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Genome-wide analysis of small nucleolar RNAs of *Leishmania major* reveals a rich repertoire of RNAs involved in modification and processing of rRNA

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Trypanosomatids are protozoan parasites and the causative agent of infamous infectious diseases. These organisms regulate their gene expression mainly at the post-transcriptional level and possess characteristic RNA processing mechanisms. In this study, we analyzed the complete repertoire of *Leishmania major* small nucleolar (snoRNA) RNAs by performing RNA-seq analysis on RNAs that were affinity-purified using the C/D snoRNA core protein, SNU13, and the H/ACA core protein, NHP2. This study revealed a large collection of C/D and H/ACA snoRNAs, organized in gene clusters generally containing both snoRNA types. Abundant snoRNAs were identified and predicted to guide trypanosome-specific rRNA cleavages. The repertoire of snoRNAs was compared to that of the closely related *Trypanosoma brucei*, and 80% of both C/D and H/ACA molecules were found to have functional homologues. The comparative analyses elucidated how snoRNAs evolved to generate molecules with analogous functions in both species. Interestingly, H/ACA RNAs have great flexibility in their ability to guide modifications, and several of the RNA species can guide more than one modification, compensating for the presence of single hairpin H/ACA snoRNA in these organisms. Placing the predicted modifications on the rRNA secondary structure revealed hypermodification regions mostly in domains which are modified in other eukaryotes, in addition to trypanosome-specific modifications.

Introduction

RNA modification has drawn the attention of many scientists, because modifications are not only enriched on stable RNAs such as rRNA, tRNA and snRNAs but also in coding and other non-coding RNAs. The transcriptome-wide mapping of such modifications suggests that folding, stability and activity are modulated and regulated by RNA modifications.[1,2]

The two major rRNA modifications, 2′-*O*-methylation (Nm) and pseudouridine (Ψ) formation, are prevalent on rRNA and small nuclear RNAs (snRNAs) in eukaryotes and are guided by small nucleolar RNAs (snoRNAs). The 2′-*O*-methylations are guided by C/D box snoRNAs. These boxes, together with the short sequences near the 5′ and 3′ ends of the RNA, are essential for processing, localization, and stabilization of these molecules. Most of the guide RNAs carry internal boxes related to the C and D boxes, known as C′ and D′ boxes. The recognition of the target is based on complementarity of 10 to 21 nucleotides (nt) between these 2 molecules, located upstream of the D and D′ sequences (reviewed in[3,4]). The methylation site is situated 5 nt upstream from the D and D′ boxes, within the domain of interaction between the snoRNA and the substrate.[5]

In most eukaryotes, the snoRNAs that guide pseudouridylation consist of 2 hairpin domains connected by a single-stranded hinge, the H domain, and by a tail, the ACA box. A short RNA recognition motif on the snoRNA base-pairs with the target and directs the conversion of uridine to pseudouridine. The pseudouridine is usually located 14 to 16 nt upstream from the H box or the ACA box of the snoRNA.[6] Most of the well-studied C/D and H/ACA box RNAs characterized to date at both structural and functional levels are from humans, or from the yeast *Saccharomyces cerevisiae*.[7] However, studies in last decade also characterized snoRNAs in other organisms, such as the amoeba diplomonad *Dictyostelium discoideum*,[8] the parasitic protozoan *Giardia lamblia*[9] and *Entameoba histolytica*,[10] and the malaria parasite *Plasmodium falciparum*.[11] snoRNAs were also studied in model organisms such as *Drosophila*[12] and the nematode *Caenorhabditus elegans*.[13]

Trypanosomatids are parasitic protozoa that are the causative agents of several infamous diseases, such as African

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trypanosomiasis, caused by *Trypanosoma brucei*, Chagas' disease, caused by *Trypanosoma cruzi*; and Leishmaniasis, caused by *Leishmania* species. *Leishmania* spp. are obligatory intracellular parasites that cause a spectrum of human diseases, with an annual incidence of 2 million cases in 88 countries. The parasite cycles between 2 hosts, namely, the insect host, where *Leishmania* parasites grow as flagellated extracellular promastigotes; and the mammalian host, where they proliferate as aflagellated intracellular amastigotes.[14]

rRNA processing events in Trypanosomatids are unique. The large subunit rRNA undergoes trypanosome-specific cleavages during rRNA maturation, yielding 2 large rRNA molecules and 4 small RNAs, ranging in size from 76 to 226 nt.[15]

Several specific features were found in snoRNAs of trypanosomatids. Most, if not all H/ACA RNAs are composed of a single hairpin RNA and carry an AGA box instead of an ACA box.[16,17] The first discovered trypanosome H/ACA-like RNA, the spliced leader-associated RNA 1 (SLA1), guides modification of a unique short-lived RNA,[18] the spliced leader RNA (SL RNA). This RNA is the donor of the spliced leader sequence to all trypanosome mRNAs.[19] Silencing of the pseudouridine synthase (CBF5) by RNA interference in *T. brucei* provided evidence for the role of SLA1 in *trans*-splicing.[20] We proposed that SLA1 has a chaperone function and escorts the SL RNA early in its biogenesis until it is assembled with Sm proteins.[21]

Using bioinformatics and experimental tools, we recently performed a genome-scale analysis of snoRNAs that guide methylations and pseudouridylations on rRNAs in both *T. brucei* and *L. major*. Our data suggested that most snoRNAs are clustered in reiterated repeats that carry a mixed population of C/D and H/ACA-like RNAs. Predicting the modifications guided by these RNAs and using partial mapping data, allowed us to identify 57 C/D snoRNAs that potentially guide 84 Nm modifications, and 34 H/ACA like RNAs that target rRNA, suggesting a high occurrence of Nms compared to pseudouridines on *T. brucei* rRNA.[16] Based on *T. brucei* snoRNAs, we identified 23 gene clusters in *L. major* that encode 62 C/D snoRNAs that potentially guide 79 methylations, and 37 H/ACAs that can guide 30 pseudouridylation reactions. In general, the pattern of Nm modifications is highly conserved between *L. major* and *T. brucei*.[17]

Using RNA-seq of small RNPs we expanded the repertoire of *T. brucei* snoRNAs and identified 79 C/D and 63 H/ACA-like snoRNA, suggesting that these organisms also harbor a large number of pseudouridines.[22] Many H/ACA were shown to exist in clusters containing only H/ACA RNAs, and these escaped our previous screens, which identified H/ACA based on their presence in clusters with C/D snoRNAs. Abundant snoRNAs, mostly of the C/D type, were shown to function in rRNA processing.[22,23]

The analysis of modifications guided by *T. brucei* snoRNAs revealed the existence of additional species specific and increased overall modification levels at domains that are already modification-rich in other eukaryotes.[16] About 40% of the trypanosome-specific modifications are situated in unique positions outside the highly conserved domains of the rRNA.[16,17]

In this study, the *L. major* repertoire of snoRNAs was determined by RNA-seq analysis of RNA affinity selected with the C/D and H/ACA specific proteins SNU13 and NHP2, respectively. The study identified 81 H/ACA and 80 C/D; among these are newly identified 13 C/D and 44 H/ACA snoRNAs. The snoRNAs vary in their abundance as can be observed by the RNA-seq reads and Northern analyses. Among the abundant snoRNAs, we identified 13 snoRNAs predicted to function in trypanosome-specific rRNA processing. The putative role of 2 such snoRNAs in rRNA processing was studied by *in vivo* psoralen cross-linking and fractionation on RNP complexes. The predicted rRNA modifications guided by the identified snoRNAs were placed on the secondary structure of rRNA. Our data suggest the presence of hyper-modifications in domains that are also modification-rich in other eukaryotes. The repertoire of *L. major* snoRNAs is highly related to that of *T. brucei*. However, species-specific snoRNAs and modifications were also identified. The relatedness of H/ACA RNAs in *T. brucei* and *L. major* was studied, suggesting the mechanism by which snoRNAs may have been generated during evolution. Flexibility in the generation of a pseudouridylation pocket was detected, which potentially enables a single hairpin H/ACA RNA to guide more than one target, thus compensating for the presence of single-hairpin RNAs in trypanosomes compared to double-hairpin RNAs in other eukaryotes.

Materials and Methods

Oligonucleotides

The list of oligonucleotides used in this study is given in Table S-1.

RNA preparation and primer extension analysis of RNAs

RNA was prepared using TRI Reagent (Sigma). Primer extension analysis was performed as described previously,[24] using 5′-end-labeled oligonucleotides specific to target RNAs, as indicated in the figure legends. The extension products were analyzed on 6% polyacrylamide-7 M urea gels.

RT-PCR

RNA was treated with the "DNase-free" reagent (Ambion) according to the manufacturer's protocol for 30 minutes to remove DNA contamination. Reverse transcription was performed by random priming (Reverse transcription system, Promega). The samples were heated for 5 min at 70°C, followed by chilling on ice for 5 minutes. Next, 1 unit of AMV-reverse transcriptase (Promega) was added, together with 1 unit RNase inhibitor (Promega) and the elongation reaction was performed according to the manufacturer's instructions at 25°C for 10 min, and then at 50°C for 60 min (Promega kit). The resulting cDNA was used for PCR amplification using primers as specified in Table S-1.

Purification of the SNU13 and NHP2 RNPs

Tandem affinity purification was performed from whole cell extracts. The cell pellet from *L. major* (2×10^{11} cells) was washed twice with PBS and once with buffer I (20 mM Tris-HCl (pH 7.7), 150 mM KCl, and 3 mM $MgCl_2$). The cells were resuspended in 15 ml of buffer II (buffer I with 1 mM DTT and

10 μg/ml leupeptin), equilibrated in a nitrogen cavitation bomb (Parr Instruments Co.) at 750 p.s.i. N_2 for 1h at 4°C, and disrupted by release from the bomb. After release of the pressure, protease inhibitor mixture (Roche Applied Science) was added, and the extract was treated with 0.5% Triton X-100. The extract was incubated at 4°C for 15 min and cleared by centrifugation (15,000 ×g), and the supernatant was incubated while rotating for 2 h with rabbit IgG-agarose beads (200 μl) (Sigma). The beads were washed 5 times with TEV buffer (buffer I with 0.5 mM DTT, 0.5 mM EDTA) and incubated overnight in 1.5 ml of TEV buffer with 200 units of tobacco etch virus protease (Promega). After centrifugation, the supernatant was incubated with 50 μl of Strep-T actin-Sepharose beads (IBA) for 1h. The beads were washed with buffer III (TEV buffer with 2 mM CHAPS (GE Healthcare)), and the complexes were eluted with elution buffer (100 mM Tris-Cl (pH 8), 150 mM NaCl, 1 mM EDTA) containing 2.5 mM *d*-desthiobiotin (Sigma). After removing the proteins by phenol-extraction, the RNA (1–2 μg) was fragmented using the Ambion RNA fragmentation kit (AM8740). The RNA was dephosphorylated at the 3′ end using T4 Polynucleotide kinase (PNK) (in the absence of ATP). The 5′ end of the RNA was repaired using PNK in the presence of tracer radioactive [γ^{32}P]ATP. The material was separated on 15% polyacrylamide denaturing gel and the radioactive bands at a size of ∼ (25–40) nt were excised from the gel. RNA was eluted and the 3′ adaptor was ligated (3′-RAppCTGTAGGCACCATCAAT/3′DDG) with T4 RNA ligase 2, (New England Biolabs). The reaction was loaded on a 15% polyacrylamide denaturing gel, the radioactive higher molecular weight bands(40–60 nt) were excised, and the 5′ RNA adaptor (5′-ACACGACGCUCUUCCGAUCU-3′) was ligated using T4 RNA ligase. RNA was extracted and cDNA was synthesized in the presence of radiolabelled dCTP as tracer. The cDNA was purified from a 15% polyacrylamide gel and subjected to PCR using "Platinum" DNA polymerase (Invitrogen). The PCR was sequenced by Illumina sequencing, as previously described.[22]

"RNA walk"

Cross-linking was performed essentially as described in.[25] Briefly, *L. major* cells were harvested and resuspended at 5×10^7 cells/ml and washed twice with PBS. Cells ($\sim10^9$) were concentrated and incubated on ice. 4-Aminomethyl-trioxsalen hydrochloride (AMT) was added to the cells at a concentration of 0.2 mg/ml. Cells treated with AMT were kept on ice and irradiated using a UV lamp at 365 nm at a light intensity of 10 μW/cm^2 for 30 minutes. Next, the cells were washed once with PBS and deproteinized by digestion with proteinase K (200 μg/ml in 1% SDS for 60 minutes). RNA was prepared using TRIzol reagent. Approximately 250 μg of RNA was used for affinity selection, essentially as described in.[23] After affinity selection, the RNA was subjected to RT-PCR as described[26]

Mapping RNA-seq reads to the genome

The 36 nts sequence reads obtained from the Illumina Genome Analyzer were first trimmed of Illumina adapters using the FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), and reads of 15 bases or less were discarded from subsequent analysis. The remaining reads were mapped to the *L. major* draft genome (TriTrypDB-2.5; http://tritrypdb.org/common/downloads/release-2.5/Lmajor/) using SMALT v0.7.5 (http://www.sanger.ac.uk/resources/software/smalt/) with the default parameters, allowing non-unique reads to be mapped randomly to their best match in the genome. Next, the reads were imported and visualized in the IGV viewer.[27,28]

Sucrose gradient fractionation of RNP complexes

Whole cell extracts were prepared from 2×10^{10} *L. major* cells as previously described,[29] and fractionated on a 10–30% sucrose gradient. Gradients were centrifuged at 4°C for 3h at 35,000 rpm in a Beckman SW41 rotor. Then, 1 ml fractions were collected using the ISCO gradient fractionation system. The absorbance profile at 245 nm was determined to locate the positions of the 80S monosome and the polysomes.

SnoRNA quantification and annotation

Raw read counts for each snoRNA were obtained using Multicov from the Bedtools suite (v 2.17.0). For each snoRNA that appears multiple times in the genome, the counts for each genomic location were combined. Reads Per Kilobase per Million (RPKM) was utilized as the quantification method to obtain a measure of each gene's expression.[30] Reads mapping to unannotated loci were chosen as potential novel snoRNAs. In order to determine if the reads mapping to unannotated loci were derived from known annotated sequences, they were merged, extracted and further analyzed by BLAST[31] against the *L. major* known coding sequences. Those reads that were not similar to any known coding sequence were then run as input to a variety of programs to identify putative snoRNAs. snoScan version 0.9b[32] was used to identify C/D snoRNAs. snoGPS[33] and PsiScan[34] were used to test if the ncRNA candidates were likely to be H/ACA snoRNAs. In the final stage, manual examination of the remaining sequences in each library was performed to look for the classical C/D and H/ACA motifs (TGAUGA/CUGA and AGA) in the SNU13 and NHP2 libraries, respectively.

Prediction of targets on rRNA

For C/D snoRNAs, the potential targets (for 2′-*O*-methylation) in rRNA and on snRNAs were determined using BLAST[31] to search for a complementary match to an rRNA or U snRNA target. For this study, the program was used to search for complementarity to rRNA that complies with the +5 guiding rule. Additionally, the targets were also predicted based on the data available from their *T. brucei* homologues. For H/ACAs, which have a well-defined structure, sequence folding by MFOLD[35] was performed. The resulting sequences from the internal loop were extracted. A PERL script was used to scan the rRNA and UsnRNAs for a compatible target for the potential pocket based on the guiding rules established for yeast, mammals, and plants (http://www.bio.umass.edu/biochem/rna-sequence/Yeast_snoRNA_Database/snoRNA_DataBase.html; http://bioinf.scri.sari.ac.uk/cgi-bin/plant_snorna/conservation.

The annotations of the newly identified snoRNA in this study were submitted to GeneDB.

Identifying *L. major* H/ACA and C/D homologues in *T. brucei*

The repertoire of H/ACA snoRNAs in *L. major* and *T. brucei* was compared to establish homologues between the 2 species. Homologues were found for each H/ACA as detailed in Doniger et al. 2009.[36] The H/ACAs were "split," and the pseudouridylation pockets and the rest of the H/ACA were aligned independently for each homologous pair using the Needle program from the EMBOSS 6.1.0 package.[37] The repertoire of C/D snoRNAs in *L. major* and *T. brucei* was compared to establish homologues between the 2 species. BLAST[31] was used to find matching guide regions.

Results

Preparation of snoRNA-specific libraries

The recent genome wide-search for small ncRNAs in *T. brucei* doubled the number of identified H/ACA-like RNAs,[22] suggesting that the previous bioinformatic studies performed in both *T. brucei* and *L. major*[16,17] had missed a large fraction of the H/ACA-like repertoire. Previous studies had identified these RNAs based on their chromosomal location in the vicinity of C/D snoRNAs. However, the study in *T. brucei* indicated that H/ACA-like RNAs are found not only in clusters with C/D snoRNA, but also in clusters containing only H/ACA RNAs or as solitary genes.[22] The *T. brucei* RNA-seq was performed on the small RNome, and may have missed snoRNAs, especially non-abundant molecules.[22] To identify the complete repertoire of these RNAs, we established a system to specifically sequence snoRNAs by affinity selecting these RNA via their association with their cognate RNA binding proteins. To this end, the *L. major* snoRNP core SNU13 (C/D) and the NHP2 (H/ACA) were cloned into the expression vector, pSAP1, carrying a C-terminal tag composed of protein A binding domain, the tobacco virus protease cleavage site and streptavidin-binding peptide.[38] *L. major* transgenic cell lines were prepared and used to affinity purify the snoRNAs. After affinity-selection, the levels of the selected snoRNAs were compared to different small RNAs by primer extension. The results (**Fig. 1A**) demonstrated the specific selection of LM26Cs1H4 snoRNA, with no background from SL RNA or the C/D snoRNA LM26C1C1. The same experiment was performed with an SNU13 tagged cell line, and the results indicated efficient selection of LM26C1C1 with no background from the SL RNA and LM26Cs1H4 (**Fig. 1A**). Next, we scaled up the affinity purification as described in Materials and Methods, and the RNA of the last purification step was de-proteinized and fractionated on a denaturing gel and stained with silver (**Fig. 1B**). The silver stain of the NHP2-selected RNA indicated the presence of RNA longer than the majority of the selected RNAs, ranging in size from 70 to 100 nt. Most of the SNU13 selected RNAs were in the size range of 60–70 nts. Next, the affinity selected RNA was fragmentized by mild alkaline hydrolysis and libraries were prepared as described in Materials and Methods. cDNA was prepared and amplified. The fragments were sequenced and 21 and 33 million reads from the NHP2 and SNU13 libraries, respectively, were mapped to the L. *major* genome.

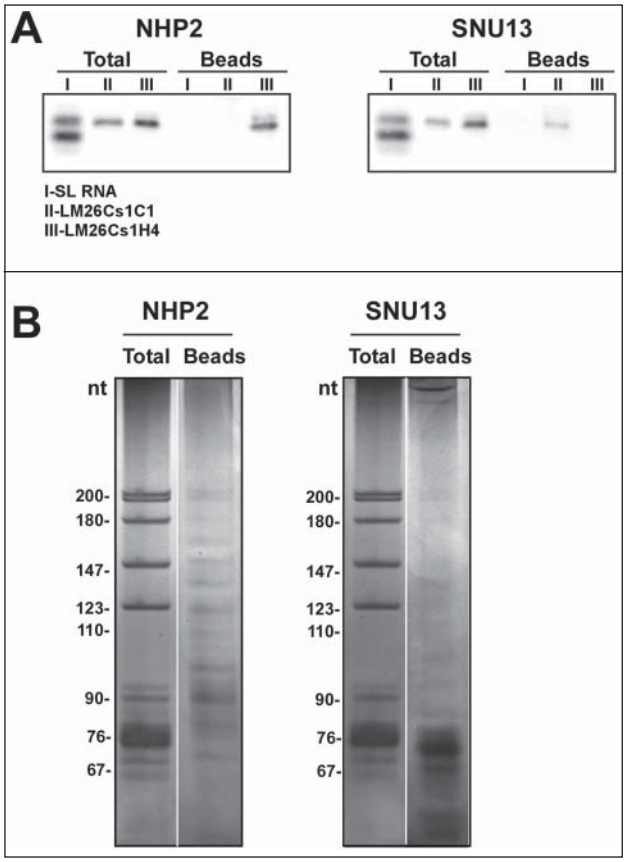
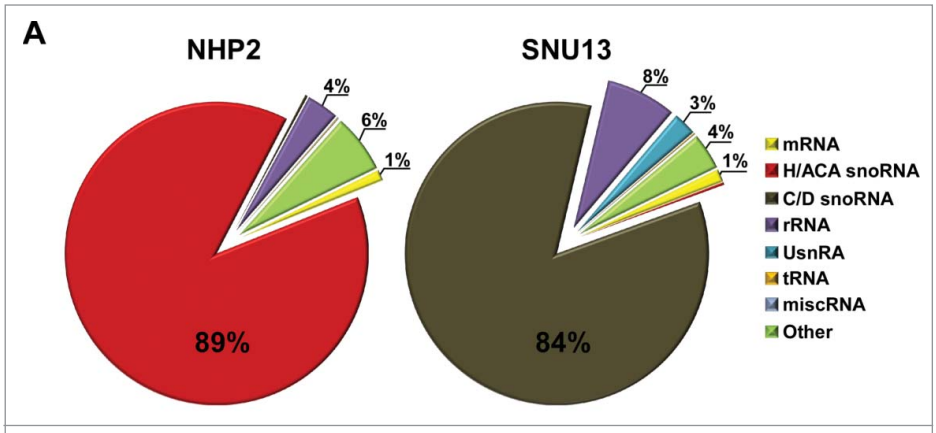


Figure 1. RNA Affinity purified with NHP2 and SNU13 tagged proteins. (**A**)The specificity of the affinity selection by NHP2 and SUN13 tagged proteins. RNA from the beads (1/10 of the amount loaded was subjected to primer extension with a sequence-specific probe (**Table S1**), and separated on a 6% polyacrylamide denaturing gel. The identities of the tagged protein are indicated and the tested RNAs are listed below the image. (**B**) Purification of RNAs associated with NHP2 and SNU13. Purification was performed using 2×10^{11} cells, as described in Materials and Methods. The purified RNPs were de-proteinized, and the RNA was separated on a 10% polyacrylamide-denaturing gel and stained with silver. The size of the pBR322 DNA-*Msp* I digest is indicated. Total RNA (1 μg) was used as a marker. Beads, the RNA extracted from the beads after the last step of purification.

Almost 90% of the reads belong to the expected snoRNA class with almost no contamination from the other snoRNA family (i.e. no C/D in the NHP2 library and no H/ACA in the SNU13 library) (**Fig. 2A**). Additionally, contamination with the most abundant RNAs (rRNA and tRNA) was minimal (**Fig. 2B**). Based on the number of reads that matched the genome (between 20 to 30 million reads), we believe that we identified the complete repertoire of snoRNAs in the cell.

Next, the reads were imported and visualized in the IGV genome browser (**Fig. 3A**). To observe the distribution of reads

within a given cluster, 3 different genomic clusters were plotted, and read distribution along the respective coding regions is presented. The results indicated that although all the snoRNA coding regions are represented, the distribution of reads was not uniform along the coding regions and certain portions of the molecules were severely underrepresented. The results clearly demonstrated that whereas the reads from the H/ACA molecules covered the entire coding region of the molecule, the reads for C/D snoRNAs were fragmented. This abnormality might be the result of the alkaline hydrolysis that was performed prior to the generation of the library. We observed that the fragmentation increased the number of reads per molecule, but the distribution of the reads was distorted. The bias observed for the C/D molecules may result from the fact the C/D snoRNA are composed mainly of single-stranded RNA, and only a very short stem is present at the termini. As a result, these snoRNAs are more sensitive to the fragmentation. In contrast, H/ACA RNA forms stem-loop structures, and thus, most of the RNA is present in dsRNA configuration except the pseudouridylation pocket and the apical loop. H/ACA molecules therefore seem to be less susceptible to degradation. Interestingly, this bias was not observed in our previous study[22] when we prepared a small RNP library, most probably because the protocol did not include the alkaline hydrolysis step. However, the fragmentation resulted in a greater number of reads per molecule (our unpublished results).



B

	NHP2	SNU13
Total	**23,135,883**	**34,218,933**
Total Mapped	**21,167,526**	**33,519,246**
mRNA	242,937	466,956
H/ACA snoRNA	18,779,964	92,638
C/D snoRNA	58,647	28,232,213
rRNA	730,148	2,518,290
UsnRNA	6,619	833,841
tRNA	25,490	48,468
misc RNA	3,182	12,087
Other	1,320,539	1,314,753

Figure 2. The quantities and identities of snoRNAs selected by NHP2 and SNU13. (**A**) The percentage of different RNAs molecules among the reads; 91% and 98% of the reads were mapped to the genome from NHP2 and SNU13 libraries. (**B**) The number of reads for each of the RNAs presented in panel A.

A very striking finding was the variation in the number of reads for the different snoRNAs ranging from 2 million counts to thousands of counts per molecule (**Table S2**). To examine the correlation between the number of reads and the abundance of the RNA, a primer extension experiment was performed. The results indicate a correlation for the H/ACA but not for C/D snoRNAs, reflecting the bias introduced due to severe degradation of the C/D during alkaline hydrolysis, as discussed above (**Fig. 3B**).

The extended repertoire *L. major* C/D and H/ACA RNAs

Next, the RNA-seq information was used to describe the repertoire of *L. major* C/D snoRNAs. 80 C/Ds snoRNAs were identified ranging in size from 70 to 150 nt (**Table 1**). The 5′ end of the molecule is indicated 3–5 nt upstream of the C box, and the 3′ end is located 2–5 nt downstream of the D box. Several long C/D molecules were revealed, and among these is LM20Cs1C2, which is predicted to guide Nm on U6. This molecule has no target on rRNA and may target other RNA classes (possibly other snRNAs or tRNAs).

Previously, we described 64 C/D molecules in *L.major*, and 30 *T. brucei* homologues were assigned.[16] Among the 80 C/D molecules identified here, 65 have homologues in *T. brucei*. Homology was determined solely on the target sites, since the sequence of the C/D outside the target site is very variable.

The proposed targets of the C/D snoRNAs are presented in **Figure 4**. Of these, 92 Nms 88 sites were identified only on rRNAs. For 8 molecules, we could not predict a target. Our target search was stringent, requiring at least a 10 bp duplex.

Eight molecules appeared to be *Leishmania*-specific, since they lack homologues in *T. brucei*, and they do have predicted targets in *Leishmania*. We cannot, however, exclude the possibility that the *T. brucei* homologues for these molecules exist but have yet to be identified. Only 21 of the *L. major* C/D snoRNAs are proposed to have 2 targets based on at least 10 nt complementarity to each of the target RNAs. Note that most of the predicted target sites are on rRNA. Searching for snoRNAs that can target Nm on snRNAs revealed a single target (see Discussion).

Among the C/D molecules, we identified highly abundant ones such as LM5Cs1C2, LM5Cs1C3, LM5Cs1C4, LM18Cs1C3, LM22Cs1C2, LM23Cs1C2, LM25Cs1C4, LM26Cs1C1, LM33Cs3C1, LM35Cs2C1, LM35Cs3C4, LM35Cs3C6, LM35Cs3C5 (**Table S2**). Since the *T. brucei* abundant snoRNAs were implicated in rRNA processing, we anticipated that their *L. major* homologues have similar functions (see below).

Next we analyzed the H/ACA RNAs that are associated with NHP2 and identified 81 molecules (**Table 2**). Our previous

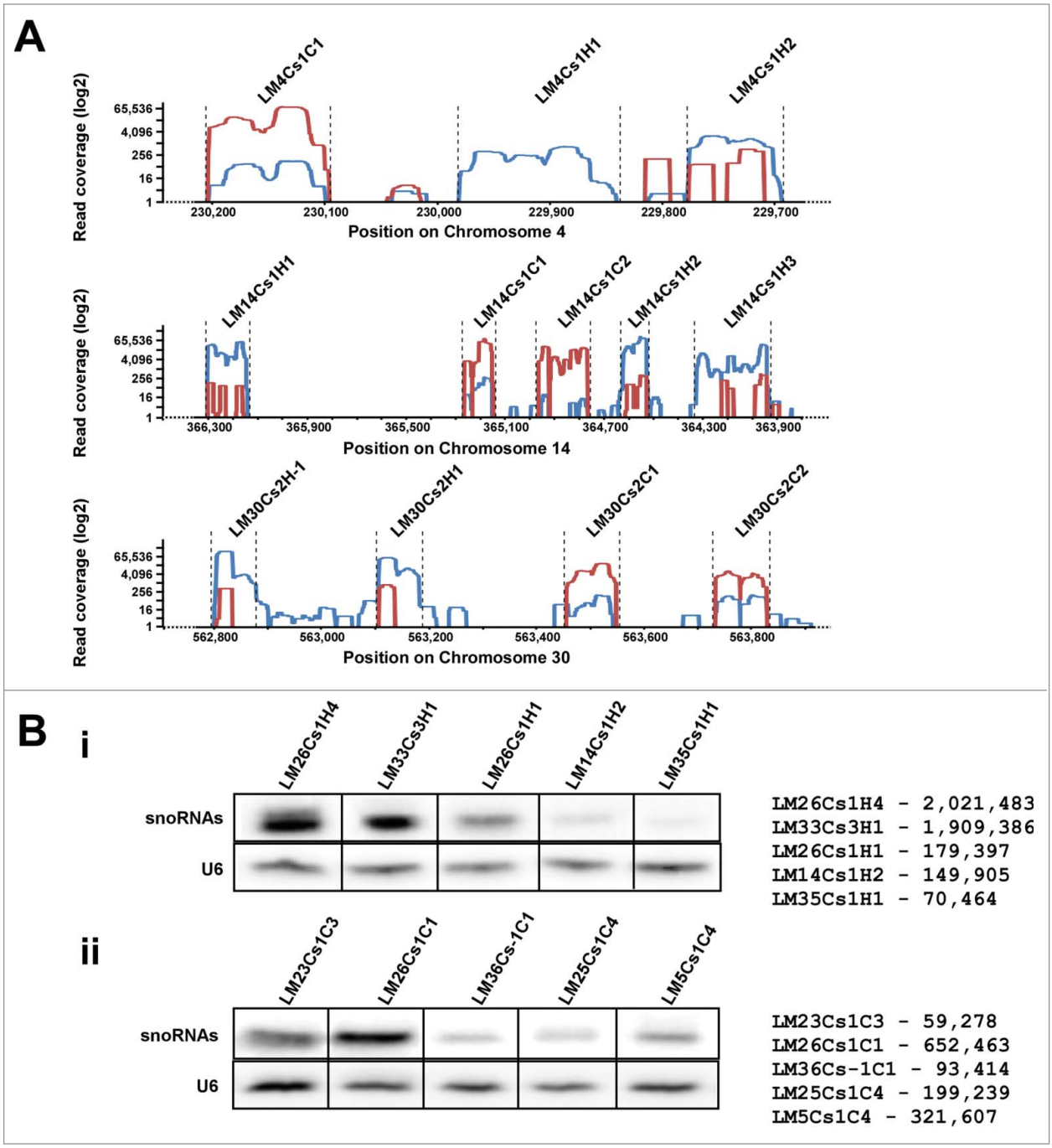


Figure 3. (**A**) Schematic presentation of the reads in distinct snoRNA clusters. The reads from the NHP2 and SNU13 are shown in red and blue, respectively. (**B**) Primer extension to validate the amount of snoRNA. Total RNA (10 μg) was subjected to primer extension. Numbers of reads corresponding to each of the snoRNA species tested are presented.

study[17] identified only 37 H/ACA RNAs. The RNAs ranged in size from 70 to ~300 nt. Of the H/ACA RNA only 67 have homologues in *T. brucei* or other organisms such as human, yeast and plants. 15 of the molecules are *Leishmania*-specific. Interestingly, the size of the H/ACA is much larger than C/D snoRNAs and molecules of sizes as large as 192 and 265 nt were also detected.

In most cases, an A is present 1 nt upstream of stem I (94% in *T. brucei* and 67% in *L. major*). In *L. major*, C can also appear in this position (about 30% of the time), while G and U are rarely found. Stem I is usually perfect and can range from 4 to 8 nt in length. In most cases, stem I is 6 to 7 bp long, and compensatory changes are often found to support the integrity of the stem. The pseudouridylation pocket varies in size from 12 to 17 nt. Stem II

Table 1. The sequence of the L. major C/D snoR N As. The boundaries of the molecules are based on the R NA-seq data of the R NA selected with S NU13 The boxes are indicated in bold and the domain of complementarity to the target is underlined. The homolog in *T. brucei* and the targets are indicated, and the source for the identification of the molecule is given. D S, deep sequencing from the current study.

Name	Sequence	Homolog	Size	Target	
LM4Cs1C1	CGCA**UGAUGC**GACACAUCCCUUAG CACUCGAGUGAAGUUAGCCCUUCA AU**CGGA**CCCAUUCACAUGAGCGAC UGCUUUCAGUUCCGUUUCAAGUUC AAGCCUUGG**CUGA**C	TB11Cs4'C1 (29.3%)	110	LSU3-A628	DS-SNU13
LM5Cs1C1	AUCGG**UGACUU**CUCCUCGACACGC UAAGCACAUGGAGAAC**UUGA**CACG AGUA**UCAGGA**GCCCCCUGACUGAG AGACUAUCAUGCACCACU**CUGA**GG	TB9Cs3C1 (54.2%) LM5Cs1C5 HuU32A AtSnr21 ScsnR40	96	SSU-G1623	Liang_et_al_2007
LM5Cs1C2	ACAGA**UGAUGA**UUGACUGUAACAU CACAGACU**UUGA**GUCGCGAUGAUA GCAACCAUGUCGCCCCAGU**CUGA**C G	TB11Cs2C1 (70%) HuU31 AtU31 SCsnR67	73	SSU-U1833 LSU3-G1046	Liang_et_al_2007
LM5Cs1C3	ACA**UGAUCG**ACACCUAGGCUGAUG UAAAGCCGUCGCAGAUGGACGUUG AGUGGCGCACGCCGUGAAACCAAC CCCGCCUCGU**CUGA**UCG	TB11Cs2C2 (59.5%)	89		Liang_et_al_2007
LM5Cs1C4	GCGGG**GGAUGA**CCGACAACACGAC AACGAAGCACAGUUUGAG**CUGA**UG CAUCGC**UGAUGG**ACAACGUCACAC AUGAGCUAACUCUG**CUGA**CGC	TB9Cs3C2 (56.1%)	93	SSU-A2021	Liang_et_al_2007
LM5Cs1C5	CGCA**GGAUGC**ACCAAACUGAUUCA GGCGUGCGGAUGCACGCAUCAGGA GCCCCCUGACUGAGAGACUAUCAU GCACCACU**CUGA**GG	TB9Cs3C1 (54.2%) LM5Cs1C1 HuU32A AtSnr21 ScsnR40	86	SSU-G1623	DS-SNU13
LM14Cs1C1	CGUUG**UGAUGU**CAACCUCACUGGA CCAUUAUUGCAAUAUACGAAUGCC UUUAGAUGCCCCGCAUCGAGGAAG AGACCCCAAUCGAGAAAUCCGCAG AGAAGGA**CUGA**ACGCU	TB7Cs1C1 (35.4%) HuU61 AtU61	112	SSU-U1777	Liang_et_al_2007
LM14Cs1C2	CUCCA**UGACGA**CACACCACUAUUU GCACGUCAGU**UUGA**CUUAUAAUUU ACUCG**GCAUGA**UACAAGCAUAGCU GUCCCACCCCCACCCACACGCACA UGCA**CUCA**CACUC	TB9Cs5C2 (40.1%) AtSnoR72Y ScsnR72	109	LSU5-A927	Liang_et_al_2007
LM14Cs1C3	**UGAUGU**GCAAGUGGGGUUGAGUU UGUGGAGGGGGUGCGUGUGUGUG UGUGCGGGGGUAUGGGUUUUA**CU GA**CGA		74		DS-SNU13
LM18Cs1C1	CGCUA**UGAUGA**CAAACAUCUCCUU AACGACGAGCGGA**CCGA**CACGCAC CCAUGCGGACAGUUCGAAUGUGUA CAAAUG**UUGA**GCC	TB10Cs4C3 (77.9%) **SSU-C2059** HuU43 AtU43 ScsnR70	85	SSU-U2048 SSU-C2059	Liang_et_al_2007
LM18Cs1C2	GCA**GGAUGA**GAGACAUUCAACGGA UCCCAAUCAGAGCACUGUGCA**CUG A**AACUCCGUCC**UGAUGC**UAAUCC**C UGA**CGC	HuHBII-180A SCsnR76	90	LSU3-C443	Liang_et_al_2007
LM18Cs1C3	AGC**UGAUGC**CAAACACUCGUAGUGC GCGU**CUGA**CGCUGCG**UGACUGA**UAC AAGACGAUGCGGAGCCGGGAAGACA UCCCGCGCCUCUUUG**CUGA**CC	TB10Cs4C4 (48.8%)	96	SSU-G1865	Liang_et_al_2007

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Name	Sequence	Homolog	Size	Target	
LM20Cs1C1	CGUG**UGAUGA**UACACCAAAGCAGG GAAUCUCUUG**UCGA**GCGGCCGGC GCGCGUCGCCGGCGUGGUGACAU GACACACUUUGAGUAACCGAAUCC AUUCAUUGAAUUGC**CUGA**CGC	TB8Cs1C3 (69.4%)	115	LSU3-A570	Liang_et_al_2007
LM20Cs1C2	AAA**UGACGA**AUGUGGCAUCUCUCC ACGGACACCGCAUUCGCACUGCGG CGCGUGACAUCACUGUUGCCUUCG AACCUAAUACCAGGG**CCGA**UGUGG GC	TB8Cs1C4 (46.6%)	98	U6-A17	Liang_et_al_2007
LM20Cs1C3	GCG**UGAUGA**GCUACAGGUCUACGA CGAACAACUCGU**CUGA**UCACCCUU GUUGAUGACCCCACACACCUUUUG CUUCCUCA**CUGA**CU	TB8Cs1C1 (67.7%) **SSU-G2151** AtSnr23	86	LSU5-G1626 SSU-G2151	Liang_et_al_2007
LM20Cs1C4	GCA**GGAUUA**CCACACCAACGAGUU UCUGAUGUAAUGAGU**CCGA**GCCCU UCCGCGGCGACGAGUCGAUAACGC UGUCAA**CUGU**GCAAUGAGG	TB8Cs1C2 (30.8%) AtU57 HuU57 Scsnr51	91	SSU-A98	Liang_et_al_2007
LM20Cs1C5	ACAUGAUUCGACACCU**GGAUGAA**C UUGUCCUCCGCAGAGUGCCGCUGC UGCUCU**CUGA**AGAGACGCGGUGUG CACGAGGCUGCAUGAAAACAUC**CU GA**UGACC		103		Liang_et_al_2007
LM20Cs2C1	CGACA**UGAUGA**CGUACAACGUGAA AAUACAAAUUCCUAACAAAACGG**CU GA**AGACGCUCUUAUUUCUCAAGAG AGGAAGAAGAGAAGGAGAUGCAUA UUUUCGGUUCUGCUAUGCU**CUGA**A GU	TB7Cs2C1 (38.2%)	123	LSU5-U1659	DS-SNU13
LM22Cs1C1	GGCUG**UGACAA**UGACCCACUUACG ACGGUCUU**AUGA**CCACGAUCCCGG CCAC**GGAUGA**AGCACGAGUGCUAC GUCUGUACAACAAGGGGGCGCGCC G**CCGA**UUG	TB9Cs2C1 (60.2%) LM35Cs3C6 HuU35A AtU35 SCsnR73	104	LSU3-C1397	Liang_et_al_2007
LM22Cs1C2	ACG**UGAUGA**GAAUUCAAGCUUAGG ACACCUU**UGGA**UGCGGCGCGUGCC GCAGG**UGAAAA**UAGCGAGUUUGUU ACACAUUCGAUUCGUACU**CUGA**CG	TB5Cs1C1 (55.7%) HuU4197 AtsnoR10	96	LSU3-U1077 LSU3-C1159	Liang_et_al_2007
LM23Cs1C1	ACA**UGAUUA**CGAUCAAGGACAUCU GAGUCAAAACCAAAGUCACUGU**CU GA**ACGGAACGACUGUCGAUUGUGU CCCCCGUGU**CUGA**GC	TB8Cs3C2 (54.3%)	87	SSU-G1550	Liang_et_al_2007
LM23Cs1C2	GUGAA**UGAUGA**UGCGUGUAUUCUG CAAUG**CGGA**CUCCAC**UGACGA**GGA ACAAUACUACUACUAUGACUAUUG GCAU**CUGA**CGC	TB8Cs3C3 (64.1%) **5.8-G75** HuU96a AtSnoR39BY	83	5.8-G75 SSU-C18	Liang_et_al_2007
LM23Cs1C3	GCUGUGAUUC**UAAUGA**AACAUCUU UGCUACAG**GCGA**UGCGCGCUGCG GCGUGCG**AGAUGA**CCUUACUGUUU CCAAUCUCCAACCAAGAUCA**AUGA** GCUGCGGCUGC	TB8Cs3C1 (57.7%) HuU24 AtU24 ScU24 **LSU5-A1539** HuU76 AtU24 ScU24	106	LSU5-C1527 LSU5-A1539	Liang_et_al_2007

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Name	Sequence	Homolog	Size	Target	
LM25Cs-1C1	GCGG**UGAUAG**GCAGGGUCCUGUG GUGUGUUGGUGAUGGGGCGGAGA AUGUAAAGGAAAGCCAAGAUGCGG AGAAGAACUGUGA**CUGA**GCAU	Telomerase RNA	2112		DS-SNU13
LM25Cs1C1	GCGAC**UGAUGA**GAAACCUCAUGUU ACUGACACCUCUU**CUGA**CACUGUU GG**UGACGA**GCACACAUUUGUAUAG UUAUCCCUGU**CUGA**ACGC	TB11Cs1C2 (62.4%) **LSU3-G1231** HuU60 SCsnR48 At2783 **LSU3-G1253** HusnR38A AtSnoR38Y SCsnR38	90	LSU3-G1231 LSU3-G1253	Liang_et_al_2007
LM25Cs1C2	ACG**UGAUGA**GCCAAUCAUGUUUCA CUCUUGU**CUGA**ACCACCCGUUG**UG AUUA**CACGCACACCCGCGAGGUGU GG**CUGA**CCC	TB3Cs1C1 (40.4%) ScSnr41	81	SSU-G1478	Liang_et_al_2007
LM25Cs1C3	CCACA**UGAUGU**GGAACACACGCAA CACUCCGAUACCG**CUGA**CCGUGCU UG**UGACUGA**CACCACAUUGCGGA**C UGA**CAC	TB11Cs1C3 (69%)	78	LSU5-A955	Liang_et_al_2007
LM25Cs1C4	CACG**UGACGA**GUAUGCAACACACA ACUAGGUAAGAGCCGU**CUGA**ACGA CAUACCUCCGUCCUGUGGAACACC AACGCCUGGGGUCUGCCACCGCAA UGGCUGUGCCCUGAGU**CUGA**UGU		119		Liang_et_al_2007
LM26Cs1C1	GCA**UGAUGC**GAAAAUCGAGGCAUU UGU**AUGA**GCCCGCAACUGUGAUU**U GAACU**CCGUGUACCCAAAGCUACC UACAUACUAUACACGGGA**CUGA**GC	TB6Cs1C3 (66%) HuU15a AtU15 SCsnR13	96	LSU3-A527	Liang_et_al_2007
LM26Cs1C2	GCG**UGAUGG**CAUUGCGAUUUAAUU AUACAC**CCGA**GGCGCGCACAGCGC CGU**GGACGA**GAACCGGAGUGA**CUG A**CUG	TB6Cs1C2 (48.3%)	76	LSU3-A382	Liang_et_al_2007
LM26Cs1C3	GCG**UGAUGA**UGCUGCAGAGUCUGA GUGUACCUUUC**UUGA**GCGUGCGCU GGUGCGCGCGUGGUGAGA**UGACG U**AUUCUCGCUGUGGCUUGCGCCGC GUGCGCUGGCCGAGU**CUGA**UG	TB9Cs1C1 (49.3%) HuU34 AtU34 ScsnR62	116	LSU3-G71	Liang_et_al_2007
LM26Cs2C1	UGAA**UGAUGG**ACUGACCUGUGGAA UCUACUGCGUUCUUU**CUGA**GCGUU GCGG**CGAUGC**GAGCUUCAUUGAGU UGCAAUCUUCAACGAAA**CCUA**UGU **CUGA**CUGC	TB10Cs7C3a/b **5.8S-A43** AtSnoR9	104	5.8S-A43 5.8S-G36	Liang_et_al_2007
LM27Cs1C1	GCG**UGAUGA**AUACUAUUCACAUAG UUUCCUGUCAGU**CCGA**GCACUGCA **UGAGGU**CAUAGCAUUUUCUCAACU CAGCUCAC**A****CUGA**CGC	TB11Cs4C1 (64.6%) **LSU3-A1384** HuU29 AtU29 ScsnR71	88	LSU3-A1372 LSU3-A1384	Liang_et_al_2007

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Name	Sequence	Homolog	Size	Target	
LM27Cs1C2	GAA**UGAUGC**ACUCAUCUAUGUUGG UCCAAACAGU**UUGA**CUGGCGCA**UG AGAA**GGUACACAUACAAAUCACCU UUCGG**CUGA**CGU	TB11Cs4C2 (69%) **LSU5-A858** ScsnR39 HuU32a AtU51	84	LSU5-U847 LSU5-A858	Liang_et_al_2007
LM27Cs1C3	CCC**UGAUGG**AGAAACAAUCCUUUU UGUGCGCGAGCGAGGCU**AUGA**GC GCACAUUU**UGAGCC**AACAAACAAAA CUCAACAGGU**CUGA**GAC	TB11Cs4C3 (59.9%)	89	LSU3-G655	Liang_et_al_2007
LM29Cs2C1	CGCA**UGAUGA**UCAUCCAAGUACAA UCGCAUCUAUUUUU**CUGA**ACCGGA CGUGAAUUGAGACUUGCUUCAUUU CUUGCGUUCACAUGUGAUGUGGAC GUCU**CUGA**CUCGGA	TB3Cs3C1 (50.9%) HuU50	110	LSU3-A95	DS-SNU13
LM30Cs1C1	AUG**UGAUGA**GUCACCGUGCAUUCC UGUGGUAU**CUGA**UGCAUCCU**GCAU GA**ACACGACAACUGCAACGAGCGC CCUUCGCGACGAAGUGGUGCGAA**C UGA**GU	TB6Cs1C1 (63.5%)	101	LSU3-C1248	Liang_et_al_2007
LM30Cs1C2	UGG**UGAUGU**CACACACGAUUCUAU UACCUGUGGGAU**CUGA**UCAAGUGU UGUAU**UGAUGA**AAAGCAGAACGAU GUACGCGUGCAGCGUGUUAGA**CUG A**GG		99	SR2-C21	Liang_et_al_2007
LM30Cs2C1 (LM30C4C1)	ACA**GGAUGA**UAUCUAUUCUUUGAA UGUUCUUCG**AUGA**CGGGCCUCGG CCUG**CGAUGA**GAAGCAUAAUAGAU GAACCCUUUAACU**CUGA**AA	TB6Cs2C1 (59.3%) HuU36A AtU36A ScsnR47	90	SSU-A668	Liang_et_al_2007
LM30Cs2C2 (LM30C4C1A)	CAC**UGAUGA**AUUCGAUUCUUUGAA UGUUCC**UUGA**CCGCAGUCUUUGGC UG**UGAUGA**AAGGCUUUAUCGAUGA ACCCUUUAACU**CUGA**GCU	TB6Cs2C1A (59.8%) HuU36A AtU36A ScsnR47	90	SSU-A668	Liang_et_al_2007
LM31Cs1C1	GCA**UGAUGA**GACACUAAUGGAUCG CUGACG**CUGA**GGGCACGCGCCCAA AUGCAAGACAAGACCGUUACUCGU UGCUCAACCU**CUGA** UG		88	LSU3-A1185 SSU-C1984	DS-SNU13
LM32Cs1C1	GGGUA**UGAUGC**GUUGACAUUGACA UAGCAAUUUGCG**AUUGA**UUCACUU CGUGAUCAUGAUACCAUUAUUUAC CUU**CUGA**U	HuU16 AtU16 Scsnr87	80	SSU-A479	DS-SNU13
LM33Cs1C1	GCA**UGAUUA**CAUUGCGUCUUCCAC CUA**ACGA**CCGUCGAUGAUUACAUG CGAUGCAAGUGACUACUAUACUAC A**CCGA**UAUCCGAGGAUGACG	TB10Cs3C5 (69.8%)	92	LSU3-U710 LSU3-G703	Liang_et_al_2007
LM33Cs1C2	CUG**UGAUGA**CUGGAUGAGUUAUUG UUUGCACAUUUA**CCGA**GCCUUCGU GGCCCG**UGAGCG**ACCGUAUAACUU CUAAGAACACCAG**CUGA**UUC	TB10Cs3C4 (65%)	92	LSU5-U1107 LSU5-U1071	Liang_et_al_2007

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Name	Sequence	Homolog	Size	Target	
LM33Cs1C3	CGUG**UGAUGA**UGUACAGAGUGUGC GAAGACGCAUGUCGCGUAGCCGCC GGCUGUGCGCGCC**UGACGA**ACUAC UGUAUUUCACGGGUGAACAAUCU**C UGA**UGCG	TB10Cs3C1 (63.7%) Hu4468 ScsnR52	103	LSU3-U1359	Liang_et_al_2007
LM33Cs1C4	CGA**UGAUGC**AGACAGAUCAAUACU CACU**CUGA**CGCGUGUGUGGCGGU GGACCGCUGCGCACUGUGCGGAUA CAAGGAUAACAUGCACUACCAG**CU GA**UGG	TB10Cs3C2 (51.6%) **SSU-U1621** HuU33 AtU33 ScsnR55	100	LSU5-A235 SSU-U1621	Liang_et_al_2007
LM33Cs2C1	UUUGC**UGAUGA**UGCGUGAGGAGC GCUCCUCUCAUCCUCCGAAAGAGC AGAAUUAACCAA**CUGA**CGCUUUCA CGGCGCCGUUGCCGCCGCGCCCC UCUGCCCCCCCCGGCCGCCCUCGCA GGCGUCGGUGC**UGCUGA**GCGGCC GCUGCGGCUGCGGUGCGUGCGUG AUGCCGAGCGGUG**CUGA**AU	TB10Cs2C1 (36.9%)	183	SSU-U8	Liang_et_al_2007
LM33Cs2C2	ACCG**AGAUGA**AGCUGAUAUGGUCC GUGUUU**CAGA**UCCGUGUGACGCAC AUUUCUCUCUUAGUACAUAUCUU**C UGA**GU	TB10Cs2C2 (73.5%) HuU18a AtU18 ScU18	77	LSU5-A681	Liang_et_al_2007
LM33Cs3C1	ACA**UGAUGA**GACUCUUUUUUCGCU GCCA**CUGA**GCGCCCCAUGCGUGG GGCGGCACG**UGAUGC**ACACAUGGA UACACUACACUGUAGAACUGUCGU UUCU**CUGA**CGG	TB10Cs2'C1 (58.9%) **SSU-A512** HuHBII-234 AtHBII-234 ScSnr87	106	SSU-A512 LSU3-A604	Liang_et_al_2007
LM34Cs1C1	UUUA**UGAUGA**UAAAGUCCUCUCUA CUACUACUAGCGU**CCGA**UGCUUCG C**UGACGA**UUACCGUUUGAACUUCA UCUUUCGCUCCGAU**CUGA**GG	TB9Cs2C5 (47.9%) HuU80a AtU80 snR60	92	LSU5-G959	Liang_et_al_2007
LM34Cs1C2	GCG**UGAUGC**CAUGCAGGUAUGCUC CUUUGU**CGUA**GCGCCCCGGCGCU GUGCCGACAGACAUACCACACUCU AUCCCAAUGCUUCAUUCACUUCAG CUG		87	SSU–A912	DS-SNU13
LM35Cs1C1	CU**GCAUGA**UUUCAUCUUAGCGUCA AGAAACAUCG**CUGA**UUGUUCUUU**G GAUUU**AAACGCAAGCCGUAAGCAG U**AUGA**UCG	TB9Cs4C3 (63.9%)	80	LSU3-C359	Liang_et_al_2007
LM35Cs1C2	UGAA**UGAUGA**UCCACGUUUAACAC AAAUUCGACACUGAGU**CGGA**GCAA UAC**CCAUGA**CGCACCGUGUCUUGA CCGUGGAGGCGGCCCUCUGUGUC GCCUCUGCCCCCUCUCUCGCGGAU GGGGCGGACGCGCGCAGGGGGUA CA**CUGA**UG	TB9Cs4C2 (51.3%)	150	5.8S-A162	Liang_et_al_2007
LM35Cs2C1	CGAGC**UGAUGA**UGAUCAACAAACU UCGUAGGGUAAAAU**CUGA**UCACUC CGA**CCAUGA**CGCUAUGGAAGUACU GCA**CUGA**AAG	TB9Cs3C3 (44.4%)	82	LSU3-U1419	Liang_et_al_2007
LM35Cs2C2	CCGUC**UGAUAG**CCGCUGUGUAGU GCCGUAUCGCUU**GUGA**GUGUCAU**C AAUGA**AUUGUGGAUAGUGCCGGAA GUGGUAGG**CUGA**GGC		86	SSU-C451	Liang_et_al_2007

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Name	Sequence	Homolog	Size	Target	
LM35Cs2C3	GCACG**UGAAGC**AGGUAAAAUAAGC AACCUGUUUAUCGGUGCCGCCCAU GUGGUCUAUCAUCAA**CUGA**UGGAA **UGAACA**GGUCUUCAAGCUCUGUAA GAGCCCGCGUCGAUGCCCCCCAUC AUUGCCAUUAGCUUGGACUGG**CUG A**CUU		148		Liang_et_al_2007
LM35Cs3C-1	ACA**UGAUGA**CUUGACAACACAAUC AUUUAACAGCAACAU**CUGA**UAGACA UCAAACCGUCU**UGACGU**GAAAGCG UUCACAAUUUCU**CUGA**CAG	TB9Cs2C6 (60.4%)	92	SSU-U661	DS-SNU13
LM35Cs3C1	CGCUG**UGAUGC**GAAUACCAACACG AACGAGAUUAACAGCAACAU**CUGA** UGCGUGCGCCAAAGCGCAAAC**UCA UGA**CGCGAACUCGUUUUUACAACA G**CUGA**UGC	TB9Cs2C6 (61.7%)	104	SSU-U661	Liang_et_al_2007
LM35Cs3C2	UCG**UGAUGA**CAUCAACCGACUGGA GUCU**CUGA**CAGGCCUCU**UGAUCGA** AAACAUCGCGUUGGAAUACUCUUC UUCUUUCCCCGU**CUGA**GGC	TB9Cs2C7 (71.6%) **LSU3-U667** HuU52 AtSnor37 ScsnR78	91	LSU3-G641 LSU3-U667	Liang_et_al_2007
LM35Cs3C3	CGAG**UGAUGA**AACUGCCGAAUCCG UGUUUCAG**CUGA**GCCGUGC**UGCU GA**CAGCGUUUCUUGAUCUGUUUGA CUU**CUGA**CGCG	TB9Cs2C2 (69.9%) **LSU5-C695** AtSnoR58Y HuU104 ScsnR58	82	LSU5-C695 LSU5-A678	Liang_et_al_2007
LM35Cs3C4	CGCG**UGAUGC**ACAACAAUUCUCAU GGUCCAAACAGGUA**CUGA**CUGUCU GUUG**UGAUGA**UCACGGAGUAUGCG AAUCACCUUUCGGGA**CUGA**GGG	TB9Cs2C3 (63.6%) **LSU5-G856** HusnR39B AtsnR39B ScsnR39B	94	LSU5-G856 LSU5-U845	Liang_et_al_2007
LM35Cs3C5	GCG**UGAUGC**ACAGCUGUUUGACCA UCAGAUGCCCCCAUGAGGACACCC AAACACAGACCUGCA**CUGA**UG	TB9Cs2C4 (59.8%) HuU25 AtU25 ScsnR56	69	SSU-G1829	Liang_et_al_2007
LM35Cs3C6	CCGC**UGAUGA**GGACCCACUUACGA CGGUCUU**AUGA**CACAACACAUCCC GACCAU**GGAUUGA**AGUCAAGUGUU CAGCAAACAACUAGGGGCGCGCCA CAGGAUUUCCAGCCGGCGCGCCCU GUGA**CUGA**GGUA	TB9Cs2C1 (57%) LM22Cs1C1 HuU35A AtU35 Scsnr73	132	LSU3-C1397	Liang_et_al_2007
LM35Cs3C7	ACG**UGAUGA**GGCUGACCCUGCUCC GCUA**CUGA**CAUGUGAAACCG**UGAU CC**GAGACUCUUCAGGGCAA**CUGA**C GGC	TB9Cs4C1 (74%) HuU38a AtU38 ScsnR61	75	LSU5-G1190	Liang_et_al_2007
LM36Cs-1C1	AGAGA**CGAUGA**UCAACCCAUUUAU GCGUUCUGUUUGAG**CUGA**UUUUAU UCACUGAAGCAUCACACUGGCCUU UUCAUGGUCUGCCUCGA**CUGA**UCU GCC		99	LSU3-C554	DS-SNU13
LM36Cs-1C2	G**UUAUGA**UUCGAACCUAUCCAUGC UUUCCGUGACUUCACCCUUCAUGA GCAUACACAGAUACACCAUUGUCA UAGUUACA**CUGA**AC	TB10Cs7C2 (66.3%) HuU36C ScsnR63	86	LSU3-C502	DS-SNU13

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Name	Sequence	Homolog	Size	Target	
LM36Cs-1'C1	UCA**UGAUUU**CAGCAAUGCAAUGUA UCUCCGUUACCGGACACGGUCGCU GACGACAACCAUUGACCCAAAUAAC CAAGAUCUGCU**AUGA**AU	TB10Cs2"C3 (63%)	90	LSU5-G1524	DS-SNU13
LM36Cs-1'C2	CGCA**GGAUGA**CUAAAAUGUUUCAU UGUAGUGUGCGCUGAGCUGGACG CCUGCUGAGAAAUCGAACAUAUCG CAAAUCACUCCACU**CUGA**GG	TB10Cs2"C1 (51.9%)	91	SSU-G1647	DS-SNU13
LM36Cs1C1	CACGC**UGAUGA**UCUGUCUAUUCGC AUCGCGACACG**CUGA**CACACACAA CCC**GAGUGA**CAGGCGAACGGAACC AACAAUCGAGAAAGAACAG**CUGA**G U	TB10Cs1C3 (67.6%)	97	5.8S-U7 SSU-U1599	Liang_et_al_2007
LM36Cs1C2	GUG**UGAUGA**GUCUGCUAAGAUGUA CGCAACAUUUACAAUUGGAAGACG AGU**CUGA**ACAC**UGAUGC**ACACACU GAUAAACGGCA**CUGA**CGC	TB10Cs1C4 (63.3%) AtU15 ScsnR75	90	LSU3-G534	Liang_et_al_2007
LM36Cs1C3	CGC**UGAUGA**CAAUCCUUCACCACA AAUGUAACUCGGGAAAA**CUGA**ACC ACCACGUGUGGG**UGAUGA**GCCACC GUACGGAACAAUCGUUAGAUAGUA AGU**CUGA**GC	TB10Cs1C1 (55.7%) **LSU3-C583** Atsnor44 HuU74 ScsnR64	105	LSU3-A591 LSU3-C583	Liang_et_al_2007
LM36Cs1C4	AUG**UGAUGA**CGUACGCUUUGGAAU UGACUGCCGACCUGCGCGGUGCG CCUCGCGCGCGUCGCGUGCUGUG CGGACAACGUGCAUUGGUGUGCAU AUCCUCUACUGUUACUCAAAAUAAA ACUU**CUGA**GGC		130		DS-SNU13
LM36Cs2C-1	UGCG**UGCGUC**UAAAUGAUGGCUUA UACGACAAACUAUUGAAGAAAA**CCG A**AUGACCGGCCUGUGGAGAGUAAA CGCCAAUGCGCUUUA**CUGA**UGGAC	HuU20	97	SSU-C2140	DS-SNU13
LM36Cs2C1	UUC**UGAUGA**UCAAUGAGAACAAGC AUAUAA**CCGA**CUGCGCUGCGCGUC AGCUCGCUGUCGUGCGCCG**UCAU GA**GAUGCAACCUCUAGUCCUUGAA AU**CUGA**CA	TB8Cs2C1 (64.9%) **SSU-A28** HuU27 AtU27 snR74	103	SSU-C38 SSU-A28	Liang_et_al_2007
LM36Cs2C2	ACGCG**UGAUGC**AUAAUUAAUCGAC CAAUAAU**CGGA**GCGUGCCGCCUGC GCGGUGGCGUGCGUGAAGUCAAAC CAAUGCAAUAUAUGUGG**CUGA**CUG	TB8Cs2C0 (60.2%)	96	SSU-U1979	Liang_et_al_2007
LM36Cs2C3	GCG**UGAUGC**AGAACAUACAACAAU CACCUCGGGGGGCUGCCUCGCGG UGGCUCCCU**CUGA**AGCGCCGCUGC GUGCGGCCACU**UGACUGA**ACCCCU GUUAAGCGUCAUCCG**CUGA**GU	TB8Cs2C2 (46.8%)	116	LSU5-U1371	Liang_et_al_2007
LM36Cs3C1	GA**CGACGA**CGACAACUGAACCUCA UUUACAAUACACAGUUGGUGAGAG CCUGUCUUUCGAUGAUGAUGCACG AGUUAAUCCAUUCU**CUGA**GUCU	TB11Cs3C1 (19.3%) HuHBI-43	94	LSU3-U560	Liang_et_al_2007

(*Continued on next page*)

Table 1. The sequence of the L. major C/D snoR N As. The boundaries of the molecules are based on the R NA-seq data of the R NA selected with S NU13 The boxes are indicated in bold and the domain of complementarity to the target is underlined. The homolog in *T. brucei* and the targets are indicated, and the source for the identification of the molecule is given. D S, deep sequencing from the current study. *(Continued)*

Name	Sequence	Homolog	Size	Target	
LM36Cs4C1a	GCA**UGAUGA**UACCCUACUUGUUCU AUUCAACAGAACACACGCACCAGCA CACGCCCUCGCACACGCACUCCUU UGUGUGCGUGCGCCGGUGUGUGC UGGCGUGGGUGCAUGAUCGAACAG CCUCGUAGCAAUACGUAUCGAGAA U**CUGA**UG	TB10Cs5C1 (33.1%)	151	LSU5-U1253	Myslyuk_et_al_2008
LM36Cs4C1b	AAGGA**UGAUGA**UACUCUCUACUUG CUCAUUUUUUUGCGAACAGAUGUG CAUGGCAUCCCCACCUCUUCUGUG GAUCCGCGCAGAACGCAAGAUUGG CGGAAGCACUGCUUCACUGAUGAG CUCCAUAGCAAUACGUAUCGAGAA C**CUGA**UG	TB10Cs5C1 (42.2%)	151	LSU5-U1253	Myslyuk_et_al_2008
LM36Cs4C2	**UGAUGA**GAGGCACCCCACGAGGGC GU**CAGG**GCCCUGCACAUUCUCUCU GUGUCUGUGUGCGUGUGUGCGUG UGUGUGGGAGGGGGAAGGGAGCC ACGCAGCCCCUCCCCCGUUCCCCU AUCCCUCUGCCAAAUGCCGAGCCA CGU**CUGA**		149		Myslyuk_et_al_2008

also varies in size, but a perfect stem of 4 to 7 nt is present immediately adjacent to the pseudouridylation pocket.

The targets for the *L. major* H/ACA are given in **Figure 5A**. For 12 molecules we were unable to suggest any target. For LM23Cs1H2, we could suggest a target on U1, but this molecule also has a predicted target on tRNA, suggesting that trypanosomatid H/ACA molecules may have a flexible pocket, and that a single molecule can guide the pseudouridylation on more than one substrate (**Fig. 5Bi**). Another example is LM36Cs-1′H1which can potentially guide a Ψ on U3 and on rRNA. And the molecules, LM27Cs1H3, LM33Cs1′H1, LM33Cs2H1 that can potentially target Ψ on 2 different rRNA domains (**Table 2**). An H/ACA (LM36Cs-1′H1) can guide Ψ on 3 different targets (**Fig. 5Bii**). An additional such RNA is LM26Cs1H6 which can potentially target 3 different Ψs on rRNA.

Interestingly, we identified 14 molecules which are longer than average (100 nt and more). Of these, 11 have a target on rRNA, but because of their size these may target modifications on additional RNAs (see below). Interestingly, long H/ACA molecules were not identified in *T. brucei*. However, each *L. major* long H/ACA molecule carries a region that is homologous to short *T. brucei* molecules. For instance, a portion of LM14Cs1H1 is homologous to TB7Cs1′H1, which targets a Ψ site that is conserved in evolution. In addition, a portion of LM14Cs1H3 is homologous to TB9Cs5H1 and the modification proposed to be guided by this RNA is also conserved in evolution. It is possible that these long molecules maybe the outcome of a fusion of more than one H/ACA molecule, and hence, may target more than one type of RNA. However, we could not find evidence that the long snoRNAs resulted from the fusion of snoRNAs located adjacent to the homologous *T. brucei* snoRNA.

The presence of 19 H/ACA snoRNAs which are found in *Leishmania* but not in *T. brucei* was surprising. Although, we have recently expanded the repertoire of *T. brucei* snoRNAs, this study identified 81 H/ACA in *L. major*, whereas only 63 H/ACA are known in *T. brucei*,[22] suggesting that the published *T. brucei* repertoire is not complete.

Genomic organization of snoRNA clusters and differences between *T. brucei* and *L. major*

Functional information can at times be derived from the genomic organization of the snoRNAs. One such an example is the SLA1 locus that carries SLA1, snR30,[20] as well as TB11Cs1C1 and TB11Cs1C2, which were demonstrated to be trypanosome-specific snoRNAs involved in rRNA processing.[22,23] We therefore examined the organization of the snoRNA clusters to identify clusters that may carry snoRNAs with special function such as rRNA processing. The *L. major* snoRNAs are organized in 49 chromosomal loci (**Fig. S3**) compared to the 23 loci previously described.[17] The majority (32) are mixed clusters with both types of snoRNAs. However, we also detected solitary snoRNAs (19 H/ACA and 10 C/D). In contrast to *T. brucei*, where the majority of the snoRNA clusters are repeated multiple times, in *L. major* only 22 loci are reiterated (**Fig. S3**). Interestingly, in some chromosomal loci, only certain portions of the cluster are repeated (such repeats are indicated by yellow squares in **Fig. S3**). For instance, only part of LM18Cs1 is repeated 10 times (the A repeat). Another complex genomic arrangement is seen in LM27Cs1, which is composed of 2 clusters, C and D, which appear in alternate order and are repeated different numbers of times in each position.

Trypanosome snoRNAs implicated in rRNA processing

The synteny analysis of *T. brucei* and *L. major* snoRNA clusters (**Fig. 6**) revealed clusters that contain the same set of

Table 2. The sequence of the L. major H/ACA snoRNAs. The boundaries of the molecules are based on the RNA-seq data of the RNA selected with NHP2 The AGA is shown in bold. The homolog in *T. brucei* and the targets are indicated, and the source of the identification of the molecule is given. DS, deep sequencing from the current study.

Name	Sequence	Homolog	Size	Target	
LM4Cs1H1	AUGAUGGC**CUUUC**CUCGACCGCUG UCUCCGCUGUUGCUUGCGCCACAU AGACGACCACCGCGCGUGUGCGCA CGCGUGUGUGUGCGUUGGUCGA**A CAAAAC**CCAUCAG**AGA**GUG	TB7Cs3H2 (40.6%)	114	LSU5-Ψ1664	DS-Nhp2
LM4Cs1H2	CACGUGCUU**GUGUAA**GCAGACUGU GCAGAGCUGUGCAUCCGUUGUGCA GUCUGU**GAUA**GCAGCAACGG**AGA**U GC	TB11Cs4'H1 (57.7%)	74	LSU3-Ψ1058	DS-Nhp2
LM5Cs1H1	CACUGGCCCUGUCACCUUGAGGGC GCCCGUGCCGCUUGUCGGCGCAU GCUGGGCGUACGGCCUCGAGUUG CUGCGGCGCGGGCGCACGUGCGU GCAUGUGCCGUCUGCGCUGCGAUU CGGGGGGCCACAGAGCAAGGGGU GCGAGCGCGCGGUGUGAUGCUCG GCGUGCUGCUCGCGCGCCUGACG CACCGCCGCGCGCCGCGGUUCCC GUUACGCAUAUCCUGUGUGCGCUC CAGCCCCGUCGGGGCCAUGACGAG UUCGCAACCAACCAUGCGUUAC**AC A**UUG	TB11Cs2C3 (57.5%) snR30	285		Liang_et_al_2007
LM5Cs1H2	ACGCGCUCACUGUAGUGCGCGUGC CGCAAGACCCACAGGCACCGUGCA UGAAACUGGAGCGCU**AGA**UGG	TB11Cs2H1 (64.4%) SLA	69	SL-RNA	Liang_et_al_2007
LM5Cs1H3	GCAGUACGAUUAAA**CAC**GGACGCG CCGCUGCAUACGCUUGGUGCUGGC GCCGUCC**GCCAGU**GUCGUACG**AG A**UGU	TB9Cs3H1 (65.4%) LM35Cs2'H2 HuU65 AtU65 ScSnR34	75	LSU3-Ψ1264	Liang_et_al_2007
LM5Cs1H4	GCAGUGCCGU**CAGGUA**AAGUGACG AUGGUGUGUCCCAUCCGCGCCACU G**GUUCA**AGCGGCACG**AGA**GCG	TB9Cs3H2 (64.5%) Hu1523 Atsnor77	69	LSU5-Ψ870	Liang_et_al_2007
LM8Cs1H1	UGAGCAGC**UUUCGC**CGAAUGCACG CUCACCACUCAUAGGUGCCUUCG**U AUAG**AACUGCCCU**AGA**UGU	ScSnR82	67	LSU3-Ψ597	DS-Nhp2
LM9Cs1H1	ACUGCACAGAGCAGAGGGUAAGAU AGUGAAUGAGUCACUGUCGGCUGA UGCUCACGUGCA**AGA**GAGCU		68		DS-Nhp2
LM11Cs1H1	CAUGAACGUUUCGUGUCUCCCGUG GCGUCCUUUACUCAUCGCACGAGG UAGGCAGAUAGCGUUC**AGA**GAUUU	TB8Cs6H1 (57.3%)	72	LSU3-Ψ1060	DS-Nhp2
LM11Cs2H1	AGCGAAGACAACAACAGGAGCAGC ACCCUCGAGAGAGCAACGAAAACG GCAGCGUUCUGGGAGACACCCACG CC**AGA**CA		79		DS-Nhp2
LM14Cs1H1	AACGAGUGAUUAGUCAGCUGUGCC UUGCCCUUGCGUGCGUGUGUGCG UGCCUUUCGCUUCUGAUCGUGUCU GUUUGGCUCGAUGAUGAGCGAUCG GCCUCGCGCCCUCACCUCUUUCAA AGUAGGCGGCUGCAGCGUUACAUC GCUCGG**AGA**GAU	TB7Cs1'H1 (36.3%) HuHU3741 Atsnor79 Scsnr191	155	LSU3-Ψ504	DS-Nhp2
LM14Cs1H2	AAGCGUUUCUUUGAGUCGUUGUGC GUCUGCACGCCUUGCGGGUACCUG UGCGGCGUGCCUGUGAUGCUUCAA UGAAUUAGGAAAGGCG**AGA**UGC	TB1Cs1H1 (37%) HuACA36 SCsnr36	94	SSU-Ψ1539	DS-Nhp2

(*Continued on next page*)

Table 2. The sequence of the L. major H/ACA snoRNAs. The boundaries of the molecules are based on the RNA-seq data of the RNA selected with NHP2 The AGA is shown in bold. The homolog in *T. brucei* and the targets are indicated, and the source of the identification of the molecule is given. DS, deep sequencing from the current study. *(Continued)*

Name	Sequence	Homolog	Size	Target	
LM14Cs1H3	UGCCUUCUCUAGAGUCAUCGCAGC UGAACUGUUGGCCUCUUCCCUCCC GCCCUUCCCUCCCUCUCCUCCUUG CAUCUCCUUCUGCGUGCGCCACAC GUGACUACACACCAUCUCCGCUGC GCUCCAUCCGCGCCGCUUGCCUCU UUCCUCUGGAAAGAUGUUGACAGG GGGUGGGUGCCGUGCGGAAGGAU ACAGAGGAGGAAAGAGCUGGGGGC GAGCGGGGUGGCGAAUCGGAGGG CGACAUCGUCACUGUUCCUGCGAU GUAGUUAGGAGGGCU**AGA**UGU	TB9Cs5H1 (14.6%) HuU19 AtU19 ScSnr191	283	LSU3- Ψ506	DS-Nhp2
LM18Cs1H1	AGGAGAACCGUAGCCGCUGCAACU CCGCUAGGUGAGAUGCCGCGGGU GAUACUUCUCCA**AGA**GCU	TB10Cs4H4 (61.8%)	65	SSU-Ψ455	Liang_et_al_2007
LM18Cs1H2	ACGACGUCUCUGUCUAACGGGGUG CGACGCUGCUCUGUCGGCGCACCU CGAUUUCAGGAGAUGG**AGA**UGA	TB10Cs4H2 (57.8%) HuACA14a	70	SSU-Ψ1156	DS-Nhp2
LM20Cs-1H1	CUUGUGCGCCCUAACAUUUUUCUG CUGCUGUUGGCCAUCGUCCACCUU GCAGACUUGCCCUUAGCAC**AGA**UC A		73		DS-Nhp2
LM22Cs-1H1	AGUCACAGCGCCGCCAUCCCCCUC UCACUUUGUCUUUCAGUUUUGAGG UGUGGGUGCACGGCUGAUUUUAGC GCGUGACGAGAGGAGGAGCGAGC G**AGA**GAGAGAACA		108		DS-Nhp2
LM23Cs1H1	CGAUGGGGUUCGAAUGCGCUGGU CGAAACCGGUUUGCCCUAGCGUGG GAAACGCCCCAA**AGA**GCC	TB8Cs3H1 (65.8%) Different target	65	SSU-Ψ1374	Liang_et_al_2007
LM23Cs1H2	GCGAAGAAGGACGAAAAGGUCGUU CGCGGUGAACCGGUGCCGUGCAC GCGGGACAGUCCUGCG**AGA**GA	TB8Cs3H-1 (56.2%)	68	U1-Ψ68 tRNA-Glu-Ψ43	DS-Nhp2
LM26Cs1H1	UCAGCGCACGCACCAGCCGCGCGC AGUUGAUGUUGGUUGCGCAUCCGG CCUUGUCGUGACGCG**AGA**GAC		69	SSU-Ψ607	Liang_et_al_2007
LM26Cs1H2	CCAGCGUGCUCCGUGGGUGAGGC AGCGGACUCUACUACCCUUGGUGC CGUACAUUGCGCCUCAAUCGGGCU CGCG**AGA**GCU	TB6Cs1H2 (67.8%) Atsno75 Hu4470 SCsnr10	81	LSU3-Ψ1361	Liang_et_al_2007
LM26Cs1H3	AGUCCACCUUCCUCCCAGCCGCAC GUGGUGUCGGCUGCGGCUGCCCA CUGCUGUGUGCGUGCGCGUGGCU UGAAGGGUGGCCG**AGA**GAU		89		Liang_et_al_2007
LM26Cs1H4	AACCAGGAGUAGCACCGUUGCGGA ACAGGACCCAUUGAUCUAUCGGCG GACUUGCUCUUGGG**AGA**CGU	TB6Cs1H3 (70.8%) HuACA24	68	SSU-Ψ609	Liang_et_al_2007
LM26Cs1H5	CCCGCCGCGCAUUCAAGGUCCCUU GGCGGCGAUUAUAUACGCUCGAUG CUGCUUCACAGGAUACCCAGCGUG GCC**AGA**GCU	TB6Cs1H4 (68.2%)	81		Liang_et_al_2007
LM26Cs1H6	CGCGCAGUUCAAGAAGCCGGUGCA UGCGUGUGACUGUGUGCUGCACC GGUGCCGAGACUGCGCA**AGA**GCC	TB9Cs1H1 (63.5%) AtU65 HuU65 SCSnR34 LM26Cs1H9	70	SSU-Ψ1192 LSU3- Ψ1318 SSU-Ψ941	Liang_et_al_2007

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Table 2. The sequence of the L. major H/ACA snoRNAs. The boundaries of the molecules are based on the RNA-seq data of the RNA selected with NHP2 The AGA is shown in bold. The homolog in *T. brucei* and the targets are indicated, and the source of the identification of the molecule is given. DS, deep sequencing from the current study. *(Continued)*

Name	Sequence	Homolog	Size	Target	
LM26Cs1H7	GGCAGGUCAGCGAAAACACAGUCG AAGUGGUAUUGACCCCGUCAUUUC GCCUGCACCUGUGCUGUCCG**AGA** GAG	TB6Cs1H1 (62.8%) HuACA27 SCsnr42	74	LSU3-Ψ1413	Liang_et_al_2007
LM26Cs1H8	CCAACGCACUAUUGCUUCCGCUGC UGCGUGUCGAUGGGCAGCGGGAU CCUGAGGUGCGUG**AGA**UGC	TB9Cs1H3 (55%) HUACA9 SCsnR43	66	LSU5-Ψ1017	Liang_et_al_2007
LM26Cs1H-9	CGAACGGGGCAAUCCGAGGUGCAC GUGGCUUGCGCCCGCUCGCGGGA CUCUGUGCCGUGGUGCGACCUUCU GGUAGCGCCGG**AGA**GCC	TB9Cs1H2 (65.2%)	88	SSU-Ψ1566	DS-Nhp2
LM26Cs1H9	UGAGCGCAGCCUAGGAAACCAGUG CAUGCAAGCCCUUGUGCUGCUCUG GUGCCGAAACUGCGCG**AGA**GCU	TB9Cs1H1 (64%) LM26Cs1H6 AtU65 HuU65 SCSnR34	70	LSU3-Ψ1318	Liang_et_al_2007
LM26Cs2H1	GUGCAUGCGUACGCGCCUGCUCAA CUCCCGUCUGUUCACGCUGCAGGC UGUGGGAGUCGGUGGUGCGGCCG GCAGGCCAGCAUGA**AGA**AGC		91		Liang_et_al_2007
LM26Cs2H2	AAAGCAGCUCGUGUUACCGCUCGA AUGGAUGCGCUGUGCAUCUGCUCC CGUAUUCGAGGUUUUGUGUGUCAA AGGGCUGCA**AGA**CGA	TB9Cs1'H1 (61.5%) SCsnR35 HuACA13	87	SSU-Ψ1543	Liang_et_al_2007
LM27Cs1H1	CAUCUUGACCUGUCUGGCACCUGU CUUUACACCUGUCAGUGACAGAAC ACGCCCGAAAGUUCAAG**AGA**GACA A	TB11Cs4H1 (61.8%)	73	LSU3-Ψ1403	Liang_et_al_2007
LM27Cs1H2	CGCACAACUCCCUUGGAAUGGUGG GUGCCUCAUCCCACGCAAGUCCUU GUCCUGUUGUGCC**AGA**GCG	TB11Cs4H3 (45.4%)	67	LSU3-Ψ611	Liang_et_al_2007
LM27Cs1H3	GAACGGAGUGCAUUGAGUCCCACC GCUCCCCACCAGUACGCGCGUGGG AGCAUCAACUCUCCU**AGA**UGC	TB11Cs4H2 (70.4%)	69	SSU-Ψ1841 SSU-Ψ33	Liang_et_al_2007
LM27Cs1H4	CCGCGGAAUUCCCUGGGCGUGAG GGUCAGCAAUCCCUCUCCGUGCCC GUGUAUAUUGCCGCU**AGA**ACUA		69	LSU3-Ψ78	DS-Nhp2
LM29Cs1H1	GGGCGAAUGGCUCUAUUCUGGUAU CGCAUCCCCCGCUCCUGUGUGCAU GUGUGUAGGUGUGUGCGCGUCUC UGUGUAUGAGCACUGCCtCCGUGA CUGGCGUACUCUACACCCCUGCAU GCAAGGGUGUCUGAUGGGUGCGA UACAUUGAAGGGGUC**AGA**GAUUG	TB3Cs2'H1 (24.6%) SCsnR43	165	5.8S- Ψ74	DS-Nhp2
LM29Cs1H2	CACAAAGGACAAAGAACUGGAGUA CGGCGGAGAAGGUGGUGGAGGCG GGAGAGACCCGGGAGACGGCAUGU GUGUAAAGCGAUGGGUGGUGAGAG GGAAGGUGGAGGAGGACAUGCUCG CGUCCGCGCUGCUGCUUCGCCUUC CUUCCCUCUCCACACGCCUUUCGC UCAGCACUGCCGCCUCUCUUCGCA UCUUGCCCUCGCAAUCGCCUGCUC GCUUUCUCUGCUGCUUCCCUUCAC CUCUUCGAUGGGGCCUUCAGUGUC AUAGCCUUUGG**AGA**UAC	TB3Cs2H1 (13.5%) HuU3747 SCsnr3	280	LSU3- Ψ510	DS-Nhp2

(*Continued on next page*)

Table 2. The sequence of the L. major H/ACA snoRNAs. The boundaries of the molecules are based on the RNA-seq data of the RNA selected with NHP2 The AGA is shown in bold. The homolog in *T. brucei* and the targets are indicated, and the source of the identification of the molecule is given. DS, deep sequencing from the current study. *(Continued)*

Name	Sequence	Homolog	Size	Target	
LM29Cs1H3	AGCGAGGAUGUUUGAGGGGCCGU CGUCCUUUUGUUAUCAUGGUGACG CCUUGCUCCAUAGGAACUUCGCG**A GA**AGCGA	TB3Cs2H2 (54.6%) HUACA9 SCsnR33	78	LSU5-Ψ1093	DS-Nhp2
LM30Cs1'H1	CCAGUACGGUUAAUCGUGGCACUU CGAACUCUCCUUCUCGAUAGAGGU UGUUGUCAUCAAGGCCGUACG**AGA** UGC	TB6Cs2H1 (46.1%)	75	LSU3- Ψ1144	DS-Nhp2
LM30Cs1'H2A	AGCUUUCACCAACCAGCUUCUCAU CUCCGCAAAGGGCUGGCGCGCCAU CGUGCCCUCCAGCUCUUGCCAACA UAGAUGAACUGAAGUCAAAGUGAA GAGCG**AGA**AGC	TB11Cs5H1 (42.9%)	107	SSU-Ψ1657	DS-Nhp2
LM30Cs1'H2B	AGCUUUAACCAACCAGCUUCGUAU CUUGGGAGCUGGGGCUUGCGAGU ACGCAUCAGCCGCACUCUCCGGUA GAUACUUGAGGUCAAAGUAAAGAG CA**AGA**CGG	TB11Cs5H1 (44.5%)	103	SSU-Ψ1657	DS-Nhp2
LM30Cs1'H3	CGCUGCAUCAGUACAGCGACAUCA UCUUUCCGAGUGGUGAUGCCUGCU GGCGAUGCCAGCA**AGA**GUC	TB11Cs5H2 (49.4%)	67	SSU-Ψ2046	DS-Nhp2
LM30Cs2H-1	CCAGCAUAAUUCGAAGGCCGUCAU UCCUCCUAUUCCUUGUGGAAUGUA ACCGGUUCAAACAUUGUGCC**AGA**A UG	TB11Cs5H3 (54.1%) LM30Cs2H1 HuACA16 SCsnr46	74	LSU3-Ψ1303	DS-Nhp2
LM30Cs2H1	CCGCACAGUUCGAAGGCCGUCAUC CCCUGUACUACAUCUUAGGGUGCU UUCGGUUCAAAGGCUGUGCC**AGA**U AG	TB11Cs5H3 (62.5%) LM30Cs2H-1 HuACA16 SCsnr46	74	LSU3-Ψ1303	Myslyuk_et_al_2008
LM30Cs3H1	AAGCCCGACAUCAGCACCACUGCA AUAGGUGGCUACAUGGACAUUUUA CUCCCCUUCCAUAGUCACCUACCC GUGCGUGCG**AGA**UAU	HuU69	87	5.8S-Ψ69	DS-Nhp2
LM32Cs2H1	AUUCGAUUUCUAAUCCUUCACUGC CUCAAUUCCUUUGUAGGCUUUCAC AUUGUGUGAGCGAAGGUUUGCUGA UCGAAG**AGA**GUU	TB11Cs4"H1 (59.6%) Hu1731 SCsnR5 ATsnoR98/ snoR81	84	LSU5-Ψ1055	DS-Nhp2
LM32Cs3H1	CCAUCUCAUACAUUAAGGGUUCAA CACUGAGAGGGAUGUCUGUGUGUG UGUGUGUGUGUGUGCGUGUGU GUGUGUGUGUGUCCGUGUGGUCU GGUCGAAGGGGCGUGCUCGGGCG CGCUCUUCCGCCCUCUUCGCGCUC CACACACACACACACACACACACAC AUGCACACACCCUUGCACAGACCU CUGCCCCCUCUCGCCUUUCUUCCA UAGUGUUUGCCUGAGUCAUGUGAG AG**AGA**GCG	TB11Cs6H1 (10.3%) Hu3749 SCsnr84	246	LSU3- Ψ512	DS-Nhp2
LM33Cs-1H1	CACACCUACGGAGCCGAUGCUGAA CUUUCCUUGUUGCUGGGCGAGGC GUUUUGGUGCACCACGUAUUCGUU GUUGGUGG**AGA**UUG		85		DS-Nhp2

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Name	Sequence	Homolog	Size	Target	
LM33Cs1H1	UGCACCUUCCGGGCAGGAUGACGC CCUUGUGCCCUUGGCGACUGUGUC CAAUCGGUGGUGCG**AGA**UGC	TB10Cs3H2 (73%) HuU3674 SCSnR32	68	LSU3-Ψ437	Liang_et_al_2007
LM33Cs1'H1	AACCACAGACAUAGUACCCUUCAC GUGCGUUCGAUAGACCACCAAGCG AUGAGGCGAAAGGGGGCGCUGAG GAGCAAGAUGGAAAUCAGGGAGGG CUGGAAAACGCGGAGGACUGAGGU GCGUACGCGCUUCGGACCCCCUCU UUUUCUCCCCUAGCUCUUUUCUAC GUUGCUUCCUCCUCCUUUUCCCCU CUGCCGGUGUCUUGAGAACACUUC UUGUGCUGCUGUCGGGCCUCCCU CUCGUGGAAGA**AGA**	**LSU3-Ψ500** HuACA23 AtSnoR92	252	LSU3-Ψ500 LSU3-Ψ699	DS-Nhp2
LM33Cs2H1	ACGGUGUUGUCGGUUGGGCGUGA CUCCUUGCACCUCGUAGGUACGAC CCUAUCUCGCAACCCCC**AGA**UUC	TB10Cs2H1 (60.5%) **LSU3-Ψ1213** Hu4323 **LSU3-Ψ472** HuACA19	70	LSU3-Ψ1213 LSU3-Ψ472	Liang_et_al_2007
LM33Cs3H1	CGAGGUCUUCGUGUACCCCGUGCG CCCCCGCUCGUGGCGCACACAUGG GGAAUGGUCAAGACCG**AGA**GAC	TB9Cs4H1 (61.8%) HuU70	70	SSU-Ψ2048	Liang_et_al_2007
LM33Cs3H2	AGUCCCAUUUCGCUCGUCCCGCUC CGCACCGUUGAGCUGCCGUCGAUG AACAAUGGGCCG**AGA**CGU	TB8Cs2H1A (44.4%) LM36Cs2H1-diffrent target HuU68	66	LSU3-1284	Liang_et_al_2007
LM33Cs4H1	GGCCCCGCCCUUUCACAUGGCGGU UGUUGUUGUCGCGGAUUCCUCCUG GGAUGGUGGGGU**AGA**GCA		66	SSU-Ψ721	DS-Nhp2
LM34Cs1H-1	ACGCUGUCUCGAAUAGGCGCUGGA GGUCAACCUUGCCGAAUACCUUCU GGCGUAAGUUAGGACAGCA**AGA**UC G		73	SSU-Ψ1371	DS-Nhp2
LM34Cs1H1	GAAACGUCAGCUACGCAGUGUGAG CUGAUAGUGUCUGUUCGGCUACGU GCGCUACCAAAGCGGCGUG**AGA**GC G	TB10Cs1'H2 (62.8%)	73	LSU5-Ψ1533	Liang_et_al_2007
LM34Cs1H2	GUGACGUCACUCCAACCGCUGCUC AUACGUCGUUGCCCAUCGUAUAGC GGUGAUAUCUGUGGACGG**AGA**UG G	TB10Cs4H3 (67.1%)	72	LSU5-Ψ1528	Liang_et_al_2007
LM35Cs1H1	CGCUCUUGUCGCUUCCCACUGCUG UCUCAUUUUAUCAUCAGCUGUCCA CUUUGGGUUAGUAUACUUGAGCA**A GA**UUU		77		DS-Nhp2
LM35Cs2'H1	CCCUUUCUCAGAGCUCUUUGGCUU UCGGCAUGAAUGAAAGGUGGAGGA GAUGUCAAGGUGGAGGG**AGA**GAU		71	LSU3-Ψ122	DS-Nhp2
LM35Cs2'H2	UACGCAGCAGAAUUAAACACGGAU CUGUGGGACGUACUUCAGUGCUGG UGAGAUCCGCCUGUGUUUUGCA**AG A**UGA	TB9Cs3H1 (41.9%) LM5Cs1H3 AtU65 HuU65 SCSnr34	76	LSU3- Ψ1264	DS-Nhp2

(*Continued on next page*)

Table 2. The sequence of the L. major H/ACA snoRNAs. The boundaries of the molecules are based on the RNA-seq data of the RNA selected with NHP2 The AGA is shown in bold. The homolog in *T. brucei* and the targets are indicated, and the source of the identification of the molecule is given. DS, deep sequencing from the current study. *(Continued)*

Name	Sequence	Homolog	Size	Target	
LM35Cs3H1	GUGCGGCCCACAUUGGAGUUGUGU CCUGGCCCCAGCAGUGGCCCUGUC UGCACACUCGCGUGGGCCA**AGA**GA U	TB9Cs2H1 (72.2%) HuACA3 SCsnr11	73	LSU3-Ψ662	Liang_et_al_2007
LM35Cs3H2	CGUCCCACCAGCUACCACGCGGAG UGCAUACCCUUUCAGUCUUCGCGG UCGGUCGUUUUCUGGGCCA**AGA**U GC	TB9Cs4H2 (71.4%) HuACA10 SCsnR37	73	LSU3-Ψ1382	Liang_et_al_2007
LM35Cs3H3	UUGCGGUGAAGCGGCUUUAGCCUU GGCGCGGCUUUCACACCCCUGUGC CGUGUGCCAUCUUGGUGCGCCUC G**AGA**UCG	TB9Cs2H2 (57.8%)	78	LSU5-Ψ1171	Liang_et_al_2007
LM36Cs-1H1	UCGAAGGGUGAUGCUUACCGCGGA GGCUGCCUUCCAUUAGUGGAUCGC UUCGCAGAAAAUAAACCCU**AGA**UU G	TB7Cs3H1 (58.2%) Hu1849 ScSnr5	73	LSU5-Ψ1181	DS-Nhp2
LM36Cs-1H2	CGAUGCCUUUAGGUCCUUCGCCGU GUUCUUUCGGUGCACGGCAAAGCU AUUGUGCUGCAUCU**AGA**UUU	TB9Cs8H1 (54.4%)	68	LSU5-Ψ1084	DS-Nhp2
LM36Cs-1'H1	GACGCUGUUAUAAUCCCGUGCCGC GCAUCUGAUCUGUGAGCGGGACGA UUCAUGCACAGCGCAGAUGG	TB10Cs2"C4 (68%) **LSU3-Ψ560** HuACA48 Atsnor83 SCsnr86	68	LSU3-Ψ560 SSU-Ψ1292 U3-Ψ24	DS-Nhp2
LM36Cs1H1	CGGAACGUGGCAGGCUGCGGAGG GCACACCCAUCCCUCAUACGCGGU UCAACACAUGA UCCC**AGA**GCU	TB10Cs1H3 (71.4%)	75	SSU-Ψ12	Liang_et_al_2007
LM36Cs1H2	CAUCCCUUGUUGAUAGCGAGCAGU CCACCCGCAUCCCCGCAAGCUGCU CGUCUACACCGAGGGAG**AGA**AAG	TB10Cs1H1 (64.2%)	71	LSU3-Ψ704	Liang_et_al_2007
LM36Cs1H3	AAGCGCUCAUUUUCAAGCCGCACG CGGGCGUGUUGCUGGUGCCCCUG CGGUCGGGUGAUGCGCG**AGA**GCG	TB10Cs1H2 (65.8%)	70	LSU5-Ψ672	Liang_et_al_2007
LM36Cs1H4	AGCAGUGUCGCUAUGCGUCUCCGU CUCACCUCGAUGCGGAGCAACGGU ACGAAACACGUGCG**AGA**UGC	TB10Cs3H1 HuSnorA63 (76.1%)	68	LSU3- Ψ1281	Liang_et_al_2007
LM36Cs2H1	CCACCUGCACUUCGCUCGUCCCGC UCGCGUGUCGAUGAGUGGUGCCG AUGGAUGGUGUGAGGG**AGA**GCG	TB8Cs2H1B (60.3%) / LM33Cs3H2 different target	69	LSU3-Ψ1194	Liang_et_al_2007
LM36Cs2'H1	GAAGAUCACAUUUCCAAGCGGAGA GGUCGGUUUCGGGAGGGGAGGGC GGGGGGCACGAGGGAUGGGCGGU GAGGGGCAGAUAGAAGCGGGAGAA ACUUCUUCUGUCUGCCUCUCCCUC CCUCCUGGUCACACUCCCCCCUCU CUCUUUCCGUUCUUCAUACCUUCG UCGCUAAGUGCAGUGAUCUG**AGA**U GU		192		DS-Nhp2

(*Continued on next page*)

Table 2. The sequence of the L. major H/ACA snoRNAs. The boundaries of the molecules are based on the RNA-seq data of the RNA selected with NHP2 The AGA is shown in bold. The homolog in *T. brucei* and the targets are indicated, and the source of the identification of the molecule is given. DS, deep sequencing from the current study. *(Continued)*

Name	Sequence	Homolog	Size	Target	
LM36Cs2"H1	UUGCUCAUCUGUCUUUUAGAACCU CUUUGGCUGCCUCUCCGCCGUUUG CCUUCCCUUGCCUCCCUCUCUCCU UCAUCACACCACUCUUUUUCUGCC CUCUGUAUCUGUGCAUUCCCCGCC CCUGGAGUAGUGUGUGCCAGGAG GGGGAGAAAGGGGUCCGAUCCGU GGUGCAGGGAGUGGGGGGGGGAC GAAGGGGGCGGCGGCGGCGGAGU CGCACAUGGAACGUUGCCUAUUCU CGAAGAGGUGUAACGUGGCAGAA**AGA**AAA	TB9Cs1"H1 (23.2%) HuE2	265	LSU3- Ψ593	DS-Nhp2
LM36Cs3H-1	AAAAGCACAGCCAUCGGACACAGG GAGGCUGCACUCAGUGCCUUCCUG UGAUGAAGUCAGGCCGGCG**AGA**AU CU	TB11Cs1'H-1 (37.7%) Same position different target	74	SSU-Ψ2202	DS-Nhp2
LM36Cs3H1	CCGGCUCUCACGAACAAUCCCGCC CGGAGUUUACCGUACUUGAUCUCU UGCGCGGACCUACGGGUGCCC**AG A**UGC	TB11C3H1 (68.8%)	75	LSU3-Ψ1354	Liang_et_al_2007
LM36Cs3H2	UGUCAAAUUCCUUGCAGCGGCUGG UGCGAGAUGGUGGGACGGGGAAC UUUCUCUCUCAUGCCCUAUCAUCC GUGUCGGUGCCGUGCCAGGACCG UAUGACUCCA**AGA**GCC	HuACA5 SCsnR85	110	SSU-Ψ1533	Liang_et_al_2007
LM36Cs3H3	CUGCCACAAACAAAGGAACACGGG GGGACUCGAAACAUCAUCUAUUCC CCUGCGUGAUGGAACUGUGGCG**A GA**CGG	TB11Cs1'H1 (50%)	76	LSU5-Ψ1402	DS-Nhp2
LM36Cs4H1A	ACUACAACUCUCUGGAUUCCUCGA GUCAUUUAAUUCUAGUGCAAUCCU CAUCGGUUGUCGA**AGA**GU	TB10Cs5H3 (62.2%) HuACA8 AtSnor5 ScSnr31	66	SSU-Ψ1246	Myslyuk_et_al_2008
LM36Cs4H1B	ACUACAACUCUCUGGAUCCCUUGG GUCAUUUCUCAUUCCCAGUGCGAU CCUCAUCAGUUGUCGA**AGA**AGG	TB10Cs5H3 (56.8%) HuACA8 AtSnor5 ScSnr31	70	SSU-Ψ1246	Myslyuk_et_al_2008
LM36Cs4H2	AGCCGACCACGUCUGUGUGCUGCG CGGAAACGGCUUGUCGUUCUUCCA UGCGCUUCUCACUGUAAGAGUCGG CA**AGA**AAA	TB10Cs5H2 (62.2%) HuACA36 ScSnr44	80	SSU-Ψ104	DS-Nhp2
LM36Cs5H1	CGGUGAGAGCUUUUCCCUCAUCUU CUGCCGAAGUCUUCUCUUCUCUCU AGUGCAGGUGAAGGUAUUCAAGCU CACCC**AGA**UUU		83		DS-Nhp2

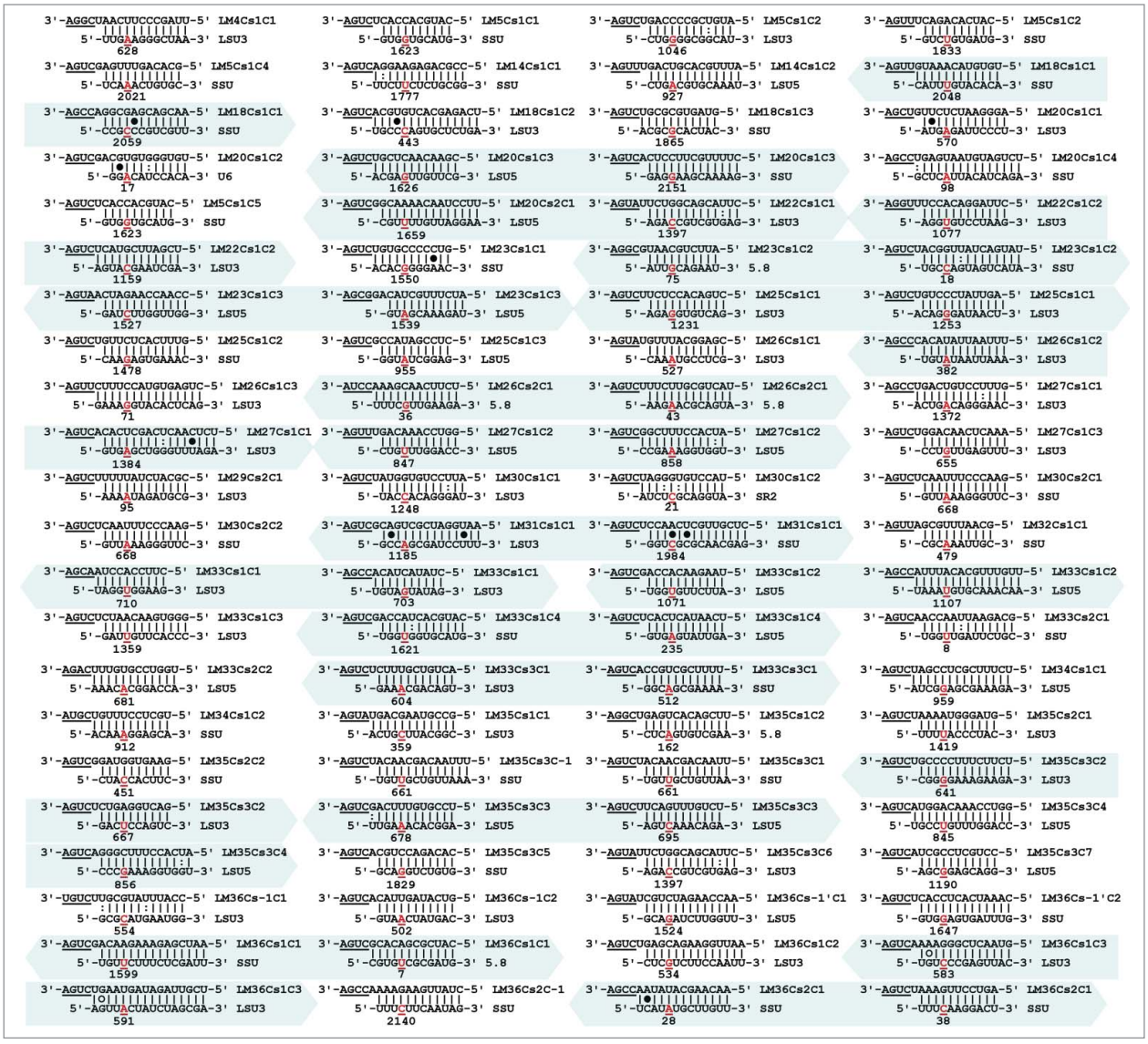

Figure 4. Potential base-pair interactions between the C/D snoRNAs and their targets. For each snoRNA and target pair, the name of the snoRNA, the domain of complementarity, and the nucleotide to be modified on its target are indicated in red. SSU, small subunit; LSU 5 or 3 indicate the 5′ and 3′ ends of the large rRNA subunit. The snoRNAs that direct the 2 Nms are shaded by blue background and if these exist in 2 lanes the shaded boxes are marked with arrowheads. The D or D′ boxes are underlined.

homologous snoRNAs despite the fact that their order within the cluster differs; such a case is LM36Cs4 and its homolog TB10Cs5. However, the LM cluster contains a unique C/D snoRNA, LM36Cs4C2 (*L.m* specific snoRNA) (**Figs. 6Ai**). The same relatedness applies to LM27Cs1 and TB11Cs4, and also in this case, the cluster contains an LM specific snoRNA, LM27Cs1H4 (**Figs. 6Aii**). In other cases, it seems that an *L. major* cluster emerged from fusion of 2 *T. brucei* clusters; this is the case for LM26Cs1, which is related to 2 *T. brucei* clusters, TB6Cs1 and TB9Cs1 (**Fig. 6B**). However, more complex relationships also exist, such as 2 LM clusters (LM30Cs1′, LM30Cs2) which are related to 2 TB clusters (TB6Cs1, TB11Cs5); the clusters in each species are related to 2 clusters in the other species (**Fig. 6C**). Interestingly, the *Leishmania*, related clusters are found next to each other, while they are encoded on 2 different *T. brucei* chromosomes. The most complex relationship was found for LM33Cs1, which shares 5 out its 6 snoRNAs with TB10Cs3; this cluster has one snoRNA that has an homolog in LM36Cs1, but the latter cluster is most closely related to TB10Cs1. Interestingly the clusters related to the LM cluster are localized on the same *T. brucei* chromosome (**Fig. 6D**).

Interestingly, snoRNAs involved in rRNA processing are found in distinct clusters. The SLA1 locus in *T. brucei* (TB11Cs2) is homologous to LM5Cs1 and contains abundant snoRNAs with special functions including rRNA processing (**Figs. 7Ai**). An additional cluster of this type is LM35Cs3, which is homologous to TB9Cs2, which contains the abundant snoRNA TB9Cs2C1, implicated in rRNA processing[22] and is homologous to

LM35Cs3C6. In addition, the LM35Cs3 cluster contains LM35Cs3C4, which is homologous to the abundant TB9Cs2C3 snoRNA. This cluster carries another abundant C/D snoRNA, LM35Cs3C5, which is implicated to direct cleavage of 5′ the ETS but seems to be a *Leishmania*-specific snoRNA (see below) (**Figs. 7Aii**). LM36Cs1 is another such cluster (**Fig. 7A-iii**) that carries 2 C/D snoRNAs implicated in rRNA processing, LM36Cs1C3, which is homologous to TB10Cs1C1, and

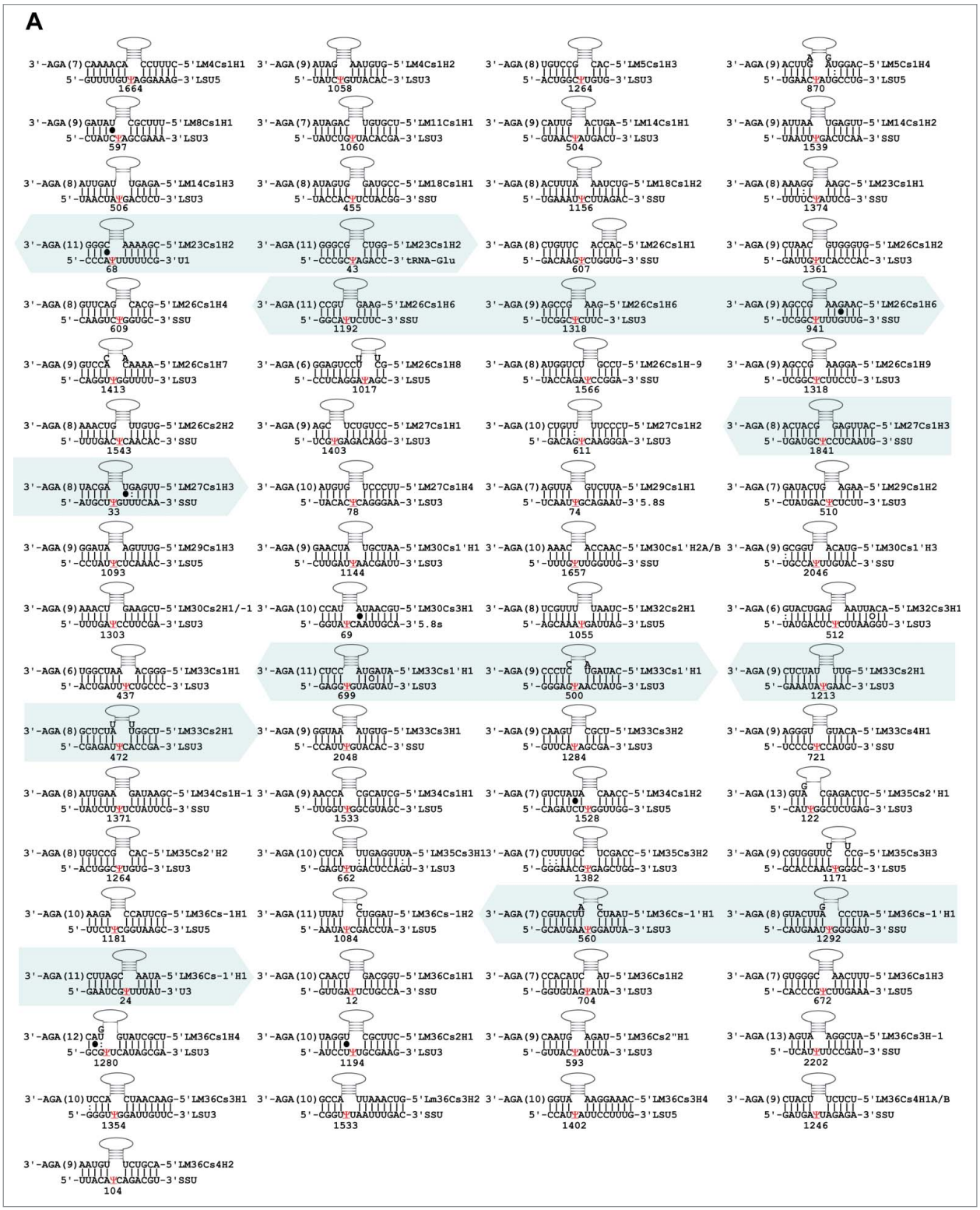

Figure 5. For figure legend, see page 1245.

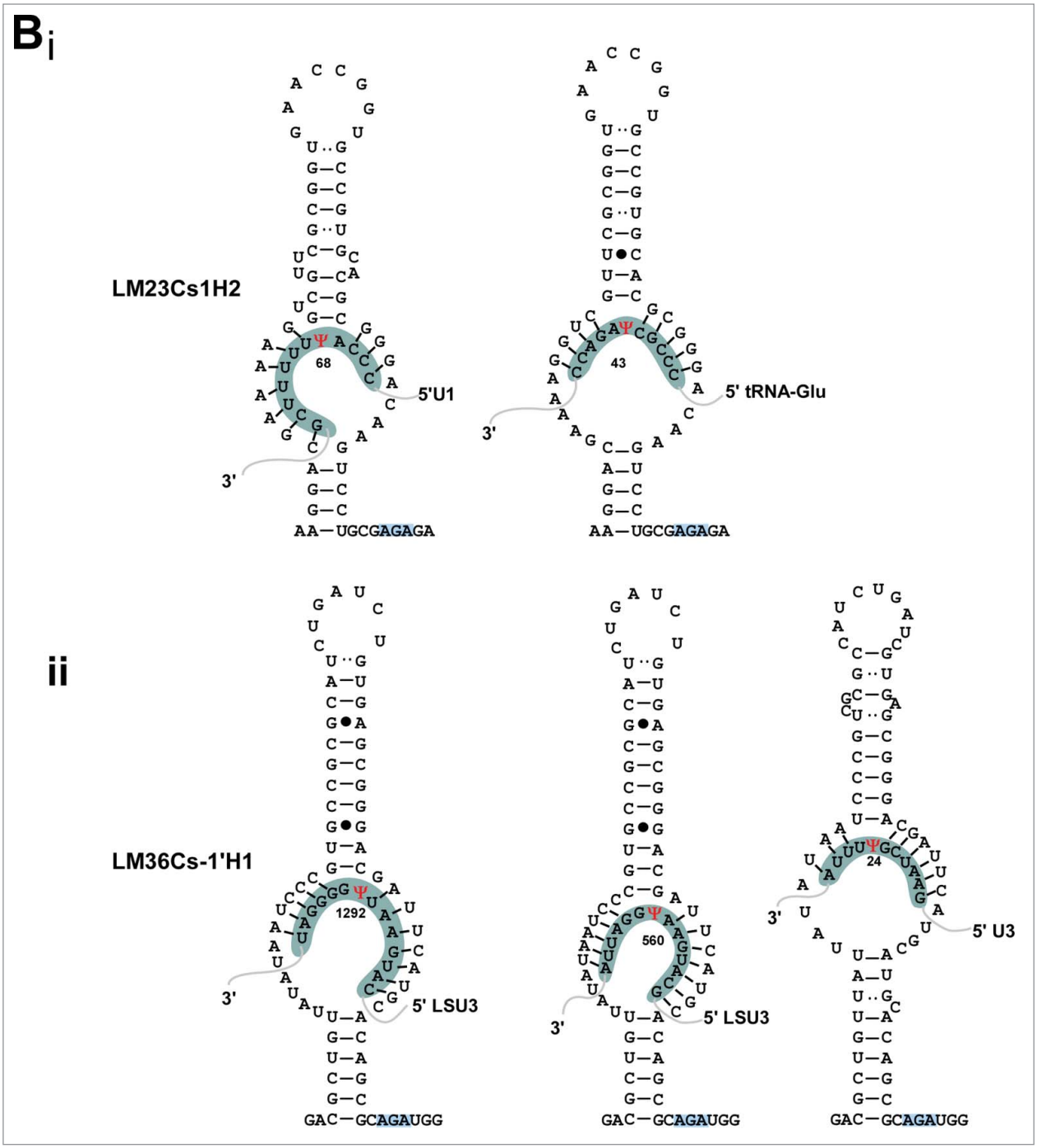

Figure 5. (**A**) Potential base-pair interaction between the H/ACA snoRNAs and their targets. The target sites are indicated in red and the positions are given. The designation of the targets is as detailed in Figure 4. The snoRNAs shaded with blue background and arrow heads can potentially guide more than a single modification. (**B**) H/ACA snoRNAs which can potentially guide pseudouridylation on more than a single site; **i**, LM23Cs1H2; **ii**, LM36Cs-1′H1.

LM36Cs1C2, which is homologous to TB10Cs1C4 and was shown to be involved in trypanosome-specific rRNA processing.[22] Additional snoRNAs implicated in rRNA processing are present in LM22Cs1, LM23Cs1, and LM26Cs1 but these are not organized in the same way as in *T. brucei*.

Among the snoRNAs involved in rRNA processing/maturation are snoRNAs that are suggested to function in processing of the small subunit rRNA (SSU) and the large subunit rRNA (LSU) such as LM5Cs1C3 (homologous to TB11Cs2C2), and those that are implicated in SSU processing (LM5Cs1C1). To examine whether 2 distinct large RNP complexes exist for LSU and SSU processing, whole cell extracts were prepared from *L. major*, fractionated on 10–30% sucrose gradients, and the fractions were analyzed by Northern analysis using the probes

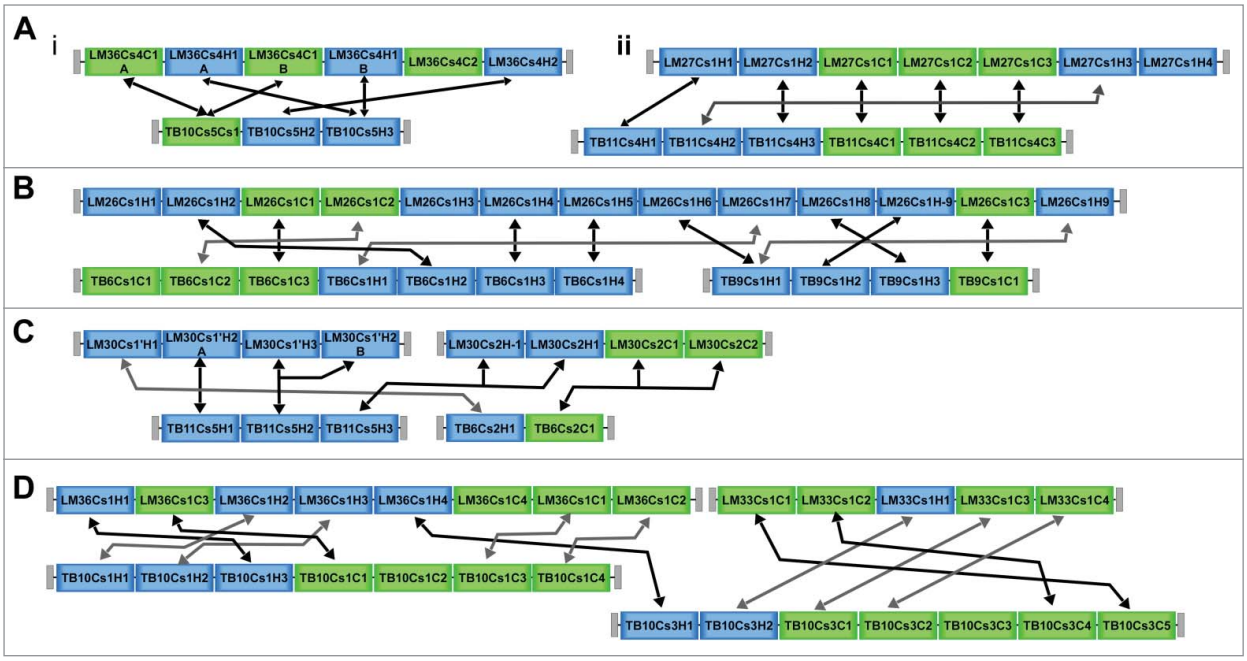

Figure 6. The relatedness between genome organization of snoRNA in *L. major* and *T. brucei*. (**A**) **i** - Genomic organization of the related clusters LM36Cs4 and TB10Cs5; **ii** - LM27Cs1 and TB11Cs4. (**B**) Relatedness of LM26Cs1 to the TB6Cs1 and T9Cs1 clusters. (**C**) Relatedness of LM30Cs1′ LM30Cs2 to TB6Cs2 and TB11Cs5. (**D**) Complex relationship of LM36Cs1 and LM33Cs1 to the *T. brucei* clusters TB10Cs1 and TB10Cs3.

specified. The results (**Fig. 7B**) indicated that U3, which is implicated in SSU processing, peaked in factions 11–17, and LM5Cs1C2 which is also implicated is SSU processing peaks in fractions 9–13, but LM5Cs1C3 which is the homolog of TB11Cs2C2 and is implicated in LSU processing[23] was also found in the higher S value complexes (a peak in fractions 9–13 and a second peak in fractions 19–23). In contrast, LM23Cs1H2 which is implicated in tRNA modification was found only on small RNPs and at the top of the gradient (fractions 3–7). The results implied that fractionation of RNPs may be used to suggest the function of a snoRNA and its involvement processing of SSU, LSU or both or even other targets.

Of special interest are those snoRNAs which are *Leishmania*-specific, such as LM25Cs1C4, and LM35Cs3C5. The bioinformatic predictions suggested that LM25Cs1C4 potentially interacts with the 5′ETS, LSUα, and ITS6 (**Figs. 7Ci-1**). We therefore examined whether this snoRNA interacts with these domains by "RNA-walk" a method that we used previously to map the interaction of snoRNAs with their target site.[22,23] In brief, cells are treated with AMT-psoralen, which intercalates between the duplex, and upon UV treatment a covalent linkage is introduced. The *in vivo* cross-linking enables capture of interactions that take place in cells. To select for the small RNA-target duplexes, they are purified by affinity selection using an antisense oligonucleotide complementary to the small RNA. The site of interaction between the small RNA and its target does not allow the reverse transcriptase to copy this domain, and as a result cDNA prepared with random-primers cannot be amplified by PCR using specific primers covering the cross-linked adduct.

The four possible target interactions of LM25Cs1C4 were examined using RT-PCR on different domains along the pre-rRNA. The results (**Figs. 7Ci-2**) indicated specific reduction in the level of 5′ETS, LSUα and ITS6, but not in other domains on the rRNA, thereby supporting the bioinformatic prediction that this snoRNA interacts with pre-rRNA, possibly for processing. Next, we examined the interaction of LM35Cs3C5 with its targets. The bioinformatics predictions suggested that LM35Cs3C5 interacts with SSU, and potentially guides the methylation at position Gm1829. In addition, this snoRNA potentially interacts with the 3′ ETS (**Fig. 7Cii-1**). 'RNA walk' was used to verify these interactions by examining the amplification of different domains along the pre-rRNA. The results supported the interactions with both SSU and the 3′ETS (**Fig. 7Cii-2**). Interestingly, this snoRNA may direct cleavages to liberate both SSU and LSU (**Fig. 7C-iii**). Our results (**Fig. 7**) suggested that the LSU processing is mediated by a complex that is distinct from the SSU processome, extensively studied in other eukaryotes (see Discussion). Although most of the snoRNAs implicated in rRNA were predicted to either process SSU or LSU, several snoRNA were predicted to be involved in processing both subunits (**Fig. 7C-iii**).

The rich repertoire of both Ψs and Nms in trypanosomatids compared to other eukaryotes

The rich repertoire of snoRNAs suggested the existence of an unusually high level of modifications that maybe related to parasites cycling between the 2 hosts.[39] Although the repertoire of H/ACA identified in this study almost doubled the amount of

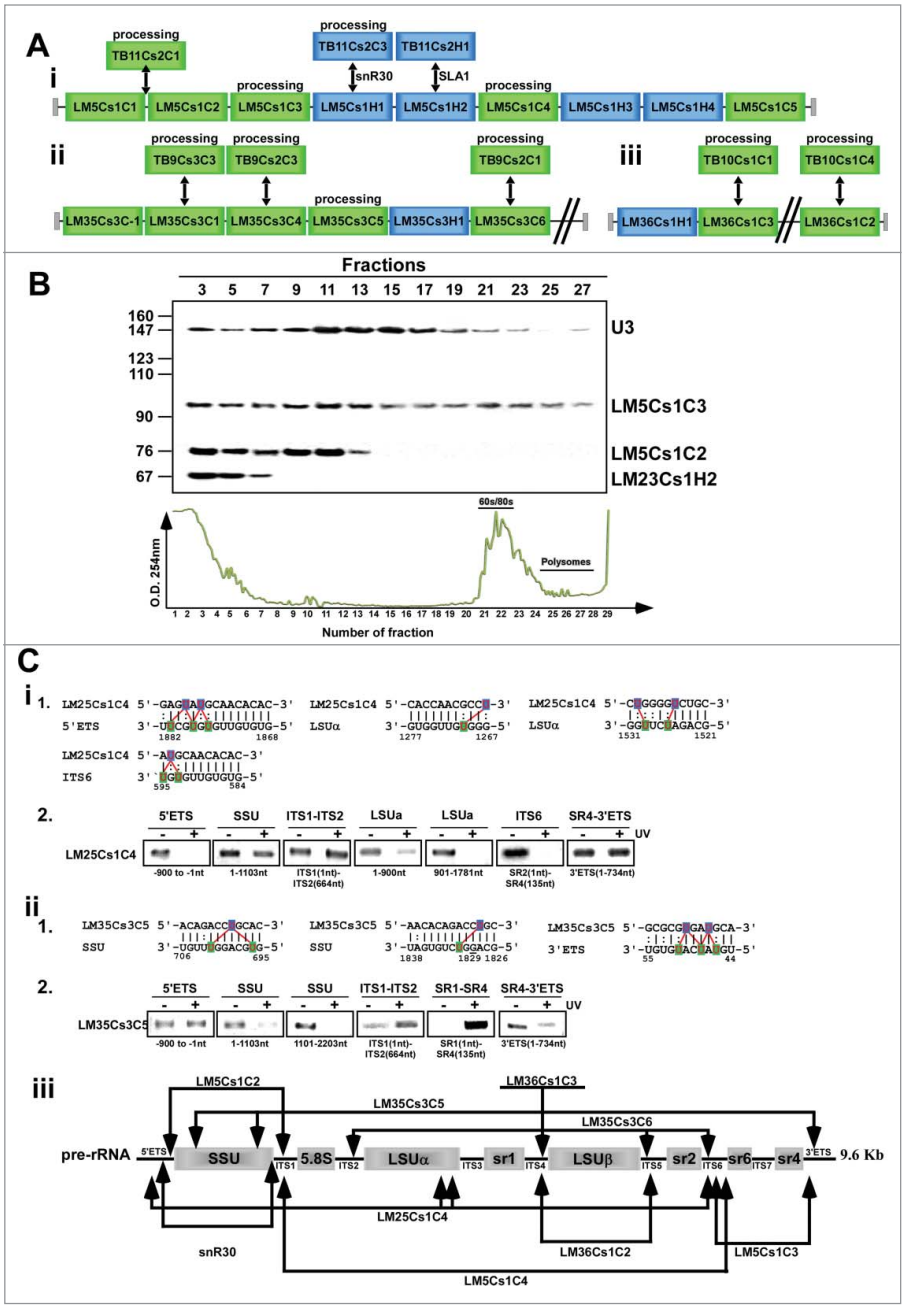

Figure 7. *L. major* snoRNAs implicated in rRNA processing. (**A**) *L. major* homologues to *T. brucei* snoRNAs implicated in rRNA processing. (**B**) Genomic relatedness between clusters encoding snoRNAs involved in rRNA processing. i. LM5Cs1 and TB11Cs2; ii. LM35Cs3/TB9Cs3; iii. LM36Cs1/TB10Cs1. B - Migration of the snoRNAs on sucrose gradients. RNPs were prepared and fractionated as described in Materials and Methods. RNA was extracted from the fractions and separated on 10% polyacrylamide-denaturing gel, and subjected to Northern analysis with the indicated probes. The sizes of pBR322 DNA-*MspI* digest marker are indicated on the left, and the identity of the RNAs on the right. (**C**) "RNA walk" analysis to validate snoRNA-rRNA interactions. i-1- The proposed cleavage sites of LM35csC5 and LM25Cs1C4 and proposed interaction domain of LM25Cs1C4 and pre-rRNA with rRNA. i-2- "RNA walk" analyses of the interaction site of the 2 snoRNAs. RT-PCR of rRNA domains interacting with TB25Cs1C4. cDNA was prepared from RNA affinity selected with LM25Cs1C4 from total RNA prepared after AMT cross-linking. The domains and the size of the amplified fragments are indicated. ii (1 and 2). The same as in (i) but for LM35CSC5. iii. Schematic representation of the interaction site and potential cleavages by snoRNA implicated in rRNA processing. The proposed cleavage site of pre-rRNA is indicated by arrows.

predicted Ψs from 37 to 69, the number of predicted Nms is 93, hence higher than the number of Ψs.

The repertoire of Ψs and Nms on SSU and LSU compared to the modifications present in yeast, human, plants, and *Euglena* are presented (**Fig. 8**). Among the 88 predicted Nms sites, 47 were found to be modified in either yeast, mammals or plants. 47 Nm sites are shared with *Euglena*. 75 Nms are shared between *T. brucei* and *L. major*. Among the 67 Ψs, 35 are found in yeast, mammals or plants, 55 are shared between *L. major* and *T. brucei*, and 28 Ψs are shared between *L. major* and *Euglena*. Interestingly, trypanosomatid-specific modifications were not evenly distributed among the rRNA: SSU (11Nm, 9Ψ), LSU 5′ (12 Nm, 2Ψ), LSU 3′ (19 Nm, 14Ψ) and included more specific Nms than Ψs. In several domains, modifications were found exclusively in trypanosomatids and *Euglena*; LSU 5′ positions 1253 −1373 contained 2 modifications shared with trypanosomatids, and 14 *Euglena*-specific ones, and positions 1659–1725 (ES19L) contained 2 trypanosomatid-specific and 2 *Euglena* modifications. In the LSU3′, most of the trypanosomatid and *Euglena* specific modifications existed in highly modified domains. However, position 699–740 contains 4 trypanosomatid-specific but 7 *Euglena*-specific modifications. Thus, the data suggested that the hypermodification in trypanosomatids and *Euglena* is found mostly in modification-rich domains. Interestingly, species-specific modifications also exist and may play a role in ribosome function in these organisms (see Discussion).

How could different snoRNAs arise: A lesson from comparing *L. major* and *T. brucei* homologues

When comparing homologous H/ACA snoRNAs in *L. major* and *T. brucei*, we noticed that the pseudouridylation pocket, the functional heart of the molecule, and the rest of the molecule, the body, evolved at different rates, yielding different types of snoRNA homologues. The first group is composed of homologous pairs that share overall identity and guide the same Ψ sites (for instance

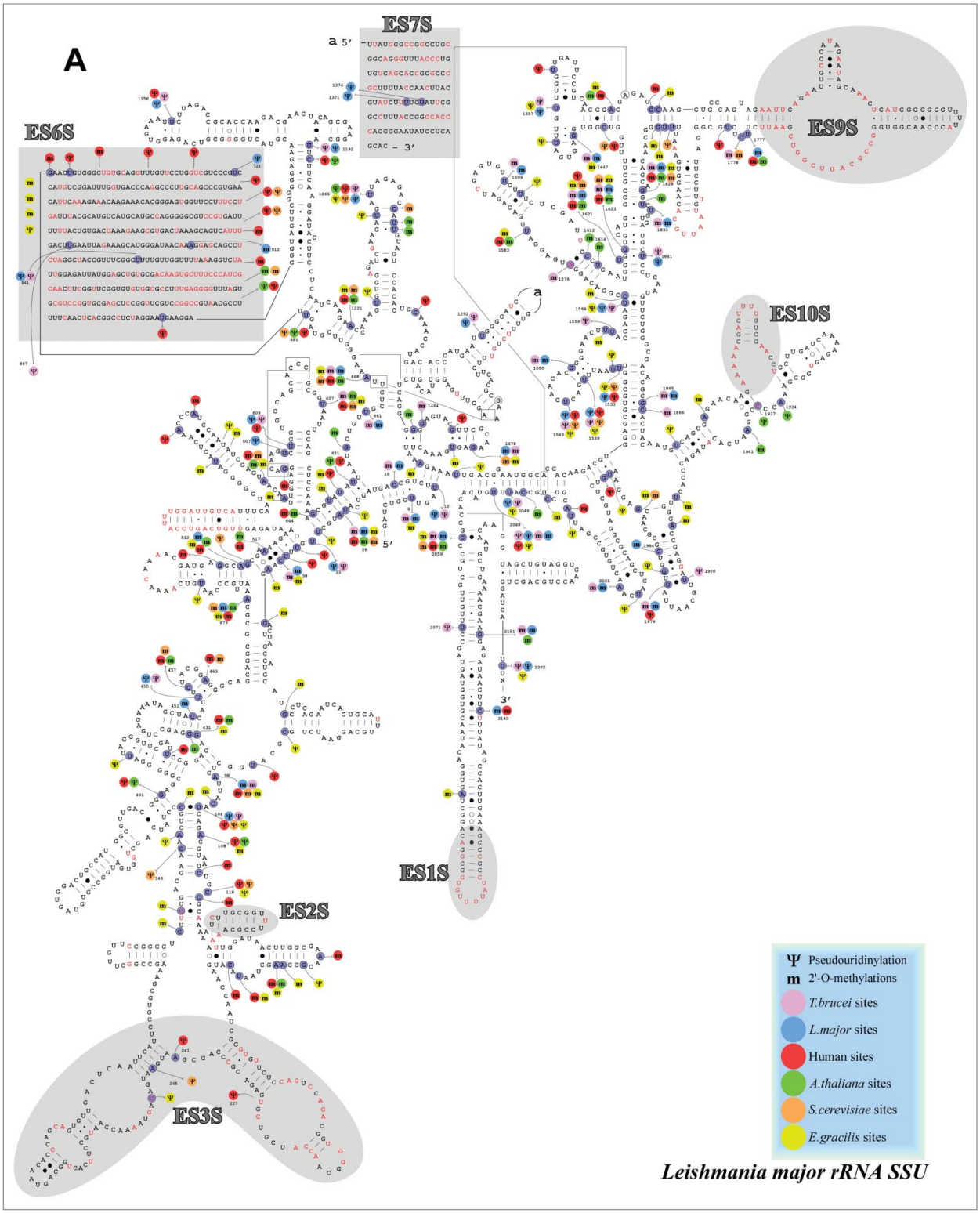

Figure 8. For figure legend, see page 1250.

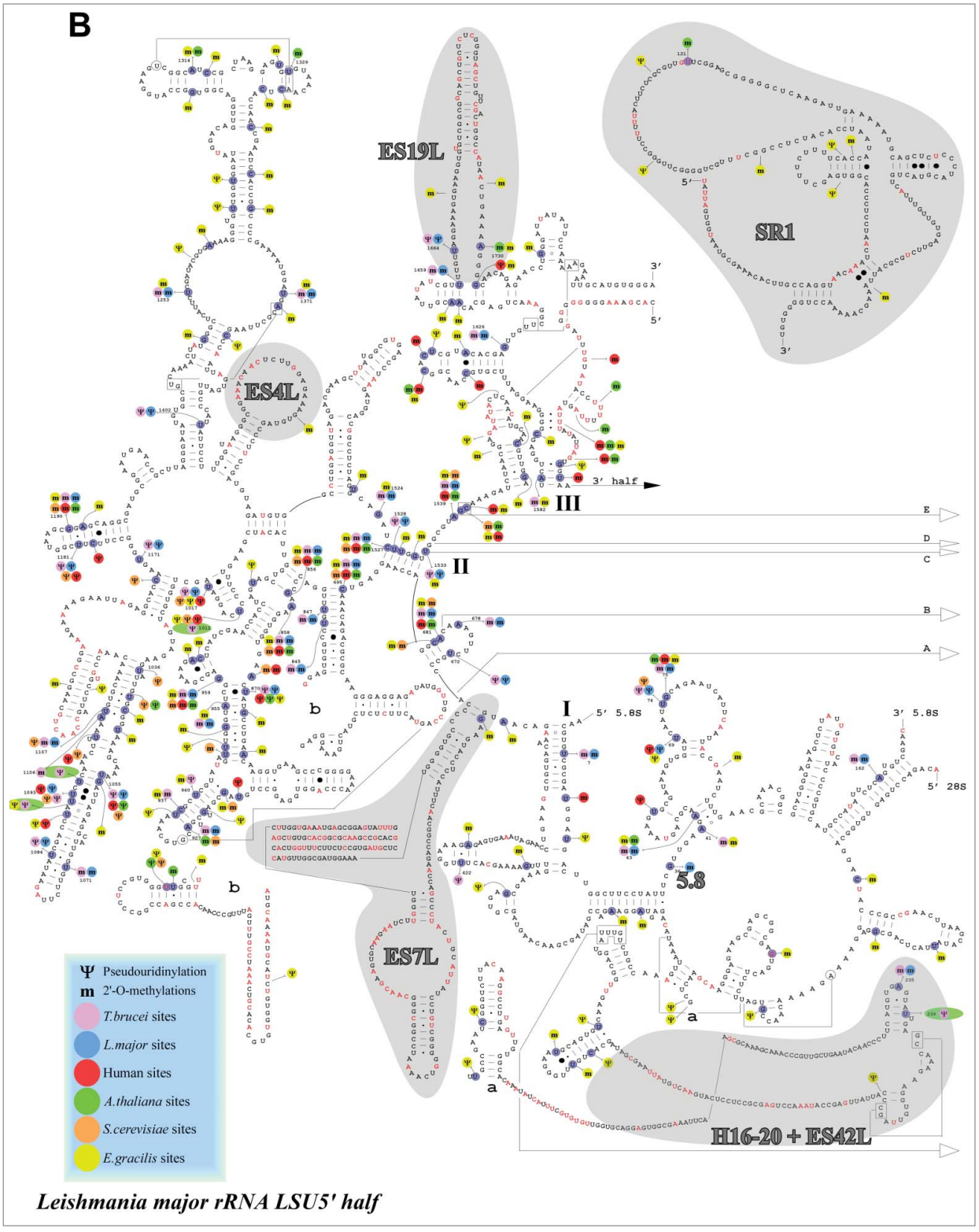

Figure 8. For figure legend, see page 1250.

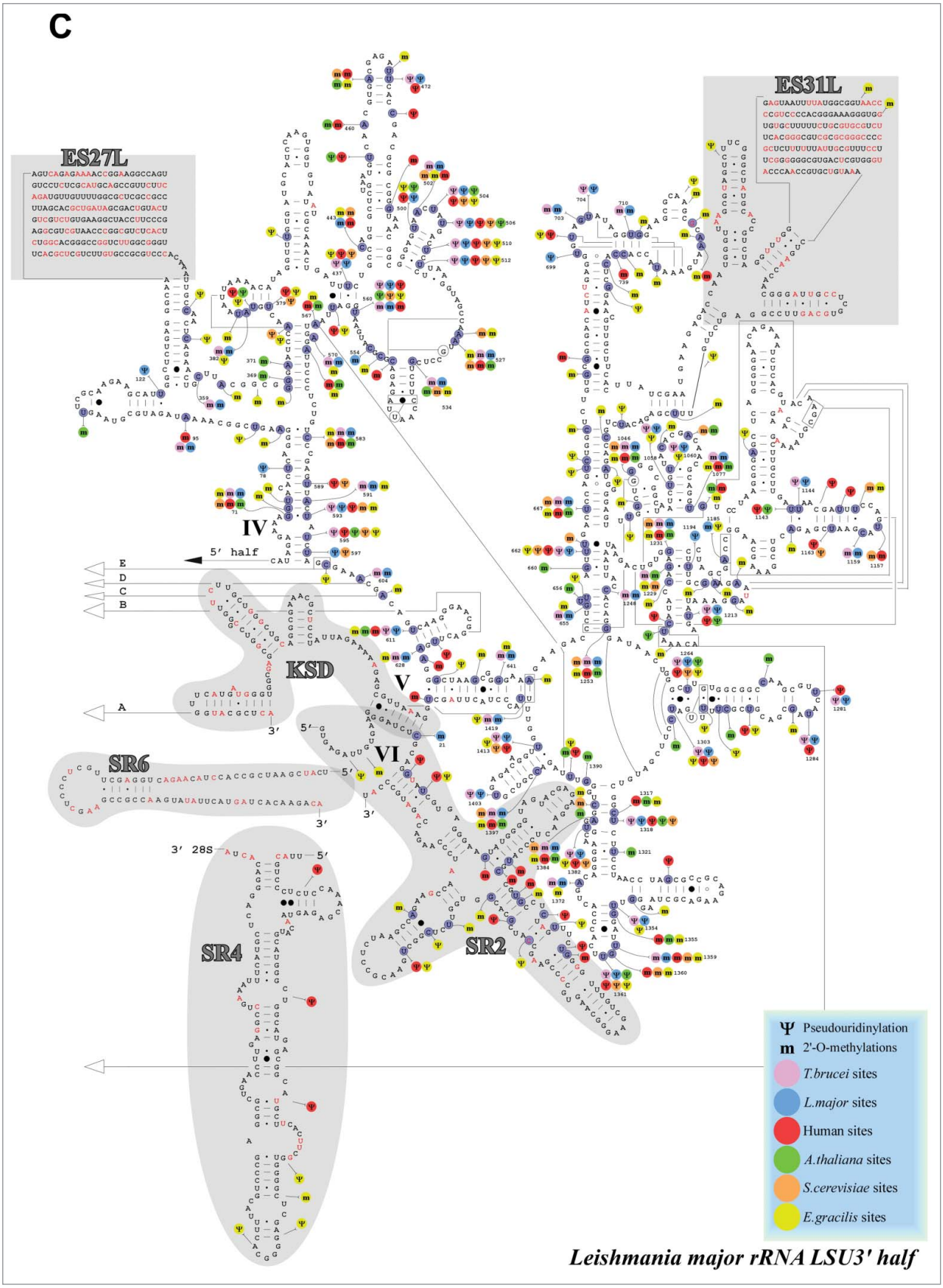

Figure 8. Localization of modified nucleotides on the secondary structure of rRNA. (**A**) Modification of SSU. Location of modified nucleotides on the structure of rRNA. The Nm are marked as m, and the pseudouridines as Ψ. The secondary structure was predicted based on the structure presented for *T. brucei* at htttp://www.icmb.utexas.edu, adjusting it to the *L. major* rRNA sequence. The identity of the small rRNA fragments and distinct domains is indicated and shaded. The modifications in different eukaryotes are designated by different colors, as indicated to the right. (**B**) As in A but for the 5′ half of LSU. (**C**) The same as in A and B, but for the 3′ part of LSU. The domains of the rRNA are indicated.

TB10Cs3H1 and LM36Cs1H4) (**Fig. 9A**). These homologues share 65% identity or greater in the pseudouridylation pocket and 55–65% identity over the rest the H/ACA. These account for 29 of the homolog pairs. The second group includes snoRNAs that share low similarity in their body region ($\leq$ 55% identity) but share almost identical pockets (90 – 100%) i.e., the sequence and even the structure (length of the second stem) are different between the homologues, but these are predicted to guide the same modification i.e. carry out the same function (e.g. TB1Cs1H1 and LM14Cs1H2) (**Fig. 9B**). Eighteen snoRNAs pairs are associated with this group. Interestingly, the last group is composed of snoRNAs that share low similarity in their body region ($\leq$ 55% identity), with differences in the pseudouridylation pocket (55–85% identity) but guide the same Ψ site (LM36Cs3H-1 and TB11Cs1pH-1). There are 14 snoRNAs pairs associated with this group (**Fig. 9C**). Kruskal-Wallis tests revealed significant differences in pocket identity ($P < 0.001$) and body identity ($P < 0.001$) as well as the interaction between them ($P < 0.001$) among these 3 groups.

In most cases, we can detect a clear ortholog in which the body and/or pocket are maintained. However, there are several cases in which we suspect a more complicated evolutionary history. We have identified 15 H/ACAs in *L. major* as likely paralogues (identity in body $\geq$ 60%). This may complicate identifying a single *T. brucei* homolog for each *L. major* H/ACA (**Fig. S4A**). Nonetheless, most of these paralogues seem to have arisen as a result of a duplication event in *L. major*, and thus map to a single *T. brucei* snoRNA. Such a case is TB10Cs5H3, which has 2 paralogues, LM36Cs4H1a that has an identical pseudouridylation pocket and 34.6% identity across the body, as well as LM36Cs4H1b, which has the same pocket but shares an overall identity of 56.8%. A few additional examples are presented in (**Fig. S4B**). Interestingly, the TB9Cs3H1 has 2 homologues LM5Cs1H3 and LM35Cs2′H2, which have almost the same pocket, suggesting that 2 different snoRNAs can be modified to target the same nt and this may be a mechanism whereby new snoRNAs are generated i.e., by only modifying the pocket after duplication of an existing snoRNA. In fact, it was possible to find 2 *Leishmania* paralogues for a single *T. brucei* snoRNA. Generation of paralogues from different snoRNA was also found in *T. brucei*. Such a case is LM18Cs1H2 and LM33Cs3H1 (61.5% identity), which we implicated as paralogues yet appear to have different homologues in *T. brucei* (TB10Cs4H2, TB9Cs4H1). This may present an example of a duplication event in a common ancestor (**Fig. S4C**).

Another interesting case involves a snoRNA in *T. brucei*, TB8Cs2H1A and B. It appears in the *T. brucei* genome in 2 forms, which are almost exactly identical copies with a 2 base pair change in the pocket area. Each pocket matches a different LM homolog (LM33Cs3H2 and LM36Cs2H1). Several of the paralogues (LM5Cs1H3 and LM35Cs2′H2, LM30Cs2H-1 and LM30Cs2H1, LM26Cs1H9 and LM26Cs1H6) guide the same

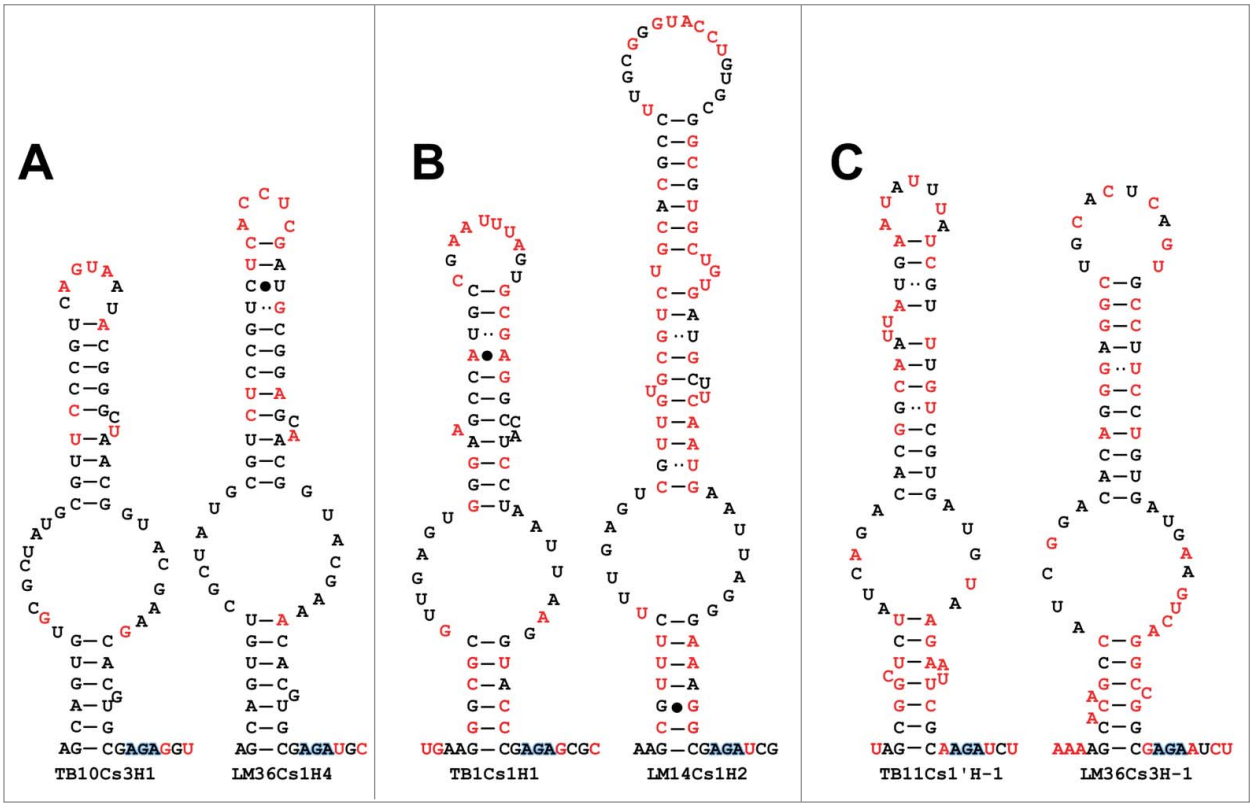

Figure 9. The relatedness between the *L. major* and *T. brucei* snoRNAs. (**A–C**) Secondary structure of the snoRNA in each of the 3 groups. The nts that differ in sequence are shown in red. The identifiers of each group are detailed in the text.

modification on rRNA. Although the paralogues may guide the same modification, we cannot rule out the possibility that these may undergo conformational changes and direct the modification on another target. Indeed, we have found the example of LM27Cs1H3, in which a single snoRNA can potentially guide 2 different modifications on rRNA.

Discussion

This study describes comprehensively the repertoire of *L. major* H/ACA and C/D snoRNAs, identifying potential targets of 92 Nm and 73 Ψ sites on rRNA. Based on these studies, the number of predicted modifications is much higher compared to yeast, which has approximately 50 modifications of each type, despite having the same genome size. The high number of modifications in trypanosomes may suggest a role in maintaining ribosome function when cycling between the insect and the mammalian host.[40] One striking finding is that except for 2 cases, all the snoRNAs described in this study have the potential to guide modifications on rRNA. Interestingly, the single-hairpin H/ACA RNA have flexibility in generating more than a single pseudouridylation pocket, and hence, a single hairpin RNA can potentially direct modifications on more than one site. The comparison between the repertoire of snoRNAs in *T. brucei* and *L. major* demonstrated how related snoRNA were engineered during evolution to direct modifications on the same or different sites. Duplication of snoRNA genes was needed to give rise to novel and species-specific snoRNAs. Abundant snoRNA emerged in both species to carry out trypanosome-specific rRNA processing events. The flexibility in the structure and function of trypanosome snoRNAs enables a relatively small repertoire of RNAs to guide a rich repertoire of modifications and carry out rRNA processing activities that are trypanosome-specific.

snoRNAs involved in trypanosome-specific rRNA processing: the LSU processome and snoRNAs with dual functions

One of the unique and striking properties of trypanosome rRNA is the fact that it is generated by cleavage of the LSU, resulting in small rRNA fragments that are held in the ribosome by base pairing. It was not clear for a long time why this fragmentation takes place, and how it is mediated. A recent study using high resolution cryo-electron microscopy solved the structure of the *T. brucei* ribosome.[41] The results revealed that the rRNA expansion regions that are highly variable in sequence and in size are extended in *T. brucei*. This is also true for *L. major* (**Fig. 8**). It was suggested that the cleavages of the rRNA fragments is necessary to accommodate for the increase in size of rRNA due to an increase in rRNA expansion domains. Our previous study supported the notion that trypanosome-specific snoRNAs evolved to direct these cleavages. Indeed, we have previously demonstrated the role of TB11Cs2C1 and TB11Cs2C2 in rRNA processing[23] and also the role of TB10Cs4C4, TB6Cs1C3, TB0Cs2C1, in trypanosome-specific rRNA fragmentation.[22] We suggested that TB11Cs2C1 may be the homolog of U14. Such special snoRNAs may also exist in *Euglena*, which also undergoes extensive rRNA processing.[42] However, despite the description of C/D and H/ACA RNAs in this organism, none of these have yet been implicated in rRNA processing. Interestingly, although the *Euglena* snoRNA repertoire resembles that of trypanosomes, it does possess a U14 homolog.[43]

snoRNAs involved in rRNA processing are clustered together in both *T. brucei* and *L. major* (**Fig. 7**). Of special interest is the finding that not all of the snoRNAs species implicated in rRNA processing are homologous in the 2 organisms. Among the 13 molecules implicated in rRNA processing[44] in *T. brucei*, only 9 have homologues in *Leishmania* while the rest are *T. brucei* specific, suggesting that different snoRNAs in these 2 species were selected to carry out these special processing functions. Indeed, all the snoRNAs implicated in rRNA processing also guide modifications on rRNA and thus these special snoRNAs may have emerged from snoRNAs involved in modification. The question remains how snoRNAs were selected for this additional function. It is possible that these snoRNAs were selected because of their chromosomal location and abundance. snoRNAs involved in rRNA processing were shown to be abundant in both *T. brucei* and *L. major*.

It is not currently known how the trypanosome differentially regulates the level of snoRNAs. The level of a snoRNA might be influenced by a post-transcriptional modification such as polyadenylation (our unpublished results).

The data presented in this study suggest that the SSU processome may be distinct from the LSU processome. However, relatively little is known about the biochemistry of the LSU in other organisms.[45] Recent bioinformatics analysis identified many factors involved in 40S processome and 60S processome function in *T. brucei*.[44] Tagging of factors from these complexes, and affinity purification and mass-spectrometry analyses should shed light on whether these 2 processomes function in a coordinated manner. Interestingly, we identified snoRNAs (**Fig. 7**) which are implicated in both SSU and LSU processing. It will therefore be interesting to identify possible cross-talk between the SSU and LSU processome, especially before the separation of the pre-40S complex from the pre-60S complexes.

The rich repertoire of trypanosome rRNA modifications

Recent studies suggest that Ψ modification on rRNA is important for the translation of a distinct subset of mRNAs such as mRNA harboring an internal ribosome entry site in mammals.[46] We have recently begun to analyze the Ψ at the whole-transcriptome genome level using a methodology similar to that used in recent studies that performed whole transcriptome mapping of this modification.[47] It will be of great interest to compare the pattern during the 2 life stages of the parasite and examine if these changes are correlated with growth at different temperatures, and/or are essential for preferential translation of distinct mRNAs which are developmentally regulated.

Of special interest are the specific modifications in the expansion regions that are highly expanded in the trypanosome ribosome. It is not currently known why these domains are extensively expanded in trypanosomes. One possibility is that

these domains bind factors involved in mRNA stability and translation, and thus regulate the stability and translation of mRNAs that are developmentally regulated in these parasites. Indeed, modifications were also found in these expanded domains in other eukaryotes but distinct positions are modified in the trypanosome rRNA (**Fig. 8**).

We suggested that the number of Nms is much higher than the number of Ψs in these organisms.[17] Even after we revealed the high number of H/ACA RNAs, there are still more expected Nms guided by snoRNAs compared to Ψs on rRNA. The Nms have been suggested to confer stability to rRNA and are found in thermophylic Archaea.[48] Indeed, studies in *T. brucei* showed that certain Nm positions are more extensively modified in the bloodstream form of the parasite than in the procyclic stage, which propagates in the fly.[40] Transcriptome-wide mapping of Nm is required to examine whether the changes in Nm are typical only of rRNA, or of other RNAs such as snRNAs or even mRNAs. The recent whole transcriptome mapping of Ψ suggests that this modification is also prevalent on mRNAs and is not only present on stable RNAs.[47] The Ψs were also shown to be induced under heat-shock.[47] Studies are underway to map the transcriptome-wide Ψ and Nms in the 2 life stages of the parasite, since both modifications are known to confer structural rigidity on localized RNA structure.[49] Indeed, these 2 modifications influence the RNA structure by favoring the C3′-endoribose conformation, diminishing the distance between the bases and enhancing stacking, which contributes to RNA stability.[50]

In *Euglena*, an organism that is evolutionarily related to trypanosomes, the LSU is fragmented to 14 pieces[51] and the degree of modification (350 modified nt on rRNA) is correlated with the level of rRNA fragmentation. In addition, the mismatch level in helical regions is 3-fold higher than in the same domain in human rRNA, and indeed, these domains are highly modified in *Euglena*.[51] Thus, breaking of helical stem structures in rRNA during evolution may have forced the generation of species-specific modification in trypanosomatids, and may explain the need for the development of species-specific snoRNA to direct these compensating modifications. Interestingly, trypanosomatids exhibit modifications which are common only to *Euglena* and in domains outside the domains enriched in modifications in other eukaryotes. It is plausible that both gain and loss of modifications during evolution shaped the repertoire found in *Euglena* and trypanosomes. The loss is represented by the absence of modifications which are conserved in other eukaryotes (**Fig. 8**). However, each of these organisms has acquired large number of species-specific modifications which are present in variable regions and may represent gain over evolutionary time.[51] However, it seems that the enhanced modifications found in both trypanosomes and *Euglena* may have evolved to cope with the fragmentation of the LSU. It will be of great interest to examine which of these modifications are constitutive or are induced under certain conditions.

The evolution of snoRNAs, a lesson from *T. brucei* and *L. major*

The comparison between *L. major* and *T. brucei* snoRNAs revealed that in 48% of the cases, H/ACA snoRNAs are true homologues, since these guide the same modifications in both species and the snoRNA share a high level of sequence similarity. Included in this family are the snoRNAs which share high similarity, but the sequence of the pseudouridylation pocket was changed to accommodate for differences in the rRNA sequence between the 2 species. However, a larger group of snoRNAs belong to a family of 43 (52%) snoRNAs which guide the same Ψ but share little sequence similarity. This may suggest how new snoRNAs can be generated by copying an existing snoRNA and "changing" the pseudouridylation pocket. This scenario resulted in different snoRNAs that guide the same modification. Indeed, we revealed cases in which 3 different snoRNAs are implicated in guiding the same modification. It will be interesting to determine if these modifications are critical for the function of the ribosome, and if they are guided by snoRNAs that are themselves developmentally regulated. Indeed, recent studies of snoRNAs in cancer demonstrated that certain snoRNAs are changed during the neoplastic process to guide a different set of modifications by minor sequence changes in the pseudouridylation pocket. It was suggested that the translation of distinct mRNA species requires different types of modification and that a particular pattern is favored to translate mRNAs that are essential for the survival and metastasis of cancer cells.[46] Thus, novel snoRNAs with different function may arise in all organisms from *Leishmania* to man by just changing the pocket. Indeed, in *Euglena* as well, recent studies suggest that frequent gene duplication is a common mechanism driving snoRNA emergence leading to both a large number of snoRNAs and clustered patterning of rRNA modification.[51]

One of the most striking observations made in our snoRNA studies[16,17,22] is that the vast majority of snoRNAs described are predicted to guide modifications on rRNA but not on snRNAs. Our recent mapping of Ψs on rRNA (unpublished) indicates that all the sites suggested to be guided by the H/ACA are valid, since these modifications exist on rRNA. We have also mapped Ψ and Nms on U snRNAs by primer extension sequencing and showed that at least 22 of the Ψs are guided by snoRNAs (our unpublished data). The question remains which snoRNAs guide these modifications and whether small Cajal body RNA (scaRNA) may be involved. The only scaRNA-like molecule identified so far is SLA1, which guides modification on the SL RNA.[18] This RNA is localized in a distinct site in the nucleus and outside the nucleolus.[21] In addition, SLA1 is bound by the protein MTAP[52] which exhibits homology to WD40 protein bound by scaRNA in mammals.[53] Thus, MTAP may specify the scaRNA-like RNAs in trypanosomes as well.

snoRNAs in pathogenic protozoan parasites

Are the specific features of snoRNAs and their guided modifications related to their parasitic life especially cycling between 2 hosts. To answer this question we compared the features of trypanosome snoRNA to those found *Plasmodium* (malaria). In malaria, as in humans, many snoRNAs are encoded in introns and are associated with genes involved in ribosome metabolism.[11] It was suggested that malaria snoRNAs were duplicated in evolution via retroposition. However, no information is

available on differences between snoRNA expression and modifications in the parasite propagating in the 2 hosts and this aspect will be interesting to study.

Since many of the protozoan parasites diverged early in evolution from the eukaryotic lineage it was interesting to compare their snoRNA properties to those of trypanosomes. As in trypanosomes, in *Entameoba histolytica* snoRNAs are single hairpin RNAs, but these possess an ACA rather than an AGA box. The *E. histolytica* snoRNAs are more related in sequence to yeast and human than to *Plasmodium* and trypanosomes.[10] Studies from *Giradia lamblia*, one of the most ancient eukaryotes, suggest that, as opposed to trypanosomes, their C/D snoRNAs are only single guiders and all H/ACAs are composed of double-stem-loop hairpins in contrast to trypanosomes. Thus, *Giardia* snoRNAs are more related to the snoRNA found in fungi and metazoans[9] and the single hairpin snoRNA is not typical to ancient eukaryotes and is specific only to certain sub-groups.

The study presented here describes the comprehensive repertoire of snoRNAs in *L. major*, and suggests how snoRNAs may have evolved to carry out different functions. Whereas the snoRNAs identified here are proposed to carry out rRNA modification, we have yet to identify the snoRNAs guiding the modifications on snRNAs. The simple rules that were applied to identify modifications on rRNA do not seem to apply to snRNAs, and as suggested above, trypanosomes may have the ability to utilize single hairpin H/ACA snoRNAs to guide more than a single modification. This study also identified the snoRNAs implicated in trypanosome-specific rRNA processing. The peculiar properties of trypanosome snoRNAs are not related to their parasitic life, but rather to the need for rRNA fragmentation, as *in Euglena*. However, we are only at the beginnings of understanding rRNA processing in these organisms, as well as the extent by which modification mediated by snoRNAs or enzymes regulate gene expression and ribosome function. The trypanosome genome encodes for numerous pseudouridine synthases and methyltransferases, which should also contribute to the complexity of RNA modifications in these important parasites.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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