heating to 75°C for 10 min. Purification of
851 mRNA was performed with NEBNext mRNA isolation module (NEB E7490),
852 following the manufacturer's instructions. The RNA that did not bind to the
853 poly(T)-beads was ethanol precipitated and saved. This RNA fraction was used
854 as A- RNA.

855 cDNA was generated using a SuperScript II Reverse Transcriptase
856 (Invitrogen 18064-014), using random hexamers, according to manufacturer's
857 protocol. Quantitative PCR (qPCR) was performed using SYBR Green PCR
858 Master Mix (Applied Biosystems 4368702 Power SYBR). Primer efficiencies
859 were determined using standard curves. Relative quantification was performed
860 based on the CT (cycle threshold) value and the method of Pfaffl⁴⁰. Amplifications
861 were normalized to 18S ribosomal RNA transcript. Primers are indicated in
862 Supplementary Table 2.

863

864 **m⁶A Immunoblotting**

865 DNase I-treated RNA samples were suspended in formaldehyde loading
866 buffer (30% formamide, 1,2 M formaldehyde, 1X MOPS buffer), denatured by
867 heating to 70°C for 5 min and immediately transferred to ice. 2 µg of total RNA or
868 50 ng of mRNA were typically loaded per lane. Samples were resolved on a
869 denaturing agarose gel (1.4% agarose, 2,2 M formaldehyde, 1X Mops buffer) for
870 1 h at 100 V at 4°C. RNA was transferred to a nylon membrane (GE Healthcare
871 Amersham Hybond-N+) by downward transfer with 10X SSC buffer (1,5 M NaCl
872 150 mM sodium citrate, pH 7.0) for 4-5 h. RNA was UV-crosslinked to the
873 membrane with a Stratalinker 2400 crosslinker (120 mJ cm⁻²). Membranes were
874 stained with 0.02% methylene blue (Sigma M9140, diluted in 0,3 M sodium
875 acetate pH 5.5) for 5 min and washed in RNase free water. After imaging,
876 methylene blue was removed by incubation in de-staining solution (0,2X SSC 1%
877 SDS) and washed 3 times in PBST (1X PBS pH 7.4 with 0,1% tween20). Nylon
878 membranes were blocked by incubation in 5% skimmed milk in PBST for 1 h and
879 then incubated overnight with 1µg/mL rabbit anti-m⁶A antibody (Abcam
880 ab151230, 1:1000 in 2.5% skimmed milk in PBST) at 4°C. Membranes were
881 washed in PBST three times, 10 min each, and then incubated with HRP-
882 conjugated donkey anti-rabbit IgG (GE Healthcare, NA934), diluted 1: 2500 in
883 2,5% skimmed milk in PBST for 1 h at room temperature. Membranes were
884 washed in PBST, three times, 10 min each and signal developed with Western

885 Lightning Plus-ECL, Enhanced Chemiluminescence Substrate kit (PerkinElmer
886 ref. NEL103E001EA). The percentage of m⁶A in the main “band” co-migrating
887 with the 2 kb molecular weight standard was measured by the intensity of the
888 “band” divided by the signal intensity of the whole lane.

889

890 **Estimation of m⁶A per VSG mRNA.**

891 The number of m⁶A per VSG mRNA molecule was estimated based on the
892 enrichment of m⁶A 1) in the VSG transcript (0,06%), 2) on the fraction of m⁶A
893 present in VSG mRNA (0,50) and 3) on previously quantified parameters
894 (detailed below). The number of mRNAs per *T. brucei* bloodstream forms cell is
895 20 000⁴¹. The number of VSG mRNAs per cell is 1000⁴¹ (assuming that
896 correspond to 5% of the mRNA). The average mRNA length is 2000 nt⁴². The
897 approximate length of the poly(A) tail is 100 nt⁴³. The approximate frequency of
898 adenosine in the transcriptome is 0,25.

899

900 The number of adenosines per mRNA molecule correspond to:

901 Number of adenosines (An) = (length of mRNA X frequency of adenose) + poly(A) tail
902
$$An = (2000 \times 0,25) + 100 \leftrightarrow An = 600 As$$

903

904 The total number of adenosines in a bloodstream forms cell correspond to:

905

906 An in bloodstream forms = mRNAs per cell X An per mRNA

907

908
$$An \text{ in } BSF = 20\,000 \times 600 \leftrightarrow An \text{ in } BSF = 12\,000\,000 As$$

909

910 The amount of m⁶A per cell in mRNA is:

911

912 m⁶A in bloodstream forms = (adenosines per cell X Frequency of m⁶A in mRNA (%)) /

913 100

914
$$m6A \text{ in } BSF = (12 \cdot 10^6 \times 0,006) / 100 \leftrightarrow m6A \text{ in } BSF = 7200$$

915

916 The amount of m⁶A in VSG mRNA molecules correspond to:

917

918 m⁶A in VSG mRNA = m⁶A in bloodstream forms X Fraction of m⁶A in VSG mRNA

919

$$m6A \text{ in } VSG = 7200 \times 0,5 \leftrightarrow m6A \text{ in } VSG = 3600 m6As$$

920

921 The amount of m⁶A per VSG mRNA molecule correspond to:

922

923 m⁶A per VSG = m⁶A in VSG/ VSG mRNA copies

924

925
$$m6A \text{ per } VSG = 3600 / 1000 \leftrightarrow m6A \text{ per } VSG = 3,6 \sim 4 m6A \text{ per } VSG \text{ mRNA}$$

926

927 Therefore, every VSG mRNA molecule harbors around 4 m⁶A per molecule.

928

929 Following the same logic, the amount of m⁶A distributed in the non VSG mRNAs
930 correspond to:

931

932 m⁶A per non-VSG = m⁶A in non-VSG/ non-VSG mRNA copies

933

$$m6A \text{ per non } VSG = 3600 / 19\,000 \leftrightarrow m6A \text{ per non } VSG = 0,19 \sim 0,2 m6A \text{ per non } VSG \text{ mRNA}$$

934

935 Therefore, in the non-VSG transcriptome there are around 1 m⁶A per 5 mRNA molecules.

936

937 **RNA ligase mediated poly(A) tail assay (PAT assay)**

938 5' phosphorylated DNA oligo adaptor (500 pmol) was linked to the free
939 hydroxyl group at the 3'-end of transcripts (1 μ g of total RNA) by T4 RNA ligase
940 1 (NEB M0204) in the presence of 15% (w/v) PEG 8000 at 25°C for 4 h. Note that
941 the adaptor oligo has a 3'-end dideoxyadenosine to avoid ligation between the
942 adaptors. The ligated RNA (250 ng) was used to generate cDNA with the
943 SuperScript II Reverse Transcriptase (Invitrogen 18064-014) according the
944 manufacturer's protocol using a primer complementary the adaptor. A VSG
945 specific forward primer and an adaptor specific reverse primer were used for the
946 subsequent PCR amplification using Phusion High-Fidelity DNA polymerase
947 (ThermoScientific) with increased MgCl₂ concentration (3.5 mM) and reduced
948 extension temperature (60°C). The amplification products were diluted 1:500 and
949 used as a template in a second amplification (nested PCR) with a second VSG
950 specific forward primer and a second adaptor specific reverse primer. The A0
951 amplification product (Fig. 6) corresponds to the amplification product without a
952 poly(A) tail. It results from a PCR amplification in which the reverse primer
953 anneals at the end of the 3' UTR immediately upstream of the poly(A) tail.
954 Amplification products were resolved on a 6% TBE-PAGE (polyacrylamide gel
955 electrophoresis) for 1 h at room temperature and stained with gel red for
956 visualization. The length of the poly(A) tail estimated by co-migration of the
957 amplification products with a DNA ladder minus the length of the A0 amplification
958 product.

959

960 **Statistical analysis of decay curves**

961 Data of the decay of mRNA, length of poly(A) tail and m⁶A signal were
962 fitted in GraphPad Prism to either "Plateau followed by one phase decay" or "one
963 phase decay". The first were used when the variable decay started after an initial
964 constant period. The "one phase decay" method was used when the measured
965 variable decreased since the beginning of the experiment. The curves were
966 adjusted to the data by the method of least squares, from which the decay
967 constant K is estimated. The half-life was calculated by the $\ln(2)/K$. This data is
968 summarized in Supplementary Table 3.

969

970

971 **RNase H digestion**

972 2 μ g of total RNA was mixed with a complementary DNA oligo (0.2 pmol)
973 in water and incubated 5 min at 70°C, after which temperature was reduced 1°C
974 per min until 37°C. RNA:DNA hybrids were digested by RNase H (Ambion
975 AM2293) for 30 minutes. The reaction was stopped by adding formaldehyde
976 loading buffer and incubating at 70°C for 5 min. For RNase H digestions of VSG2
977 transcript (Fig. 5), a thermostable RNase H (NEB M0523) was used to reduce
978 unspecific digestion of the abundant VSG117 transcript. Also mRNA, instead of
979 total RNA, was used as the substrate to reduce the background and increase the
980 sensitivity in the detection of VSG117 transcript. The digestion mixture was
981 prepared on ice with 50 ng of mRNA, (0.2 pmol) complementary DNA oligo, and
982 Thermostable RNase H. The reaction mixture was transferred to the pre-warmed
983 thermoblock at 50°C and incubated for 25 minutes.

984

985

986 **Immunofluorescence assays**

987 Parasites were pelleted by centrifugation (800 g, 3 min, room
988 temperature), resuspended in the remaining medium and transferred to an
989 microcentrifuge tube. Pellet was resuspended in PBS and immediately fixed 4%
990 v/v formaldehyde with gentle agitation by inversion. Fixed cells were centrifuged,
991 resuspended in PBS and settled on poly-L-lysine coated coverslips for 1 h (for
992 cells to settle by gravity). Parasites were permeabilized in 0,2% Nonidet P-40 in
993 PBS for 5 min and washed in PBS three times for 5 min each. Cells were blocked
994 in 1% BSA, 25 mg/mL glycine, in PBST for 1 h and then incubated with anti-m⁶A
995 antibody (Abcam) 1: 250 (4 µg/ml⁻¹ final concentration with 1% BSA in PBS) for
996 3 h at room temperature. After washing cells three times with PBS for 15 min
997 each with agitation, cells were incubated with secondary anti-rabbit antibody
998 (Alexa Fluor 488, A11034) for 30 min (protected from light). At the end, cells were
999 washed three times with PBS for 15 min each and DNA stained for 1 min with 1
1000 µg/ml Hoechst. Slides were mounted using Vectashield.

1001

1002 **Image acquisition and analysis**

1003 Confocal images on fixed *T. brucei* parasites were acquired using a Zeiss
1004 confocal Laser Point-Scanning (LSM) 880 Microscope equipped with the Zen 2.1
1005 (black) software with a Plan Apochromat 63x NA 1.40 oil immersion DIC M27
1006 Objective. The laser units used were a Diode 405-430 to excite the 405nm
1007 wavelength (corresponding to Hoechst), and an Argon laser to excite the 488nm
1008 wavelength (GaAsP detector 525/50 nm) (corresponding to AF488 used as a
1009 secondary antibody for m⁶A labelling). Images were acquired in a two-track
1010 mode. A pinhole diameter of 1AU for the 488 laser track was used. DIC images
1011 in confocal mode were obtained using the 405nm laser line. A digital zoom of
1012 1.2x was used for general quantification, or a digital zoom of 4x for sub-
1013 localization analyses. For z-stack acquisition, on average 7-14 slices were
1014 obtained to cover all parasite areas per field, with a stack slice of 0.3µm. Images
1015 were acquired in multiple fields of view to enable quantification of at least 100
1016 parasites per condition per experiment repeat. Images were analysed using
1017 Fiji/ImageJ (imagej.nih.gov/ij/) for background correction, MFI determination and
1018 segmentation analysis. 3D rendering and 3D quantifications of m⁶A were
1019 performed using Imaris 9.1.0 (BitPlain). Statistical significance was determined
1020 using GraphPad Prism (GraphPad Prism Software version 6). Statistical tests
1021 used are mentioned in the corresponding Fig. legends. p<0.05 was considered
1022 significant.

1023

1024