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Epitranscriptome machinery in Trypanosomatids: New players on the table?

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Abstract

Trypanosoma and *Leishmania* parasites cause devastating tropical diseases resulting in serious global health consequences. These organisms have complex life cycles with mammalian hosts and insect vectors. The parasites must, therefore, survive in different environments, demanding rapid physiological and metabolic changes. These responses depend upon regulation of gene expression, which primarily occurs post-transcriptionally. Altering the composition or conformation of RNA through nucleotide modifications is one posttranscriptional mechanism of regulating RNA fate and function, and modifications including N6-methyladenosine (m6A), N1-methyladenosine (m1A), N5-methylcytidine (m5C), N4-acetylcytidine (ac4C), and pseudouridine (Ψ), dynamically regulate RNA stability and translation in diverse organisms. Little is known about RNA modifications and their machinery in Trypanosomatids, but we hypothesize that they regulate parasite gene expression and are vital for survival. Here, we identified Trypanosomatid homologs for writers of m1A, m5C, ac4C, and Ψ and analyze their evolutionary relationships. We systematically review the evidence for their functions and assess their potential use as therapeutic targets. This work provides new insights into the roles of these proteins in Trypanosomatid parasite biology and treatment of the diseases they cause and illustrates that Trypanosomatids provide an excellent model system to study RNA modifications, their molecular, cellular, and biological consequences, and their regulation and interplay.

KEYWORDS
ac4C, m1A, m5C, pseudouridine, RNA modification, Trypanosomatids

1 | INTRODUCTION

Trypanosomatids are unicellular protozoan organisms in the class Kinetoplastida. Most of the known species are parasites belonging to the *Trypanosoma* and *Leishmania* genera that have a huge public health and economic impact. The diseases that these parasites cause are considered some of the most important neglected tropical diseases, affecting nearly 22 million people globally (Altamura et al., 2020; Buscher et al., 2017; Filardy et al., 2018; Okwor & Uzonna, 2016; Perez-Molina & Molina, 2018).

Trypanosomatid parasites have complex life cycles with mammalian hosts and insect vectors, meaning that the parasites must be

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able to survive in vastly different environments. These environmental changes demand and result in rapid and large-scale physiological and metabolic changes across different parasite life cycle stages. These changes in turn require efficient gene expression regulation in order to modulate the levels of different proteins present in each life cycle stage (Bringaud et al., 2007; Cruz-Saavedra et al., 2020; Dillon et al., 2015, Dumoulin & Burleigh, 2018; Fernandes et al., 2016; Goldman-Pinkovich et al., 2016, Gonzalez-Marcano et al., 2020; Lahav et al., 2011; Li et al., 2016c; Melo et al., 2020; Saunders et al., 2010; Smith et al., 2017).

Regulation of gene expression in Trypanosomatids is different from other eukaryotes because most genes lack individual promoters and are transcribed together in polycistronic units (El-Sayed et al., 2005). Processed transcripts from the same unit vary in abundance and are differentially expressed in parasite life cycle stages, demonstrating that posttranscriptional mechanisms, including at the levels of mRNA processing, splicing, stability, localization, editing, and translation are key regulators of gene expression and development (Clayton, 2019; Kramer, 2012). Furthermore, Trypanosomatid RNA-Binding Proteins (RBPs) coordinate major developmental shifts by regulating expression of groups of mRNAs that encode functionally and developmentally related proteins (Das et al., 2012; De Pablos et al., 2016, 2019; Kolev et al., 2014; Romagnoli et al., 2020; Trenaman et al., 2019).

While the impact of protein modifications on cellular processes are widely appreciated, chemical and structural modifications to the nucleotides in diverse RNA molecules can also significantly impact posttranscriptional processes via changes to RNA structure, recognition, and function. RNA modifications including N6-methyladenosine (m6A), 5-methylcytosine (m5C), and pseudouridine (Ψ) were originally identified in abundant noncoding RNAs such as rRNAs and tRNAs and are essential for RNA splicing, translation, and cell survival. Recent technological improvements in nucleic acid sequencing methods have now revealed these and many other modifications such as N1-methyladenosine (m1A) and acetylcytidine (ac4C) within mRNAs throughout phylogeny, and there are indications that RNA pseudouridylation, methylation, and acetylation are dynamically regulated through development, differentiation, and environmental adaptation in many organisms (Frye & Blanco, 2016; Kadumuri & Janga, 2018; Roundtree et al., 2017; Roundtree & He, 2016; Seo & Kleiner, 2020; Zhao et al., 2017). Furthermore, the modifications m6A and ac4C can promote translation and stability of modified mRNAs (Arango et al., 2018; Boo & Kim, 2020; Ru et al., 2020; Wang et al., 2015). For example, interactions between the eukaryotic translation factor eIF3 and the m1A and m6A-modified mRNA-binding protein YTHDF1 increase the translational efficiency of modified transcripts (Wang et al., 2015).

The RBPs that comprise the RNA modification machinery can be classified as "writers," which are enzymes responsible for catalyzing addition of the modifications; "readers," which are proteins that recognize and bind RNA sequences containing modified nucleotides; and "erasers," which remove the modifications. These RBPs are further subject to complex and dynamic mechanisms that regulate addition, interpretation, and removal of RNA modifications and their impact on gene expression control. An important aspect of the RNA modification machinery is as a therapeutic target, as several proteins are targets for drugs and small molecule inhibitors. For example, the RNA methyltransferase METTL3 is inhibited by neplanocin A, which significantly impacts the success of some cancer treatments (Uddin et al., 2019) and has antiviral activity (De Clercq, 2015; De Clercq et al., 1989). Furthermore, the benzoxaborole AN5568, which is a lead compound awaiting phase III clinical trials for treatment of human *Trypanosoma brucei* infection (Steketee et al., 2018), appears to have a similar mechanism of action as the pan S-adenosyl-L-methionine (SAM)-dependent methyltransferase inhibitor sinefungin, which inhibits methylation in an essential noncoding RNA required for mRNA trans-splicing. This highlights the potential therapeutic of mRNA modification in Trypanosomatid pathogens.

Given the reliance of Trypanosomatids on posttranscriptional control of gene expression, we hypothesize that modifications within diverse RNAs, the enzymes that catalyze the formation and removal of these modifications, and the RBP readers of these modifications are vital for parasite survival and life cycle progression. There are currently few published studies related to RNA modifications, particularly of mRNA, and the modification machinery, or their impact on gene expression control in Trypanosomatids. Therefore, many questions remain outstanding, including the identities of candidate Trypanosomatid writer, reader, and eraser proteins, what Trypanosomatid RNAs are modified, and whether RNA modifications modulate key posttranscriptional processes and impact parasite biology as a whole, including infectivity and virulence.

Here, we use bioinformatics approaches to identify candidate Trypanosomatid homologs for m1A, m6A, m5C, ac4C, and Ψ writers, specifically focusing on those parasites that cause serious human disease. We analyze the evolutionary relationships of these proteins, systematically review the evidence for their function, and assess their potential use as therapeutic targets, thereby providing new insights into the roles of these proteins, and of the modifications they catalyze in Trypanosomatid biology.

2 | RNA MODIFICATION MACHINERY IS PRESENT IN TRYPANOSOMATIDS

Residues in all known RNA species are modified, with ribosomal RNA (rRNA) and transfer RNA (tRNA) being the most characterized. Over 100 and 200 residues are modified in yeast and human rRNAs, respectively, with the most prevalent modifications being 2′-OMe and Ψ. There is a large diversity of potential modifications and over 100 RNA modifications have been detected to date in different RNA species (Boccaletto et al., 2018). For example, the density and diversity of described modifications is higher for tRNA than for rRNA, and over 50 unique modifications have been identified in ~17% residues in mammalian tRNA (Jackman & Alfonzo, 2013; Kirchner & Ignatova, 2015). A set of 18 modifications occurs in tRNAs spanning Archaea, Bacteria, and Eukarya, and include m1A, m5C, Ψ, and

ac4C (Jackman & Alfonzo, 2013). Although backbone modifications do exist, for example, 2′-OMe, most modifications are made to the RNA bases. All four RNA bases can be modified, although uracil (U) and guanine (G) have a higher number of described modifications (Boccaletto et al., 2018) (Figure 1a). Indeed, the most frequent chemical group present in these modifications is the methyl group, followed by aminoacyl, geranyl, glycosyl, aminoakyl, and acetyl groups (Figure 1b), which are differentially distributed among RNA bases (Figure 1b, right panel).

Trypanosomatid rRNAs contain 2′-OMe and Ψ sites (Chikne et al., 2016; Eliaz et al., 2015) that are conserved between *T. brucei* and *Leishmania major* and partially shared with humans (Chikne et al., 2016; Eliaz et al., 2015; Liang et al., 2007) (Figure 1c). Furthermore, we find high conservation of human m1A, m5C, Ψ, and ac4C target sites compared to *L. major* tRNA (Figure 1d), which were not previously described in Trypanosomatids. Based on these findings and the published works cited above, it is therefore very likely that Trypanosomatids have the machinery to write, read, and erase diverse RNA modifications.

To investigate this, we performed homology searches for the writer enzymes responsible for m1A, m6A, m5C, Ψ, and ac4C modifications in different species of Trypanosomatids (*Leishmania* species, Trypanosomes, and *B. saltans*). We found candidate enzymes for all these modifications, except for m6A (Figure 2). The number of family members found among the Trypanosomatids are generally very similar, except that *B. saltans* has only one member for NSUN2 (m5C) and five members of the RluA pseudouridine synthase family, while *Leishmania* species and Trypanosomes have two and seven members, respectively (Figure 2). Similarly, the number of family members found in Trypanosomatids compared to other protozoans or humans are also very similar, again with the exception of the RluA pseudouridine synthases (Figure 2). All the methodologies and data base used to perform these analyses are described in Supplementary file 1.

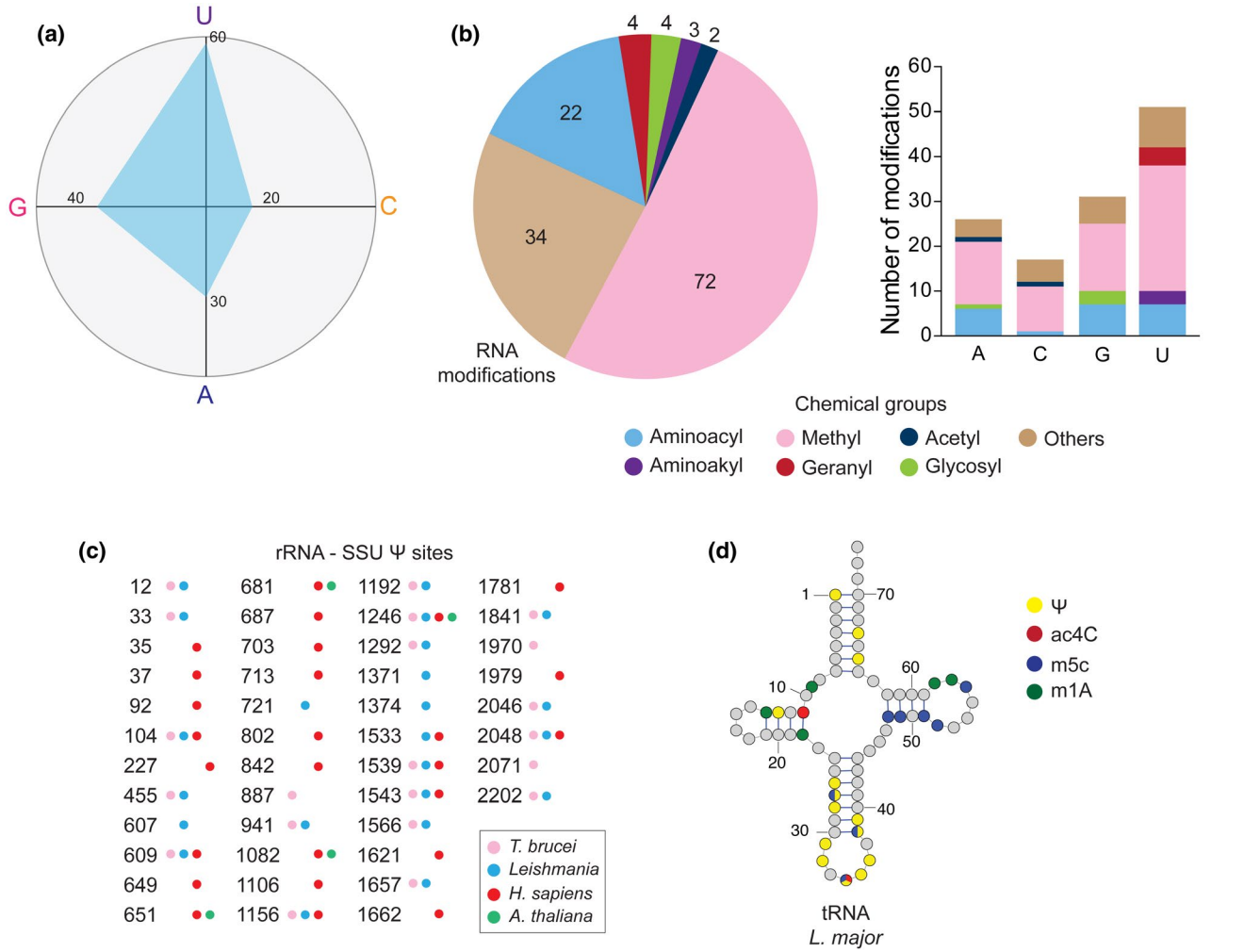

FIGURE 1 Distribution of modified ribonucleotides, chemical groups, and conservation in Trypanosomatids. (a) Number of different chemical modifications identified for each ribonucleotide based on published studies. (b) Total number of modifications classified based on their chemical groups and for each ribonucleotide. (c) Degree of conservation of rRNA-SSU Ψ sites among *T. brucei*, *L. major*, *Homo sapiens*, and *Arabidopsis thaliana*. (d) Potential m1A, m5C, Ψ, and ac4C modification sites in *L. major* tRNA based on modified sites in human tRNA

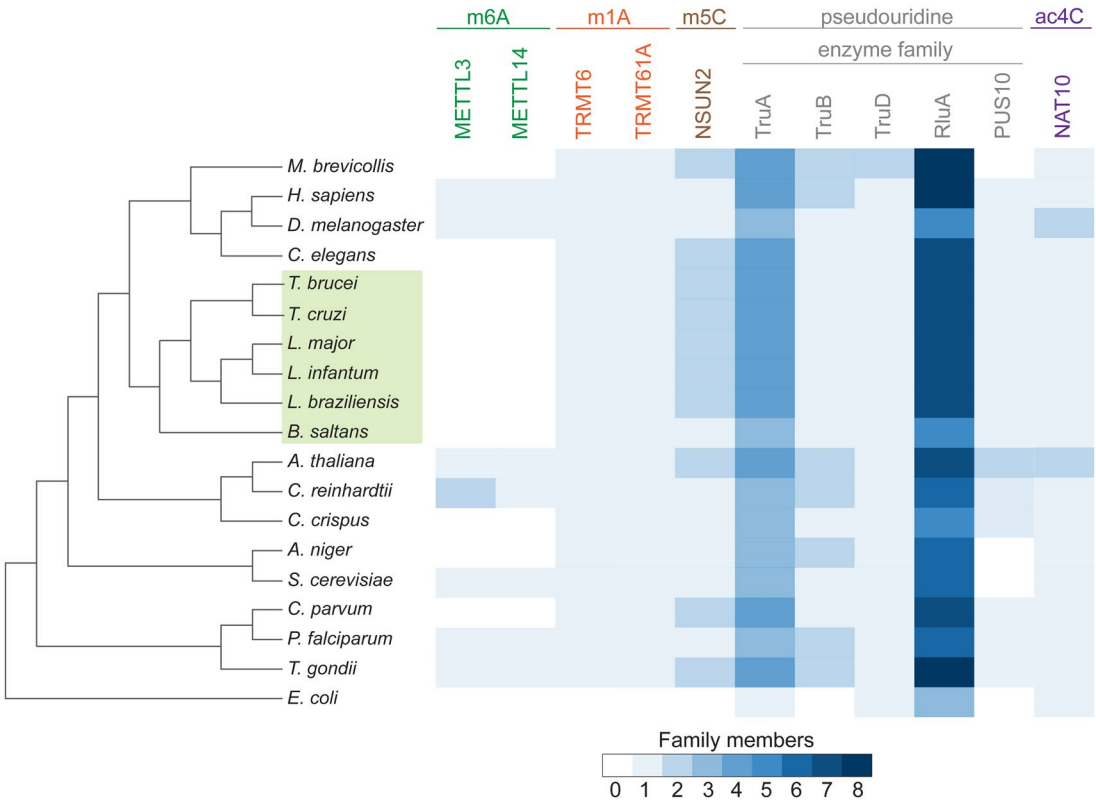

FIGURE 2 Evolutionary trajectories for RNA modifying writer enzymes in Trypanosomatids. The presence of several RNA modification enzymes was assessed in an evolutionary tree for a diverse set of organisms built using several different markers. In general, Trypanosomatids have a similar profile of enzymes when compared to the other organisms

3 | CANDIDATE TRYPANOSOMATID WRITER ENZYMES FOR m6A, m1A, m5C, Ψ, AND ac4C

3.1 | m6A (N6-methyladenosine)

m6A is the most prevalent mRNA modification described in eukaryotes, often detected near stop codons and in 3′ UTR regions, usually at an average of three sites per given mRNA molecule (Desrosiers et al., 1974; Dominissini et al., 2012; Perry et al., 1975). Studies suggest that m6A has a regulatory role in mRNA degradation and translation initiation or efficiency, and it is involved in cell fate determination in yeast, plants, and mammalian stem cells. METTL3 has been identified as the central catalytic subunit of a large mammalian m6A-methyltransferase writer complex (MTC) and is conserved in other eukaryotes. The methyltransferase accessory factor METTL14 and the splicing regulators WTAP, KIAA1429, and RBM15/RBM15B also associate with the MTC and are functionally required for normal accumulation of m6A (Wang et al., 2016a, 2016b). Recent structural studies clarify that METTL14 binds directly to METTL3 but lacks methyltransferase activity and instead serves a structural role in the MTC (Sledz & Jinek, 2016; Wang et al., 2016a, 2016b). WTAP appears to scaffold the MTC and RBM15/RBM15B together recruit the MTC to target sites of modification (Liu et al., 2014; Patil et al., 2016; Ping et al., 2014).

Direct orthologs of MTC components were not identified in Trypanosomatids in our analyses and those of others (Balacco & Soller, 2019). Despite this, a homolog of the m6A reader YTHDC was identified in *T. brucei* and *B. saltans* (Balacco & Soller, 2019), and m6A mRNA modifications themselves were recently identified in both the insect procyclic (PF) and mammalian bloodstream (BSF) stages of *T. brucei* (Liu et al., 2019; Viegas et al., 2020). Furthermore, m6A was detected in a greater diversity of transcripts in PF, suggesting a regulatory function for this modification. Strikingly, over 50% of m6A in BSF was found in the transcript encoding the essential variant surface glycoprotein (VSG). Here, m6A is localized to the poly(A) tail (Viegas et al., 2020), which is so far unique to trypanosomatids and promotes VSG mRNA stability potentially by blocking deadenylation.

Thus, m6A plays an important role in *T. brucei*, particularly in the mammalian bloodstream stage, and is likely added by an unusual, highly divergent RNA methyltransferase that cannot be identified using standard homology searches. This is not surprising if we consider that *T. brucei* is an early diverging eukaryote, where many proteins that act in fundamental cellular processes, including RNA processing, differ from their counterparts in other eukaryotic organisms and even protozoa, for example, basal transcription factors (Schimanski et al., 2006) and mRNA export factors (Dostalova et al., 2013). Alternatively, m6A could be incorporated during transcription, as suggested by Viegas et al., which requires the presence of m6ATP inside the cell. It will therefore be important for future studies to define at what stage m6A is added to

transcripts, and to identify the enzymes and proteins involved in adding, reading, or removing this RNA modification in Trypanosomatids, as they may provide therapeutic targets that are unique to the parasite and absent from their mammalian hosts.

3.2 | m1A (N1-methyladenosine)

m1A was first detected in tRNAs (Anderson et al., 1998) and rRNAs (Sharma et al., 2013) where it is implicated in the maintenance of the proper RNA structure and, therefore, function. For example, most human cytoplasmic and mitochondrial tRNAs are modified by m1A at position 58 and 9, respectively (Ozanick et al., 2005; Suzuki et al., 2011). More recently, mammalian transcriptome-wide mapping analyses identified m1A in mRNA, predominantly in 5′ UTRs within regions close to start codons, and also to a more minor extent in coding regions and 3′ UTRs (Dominissini et al., 2016; Li et al., 2016b, 2017). Human mitochondrial mRNAs are also m1A modified in their coding regions (Li et al., 2017). m1A sites close to start codons are specifically associated with higher translational efficiency in humans, while the presence of m1A in coding regions of mitochondrial transcripts are linked to translational inhibition (Li et al., 2017). Indeed, m1A levels vary under certain physiological conditions including stress, suggesting a direct link of this modification with environmental adaptation (Dominissini et al., 2016).

In Trypanosomatids, m1A was recently identified in liquid chromatography-tandem mass spectrometry analyses of total and poly(A) depleted RNA samples from *T. brucei* (Viegas et al., 2020). Moreover, it is important to note that m1A-modified positions in human tRNAs are also conserved in Trypanosomatids (Figure 1d). Together, these findings suggest that the methyltransferase enzymes responsible for m1A addition also exist in these parasites.

m1A addition is catalyzed by a methyltransferase complex that is a heterodimer of TRMT6 and TRMT61A (Anderson et al., 1998). TRMT61A is the catalytic subunit, while TRMT6 is a structural accessory subunit that is critical for methyltransferase activity in vitro and in vivo (Anderson et al., 1998). This is conceptually very similar to the relationship between the m6A methyltransferase METTL3 and its accessory factor METTL14. The formation of the TRMT6/TRMT61A heterodimer is postulated to increase binding affinity of the catalyst to its substrates, and to assure specificity among almost identical tRNA substrates (Guy & Phizicky, 2014).

We searched for TRMT6 and TRMT61A Trypanosomatid homologs by conducting searches using the human protein sequences and found one distant member for each protein in all Trypanosomatid species analyzed (Figure 2), sharing ~23% and 30% of identity with the human TRMT6 and TRMT61A homologs, respectively. The RNA substrate binding Gcd10p and GCD14 domains, as well as almost all the critical residues for catalysis are conserved in the Trypanosomatid TRMT6 and TRMT61A proteins (Finer-Moore et al., 2015) (Figure 3a). We also modeled the protein structures for *T. brucei* TRMT6 and TRMT61A and their heterodimer. These models reveal that the *T. brucei* heterodimer is very similar to that formed by the human homologs (Figure 3b), including the RNA substrate interaction pocket.

The detection of m1A in total mRNA samples and the presence of writer homologs with high predicted protein structure similarity to human proteins in Trypanosomatids, opens the opportunity to explore the function of this modification in the regulation of mRNA stability and protein synthesis in these parasites.

3.3 | m5C (5-methylcytosine)

5-methylcytosine (m5C) has long been studied as an epigenetic modification in DNA and it was detected more than 40 years ago in mRNA (Dubin & Taylor, 1975). The presence of m5C varies among different organisms, for example, it is found in tRNA and rRNA of archaea and eukaryotes but is not detected in tRNA from eubacteria. tRNA m5C is frequently found at positions 48 and 49 and is associated with RNA stability and protein synthesis in eukaryotes (Tuorto et al., 2012). Although it is present in lower levels in mRNA than m6A, m5C-modified sites are enriched in 5′ and 3′ UTRs and are especially prominent in proximity to the translation start codon (Hussain et al., 2013a). It is implicated in multiple aspects of gene expression control including RNA stability, translation, RNA export, and ribosome assembly (Bohnsack et al., 2019). m5C has previously been detected in *T. brucei* RNAs, including in total RNA and tRNA (Militello et al., 2014; Viegas et al., 2020) but the extent of the presence of m5C in the Trypanosomatid transcriptome is still unknown. The main enzyme responsible for catalyzing m5C modification of mRNA is called NOP2/SUN RNA methyltransferase family member 2 (NSUN2) (Hussain et al., 2013b; Khoddami & Cairns, 2013).

We identified two groups of Trypanosomatid orthologs of human NSUN2, each with ~ 23% of identity to the human protein (Figure 4a). Human NSUN2 is a S-adenosylmethionine (SAM)-dependent methyltransferase and contains a catalytic rRNA methyltransferase RsmB/F domain and RNA-binding RRM motif. Its activity depends on two catalytic cysteines (C308 and C359) found in two regions called Motif IV and Motif VI of the RsmB/F domain (Bohnsack et al., 2019). The catalytic domain and the motifs are well conserved in the Trypanosomatid proteins (Figure 4a), and both cysteines (C238 and C288) are completely conserved (Figure 4b). Moreover, NSUN family proteins use specific proline (P306) and aspartate residues (D305) in motif IV to position the substrate nucleotide in the catalytic site (Bohnsack et al., 2019). These residues, particularly the aspartate, are also highly conserved in the Trypanosomatid orthologs. Given how varied the presence of m5C seems to be across evolution and in different RNAs, and considering that Trypanosomatids diverged early during evolution, it will be important to globally map m5C RNA modifications in Trypanosomatid RNAs.

3.4 | ac4C (N4-acetylcytidine)

There are three types of acetylated nucleotides in RNA: N4-acetyl-2′-O-methylcytidine (ac4Cm), N6-acetyladenosine (ac6A), and N4-acetylcytidine (ac4C). The first two are present only in thermophilic

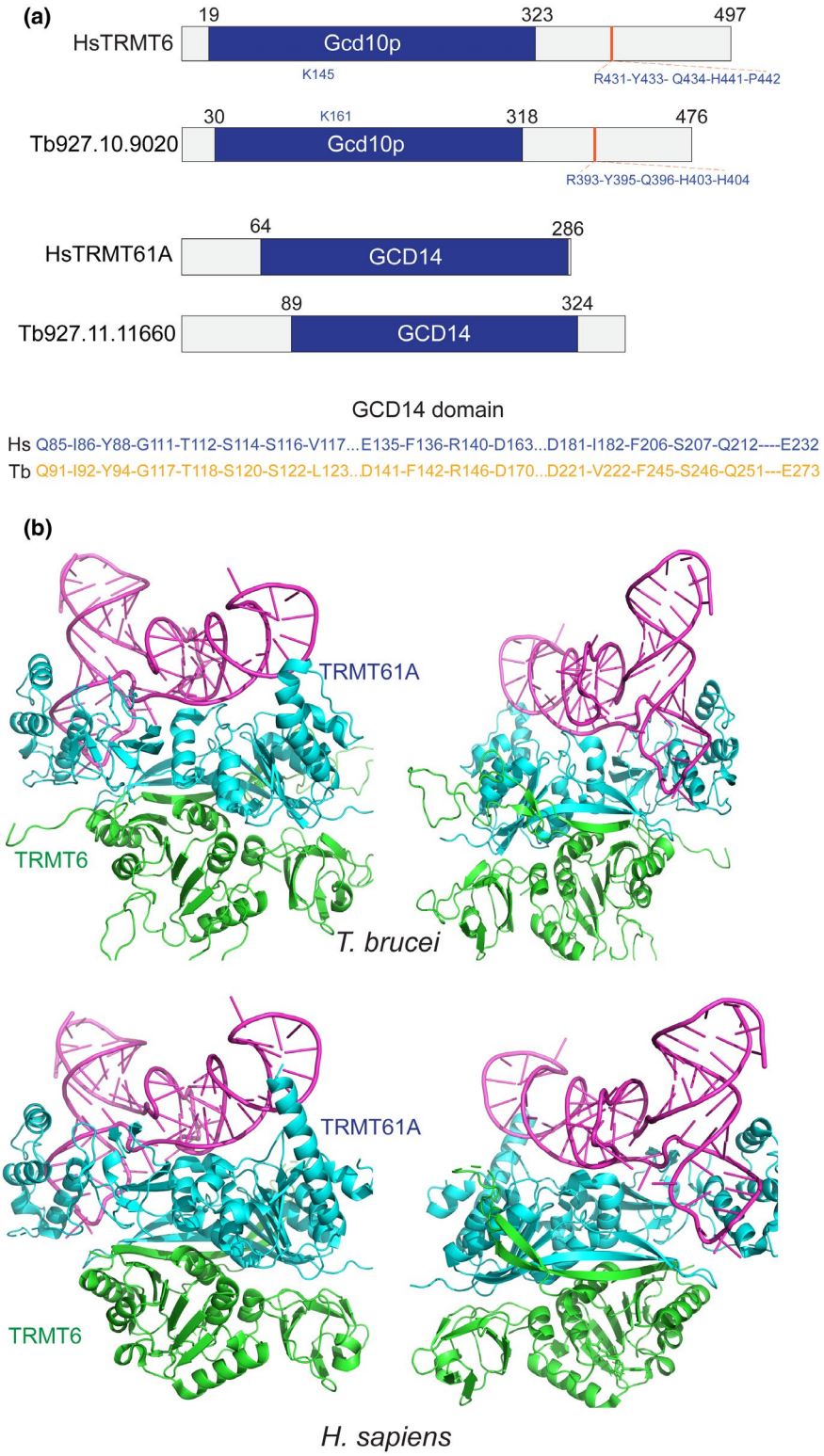

FIGURE 3 m1A writers orthologs in *T. brucei*. (a) Domain structure conservation between homolog subunits of TRMT6/61A complex from *T. brucei* and *H. sapiens*. The amino acids from GCD14 domain important for enzyme activity are highlighted (purple and orange). (b) Protein structural conservation of *T. brucei* TRMT6 (light green) and TRMT61A (light blue) compared to human proteins. The heterodimer is presented in association with an RNA molecule (pink)

archaea, and the last one is found in all domains of life (Boccaletto et al., 2018). ac4C was first detected in archaea, bacterial, mammalian, and yeast tRNA (Kowalski et al., 1971; Oashi et al., 1972; Staehelin et al., 1968) and was later also observed in archaeal 5S rRNA (Bruenger et al., 1993), and in mammalian, yeast, and plant 18S rRNA (Ito et al., 2014; Sharma et al., 2015; Taoka et al., 2014; Thomas et al., 1978). ac4C modification of 18S rRNA has been implicated in promoting translational accuracy (Chernoff et al., 1996), and together with other rRNA modifications also appears to stabilize interactions between ribosomal subunits (Sharma et al., 2015). Similarly, the presence of ac4C in tRNA impacts translational fidelity by favoring correct codon recognition and through the maintenance of tRNA tertiary structure. Recently, this modification was described in mammalian, yeast, HIV virus, and hyperthermophilic archaeal mRNA

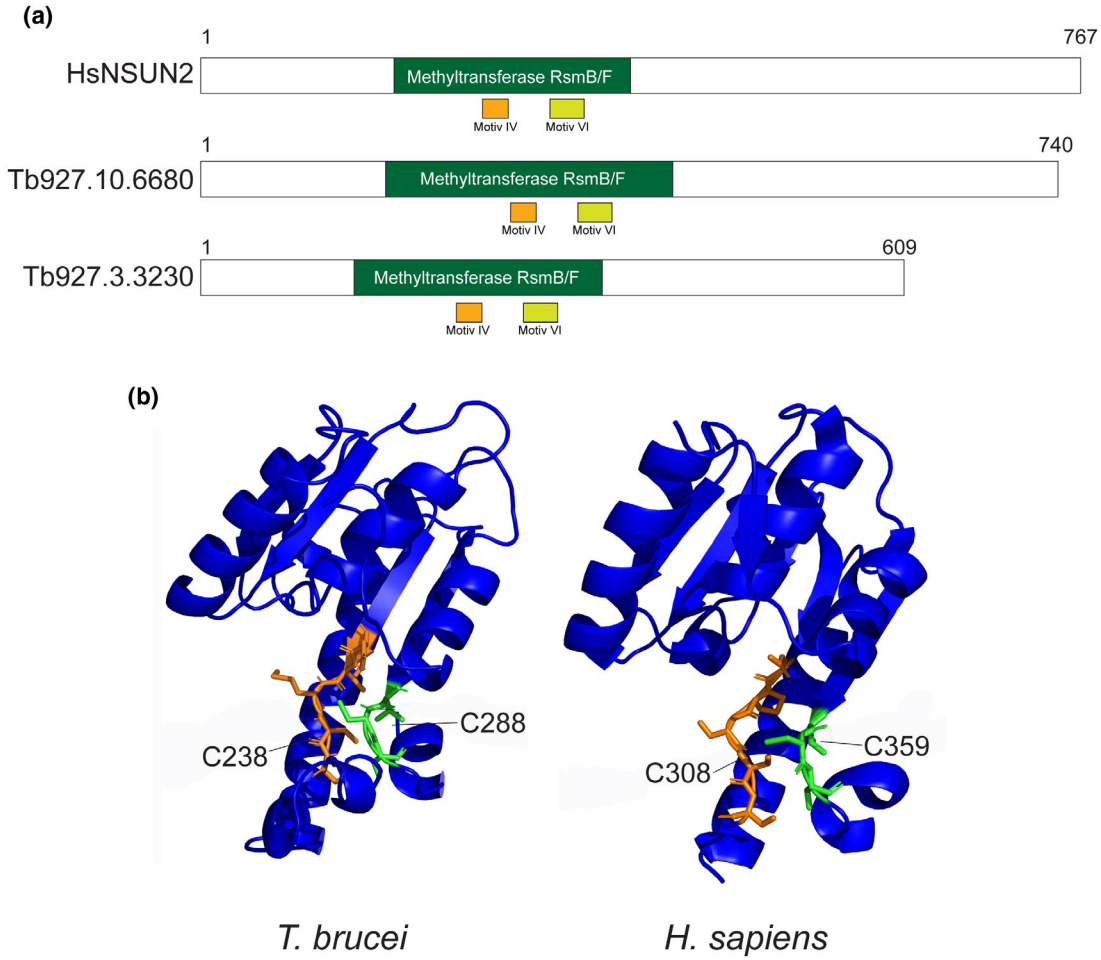

FIGURE 4 NSUN2 orthologs in *T. brucei*. (a) Domain structure of human and *T. brucei* NSUN2 orthologs. The main motifs Motif IV and VI, including the cysteine residues (C238 and C288, respectively), important for methyltransferase activity are conserved in the *T. brucei* orthologs. (b) Predicted *T. brucei* and human NSUN2 protein structure highlighting motifs IV (orange) and VI (green) and the cysteine residues key for enzymatic activity

(Arango et al., 2018; Jin et al., 2020; Sas-Chen et al., 2020; Tardu et al., 2019; Tsai et al., 2020). In mammalian and yeast mRNA ac4C is found at 5′ UTRs, 3′ UTRs, and coding sequences (CDS), where it increases translational efficiency and mRNA stability, while in HIV viral mRNA ac4C is also detected at 5′ UTRs and CDS, but not at 3′ UTRs (Tsai et al., 2020). Interestingly, ac4C levels vary in response to environmental changes, for example, ac4C levels in hyperthermophilic archaea and yeast mRNAs are augmented by increasing temperature or oxidative stress conditions, respectively (Tardu et al., 2019; Sas-Chen et al., 2020).

The enzyme that catalyzes the formation of ac4C in rRNA, tRNA, and mRNA is known as NAT10 in humans (Arango et al., 2018), tRNAMet cytidine acetyltransferase (TmcA) in *E. coli*, and Kre33 in yeast (Ikeuchi et al., 2008; Ito et al., 2014) and contains a classical Gcn5-related N-acetyltransferase (GNAT) domain. Beyond the GNAT domain, NAT10 has three other domains: DUF1726, RNA helicase RecD, and a tRNA-binding domain. The RecD domain may function to deliver the substrate base to the active center in the GNAT domain, as demonstrated for TmcA (Ikeuchi et al., 2008).

NAT10 orthologs are widely distributed among prokaryotes and eukaryotes but have been characterized in only few organisms. NAT10 null yeast and mammalian cells are not viable (Arango et al., 2018; Taoka et al., 2014), and yeast cells expressing a version of NAT10 with a mutated RNA helicase domain have a slow-growth phenotype and lack ac4C in rRNA (Taoka et al., 2014). Human NAT10 shares more than 50% of identity with the yeast protein and was initially described as involved in the regulation of mitotic chromosome decondensation, cytokinesis, and telomerase expression (Chi et al., 2007; Shen et al., 2009; Taoka et al., 2014).

We found Trypanosomatid orthologs of human NAT10 in several species of *Leishmania* and *Trypanosoma* in addition to other protozoan parasites including *T. gondii* and *P. falciparum* (Figures 2 and 5a). *T. brucei*, *T. cruzi*, and *Leishmania* species proteins share 43.7%, 41.9%, and 38.7% amino acid identity with human protein, respectively (Figure 5b) and contain all four NAT10 domains (Figure 5c). We found a high degree of conservation in N-acetyltransferase domain (GNAT) residues that are important for NAT10 catalytic activity, including the key glycine residue G641 from *H. sapiens*, which is fully conserved in the Trypanosomatids

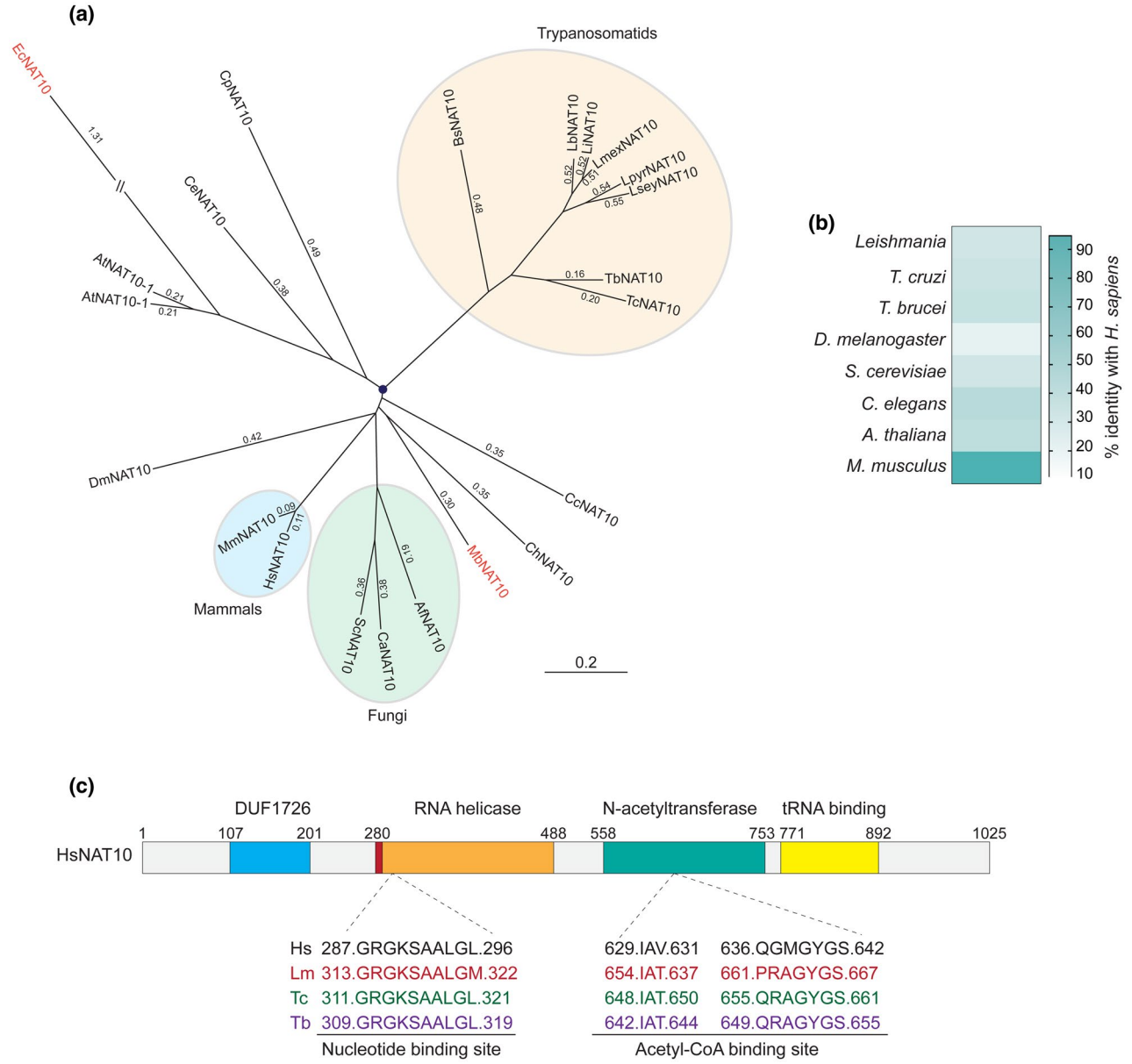

FIGURE 5 Overview of homologs of enzyme NAT10 responsible for ac4C modification. (a) Evolutionary relationships among NAT10 homologs present in different organisms including Trypanosomatids. (b) Degree of amino acid conservation (% identity) among selected NAT10 homologs. (c) Schema presenting the conservation of domain structure and main binding sites of NAT10 protein for Trypanosomatids, including the key glycine residue G641 (*H. sapiens*) in the N-acetyltransferase domain (green)

(Figure 5c). Also, residues $_{287}$GRGKSAALGL$_{296}$ that comprise the nucleotide-binding site in the RNA helicase domain are completely conserved between human, *T. cruzi* and *T. brucei* proteins, with only a single conservative substitution observed in the *Leishmania* protein at position 296 (Figure 5c). Preliminary data from our group indicates that *L. mexicana* and *T. brucei* NAT10 are essential as demonstrated previously for the human and yeast proteins (Maran et al., in preparation).

Considering that Trypanosomatids face environmental changes during their life cycles it is reasonable to consider that ac4C would play an important role in maintaining stability and translation of specific mRNAs to facilitate the adaptation of these parasites during the transition from one host to another. Moreover, the finding that *L. mexicana* NAT10 is essential opens the opportunity to explore this enzyme as potential drug target in Trypanosomatids.

3.5 | Pseudouridylation

Pseudouridine (Ψ) was one of the first RNA modifications to be discovered (Cohn, 1960) and is thought to be one of the most abundant, found in all domains of life. Ψ is a seemingly simple modification derived from base-specific isomerization of uridine (U) and occurs via a snoRNA-guided mechanism requiring H/ACA RNPs containing the Cbf5/dyskerin enzyme in yeast and humans, respectively, or snoRNA-independent mechanisms catalyzed by pseudouridine

synthases, also called Pus enzymes in eukaryotes (Li et al., 2016a; Rintala-Dempsey & Kothe, 2017; Spenkuch et al., 2014). The enzymes that catalyze pseudouridylation are classified into six families TruA, TruB (which includes Cbf5), TruD, RsuA, RluA, and Pus10. However, RsuA-type enzymes have so far only been described in bacteria while the Pus10 enzymes are present in only a few archaeal and eukaryotic organisms.

When incorporated, Ψ can alter the chemical and physical properties of RNA via its capacity to form an extra hydrogen bond compared to uridine, which can change RNA secondary structure via increased base stacking and altered base pairing (Arnez & Steitz, 1994). Ψ modifications have traditionally been studied in the context of noncoding RNAs, including tRNA, rRNA, and snRNAs. The TΨC arm of tRNAs contains a conserved Ψ at position 55 that suggests a fundamental role in translation, while rRNAs have numerous Ψ modifications near the peptidyl transferase center, the subunit interface of the ribosome and the decoding site, which are thought to impact the accuracy of translation and ribosomal assembly (Liang et al., 2009). Indeed, replacement of U with Ψ in synthetic RNAs results in increased protein expression level (Kariko et al., 2008), while artificially incorporated Ψ in mRNAs mediates recoding by facilitating unusual base pairing in the ribosome, thus, also demonstrating how this modification could also possibly generate protein diversity (Fernandez et al., 2013; Karijolich & Yu, 2011). snRNA pseudouridylation is essential for spliceosome biogenesis and activity and may also be important for branch site recognition during splicing (Newby & Greenbaum, 2002; Wu et al., 2016; Yu et al., 1998). As for other modifications, more recent technologies for identification and mapping of Ψ have also revealed its presence in mRNAs (Antonicka et al., 2017; Carlile et al., 2014; Li et al., 2015; Schwartz et al., 2014). Notably, a large proportion of Ψ modifications identified in yeast and human mRNA appear to be regulated in response to environmental signals. However, the cellular functions of pseudouridylation beyond translation and splicing remain largely unexplored.

To date in Trypanosomatids, Ψ has been mapped on rRNAs (Chikne et al., 2016) and snRNAs in *T. brucei* (Rajan et al., 2019). In both cases, Ψ sites differed between BSF and PF parasites, indicating that the modifications are developmentally regulated. Furthermore, there is evidence that Ψ strengthens RNA–RNA and RNA–protein interactions within ribosomes and spliceosomal snRNPs in *T. brucei* at elevated temperatures (Chikne et al., 2016; Rajan et al., 2019), thus, indicating that these modifications are likely to be essential for parasite adaptation to changes in temperature in the different environments of their mammalian hosts and insect vector. The only pseudouridine synthase studied to date in Trypanosomatids is Cbf5 (Barth et al., 2005; Chikne et al., 2016; Rajan et al., 2019), which is an ortholog of Cbf5/dyskerin found in H/ACA RNPs. Silencing of Cbf5 in *T. brucei* results in destabilization of H/ACA snoRNAs, including the spliced-leader-associated RNA1 (SLA1), with reductions in the levels of all detected Ψ on snRNAs, defects in spliced-leader RNA and specific rRNA Ψ site modification and maturation, and inhibition of trans-splicing. These results indicate that *T. brucei* Cbf5 functions much like that in other eukaryotes. However, snoRNA-independent pseudouridylation and other Pus enzymes have not been described in Trypanosomatids.

Here, we found multiple Trypanosomatid orthologs of Pus enzymes in addition to Cbf5 that together represent the major eukaryotic families TruA, TruB, TruD, RluA, and PUS10 (Figures 2 and 6). All Pus enzymes share a catalytic aspartate, but different families and enzymes vary in the amino acids surrounding this residue. The Pus families are also distinguished by the presence or positioning of specific domains and loops, including the RNA-binding PUA and THUMP domains and forefinger and thump loops, and individual enzymes may also have additional extensions. The domain architectures and amino acid sequences that characterize each Pus family are highly conserved in the Trypanosomatid orthologs that we identified. The sequences and lengths of extensions beyond defined domains are less well-conserved between Trypanosomatid orthologs and other organisms (Figure 6).

Pus enzymes within each of these eukaryotic families can have a few specific to numerous target uridines within rRNAs, tRNAs, snRNAs, other noncoding RNAs, and mRNAs. Furthermore, members within each family are active in the nucleus, cytoplasm, or in mitochondria reflecting localization of different family members. Additionally, individual enzymes may also be found in multiple cellular compartments (Rintala-Dempsey & Kothe, 2017). Eukaryotic Pus enzymes, therefore, likely play broad roles in the regulation of gene expression, and their activities may be regulated by environmental conditions (Schwartz et al., 2014). We expect that the newly identified Trypanosomatid Pus enzymes will also have such wide-ranging functions in the regulation of gene expression, particularly in the changes that occur between different parasite life cycle stages. Indeed, *T. gondii* has a TruA family member TgPUS1 that is critical for parasite differentiation, supporting our hypothesis (Anderson et al., 2009; Nakamoto et al., 2017).

3.6 | Regulation of expression of the putative RNA modification machinery

We hypothesize that RNA modifications are a key mechanism by which Trypanosomatids regulate gene expression during their life cycle. The expression of the putative RNA modification machinery may, therefore, also be dynamically regulated in different parasite stages. To test this prediction, we used available expression data to investigate the dynamics of representative writer enzymes responsible for these modifications during the *T. brucei* (Figure 7a) and *T cruzi* (Figure 7b) insect and mammalian life cycle stages, and during specific aspects of *Leishmania* stages (Figure 7c).

Genes encoding the m1A, m5C, ac4C, and Ψ writers have approximately similar mRNA levels in *T. brucei* BSF and PF forms (Figure 7a). However, ribosome profiling experiments (Jensen et al., 2014) revealed significant differences in the translational efficiency (TE) of the mRNAs encoding some of these enzymes, particularly the TruD PUS family member Tb927.10.9050 and NAT10,

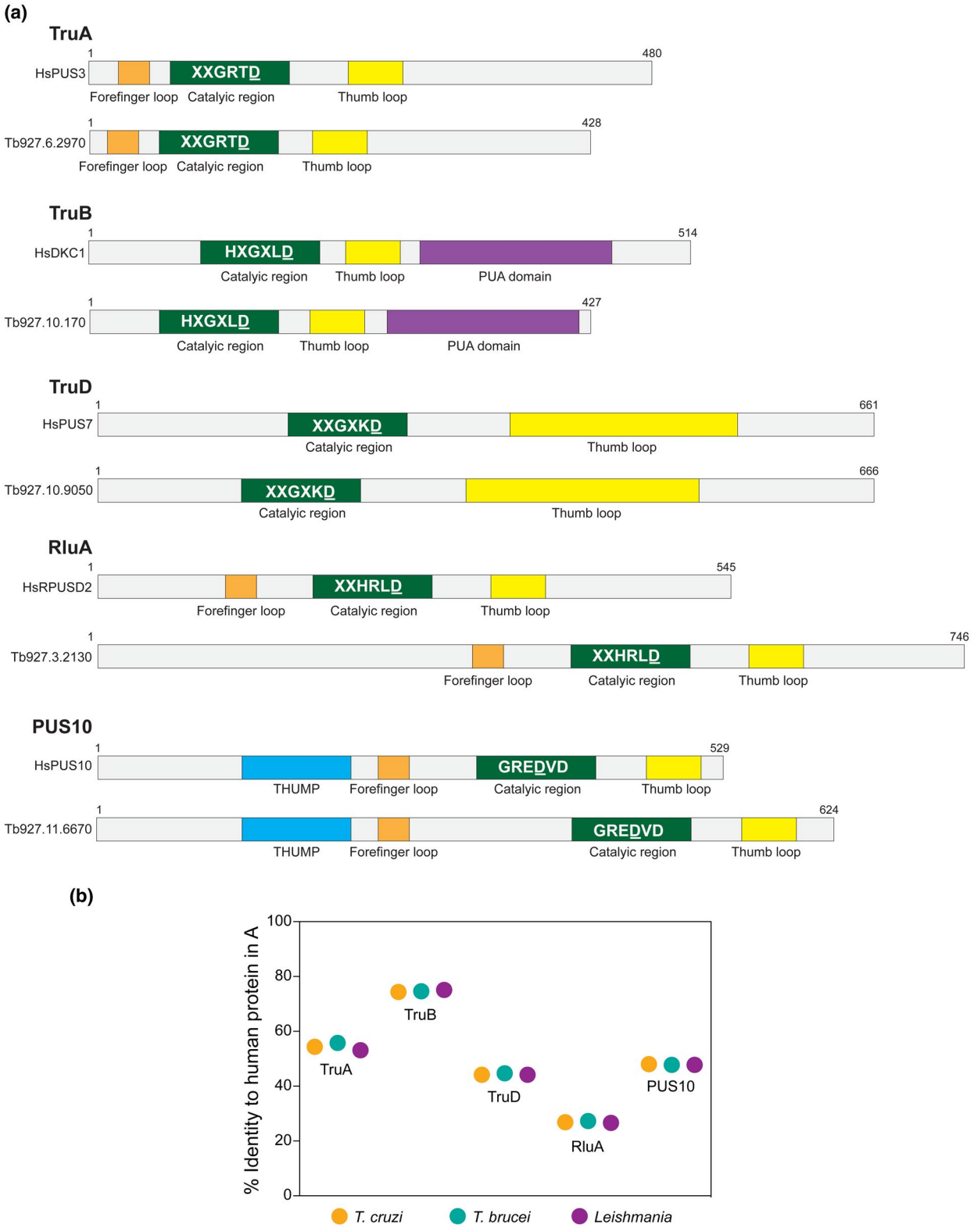

FIGURE 6 (a) Domain organization of representative human and *T. brucei* ortholog pseudouridine synthases from the five major eukaryotic families, TruA, TruB, TruD, RluA, and PUS10. Conserved domains are represented as colored boxes, and consensus sequences for catalytic sites are shown. The conserved catalytic aspartate (d) across all pseudouridine synthases is underlined, X indicates any amino acid. (b) Amino acid identities between the human pseudouridine synthases shown in (a) and their *T. brucei*, *T. cruzi*, and *L. major* orthologs

which have a higher TE in PF, and also TRMT61A which has a higher TE in BSF. Furthermore, the TE differences between BSF and PF for these genes were also independently observed in a separate set of ribosome profiling experiments (Vasquez et al., 2014), indicating differential protein expression levels for these enzymes between *T. brucei* life cycle stages.

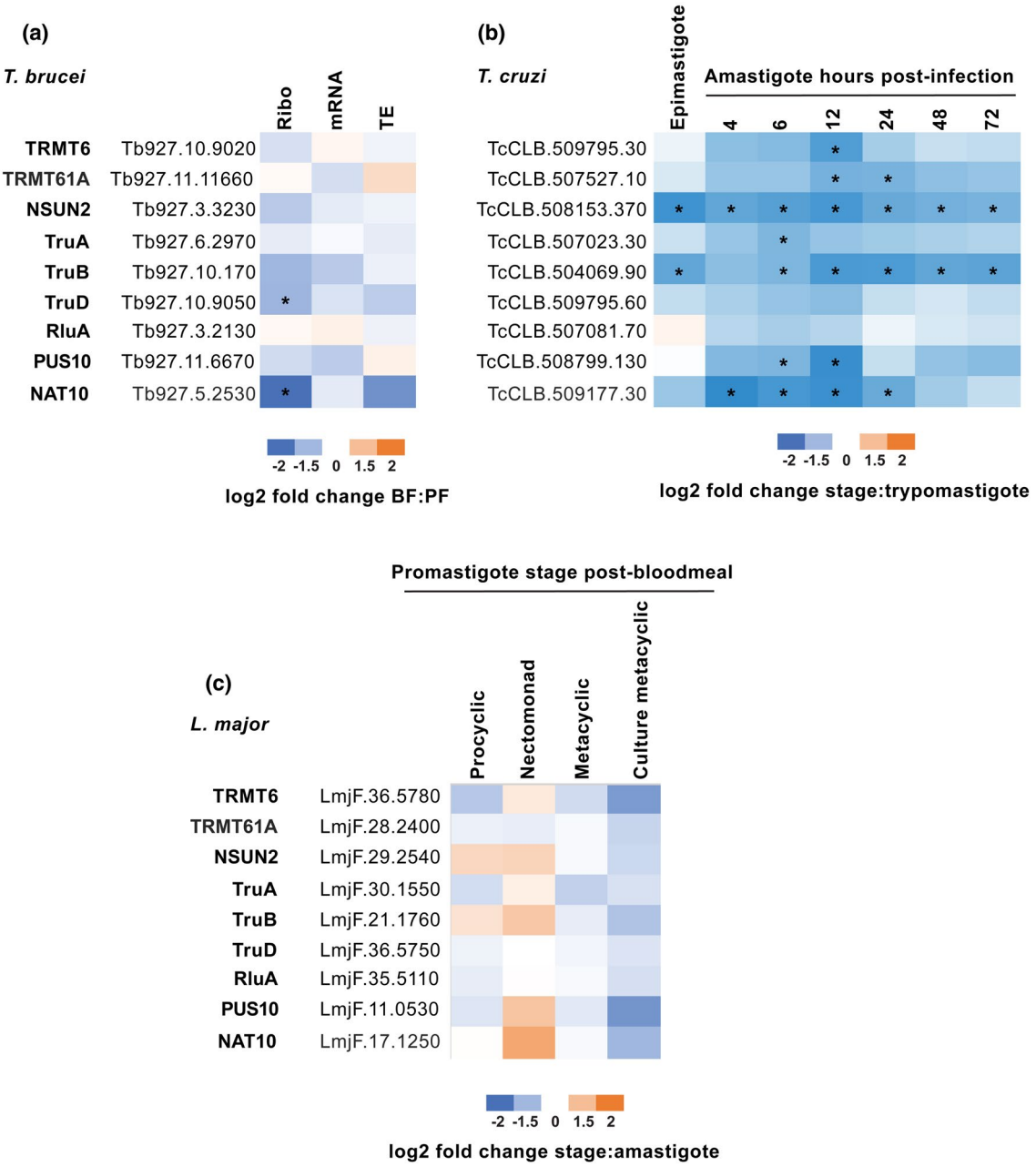

FIGURE 7 Relative expression profiles of RNA modification enzyme orthologs in different parasite life cycle stages. (a) Log2 normalized fold changes in read counts for ribosome footprint (Ribo), mRNA, and the translational efficiency (TE) between bloodstream (BF) and procyclic form (PF) of *T. brucei*. (b) Log2 normalized fold changes in read counts for mRNA between *T. cruzi* epimastigotes or amastigotes at different developmental time points compared to trypomastigotes forms. (c) Log2 normalized fold changes in read counts for mRNA between *L. major* cultured metacyclic, procyclic, nectomonad, and metacyclic promastigotes derived from sand flies compared to amastigotes. (a–c) Asterisks indicate comparisons where fold changes ≥2, and FDR ≤ 0.01 in (a) or adjusted *p* value ≤ 0.05 in (B) among replicate experiments in the cited studies

Comparable gene expression levels of representative PUS TruD and RluA family Ψ writers are observed in the replicative insect epimastigote, and vertebrate intracellular amastigote forms of *T. cruzi* compared to non-replicative trypomastigotes (Li et al., 2016c) (Figure 7b). The TruB family representative TcCLB.504069.90 is expressed at significantly lower levels in epimastigotes and throughout amastigote development than in trypomastigotes (Figure 7b), while the TruA and PUS10 representatives TcCLB.507023.30 and TcCLB.508799.130, respectively, are significantly reduced at specific times during amastigote development, corresponding to when the parasites are beginning to multiply in their host cell cytoplasm. TRMT6 and NAT10 transcripts are also significantly lower in the initial multiplication stages of intracellular amastigotes than in trypomastigotes (Figure 7b). Both m1A and ac4C, catalyzed by TRMT6 and NAT10, respectively, increase mRNA stability in response to environmental change, which suggests that dynamics in the enzymes responsible for the addition of these

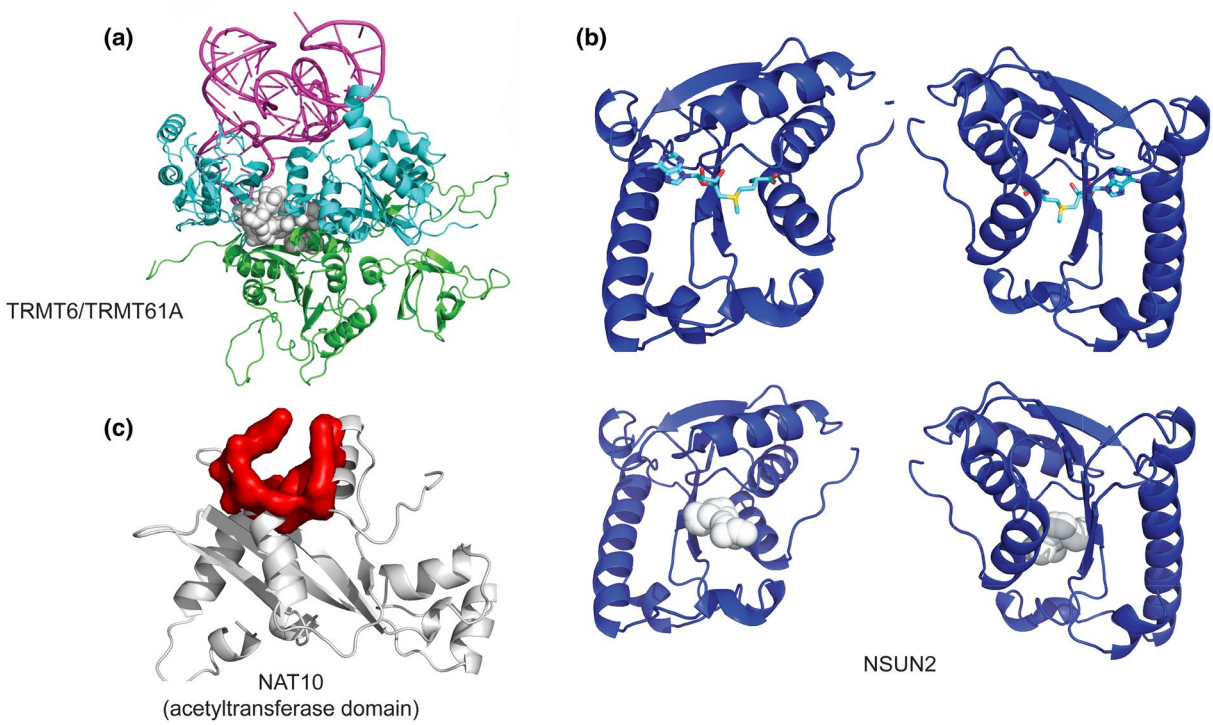

FIGURE 8 Druggability analyses of writer enzymes in Trypanosomatids. (a) Structural model of *T. brucei* TRMT6/TRMT61A heterodimer associated with RNA showing the binding pocket at the interaction interface of the subunits (gray). (b) Structural models for *T. brucei* NSUN2 exhibiting its interaction with S-adenosylmethionine (SAM) (upper panel) and displaying the prediction of a drug pocket located at the SAM-binding region (lower panel). (c) *L. major* NAT10 N-acetyltransferase domain model showing the binding pocket region (red)

modifications could be important for the development of replicative amastigote stages in *T. cruzi*.

Coordinated changes in gene expression are observed for almost all writers during *Leishmania* development in the insect host (Inbar et al., 2017). Interestingly, an increase for members responsible for all RNA modifications studied were observed in nectomonad stages compared to procyclic forms (Figure 7c). Nectomonad is one of the stages that precede the development of non-replicative infective metacyclic forms of *Leishmania* (Bates & Rogers, 2004), and an increase in RNA modification writer expression indicates that these modifications may participate in RNA stability and protein synthesis dynamics necessary for the metacyclic stage.

The fluctuations observed in RNA modification writer enzyme gene expression in different Trypanosomatid developmental stages suggest that the RNA modifications themselves may also be highly dynamic, which is supported for m6A and Ψ by previous studies (Chikne et al., 2016; Liu et al., 2019; Rajan et al., 2019). Furthermore, they suggest that RNA modification as a mechanism of posttranscriptional gene expression regulation is conserved among these parasites where it is important for adaptation to different environments, nutrients, and temperatures.

3.7 | The RNA modification machinery as a Trypanosomatid drug target

Over the past decade, a number of RNA modification enzymes have been associated with human disease, including cancer, metabolic, and infectious disease (Boriack-Sjodin et al., 2018; Li et al., 2020; Patton et al., 2005; Tsai et al., 2020). For example, NSUN2 is highly expressed in multiple tumor types (Frye & Watt, 2006; Yi et al., 2017) and knockdown of NSUN2 inhibits cell proliferation in vitro in breast cancer (Frye et al., 2010) and squamous cell carcinoma models (Frye & Watt, 2006). Mutations at TRMT6 gene are associated with colon cancers (Yeon et al., 2018), and METTL3-METTL14 is also linked to diverse cancers (Barbieri & Kouzarides, 2020). Although several studies have, therefore, demonstrated the involvement of RNA modifications in human disease, testing and clinical trials of potential inhibitors are still at an early stage. For example, the NAT10 inhibitor remodelin has just recently been demonstrated to improve the effects of the premature-aging disease Hutchinson–Gilford progeria syndrome (HGPS) (Larrieu et al., 2014) and to decrease HIV virus load in in vitro infection assays (Tsai et al., 2020), while putative methyltransferase inhibitor AN5568 is still awaiting phase III clinical trials for treatment of human *T. brucei* infection (Steketee et al., 2018).

There is a recurrent need for more affordable and less toxic therapeutics to treat diseases caused by Trypanosomatids, and the validation of new families of drug targets including RNA modification enzymes would contribute to development of new approaches. To explore the possibility of using Trypanosomatid writers as potential drug targets in the future, we performed druggability analysis to predict potential pockets for binding of small molecules in all parasite enzymes. We found pockets at the interaction interface between the *T. brucei* TRMT/TRMT61A subunits of the heterodimer

complex that would be accessible to small molecules (Figure 8a). Because both subunits together are necessary for m1A modification of RNA, these pockets provide potential targets for therapeutic drug-like compounds. We also identified pockets in the *T. brucei* NSUN2 structure, including at the predicted SAM-interaction site (Figure 8b). As NSUN2 is a SAM-dependent enzyme, molecules interacting in that region of the protein would potentially disrupt catalytic activity. Finally, a potential binding region was also found in the N-acetyltransferase domain of *L. major* NAT10, which is also the target region of remodelin (Figure 8c). Indeed, our group has demonstrated that NAT10 inhibitors significantly reduce *T. cruzi* and *Leishmania* intracellular amastigote multiplication in vitro (Maran et al., in preparation). Together, these results indicate that putative Trypanosomatid RNA modification enzymes are an important class of drug targets for these parasites.

4 | CONCLUSIONS AND FUTURE PERSPECTIVES

Gene expression regulation in Trypanosomatids relies mainly on posttranscriptional mechanisms and regulatory processes. These may include RNA modifications that are capable of dynamically changing mRNA translation and RNA stability, and that are potentially essential to parasite biology and could be exploited for candidate drug targets. We therefore bioinformatically searched for homologs of writer enzyme families that are responsible for addition of the RNA modifications m5C, m1A, m6A, Ψ, ac4C across Trypanosomatids, and identified homologs of m5C, m1A, Ψ, and ac4C writers. Notably, key amino acids responsible for catalytic activity are conserved and domain architectures are very similar across the species analyzed. m6A writer enzymes remain elusive despite studies m6A modification of *T. brucei* mRNA. Together these findings support the idea that the RNA modification machinery for m5C, m1A, Ψ, and ac4C is present and active in Trypanosomatids but that further studies are necessary to determine how m6A is added to RNA in these organisms.

Shifting between hosts and vectors and their very different environmental conditions poses a great challenge to Trypanosomatids to adapt and survive, which in turn necessitates alterations in gene expression and protein synthesis across their life cycles. Based on existing expression data, we found that the expression of the writer enzymes is dynamic across parasite life cycles, which may indicate that the modifications that they add are also dynamic. This adds to previous work showing that m6A and Ψ levels change across the *T. brucei* life cycle and supports our hypothesis that RNA modification may be a novel mechanism by which Trypanosomatids efficiently and rapidly adapt their gene expression programs for survival in these different environments.

The need to efficiently develop more affordable and less toxic treatments for the diseases caused by Trypanosomatids is ongoing and the identification of new drug targets will contribute to development of new therapeutic approaches. Thus, considering the potential crucial role for RNA modifications in these parasites, it is suitable to consider the writer enzymes we identified as potential drug targets. We tested this using different in silico approaches and found important binding pockets in the identified proteins, opening up the opportunity to screen these enzymes in further drug assays.

This work therefore brings new insights into the roles of the proteins responsible for the addition of RNA modifications in Trypanosomatid parasite biology and treatment of the diseases they cause. We also illustrate that Trypanosomatids provide an excellent model system in which to study RNA modifications, their molecular, cellular, and biological consequences, and their regulation and interplay. These are fundamental questions whose answers are poorly understood across biology, and that have the potential to provide new paradigms and change our understanding of gene regulation. A major challenge in this area is the identification of modified RNA residues and the transcripts in which they are found. However, new "third-generation" sequencing technologies including nanopore and SMRT (Schwartz & Motorin, 2017; van Dijk et al., 2018), combined with cutting-edge mass-spectrometry approaches (Kullolli et al., 2014; Wein et al., 2020), provide opportunities to directly and comprehensively identify a diversity of chemically modified nucleotides, and characterize where they are located in the transcriptome and under what conditions, thus, pushing forward our understanding of the biological functions of RNA modifications in Trypanosomatids and beyond.

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SUPPORTING INFORMATION

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

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