INTRODUCTION

The unicellular eukaryote *Trypanosoma brucei* is a useful model organism for studies of post-transcriptional regulation of gene expression. As in other kinetoplastids, transcription in *T. brucei* is polycistronic, and mRNAs are excised by co-transcriptional trans-splicing and polyadenylation. RNA-binding proteins play important roles throughout the lifetime of every mRNA, governing mRNA processing, translation, and mRNA decay (Clayton, 2019; Kolev, Ullu, & Tschudi, 2014).

*T. brucei* is always extracellular, and grows in mammals as long slender trypomastigotes. These have a variant surface glycoprotein surface coat and depend mainly on glycolysis for ATP, using enzymes that are compartmentalised in a microbody called the glycosome (Hannaert, Bringaud, & Michels, 2003). When bloodstream forms reach high density, either in culture or in a mammal, they have a quorum sensing response (Rojas et al., 2018) and transform to nondividing stumpy form trypomastigotes. Upon uptake of the stumpy forms by Tsetse, or transfer to culture at 27°C in the presence of cis-aconitate, stumpy forms develop into growing procyclic forms that are short and survive long term in the insect vector. As they develop, they convert into the intracellular tachyzoite form that infects the mammal to reinitiate the parasite life cycle. This switch from extracellular to intracellular forms is controlled by multiple factors occurring at the right time and in the right place (Clayton, 2018). The ZC3H20 and ZC3H21 zinc finger proteins are closely related, with two C_(x)8C_(x)5C_(x)3H zinc finger motifs. ZC3H20 is present at a low level in replicating mammalian-infective bloodstream forms, but becomes more abundant when they undergo growth arrest at high density; ZC3H21 appears only in the procyclic form of the parasite, which infects Tsetse flies. Each protein binds to several hundred mRNAs, with overlapping but not identical specificities. Both increase expression of bound mRNAs, probably through recruitment of the MKT1-PBP1 complex. At least 28 of the bound mRNAs decrease after depletion of ZC3H20, or of ZC3H20 and ZC3H21 together; their products include procyclic-specific proteins of the plasma membrane and energy metabolism. Simultaneous depletion of ZC3H20 and ZC3H21 causes procyclic forms to shrink and stop growing; in addition to decreases in target mRNAs, there are other changes suggestive of loss of developmental regulation. The bloodstream-form-specific protein RBP10 controls ZC3H20 and ZC3H21 expression. Interestingly, some ZC3H20/21 target mRNAs also bind to and are repressed by RBP10, allowing for dynamic regulation as RBP10 decreases and ZC3H20 and ZC3H21 increase during differentiation.

**KEYWORDS**

mRNA, *Trypanosoma*, translation, binding, RNASeq

**1 | INTRODUCTION**

*Trypanosoma brucei* is a useful model organism for studies of post-transcriptional regulation of gene expression. As in other kinetoplastids, transcription in *T. brucei* is polycistronic, and mRNAs are excised by co-transcriptional trans-splicing and polyadenylation. RNA-binding proteins play important roles throughout the lifetime of every mRNA, governing mRNA processing, translation, and mRNA decay (Clayton, 2019; Kolev, Ullu, & Tschudi, 2014).
trypanosomes, which rely mainly on mitochondrial pathways of ATP generation (Bringaud, Rivière, & Coustou, 2006; Mantilla et al., 2017). A protein called PAD1 (protein associated with differentiation), which is expressed by stumpy forms, is implicated in cis-aconitate sensing (Dean, Marchetti, Kirk, & Matthews, 2009). The trypanosomes later migrate from the midgut, via the proventriculus, to the salivary glands, becoming first epimastigotes, then nondividing metacyclic trypomastigotes which have variant surface glycoprotein and are infectious for mammals (Rotureau & Van Den Abbeele, 2013). All of these transitions are accompanied by changes in RNA-binding proteins, and in some cases are known to be governed by them (Kolev, Ramey-Butler, Cross, Ullu, & Tschudi, 2012; Kolev et al., 2014; Mugo & Clayton, 2017).

One example of a controlling RNA-binding protein is ZC3H11, which has a single CCCH zinc finger domain (consensus: C(x)7C(x)5C(x)3H) and promotes the expression of chaperone mRNAs. ZC3H11 binds (UA(U)6) in mRNA 3′-untranslated regions (3′-UTRs), and is required for stabilisation of its target mRNAs after heat shock (Droll et al., 2013). ZC3H11 recruits another protein, MKT1, via an interaction motif, (Y/W/T)(R/T/Q)H(N/D)PY. MKT1 is part of a protein complex that promotes mRNA translation and stability (Singh et al., 2014).

A second RNA-binding protein, RBP10, is essential to maintain the differentiation state of bloodstream form T. brucei. Binding of RBP10 to its target mRNAs, probably via the sequence UA(U)6 in 3′-UTRs, targets the mRNAs for translational repression and destruction (Mugo & Clayton, 2017). Levels of RBP10 protein and mRNA decline in stumpy forms (Dejung et al., 2016; Silverst, Ivens, & Matthews, 2018) and when bloodstream forms are incubated at maximal density in vitro (Mugo & Clayton, 2017). In procyclic forms, RBP10 is undetectable and its target mRNAs are generally abundant and well translated. Correspondingly, depletion of RBP10 in bloodstream forms triggers a cascade of events, starting with increases in RBP10 target mRNAs, and followed by secondary effects that switch the gene expression pattern towards that of procyclic forms. Conversely, expression of RBP10 in procyclic forms switches the cells towards the bloodstream-form pattern (Mugo & Clayton, 2017).

Among the directly bound targets of RBP10 in bloodstream forms are the mRNAs encoding three proteins with CCCH zinc finger domains: ZC3H22 (Tb927.7.2680), ZC3H21 (Tb927.7.2670) and ZC3H20 (Tb927.7.2660) (Mugo & Clayton, 2017). The 3′-UTRs of these mRNAs have, respectively, two, five and seven copies of the RBP10 consensus recognition motif (Figure 1). All three mRNAs are more abundant, and considerably better translated, in procyclic forms than in growing bloodstream forms (Antwi et al., 2016; Dejung et al., 2016; Fadda et al., 2014; Jensen et al., 2014), and their expression is also lower in metacyclic forms than in procyclic forms (Christiano et al., 2017). When ZC3H20 or ZC3H21 are tethered (via a lambdaN-peptide–boxB interaction) to a reporter mRNA in bloodstream forms, expression is stimulated, whereas tethering of ZC3H22 leads to repression (Erben, Fadda, Lueong, Hoheisel, & Clayton, 2014; Lueong, Merce, Fischer, Hoheisel, & Erben, 2016). Tagged versions of each are in the cytoplasm of procyclic forms (see www.tryptag.org) (Dyer, Dean, & Sunter, 2016), and move to messenger ribonucleoprotein (mRNP) granules after starvation (Fritz et al., 2015). Depletion of ZC3H22 was shown to inhibit growth of procyclic forms (Domingo-Sananes, Szoor, Ferguson, Urbaniak, & Matthews, 2015).

Ling et al (Ling, Trotter, & Hendriks, 2011) showed that RNAi that simultaneously targeted ZC3H20 and ZC3H21 inhibited procyclic form trypanosome growth. Microarray experiments revealed that RNAi resulted in reduced levels of six RNAs, and increases in another ten. The authors also demonstrated binding of ZC3H20 to two of the reduced transcripts. To obtain a detailed picture of the functions of ZC3H20 and ZC3H21, we have now determined their transcriptome-wide binding specificities. These, combined with analyses of expression and the effects of depletion, showed us that the two proteins affect the fates of several hundred mRNAs, and have overlapping but different roles in the trypanosome life cycle and in gene regulation.

2 | RESULTS

2.1 | Conservation of ZC3H20, 21 and 22

The ZC3H22, ZC3H21 and ZC3H20 genes are arranged consecutively in the Trypanosoma brucei genome (Figure 1a). ZC3H21 and ZC3H20 are similar in the region that includes the two zinc fingers, each of which has the structure C(x)7C(x)5C(x)3H (Figure 1b,c and Supplementary Figure S1). ZC3H22 is similar to the other two proteins only in the region containing the N-terminal zinc finger; its C-terminal one has the structure C(x)7C(x)5C(x)3H. There is at least one homologue of ZC3H20 or ZC3H21, and of ZC3H22, in Trypanosoma, Leishmania, Crithidia, Endotrypanum, Leptomonas, Paratrypanosoma and Blechomonas species (Figure 1b,c Supplementary Figure S1) but both ZC3H21 and ZC3H22 appear to be absent in the free-living Bodo caudatus. In Trypanosoma congolense and Trypanosoma vivax, for which the genomes are incomplete, all three genes appear to be present only as fragments, but longer open reading frames are obtained by adding or deleting a few nucleotides, yielding homologues containing both zinc fingers, which suggests that the fragmentation might be due to assembly errors. The phylogenetic tree (Figure 1b) suggests that duplication of ZC3H21 occurred independently in Trypanosoma brucei and in T. cruzi (TCCLB) and T. rangeli (TRSC), which are transmitted by triatomine bugs. The duplication may therefore be an adaptation to specific conditions.

Apart from the conservation around the zinc fingers, all ZC3H20 and ZC3H21 sequences have a variant of the MKT1-binding motif (Y/W/T)(R/T/Q)H(N/D)PY near their C-termini. The C-termini of the different ZC3H22s, in contrast, have little in common. Three of the ZC3H22s do, however, have poly(Q) sequences that might form disordered interaction domains (Figure 1c, Supplementary Figure S1).

The RNA-binding specificities of CCCH domains are influenced by the sequences within the zinc fingers, and by five flanking residues (Hudson, Martinez-Yamout, Dyson, & Wright, 2004; Pagano,
Farley, McColg, & Ryder, 2007). The N-terminal CCCH domains of ZC3H20 and ZC3H21 have only three differences, but the C-terminal motifs have eight (Supplementary Figure S2). This suggests that the RNA-binding specificities of the two proteins could be related, but not identical. ZC3H22 specificity is likely to differ more. A comparison with the RNA-binding regions of other T. brucei proteins with two CCCH domains revealed that each is likely to have binding specificities that differ from those of ZC3H20 and ZC3H21 (Supplementary Figure S2). The proteins with most closely related zinc finger domains are ZC3H18 and ZC3H30.

2.2 Regulated expression of ZC3H20 and 21

Unless otherwise stated, experiments in bloodstream forms were done with differentiation competent EATRO1125 strain trypanosomes. They were grown in methylcellulose-containing medium (Vassella et al., 2001) and their density was routinely maintained below 8 × 10⁵ cells/ml, in order to maintain differentiation competence. Some experiments were also done with monomorphic Lister 427 strain bloodstream form T. brucei, which cannot make stumpy forms (Vassella, Reuner, Yutzy, & Boshart, 1997). Established procyclic form cultures were either Lister 427 or EATRO1125 strain trypanosomes.

We confirmed published data concerning regulation of ZC3H20 and ZC3H21, since mass spectrometry results on total lysates have limited sensitivity. The 5′-UTRs of T. brucei genes have not so far been implicated in developmental regulation. Protein expression can therefore be assessed using N-terminally in situ-tagged versions, with the caveat that N-terminal tagging might influence protein stability. For maximal sensitivity, we used versions bearing a tandem affinity purification (TAP) tag that includes an IgG-binding domain (Vassella et al., 2001).
domain, allowing detection by direct IgG binding (Puig et al., 2001). Functionality of the tagged protein was later confirmed by deletion of the remaining wild-type allele (Figure 2b, Supplementary Figure S3B,H). TAP-ZC3H20 was just detectable in long slender differentiation-competent bloodstream form trypanosomes. As predicted by proteomic results for stumpy forms (Dejung et al., 2016), TAP-ZC3H20 increased at maximal density and was more abundant in trypanosomes that had been further incubated at high density at 37°C (Figure 3a,b and Supplemental Figure S4A). This treatment induced expression of the stumpy form marker protein PAD1, as judged by western blotting (Figure 3b, Supplementary Figures S4A and S5A). However, PAD1 was not detected by immunofluorescence and most of the cells retained long slender or intermediate morphology (not shown), indicating that complete stumpy form differentiation had not taken place. We therefore suggest that our high-density cultured forms were in an intermediate differentiation state. The increase in TAP-ZC3H20 was detected before PAD1 (Figure 3b, Supplementary Figure S4A).

To induce differentiation of high-density cultured bloodstream forms to procyclic forms (Figure 3a), the temperature was decreased from 37 to 27°C, and cis-aconitate (6mM) was added. After 24 hr, the cells were transferred to procyclic form medium, which has proline as the principal energy source. Cell numbers started to increase within 2 days (Figure 3a). As predicted from a proteomic comparison of stumpy and procyclic forms (Dejung et al., 2016), the amount of TAP-ZC3H20 showed no further increase upon procyclic differentiation (Figure 3b, Supplementary Figure S4A). The transient decreases in both ZC3H20 and EP procyclin expression that were sometimes seen 1 day after the medium change (point “h” in Figure 3a,b) are unexplained but similar reversions were already documented in microarray studies (Queiroz, Benz, Fellenberg, Hoheisel, & Clayton, 2009). Interestingly, TAP-ZC3H20 also increased when monomorphic Lister 427 strain bloodstream forms attained high density (Supplemental Figure S4B). This suggests that the increase in TAP-ZC3H20 does not depend on the ability to respond to the quorum-sensing signal (Vassella et al., 1997). The increase in TAP-ZC3H20 correlated with the stationary phase of culture and was not dependent on the presence of methyl cellulose (Supplemental Figure S4B,C).

TAP-ZC3H21 was, like TAP-ZC3H20, just detectable in bloodstream forms, but it did not increase upon incubation at high density (Figure 3a,b, Supplementary Figure S4D), which is consistent with its absence in stumpy form proteomes (Dejung et al., 2016). TAP-ZC3H21 only became readily detectable upon induction of procyclic differentiation, and it was not as abundant as TAP-ZC3H20 in established procyclic forms (Figure 3a,b, Supplementary Figure S4D).

We next examined which differentiation stimuli affected ZC3H20 and ZC3H21 expression. Treatment of growing (low density) EATRO1125 bloodstream forms at 27°C alone for 6 hr caused a 2.5–6-fold increase in ZC3H20 (Figure 3c, Supplementary Figure S4E), but higher expression was obtained at 27°C in the presence of cis-aconitate (Figure 3c, Supplementary Figure S4E,F). Incubation at 19°C sensitises bloodstream forms to differentiation signals (Engstler & Boshart, 2004) and also increased TAP-ZC3H20 more than incubation at 27°C (Figure 3c, Supplementary Figure S4e); a preliminary result suggested that the mRNAs were also increased (Supplementary Figure S4f). TAP-ZC3H21 appeared once after 19-C incubation (Supplementary Figure S4G), but it was only reproducibly detected after both 27°C and cis-aconitate treatment (Figure 3c, Supplementary Figure S4G). Incubation of procyclic forms at high density did not affect TAP-ZC3H20 expression (Supplementary Figure S4H).

The ZC3H20 3′-UTR has two RBP10-binding motifs, whereas that of ZC3H21 has five. The ZC3H20 and ZC3H21 mRNAs were

**FIGURE 2** Genotypes of transgenic cell lines with altered ZC3H20 expression. The different panels are explained in the text and on the Figure. Additional genotypes and examples of PCR evidence are illustrated in Supplementary Figure S3 [Colour figure can be viewed at wileyonlinelibrary.com]
not examined separately in our previous transcriptome analyses (Mugo & Clayton, 2017). However, there were twice as many reads for ZC3H20 as for ZC3H21, and the excess reads were reproducibly threefold higher in the RBP10 bound than the unbound fraction. A pull-down confirmed that ZC3H20 mRNA is bound by RBP10 (Figure 4a). TAP-ZC3H20 expression in bloodstream forms increased after rbp10 RNAi, as expected (Figure 4b), but this could be an indirect effect.

2.3 | Activation by tethered ZC3H20 and ZC3H21

In tethering screens in bloodstream forms, full-length λN-ZC3H20 increased reporter expression, as did a C-terminal fragment starting at residue 222 (Erben et al., 2014; Lueong et al., 2016). To confirm this for procyclic forms, we expressed λN-ZC3H20-myc in cells containing a CAT-boxB mRNA that has five boxB sequences between the CAT (chloramphenicol acetyltransferase) open reading frame and the actin 3′-UTR (Figure 5a). A reporter lacking boxB served as the control. Tethering of the full-length protein (Figure 5b) reproducibly increased the CAT activity (Figure 5c) and also increased the RNA (Figure 5d).

ZC3H20 and ZC3H21 interacted with MKT1 in the yeast 2-hybrid assay (Singh et al., 2014) and all ZC3H20/ZC3H21 homologues contain a variant of the MKT1-binding motif, (Y/W/T)(R/T/Q)H(N/D)PY (Figures 1c and 5e). However, the motif in T. brucei ZC3H20, WHYNPY, which starts at position 383, does not conform to the consensus because it has an inserted tyrosine...
added and differentiation was induced without methyl cellulose, by addition of cis-aconitate and reduction of the temperature to 27°C. One day later, cells were transferred to procyclic medium. TAP-ZC3H20 was only two–fourfold depleted after 12 hr, despite the presence of tetracycline; this is probably because the cells were approaching stationary phase, during which RNA polymerase I transcription (which is required for RNAi induction) is repressed. After 1 day, when TAP-ZC3H20 was more reduced, expression of the procyclic surface protein EP procyclin was impaired in the induced lines (Figure 6a, upward arrows). This suggested that ZC3H20 was required for normal differentiation kinetics. After 5 days, the cells were growing normally as procyclic forms, but some ZC3H20 was still detectable (3%–5% of maximal expression), so its role could not be assessed.

To further investigate ZC3H20 function, we deleted both genes in bloodstream forms (homozygous or “double” knock-out, DKO) (Figure 2d, Supplementary Figure S3E,G). These cells were unable to express PAD1 at high density (Figure 6b, Supplementary Figure S5A). Also, when procyclic form differentiation was induced the cells were unable to proliferate. Although they remained alive and motile for several days, the kinetoplast remained close to the posterior end, as in bloodstream forms, instead of being roughly half-way between the nucleus and the posterior end, which is normal for procyclic forms (Figure 6c, Supplementary Figure S5B). Eventually, the cells died.

To check whether the differentiation defect in the cells that lacked ZC3H20 was indeed due to the loss of ZC3H20, rather than to some other unknown genetic change, we introduced a construct enabling tetracycline-inducible expression of C-terminally myc-tagged ZC3H20 (ZC3H20-myc) into the DKO bloodstream forms (Figure 2e). Oddly, no ZC3H20-myc was detected in the bloodstream forms after tetracycline addition, but nevertheless, we attempted differentiation. Since there is little transcription in trypanosomes that are at maximal density, we used growing cells to enable ZC3H20-myc expression; tetracycline and cis-aconitate were added and the temperature was reduced to 27°C. After a further day, the medium was changed as before. With this protocol, differentiation of wild-type cultures takes longer than with the growth-arrested cultures: full EP procyclin expression is seen after 3 days and the cells start to grow as procyclic forms on day 4 (Figure 6d). Two different cloned lines containing the inducible ZC3H20-myc construct were studied (Figure 6d). In the absence of tetracycline, clone 1 showed no expression of ZC3H20-myc, and after induction of differentiation, it never expressed EP procyclin, and was dying within 3 days. With tetracycline, ZC3H20-myc appeared gradually over 3 days, procyclin expression was restored, and survival of the cells was prolonged—but they never grew as procyclic forms (Figure 6d, Supplementary Figure S7A). This suggested that ZC3H20-myc was functional in the early stages of differentiation. Clone 2 showed similar levels of expression of ZC3H20 both with and without tetracycline, and behaved similarly to clone 1 in the presence of tetracycline. After 3 days, ZC3H20-myc expression, however, the EP procyclin expression pattern was altered, with accumulation of faster migrating variants that were present only at low abundance in

To assess the requirement for ZC3H20 in differentiation, we first attempted depletion by RNAi. We used cell lines expressing TAP-ZC3H20 in order to be able to follow protein loss (Figure 2c). Two independent EATRO1125 clones were grown to 3x10^5/ml in methyl cellulose medium and incubated for a further 12 hr, at which point PAD1 was expressed (Figure 6a). Tetracycline was then

2.4  ZC3H20 is required for PAD1 expression and for differentiation to procyclic forms

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the wild-type cells (Figure 6d, arrows). Either posttranslational modification of the protein was defective, or different procyclin genes were being expressed.

The inducible genes are transcribed by RNA polymerase I, which is at least 5 times more active than RNA polymerase II in transcription of protein-coding genes (Biebinger et al., 1996). Inducible ZC3H20-myc was therefore probably overexpressed relative to the normal endogenous level. Our results thus suggested that excess ZC3H20 might prevent establishment of growing procyclic forms. We therefore examined differentiation-competent bloodstream form trypanosomes with inducibly expressed ZC3H20-myc as well as normal ZC3H20 genes (Supplementary Figure S3 K), using the same protocol as before. Again, addition of tetracycline to the growing bloodstream forms gave no detectable ZC3H20-myc expression, and had no effect on growth (not shown). Clone 1, which had no detectable ZC3H20-myc without tetracycline, could differentiate and grow as procyclic forms without tetracycline, but this was prevented by tetracycline addition. Clone 2 expressed ZC3H20-myc without tetracycline and was unable to grow as procyclic forms (Supplementary Figure S6A).

**FIGURE 5** Enhancement of expression by tethered ZC3H20 in procyclic forms depends on the MKT1 interaction motif. (a) The cartoon shows λN-ZC3H20-myc bound to a boxB motif in the CAT-boxB mRNA, which has five boxB sequences between the CAT open reading frame and the actin 3′-UTR. (b) Cartoon of ZC3H20 fragments used in the tethering assay. Colour codes are as in Figure 1. FL is full-length protein, “N” is the N-terminal part of the protein containing the RNA-binding motifs and “C” is the C-terminal part of the protein containing some phosphorylation sites and the putative MKT1 interacting motif. The lowest diagrams show full-length or C-terminal portions with point mutations in the motif. (c) Relative CAT (chloramphenicol acetyltransferase) activities in cells expressing reporters with or without boxB (two clones each, C1 and C2). Expression of λN-ZC3H20-myc was assessed by western blot (lower panel). (d) Effect of tethering λN-ZC3H20-myc fragments. Upper four panels: Levels of CAT mRNA were assessed by detected by RT-PCR with two different numbers of cycles and with tubulin as the control. Lower panels: Expression of CAT and of the λN-ZC3H20-myc proteins, assessed by western blotting with ribosomal protein S9 as the control. For each cell line, the ratio of +tet to −tet is shown below relevant images. (e) Conservation of the MKT1-interacting motif in ZC3H20/21 homologues and paralogues. The three amino acids flanking either side of the motif are included, and the numbers of amino acids to the C-termini are shown. Full protein IDs are in supplementary Figure S1. (f) Effect of mutating the MKT1 interaction motif. Different λN-ZC3H20-myc proteins (see panel b) were used. The graph shows CAT activity, and beneath are northern and western blots. For each cell line, the ratio of +tet to −tet is shown below relevant images. (g) Tethering of λN-ZC3H21-myc [Colour figure can be viewed at wileyonlinelibrary.com]
Why was no ZC3H20-myc expression detected after induction in bloodstream forms? The results in Figure 6e show that if growing bloodstream forms were treated with tetracycline for 24 hr, ZC3H20-myc was detectable if a protease inhibitor that targets the proteasome was added, but even then the amount was lower than in similarly treated procyclic forms. A preliminary result suggested that ZC3H20-myc has a half-life of at least 6 hr in cycloheximide-treated procyclic forms (Supplementary Figure S6B), but pulse-labelling followed by mass spectrometry suggested relatively short half-lives in both forms (~1 and ~2 hr in bloodstream and procyclic forms, respectively) (Tinti, Güther, Crozier, Lamond, & Ferguson, 2019).
FiguRe 6  Depletion of ZC3H20 affects differentiation. (a) The cells used were EATRO1125 bloodstream forms expressing TAP-ZC3H20 and with tetracycline-inducible RNAi targeting ZC3H20 (Figure 2c). The cells were maintained at high density with methyl cellulose (around 3 × 10⁷/ml) for 12 hr prior to the start of the experiment. At time = 0, cells were transferred to medium without methyl cellulose (1 × 10⁷/ml), cis-aconitate was added with or without 100 ng/ml tetracyclen, and the temperature was reduced to 27°C. One day later cells were switched to MEM-pros medium. The experiment was done in two independent cultures (A and B). Cell densities for the two clones (with different symbols) are shown on the left; the arrow indicates the time at which some of the cells were harvested for RNASEq. Western blots for protein expression on the right; the arrows point to RNAi lanes with lower EP procyclin expression than the controls. S9 is ribosomal protein S9. Relative amounts of TAP-ZC3H20, and EP procyclin are shown below the relevant images. (b) Bloodstream form EATRO1125 T. brucei that lack ZC3H20 (Figure 2d, Supplementary Figure S3g) do not express PAD1 at high density. Cells were allowed to grow in methyl cellulose without dilution. The cell densities are shown above and western blots beneath. PAD1 is not expressed in long slender cells (Dean et al., 2009) but the antibody to PAD1 cross-reacts with other proteins. The bands at time point “a”, which are labelled with white arrows, must therefore be a different protein—perhaps another member of the PAD family. PAD1 is labelled with a black arrow. Results from two different experiments are shown. (c) Cells lacking ZC3H20 (DKO, Figure 2d) were grown to 3 × 10⁷/ml, then treated as in (a) except that the cells were maintained at high density for 16 hr before cis-aconitate addition at 1x 10⁵/ml. Cells with two ZC3H20 genes served as controls. The upper panel shows cumulative cell numbers—the cells lacking ZC3H20 died. The lower panel illustrates the morphologies of the cells after 60 hr (double-headed arrow). P = posterior, K = kinetoplast, N = nucleus. A higher PK/N ratio is characteristic of procyclic forms.

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2.5 | Excess ZC3H20 is toxic for procyclic forms

We next attempted to obtain procyclic forms in which all ZC3H20 expression was tetracycline dependent. To do this, we attempted differentiation in 2.5-fold serial dilutions of tetracycline. After several weeks, a single culture that had been maintained with 1 ng/ml tetracycline started to grow as established procyclic forms. This culture could then grow in the absence of tetracycline. Although ZC3H20-myc was not detectable by western blotting, it is likely that a small amount was still being expressed. When 100 ng/ml tetracycline was added to these cells, cell growth was strongly inhibited, recovering only when cells lacking detectable expression were selected (Figure 7a). After 2 days’ expression the cells became swollen (Figure 7a). Importantly, the myc tag was not responsible, because ectopic expression of the unaltered protein (on a wild-type background) had the same effect (Figure 7b). Some cells had an enlarged vacuole-like structure between the kinetoplast and the nucleus (Figure 7b, supplementary Figure S7b), which is probably the flagellar pocket. This is reminiscent of the “big-eye” phenotype seen after RNAi targeting clathrin in bloodstream forms (Allen, Goulding, & Field, 2003). Incubation with 10ng/ml tetracycline also did not allow growth (not shown).

To find out whether toxicity of overexpressed ZC3H20 was linked to its ability to promote expression of bound mRNAs, we compared lambdaN-ZC3H20-myc with the HYNPY→HYNAA mutant. LambdaN-ZC3H20-myc was toxic as expected; recovery on day 4 is presumably due to loss of expression (Figure 8). The mutant protein was better expressed than the wild-type version (Figure 8), but did not affect cell morphology (not shown). This result suggests that toxicity of excess ZC3H20 depends on its ability to activate gene expression, probably via interaction with MKT1. Nevertheless, the cells did grow somewhat slower than normal (Figure 8), suggesting that zc3h20_PY→AA has a dominant-negative effect.

2.6 | ZC3H21 during differentiation and in procyclic forms

The similarities between ZC3H20 and ZC3H21 suggested that they might have shared properties. We therefore wondered whether the absence of ZC3H20 during differentiation might be compensated by premature expression of ZC3H21. We therefore attempted to complement the ZC3H20 DKO with inducible ZC3H21-myc (Supplementary Figure S3, L). The cells completely failed to differentiate and eventually died (Supplementary Figure S7a). Ectopic expression of ZC3H21 in procyclic forms that retained their ZC3H21 genes (Supplementary Figure S3, M) caused only a mild growth defect without obvious morphological changes (Supplementary Figure S7b,c) but expression was not maintained, suggesting selection against cells that expressed ZC3H21-myc (Supplementary Figure S7d). Thus, ectopic expression of ZC3H21-myc in procyclic forms is probably also deleterious.

2.7 | Roles of ZC3H20 and ZC3H21 in procyclic forms

To find out whether either protein is required for growth of procyclic forms, we first depleted each by RNA interference, using cells expressing TAP-tagged proteins. Reduced ZC3H20 in Lister 427 procyclic forms resulted in a very slight growth defect (Figures 2c and 9a), while loss of ZC3H21 had no obvious effect (Figure 9b, Supplementary Figure S30). We were, however, unable to generate
FIGURE 7 Overexpression of ZC3H20 in procyclic forms is deleterious. (a) EATRO 1125 procyclic form cells with inducible ZC3H20-myc and no wild-type genes were treated with 100 ng/ml tetracycline to induce expression. Growth curves (three replicates) are on the left and western blots for expression of ZC3H20-myc, lambdaN-ZC3H20-myc and lambdaN-ZC3H20-myc with a mutant MKT1-binding motif (Figure 5b) are on the right. The micrographs show typical cell morphologies 2 days after induced expression of ZC3H20-myc. Results for lambdaN-ZC3H20-myc were similar whereas the mutant version had no effect. Differential interference contrast images are overlaid DNA stain (DAPI, dark blue). (b) EATRO 1,125 procyclic form cells with extra inducible ZC3H20 (Supplementary Figure S3K) were treated with tetracycline to induce the expression. Growth curves for two clones with inducible expression (C1 and C2) are shown above and morphologies of clone 2 cells, on day 2, are below [Colour figure can be viewed at wileyonlinelibrary.com]
procyclic forms that completely lacked either ZC3H20 or ZC3H21, despite several attempts, suggesting that such cells are either severely defective or nonviable. This suggests that the two proteins have important, and perhaps essential, independent functions. Loss of ZC3H21 did not result in an increase in TAP-ZC3H20 (Figure 9b).

Simultaneous depletion of both proteins (Supplementary Figure 3P) severely inhibited growth (Figure 9b). After 2 days of RNAi the cells looked normal but after 3 days, they were clearly smaller than nondepleted cells (Figure 9c). We did not see any morphological changes suggesting development of epimastigotes or bloodstream forms. Nevertheless, we tested this possibility by generating EATRO1125 bloodstream forms with the double RNAi. After 2 weeks, when procyclic form growth was well established, RNAi was induced. All of the cells died, and could not be rescued by transfer to (tetracycline-containing) bloodstream form medium either 1 of 2 days after RNAi induction (not shown).

2.8 | RNAs bound by ZC3H20 and ZC3H21

To find RNA targets of ZC3H20 and ZC3H21, we used cells expressing only TAP-tagged versions (Figure 2b, Supplementary Figure S3I). After UV cross-linking to stabilise protein–RNA interactions, we purified the proteins on an IgG column, released them with Tobacco Etch Virus protease and sequenced the associated RNA from duplicate unbound (flow-through) and bound (eluate) fractions (Mugo & Clayton, 2017) (Supplementary Tables S1 and S2). Each pull-down was done twice. Principal component analysis (Supplementary Figure S8A) indicated that the results were reproducible, and correlations between replicates were reliably very high ($R^2 > .96$) (Supplementary Figure S8A–E), while bound fractions correlated less well with the unbound fractions (Supplementary Figure S8A,F–I). Moreover in each case, mRNA encoding the target protein was strongly enriched, indicating successful selection of the cognate polysomal mRNA via the N-terminally tagged nascent polypeptide (Supplementary Figure S9A,B). ZC3H20 showed no bias towards longer or shorter mRNAs, whereas ZC3H21 tended not to bind longer mRNAs (Supplementary Figure S9C,D). The latter effect is only partially due to a lack of binding to mRNAs encoding large cytoskeletal proteins (Supplementary Figure S8H,I and S9D, "cytoskeleton"). When we compared the enriched RNAs, there was moderate correlation between the ZC3H21 and ZC3H20 data sets (Figure 10a).

We defined mRNAs as “bound” if they were at least twofold enriched in both bound fractions relative to the respective unbound fractions. By this criterion, 288 mRNAs were reproducibly at least 2x enriched in the ZC3H20-bound fractions (Supplementary Table S1, sheet 1), and 238 were ZC3H21 bound (Supplementary Table S2, sheet 1). Of these, 101 were shared (Figure 10a). This confirmed our prediction that the two proteins might have overlapping, but not identical, specificities. The products of transcripts bound to ZC3H20 and ZC3H21 were significantly enriched in mitochondrial proteins, and ZC3H20 also preferred mRNAs encoding membrane proteins (Figure 10c). Quantitative comparisons for all functional classes (Supplementary Figure S10) revealed similar biases. ZC3H20 showed no binding to mRNAs encoding ribosomal proteins (Supplementary Figures S9C, S10).

Membrane protein mRNAs that were bound to both proteins encoded amastin-like protein, trans-sialidases, GPI-anchored membrane proteins including GPEET procyclin, a flagellar membrane protein and a procyclic surface protein (PSSA-2). ZC3H21 also was found to bind the EP2 mRNA; binding by ZC3H20 was just below our cutoff. The two mRNAs that Ling et al. (2011) had previously shown to be bound to ZC3H20, encoding a trans-sialidase (Tb927.5.440) and the mitochondrial carrier MCP12 (Tb927.10.12840), were bound to both ZC3H21 and ZC3H20 in our analysis. We independently confirmed binding of ZC3H20 to the mRNAs encoding Aquaporin 1 and GPEET procyclin by pull-down followed by semi-quantitative PCR: Figure 10b shows that these two mRNAs were enriched relative to the tubulin control. The RT-PCR did not confirm enrichment of the PSSA-2 and PIP39 mRNAs, which were on average 3.3-fold and 2-fold enriched by RNASeq, but this may reflect the limited sensitivity of the PCR assay.

Since several of the identified genes belong to multigene families, we decided to distinguish between the members. To do this,
we set the alignment criteria such that all reads that mapped to the genome more than once were eliminated. Normalisation of these results is impossible because most abundant mRNAs are encoded by at least two genes: repeated sequences constitute 80%–95% of reads (Supplementary Tables S1 and S2, sheets 5 and 6, MAPQ42 alignments). For ZC3H21 in particular, normalisation of the MAPQ42 results to reads per million reads yielded results that contradicted those obtained when entire mRNAs were considered (Supplementary Table S2, sheets 5 & 6). Nevertheless, for ZC3H20, the results did suggest selective enrichment of several additional mRNAs, encoding various amino acid transporters, ESAG9 variants, a META domain protein and PAD2 (Supplementary Table...
Binding to EP and GPEET procyclin mRNAs was also revealed (Supplementary Table S1 and S2, sheet 1; Supplementary Figures S11 and S12). Neither protein bound to the mRNA encoding PAD1 nor ZC3H21 bound to trans-sialidase mRNAs (Supplementary Figures S11 and S12). Of the six mRNAs that were previously reported as decreased after ZC3H20 + ZC3H21 RNAi (Ling et al., 2011), we identified four as being bound to ZC3H20: META domain, trans-sialidase, neuraminidase and MCP12.

**FIGURE 9** Effects of depleting ZC3H20 or ZC3H20 and ZC3H21 in procyclic forms. (a) RNAi targeting ZC3H20 causes a very slight growth defect. The experiment was done with Lister427 trypanosomes with one TAP-ZC3H20 gene, one wild-type ZC3H20 gene, and tetracycline-inducible RNAi (Supplementary Figure S3, panel N). Results are for three independent clones: RNAi reduced ZC3H20 expression to averages of 29%, 17% and 31% of wild type in clones 1, 2 and 3, respectively. Expression of TAP-ZC3H20 is shown on the left, and division times are on the right. The p value is for the difference between +tet and −tet. (b) RNAi targeting ZC3H20 and ZC3H21 causes a marked growth defect. These cells expressed TAP-ZC3H20 and TAP-ZC3H21, with RNAi targeting a region shared by both genes (Supplementary Figure S3, panel P). Two different RNAi clones were used. The western blots (left) show depletion of both proteins on days 1–3 and recovery of expression on day 6. Growth curves are on the right. (c) Left—images of procyclic forms with RNAi targeting ZC3H20 and ZC3H21 either without tetracycline, or after 3 days with tetracycline. These are differential interference contrast micrographs with DAPI DNA stain in cyan. The dot plot on the right shows measured cell areas from these and similar images, with mean and standard deviation in cyan [Colour figure can be viewed at wileyonlinelibrary.com]

**FIGURE 10** Binding of ZC3H20 and ZC3H21 to mRNAs. (a) ZC3H20 and ZC3H21 show overlapping mRNA-binding specificities. The average binding ratios (RPM of eluate/unbound fractions) for each mRNA are plotted for ZC3H20 (x axis) and ZC3H21 (y axis). A list of unique mRNAs ([Siegel et al., 2010] and see Supplementary Tables) was used for the analysis. Bound mRNAs were defined as those that gave ratios of at least twofold in both experiments. mRNAs bound by both proteins are in red, those bound only by ZC3H20 are in purple and by ZC3H21 in cyan. (b) Verification of specific mRNA binding by ZC3H20. RNA was pulled down and subjected to RT-PCR. Products were analysed by agarose gel electrophoresis with ethidium bromide staining. Results for various cycle times are shown. Numbers beneath the lanes are signal relative to input with the same number of cycles (as indicated by the lines at the bottom). The data are semi-quantitative and the intensity differences between cycles were lower than expected. As expected, ZC3H20 mRNA was pulled down, presumably via the nascent polypeptide, while the ZC3H21 mRNA was not. (c) Locations of proteins encoded by mRNAs bound by ZC3H20 and ZC3H21. Locations were assigned using information in TritypDB, TrypTag (Dean, Sunter, & Wheeler, 2016) and various publications. The pie chart on the left shows locations of all proteins encoded by the unique gene list. The numbers of mRNAs are inside the segments, and the p values for significant enrichment (Bonferroni-corrected Fisher test result) are in blue [Colour figure can be viewed at wileyonlinelibrary.com]
Effects of ZC3H20 and ZC3H21 depletion on the transcriptome

To find out how loss of ZC3H20 impairs bloodstream form trypanosome differentiation, we induced RNAi 12 hr after cis-aconitate addition and temperature reduction (Figure 6a). Cells were harvested 24 hr later, when ZC3H20 levels were decreased to about 15% of normal (Figure 6a), and the mRNA was subjected to RNASeq (Supplementary Table S3). All differences were relatively minor, and they mostly reflected the fact that cells lacking ZC3H20 were failing to switch towards a procyclic form expression pattern. ZC3H20 reads were reduced only twofold, suggesting that the RNAi had inhibited translation before causing mRNA destruction. The three other mRNAs that were more than 1.5-fold significantly decreased (Supplementary Table S3, sheet 1) encode a trans-sialidase, flabarin and the haeme uptake protein LHR1. The amastin, GPEET, PSSA-2 and MCP12 mRNAs were between 1.5-fold and 1.3-fold lower after RNAi than in the control. Of the 51 mRNAs with at least 1.3-fold reductions, 19 had been shown to bind to ZC3H20. In contrast, five ZC3H20-bound mRNAs were that encode proteins of unknown function increased slightly relative to the controls (Supplementary Table S3, sheet 1). However, these very minor effects would need confirmation with additional replicates before they can be interpreted.

The effects of depleting both ZC3H20 and ZC3H21 in procyclic form Lister 427 cells were stronger. This is essentially a repeat of the microarray experiment done by Ling et al. (2011), but using the more sensitive RNASeq technology and a slightly shorter RNAi induction time (36 hr instead of 48 hr) to reduce secondary effects. One hundred and six mRNAs were more than 1.5-fold decreased, and 110 more than 1.5-fold increased (Supplementary Table S4, sheet 1). The results correlated moderately with those seen when bloodstream and procyclic form transcriptomes are compared (Figure 11a), suggesting a shift towards bloodstream form gene expression, with a trend towards lower expression of mRNAs encoding mitochondrial proteins (Supplementary Figure S13). The results from ZC3H20 + 21 RNAi in procyclic forms also showed moderate correlation with those seen after ZC3H20 RNAi in differentiating cells (Figure 11b) (Supplementary Tables S3–5). We saw decreases in all five mRNAs that had been identified by Ling et al. (2011) as being significantly reduced. The genes encoding relevant trans-sialidases, mitochondrial carrier protein MCP12, Tb927.5.4020 and GRESA4 are in the unique set. The remaining genes, members of the META gene family (Tb927.5.2160, 2170, 2200, 2230, 2260), were not in our unique gene list but were also sixfold decreased in the raw data set (Table 1). The META mRNAs were also at least fourfold enriched in the ZC3H20 pull-down (Supplementary Table S1, sheet 4 and Table 1). Since many of the decreases observed by RNASeq were below the normally accepted cut-off of twofold, we retested nine mRNAs by northern blotting. Although, in this experiment, both TAP-ZC3H20 and TAP-ZC3H21 remained detectable after RNAi, decreases were confirmed for most mRNAs tested (Table 1, Supplementary Figure S14).

To find direct effects of ZC3H20 and ZC3H21 depletion, we divided the affected mRNAs according to whether or not they were bound by ZC3H20 or ZC3H21, and focused on mRNAs that decreased after RNAi (Table 1; Supplementary Table S5, sheet 1). The majority of bound mRNAs were not affected by RNAi (Figure 11b). This could be because the depletion time was too short, depletion was insufficient or other bound RNA-binding proteins override the effects of loss of ZC3H20 and ZC3H21. Nearly all of the bound mRNAs that decreased after RNAi are procyclic specific (Figure 11c(a)). They are enriched for location to the plasma membrane (\( p = 7 \times 10^{-6} \), Fisher test adjusted for multiple testing) or the mitochondrial (\( p = .018 \)). Analysis of the 3′-UTRs from these mRNAs, in comparison with a set bound by neither protein, revealed a potential enriched motif, (G/U) UUA(U/G)CG. However, this motif was only found in about half of the bound and regulated mRNAs, so its significance is unclear. It is, for example, absent in the two regions of the EP procyclin mRNA 3′-UTR are implicated in mRNA stability and translation in procyclic forms (Hehl, Vassella, Braun, & Roditi, 1994; Wilson, Uyetake, & Boothroyd, 1999).

The mRNAs that were not bound by either ZC3H20 or ZC3H21, but nevertheless showed a significant decreases after RNAi, were predominantly procyclic specific (Figure 11c(b) and Supplementary Table S5, sheet 3); the slight decreases in mRNAs encoding proteins required for gene expression (Supplementary Figure S13) may reflect the onset of impaired growth. In contrast, mRNAs that became more abundant after RNAi were mainly bloodstream form specific (Figure 11c(c) and Supplementary Table S5, sheet 3). The latter included the ten mRNAs that were reported as increased by Ling et al. (2011). Thus, the loss of ZC3H20 and ZC3H21 provoked a change in the transcriptome towards the bloodstream form pattern. Expression of RBP6 induces differentiation of procyclic forms to epimastigotes, and hence to rodent-infective
metacyclic trypomastigotes; and expression of RBP10 switches procyclic forms to metacyclic-like forms that can proliferate as bloodstream forms. The mRNAs encoding both of these proteins were increased after ZC3H20 + 21 RNAi. There were also increases in mRNAs encoding three other bloodstream form specific RNA-binding proteins: RBP26, ZC3H32 and HNRNPH/F, and in mRNAs encoding the epimastigote coat protein BARP (Urwyler, Studer, Renggli, & Roditi, 2007) and HAP2, which is implicated in gamete fusion (Peacock et al., 2011). However, the effects of ZC3H20 + 21 RNAi correlated only weakly with those seen after ectopic expression of RBP10 (Supplementary Figure S15) and as noted above, the cells could not grow as bloodstream forms. Loss of ZC3H20 and 21 target mRNAs may therefore be too deleterious to allow further differentiation.

3 | DISCUSSION

The results we have described indicate that ZC3H20 and ZC3H21 are mRNA-binding proteins with overlapping but non-redundant
| Gene ID | Annotation | Z20 + 21 RNAi | Z20 RNAi | MA$^/$NB$^*$ | Bind Z20 | Bind Z21 | RNA PC/BS | Ribosomes PC/BS | Bind RBP10 |
|---------|------------|---------------|----------|---------------|----------|----------|-----------|---------------|------------|
| Membrane |            |               |          |               |          |          |           |               |            |
| 4.3500  | Amastin    | 1.6 (1.3)     |          | 3.6           | 2.8      | 9.7      | 2.2       | 6.8           |            |
| 11.2410 | Flabarin   | 2.5 (1.6)     |          | 3.1           | 2.7      | 1.0      | 3.9       | 3.2           |            |
| 8.1620  | MSP-B      | 1.6 (1.2)     |          | 3.3           | 3.8      | 4.6      | 3.5       | 1.2           |            |
| 10.11220| PSSA-2     | 2.6 (1.4)     | 1.6$^*$  | 3.3           | 3.8      | 4.6      | 3.5       | 1.2           |            |
| 5.440   | Trans-sialidase | 4.5 (1.7) | 2.8$^*$  | 2.9           | 1.2      | 5.6      | 4.3       | 1.6           |            |
| 8.7340  | Trans-sialidase | 3.6 (1.5) | 2.3$^*$  | 2.1           | 1.3      | 3.3      | 3.3       | 4.6           |            |
| Mitochondrion |        |               |          |               |          |          |           |               |            |
| 8.6060  | 2-Amino-3-ketobutyrate coA ligase | 2.0 (1.3) | 1.5$^*$  | 2.3           | 4.1      | 3.6      | 0.8       | 0.8           |            |
| 11.16480| Enoyl-CoA hydratase/isomerase | 1.9 (1.2) | 1.6      | 3.5           | 6.8      | 1.6      | 0.7       |               |            |
| 3.3330  | HSP20      | 1.5 (1.3)     | 1.3$^*$  | 3.3           | 2.8      | 3.7      | 1.3       | 1.4           |            |
| 6.2790  | L-threonine 3-dehydrogenase | 2.1 (1.4) | 1.1$^*$  | 2.3           | 2.2      | 6.1      | 1.1       | 0.5           |            |
| 10.2560 | Malate dehydrogenase | 2.6 (1.4) | 2.6      | 7.1           | 12.2     | 1.6      | 0.7       |               |            |
| 10.12840| MCP12      | 1.9 (1.5)     | 3.1$^*$  | 2.3           | 2.1      | 9.5      | 1.8       | 1.3           |            |
| 7.2700  | NADH-cytochrome b5 reductase | 1.9 NS | 1.6      | 2.3           | 4.5      | 0.6      | 0.6       |               |            |
| 11.1310 | NADH-cytochrome b5 reductase | 1.5 NS | 1.9      | 2.6           | 0.7      | 1.4      | 0.9       |               |            |
| 9.2320  | POMP1      | 1.6 (1.4)     | 3.3$^*$  | 3.0           | 1.3      | 5.1      | 2.3       | 3.4           |            |
| 4.1670  | POMP29     | 1.9 NS        | 1.4      | 2.9           | 2.1      | 1.6      | 0.9       |               |            |
| 9.4310  | Tricarboxylate carrier? | 1.9 (1.4) | 2.3      | 2.0           | 4.4      | 1.1      | 4.6       |               |            |
| 8.2470  | Unknown    | 1.7 NS        | 5.1      | 4.1           | 2.4      | 0.9      | 2.4       |               |            |
| Other   |            |               |          |               |          |          |           |               |            |
| 5.2160  | #META domain protein | 6.7 (1.3) | 6.6$^*$  | 4.9           | 7.2      | 8.9      | 36        | (+?)          |            |
| 9.7470  | purine nucleoside transporter NT10 | 1.9 (1.3) | 1.7      | 2.2           | 12.2     | 2.9      | 11.3      |               |            |
| 10.12260| Zn-dependent peptidase | 2.6 (1.3) | 1.1      | 2.4           | 12.1     | 1.5      | 0.5       |               |            |
| 7.2980  | Nitroreductase family | 3.5 (1.2) | 2.4$^*$  | 1.2           | 2.7      | 6.3      | 1.4       | 3.5           |            |
| 11.10060| Biotin--acetyl-CoA-carboxylase ligase | 1.7 NS | 1.4$^*$  | 1.9           | 2.5      | 8.7      | 1.0       | 0.4           |            |
| 8.7730  | Sphingosine N-acyltransferase | 1.7 NS | 2.7$^*$  | 2.1           | 2.7      | 2.3      | 1.1       | 6.8           |            |
| 11.1020 | Ribokinase | 1.9 NS        | 1.3$^*$  | 2.0           | 2.5      | 2.9      | 0.7       | 0.5           |            |
| 5.3400  | ESAG5-related | 1.5 NS | 3.0      | 3.9           | 1.1      | 1.0      | 1.0       | 0.4           |            |
| 10.12780| ZC3H37     | 2.5 NS        | 1.7      | 2.5           | 5.4      | 1.3      | 2.0       |               |            |
| 9.11840 | Pre-rRNA-processing protein PNO1 | 1.7 NS | 2.6      | 2.4           | 1.7      | 2.4      | 0.9       |               |            |
| 5.3520  | Queuine tRNA-ribosyltransferase | 1.7 NS | 3.5      | 2.3           | 0.8      | 2.1      | 0.6       |               |            |
| 10.14680| Ribosome biogenesis protein | 1.7 NS | 1.1      | 2.6           | 1.6      | 2.0      | 0.8       |               |            |

(Continues)
functions. ZC3H20 is expressed at a low level in bloodstream forms, reaches a maximum in stumpy forms, then persists in procyclic forms (Dejung et al., 2016). Some of this regulation is at the mRNA level, perhaps mediated by RBP10: but ZC3H20 ribosome footprints are only two-fold less abundant in bloodstream forms than in procyclic forms (Antwi et al., 2016). Our results with tagged proteins suggested that the remaining regulation might be accounted for by protein degradation in bloodstream forms, although the results of high-throughput protein degradation analysis did not support this (Tinti et al., 2019).

ZC3H21 shows slightly stronger (three-fold) regulation of ribosome footprints than ZC3H20, and appears only after differentiation has been initiated. Phosphorylation of both proteins has been detected (Urbaniak, Martin, & Ferguson, 2013), but whether this has any role in their function or stability is not known. The difference in behaviour of the ZC3H20 and ZC3H21 mRNAs might partially be explained by the fact that the ZC3H21 3′-UTR has five RBP10-binding motifs, while that of ZC3H20 has only two. As RBP10 levels decrease during differentiation, binding to ZC3H20 will be lost first.

The products of mRNAs that are both bound and stabilised by ZC3H20 and ZC3H21 were strongly enriched in proteins of the plasma membrane and mitochondrion, and all of those mRNAs are more abundant in procyclic than in bloodstream forms (Supplementary Table S5, sheets 1 and 2). Stabilisation of membrane protein mRNAs may explain why the cells become smaller after ZC3H20 and ZC3H21 RNAi (Figure 9c), and swollen upon ZC3H20 overexpression (Figure 7 and Supplementary Figure S6). Although procyclic forms can grow and divide without EP and GPEET procyclins (Vassella, Butikofer, Engstler, & Roditi, 2003), they may not be able to do so if they also lack trans-sialidases, PSSA-2 and amastin. Conversely, ZC3H20 overexpression might result in an overload of the secretory pathway and of protein transfer from the flagellar pocket to the outer plasma membrane, resulting in swelling of the flagellar pocket; trans-sialidases, which are regulated by ZC3H20 but not ZC3H21, seem the most likely culprits, but flabarin and another flagellar membrane protein (Tb927.11.1830), might also be implicated.

Metabolic effects might also contribute to the small size of ZC3H20- and ZC3H21-depleted cells. The RNAi caused decreases in mRNAs encoding two enzymes required for threonine conversion to acetyl coA (Figure 12)—a major source of acetate for lipid biosynthesis (Millerioux et al., 2013). Moreover, mitochondrial energy metabolism should be impacted, because of decreases in di- or tricarboxyrate carriers, the cytochrome oxidase and cytochrome b5 reductase complexes, and mitochondrial malate dehydrogenase (Figure 12). Finally, ZC3H20 and ZC3H21 target mRNAs encoding five proteins that are implicated in pre-rRNA processing, and whose loss could result in slower growth.

Cells lacking ZC3H20 were unable to express PAD1 after cultivation at high density. This may be a symptom, or a cause, of the subsequent inability of the cells to differentiate in vitro to procyclic forms. PAD1 mRNA is not bound by ZC3H20 (Supplementary Figure S11), so the effect of ZC3H20 loss on PAD1 expression is probably indirect. Mitochondrial energy metabolism is probably
FIGURE 12 Possible effects of depleting ZC3H20 and ZC3H21 on energy metabolism. Information for this Figure is from (Allmann et al., 2013; Coustou et al., 2008; Millerioux et al., 2013; Wargnies et al., 2018). The black arrows indicate metabolism in cells grown in the proline-rich, glucose-poor medium used in our study. Dotted lines are steps that are possible but may not be active under these conditions. The presence of a complete citric acid cycle has not been demonstrated under any conditions. Numbers in magenta are enzymes for which the mRNAs are bound by both ZC3H20 and ZC3H21, and which also decrease after the double RNAi. Those in cyan are bound by ZC3H20 and decreased after RNAi; green indicates binding and regulation by ZC3H21. Succinate dehydrogenase (6a) is essential in the absence of glucose (Coustou et al., 2008). Abbreviations are: Ac, acetate; AcCoA, acetyl CoA; Aco, aconitate; AOB, amino oxobutyrate; Cit, citrate; FA, fatty acids; Fum, fumarate; G3P, glyceraldehyde-3-phosphate; G6P, glucose-6-phosphate; Gly, glycine; KG, alpha-ketoglutarate; Mal, malate; OAA, oxaloacetate; PEP, phosphoenol pyruvate; PGA, phosphoglycerate; Pro, proline; PPP, pentose phosphate pathway; Pyr, pyruvate; Succ, succinate; Thr, threonine. Enzymes are: (1) proline dehydrogenase; (2) ∆1-pyrroline-5-carboxylate dehydrogenase; (3) L-alanine aminotransferase; (4) a-ketoglutarate dehydrogenase; (5) succinyl CoA synthase; (6a) succinate dehydrogenase (complex II); (6b) m fumarate redcutase; (7) mitochondrial Fumarase; (8) mitochondrial malic enzyme; (9) m malate dehydrogenase; (10) citrate synthase; (11) aconitate; (12) isocitrate dehydrogenase (NADP); (13) pyruvate dehydrogenase complex; (14) L-threonine 3-dehydrogenase; (15) 2-aminofumarate reductase; (16) acetate:succinate CoA transferase; (17) acetyl coA thioesterase; (18) cytochrome c oxidase complex; (19) aconitase; (20) MCP12; (21) tricarboxylate carrier; (22) POMP1; (i) cytosolic malic enzyme; (ii) cytosolic fumarase; (iii) pyruvate kinase; (iv) enolase; (v) phosphoglycerate mutase; (vi) cytosolic phosphoglycerate kinase; (a) glycosomal malate dehydrogenase; (b) phosphoenolpyruvate carboxykinase; (c) pyruvate phosphatase dikase; (d) fumarase/fumarate hydratase; # pentose phosphate pathway (PPP) enzymes [Colour figure can be viewed at wileyonlinelibrary.com]
(Figure 11c). We suggest that they are likely to be secondary effects. One possibility is a response to stress, although there was no correlation with the effects of heat shock (Minia, Merce, Terrao, & Clayton, 2016) or starvation (Fritz et al., 2015). Altered metabolite levels could also be responsible (Bringaud et al., 2006; Fernandez-Moya, Carrington, & Estevez, 2014; Qiu et al., 2018; Vassella et al., 2000, 2004); and it is conceivable that one or more of the directly regulated surface proteins acts as a receptor for a signal that maintains procyclic identity.

We conclude that during differentiation of bloodstream forms to procyclic forms, loss of RBP10, and of a specific degradation mechanism, causes an increase in ZC3H20, enabling trypanosomes to prepare for growth as procyclins by stabilising mRNAs that encode mitochondrial enzymes and procyclic surface proteins. We suggest that in mRNAs that bear binding sites for both proteins, the appearance of ZC3H20 within the mRNP diminishes the influence of RBP10, preventing mRNA destruction. During full differentiation to procyclins and following complete loss of RBP10, ZC3H21 appears, reinforces the effects of ZC3H20 and stabilises additional mRNAs. Our results thus not only illuminate mechanisms of mRNA control during trypanosome differentiation, but also provide an excellent general example of how mRNAs with more than one specific protein-binding site can be subject to stringent control through changes of binding proteins with competing activities.

4 | EXPERIMENTAL PROCEDURES

4.1 | Trypanosome culture

Bloodstream form T. brucei brucei 427 Lister strain were cultured in HMI-9 plus 10% foetal calf serum. Procyclic form T. b. brucei were cultured in MEM-Pros plus 10% foetal calf serum at 27°C. Tetracycline-regulated transcription was induced with 0.1 µg/ml tetracycline. The drug was re-added every day to compensate for its degradation (assumed half-life 24 hr).

Stable cell lines were generated as described in (Benz, Mulindwa, Ouna, & Clayton, 2011). All plasmid constructs and oligonucleotides are listed in Supplementary Table S6.

For differentiation, the pleomorphic EATRO 1125 cell lines were growing in medium containing 1.1% methyl cellulose to around 3 × 10⁵ cells/ml, then maintained at this density for 10–14 hr. PAD1 expression was checked by western blot. Differentiation was triggered by adding 6mM cis-aconitate and lowering the temperature to 27°C. After 24 hr, the medium was changed to MEM-Pros.

All results were obtained at least twice, unless explicitly stated otherwise.

4.2 | Protein and northern blot analyses

For protein analysis, 2–3 × 10⁶ cells were collected per each sample, resuspended in Laemmli buffer heated and subjected to SDS-PAGE gel electrophoresis. All assays of macromolecular biosynthesis and RNA processing were done at densities of less than 2 × 10⁵/ml. Antibodies used for western blots were: HRP conjugated anti-peroxidase (1:5,000, Sigma); rat anti RBP10 (1:2000); mouse anti-myc (1:1,000 Santa Cruz); mouse anti EP (1:1,000, Cedar Lane); rat anti S9 (1:2,000); and rabbit anti-PAD1 (1:1,000) (kind gift from Keith Matthews). HRP conjugated antibodies (GE Healthcare) were used at a concentration of 1:5,000. Detection was done using Western Lightning-Plus (Perkin-Elmer) followed by exposure using X-ray films.

Total RNA was extracted from roughly 5x10⁷ cells using peqGold TriFast (peqLab) following the manufacturer’s instructions. The RNA was separated on formaldehyde gels and then blotted on Nytran membranes (GE Healthcare), following crosslinking and methylene blue staining (SERVA). For mRNA detection, the membranes were incubated with [α-32P]dCTP radioactively labelled DNA probes (Prime-IT RmT Random Primer Labelling Kit, Stratagene) overnight at 65°C. After washing the blots, they were exposed to autoradiography films and detection was performed with FLA-7000 (GE Healthcare). The images were processed and quantified with Fiji (ImageJ) and/or Adobe Photoshop, using the illustrated loading controls. Ratios on the Figures are rounded either to one figure after the decimal point, or to one significant figure.

All tethering assays were done as described in (Mugo & Clayton, 2017).

4.3 | RNA pull-down and sequencing

mRNAs bound to ZC3H20 and ZC3H21 were identified as described previously (Droll et al., 2013; Mugo & Clayton, 2017). Two independent purifications were done for each protein, using EATRO1125 procyclic forms expressing only TAP-tagged versions of the relevant protein. In each replicate, 1–2 × 10⁶ cells were centrifuged down and resuspended in 25-ml 4°C cold PBS, then put in a 145-mm-diameter Petri dish on ice. The RNA–protein complexes were crosslinked by twice irradiating the cells with UV at 240 mJ/cm². All procedures were performed at 4°C or on ice unless otherwise stated. The cells were then centrifuged washed once with ice-cold PBS. After removing the supernatant the pellets were snap frozen in liquid nitrogen. Cell pellets can be stored in −70°C for several days before the RNA pull-down experiment. For the RNA pull-down, cells were broken in 1-ml lysis buffer (20 mM Tris-HCL pH7.5, 5mM MgCl₂, 0.1% IGEPAL GA-630, 1 mM DTT, 100U RNase inhibitor, proteasine inhibitor cocktail) by passing through syringe needle (20 times 21G × 1/2 then 20 times 27G × 3/4). Cell debris was pelleted by centrifuging at 10,000×g for 15 min at 4°C. The supernatant was transferred to a 1.5-ml tube and KCL was added to 150mM. The sample for input could be taken out for RNA purification or western blot if necessary. Two hundred and fifty microliters of IgG Sepharose beads (GE Healthcare) were washed 3 times with wash buffer (20mM Tris-HCL pH7.5, 5mM MgCl₂, 150mM KCl, 0.1% IGEPAL GA-630,
1 mM DTT, 20 U/ml Rnase inhibitor). The speed of centrifuging of the beads was 900 g for 3 min. The cell lysate was added to the beads, and the mixture was incubated with gentle rotation for 2 hr at 4°C. The beads were centrifuged as before and the 250 µl unbound or flow-through sample was retained for RNA purification and western blotting. The beads with bound protein and RNA were transferred to a column, washed with wash buffer 3 times (10 ml each), then transferred into a 1.5-ml tube. The beads were suspended in 500-µl wash buffer, 5-µl TEV protease was added and the mixture was incubated at 16°C with gentle rotation for 2 hr. After the reaction, beads were centrifuged down and supernatant containing the protein–RNA complexes was retained; 500-µl wash buffer was added to the beads to harvest remaining released RNA and protein. The bound and unbound fractions were then subjected to RNA purification and western blotting. Before RNA purification, the sample was treated with proteinase K, and Trifast (Peqlab) was used for RNA purification according to manufacturer’s instructions.

Before RNA sequencing, ribosomal RNA was depleted from the flow-through samples by incubation with specific oligonucleotides and RNaseH (Minia et al., 2016). RNAseq analysis was performed on eluted (bound) and flow-through (unbound) samples, and analysed as described previously (Droll et al., 2013; Leiss, Merce, Muchunga, & Clayton, 2016; Mugo & Clayton, 2017; Mulindwa et al., 2018). The same analysis method was used for RNA obtained after ZC3H20 and ZC3H20 + 21 RNAi, which was again done for two independent experiments. Briefly, after trimming of primers, the reads were aligned with the TREU927 genome using Bowtie2, allowing 20 alignments per read. Reads that mapped to open reading frames were counted (Supplementary Tables S1 and S2, sheet 4, and Supplementary Tables S3 and S4, sheet 3). For most subsequent analyses, we used a list of unique genes (modified from [Siegel, Hekstra, Wang, Dewell, & Cross, 2010]), in order to avoid giving undue weight to repeated genes and multigene families. Allowing 20 alignments means that with the unique set, the normalised read counts reflect the relative abundance of each mRNA. For pull-downs (Supplementary Tables S1 and S2), reads per million reads were counted and the eluate/flow-through ratios were calculated. An mRNA was classified as “bound” if both ratios were higher than 2. Note that these are not the real ratios because we did not normalise the values according to the amounts of mRNA in the different fractions. Differential gene expression after RNAi was assessed DeSeqU1 (Leiss & Clayton, 2016), a customised version of DeSeq2 (Love, Huber, & Anders, 2014). Statistical analyses were done using Microsoft Excel and R Studio. Correlations are classified as follows: R values above .7 are rated “good”, .5—.7 is “moderate”, .3—.5 is “poor” and less than .3 is no correlation.

### 4.4 | Microscopy

To examine cell morphology, dry smears were made and fixed with methanol for 1 min at room temperature, then DAPI stained. Randomly chosen images were quantified, blind, by an independent observer.

### DATA AVAILABILITY STATEMENT

The RNAseq raw data are available at Array Express with the following accession numbers: ZC3H20 RNAi: E-MTAB-7995; ZC3H20 + 21 RNAi: E-MATB-7996; ZC3H20 and ZC3H21 RNA pull-downs: E-MTAB-7998. Additional raw data are available from BL or CC on request.

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### AUTHOR CONTRIBUTIONS

BL and CC designed experiments, interpreted the results and wrote the paper. BL did all of the experiments except those in Supplementary Figures S5a and S14, and the PSSA2 RIP. KM designed differentiation experiments, helped BL with bioinformatics and did the unique-copy sequence alignment. CC supervised the work.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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