

Profiling of 2'-O-Me in human rRNA reveals a subset of fractionally modified positions and provides evidence for ribosome heterogeneity

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ABSTRACT

Ribose methylation is one of the two most abundant modifications in human ribosomal RNA and is believed to be important for ribosome biogenesis, mRNA selectivity and translational fidelity. We have applied RiboMeth-seq to rRNA from HeLa cells for ribosome-wide, quantitative mapping of 2'-O-Me sites and obtained a comprehensive set of 106 sites, including two novel sites, and with plausible box C/D guide RNAs assigned to all but three sites. We find approximately two-thirds of the sites to be fully methylated and the remainder to be fractionally modified in support of ribosome heterogeneity at the level of RNA modifications. A comparison to HCT116 cells reveals similar 2'-O-Me profiles with distinct differences at several sites. This study constitutes the first comprehensive mapping of 2'-O-Me sites in human rRNA using a high throughput sequencing approach. It establishes the existence of a core of constitutively methylated positions and a subset of variable, potentially regulatory positions, and paves the way for experimental analyses of the role of variations in rRNA methylation under different physiological or pathological settings.

INTRODUCTION

Nucleotide modifications are found in most major classes of RNA and in all domains of life. Currently, >140 modifications are known (1). Most are methylations, and ribose methylations (2'-O-Me) together with pseudouridines (Ψ) are the most frequent modifications (2). Ribosomal RNA (rRNA) is one of the most densely modified classes of RNA and the modifications are clustered in conserved and functionally important parts of the molecule (3). The number

of modification sites appears to increase during evolution and the relative numbers of different modifications appear to change in parallel. From inspection of two widely used public databases (4,5), *E. coli* has 19 base modifications, 11 Ψ and four 2'-O-Me. In contrast, 12 base methylations, 54 2'-O-Me and 44 Ψ are listed for yeast. The two databases have slightly different numbers for human rRNA, with approximately 10 base modifications and around 100 each of 2'-O-Me and Ψ (4,5). Although recent studies have identified additional modifications not listed in these databases, the increase in the absolute and relative importance of 2'-O-Me and Ψ over the course of evolution remains striking. The shift in modification patterns appears to be related to the advent of RNA guided modifications in Archaea and Eukarya. Here, box C/D snoRNAs (snoRDs) guide the methyltransferase fibrillarin to target sites for ribose methylation (6,7), while box H/ACA snoRNAs (snoRAs) guide the isomerase dyskerin to target sites for pseudouridylations (8,9). Ribose methylations and pseudouridylations are suggested to play roles in RNA folding and translational fidelity (3). However, it is likely that the guide RNA itself contributes to the folding function in some cases, due to its base pairing with the target, as observed with some box C/D snoRNAs (e.g. U3 and U8) that do not elicit methylation (10).

The RNA guided modification system has undergone considerable diversification. The number of box C/D guide RNAs range from 7 to 128 in different species of Archaea for example (11), also several new guide RNA families have emerged during evolution of vertebrates (12). Furthermore, in mammals, the high number of additional snoRNAs lacking a known target ('orphans') (13) testifies to the evolving complexity of the guide RNA system. This raises the question of whether the modifications in human rRNA are constitutive or variable, within or between cells.

The ribose methylation pattern in human ribosomal RNA is known mainly from experimental analysis based

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on RNA fingerprinting conducted in the Maden laboratory in the 1980s (14,15). This work used HeLa RNA labeled *in vivo* with [³²P]ortho-phosphate and [¹⁴C]methyl-methionine and thus yielded results that allowed estimation of the fraction of molecules that were methylated at any given position. Later, this was supplemented with predictions of methylation sites based on sequences of box C/D snoRNA guides, that in many cases have been validated by biochemical methods (6). This work mostly used primer extension-based assays for validation of individual methylation sites without providing quantitative information. It was shown that target methylation is introduced in the duplex formed between the guide RNA and the target at the position 5 base pairs upstream of box D (or the equivalent box D') (6,7). The modification sites in human rRNA and information on their respective guide RNAs was compiled in snoRNABase (4) that was last updated in 2007. Since then, studies have revealed a few candidate snoRDs and experimentally validated selected 2'-O-Me sites (12,16,17).

We recently developed a sequencing based profiling method for ribose methylations, RiboMeth-seq (RMS), based on the resistance of ribose-methylated RNA toward alkaline degradation (18) (Figure 1A). The method yields quantitative data that in yeast was in agreement with results obtained by a variety of other quantitative methods. Here, we use RMS supported by MALDI-TOF mass spectrometry (MS) to arrive at a comprehensive map of 2'-O-Me sites in HeLa cell rRNA that features two new modification sites. Importantly, we assign a score to each 2'-O-Me site expressing the fraction of molecules modified at the site. Re-examination of human snoRDs results in assignment of a plausible guide RNA to 103/106 2'-O-Me sites. We use this comprehensive data set to define a conservative subset of box C/D guide RNAs that are sufficient to explain the observations, and correlate the fraction score with various parameters, including snoRD expression. Finally, we compare the methylation profile of HeLa cells and HCT116 colon cancer cells and demonstrate that most sites are modified to the same extent, with a subset of sites showing variation. Our work provides a consensus view of ribose methylation in human rRNA that can serve as a platform for future studies.

MATERIALS AND METHODS

Cell cultivation and RNA isolation

The cell lines used were cervix adenocarcinoma cell HeLa S3 (ATCC CCL-2.2) and the colorectal carcinoma cell line HCT116 (ATCC CCL-247). Cells were maintained as adherent monolayer cultures in either DMEM media (HeLa S3) or McCoy's 5A media (HCT116) supplemented with 10% fetal bovine serum (FBS) and grown to ~70–80% confluence before harvest. Whole cell RNA was extracted using QIAzol (QIAGEN), according to the manufacturer's instructions, after which 15 µg of RNA per sample was subjected to native agarose gel electrophoresis and the SSU (18S) and LSU (28S) rRNA subsequently gel purified using the NucleoSpin Gel and PCR Clean-up kit (MACHEREY-NAGEL) according to the manufacturer's instructions.

Library construction and sequencing

The protocol was essentially as previously described (18). Briefly, for each replicate library, 4–5 µg of purified SSU and LSU subunit rRNA, combined at 1:2.6 weight ratios to give approximate 1:1 molar ratio, was partially degraded by alkaline at denaturing temperatures. The size fraction 20–40 nt was purified by polyacrylamide gel electrophoresis and linkers added using a system relying on a modified *Arabidopsis* tRNA ligase joining 2',3' cyclic phosphate and 5'-phosphate ends. The library fragments were then sequenced on the Ion Proton platform (Life Technologies). Experiments were done in triplicate and individual libraries varied in the range 25–36 × 10⁶ (HeLa) and 17–25 × 10⁶ (HCT116) 5' or 3' read-ends per library.

Data treatment

Data treatment was similar to that reported previously (18). We used the human ribosomal DNA complete repeating unit (Genbank acc. no. U13369, version GI:555853) as a starting sequence and corrected it according to the high coverage sequencing from the RMS analysis. Our derived reference sequence showed several differences to the GenBank and snoRNABase sequences and is more similar to the sequence reported recently in a paper describing the cryo-EM structure of the human ribosome (19). An alignment table of these four sequences is included as a separate file in Supplementary Information. To facilitate comparison with snoRNABase, we have used the snoRNABase numbering throughout the manuscript. Thus, insertions compared to snoRNABase are left unnumbered and numbers are removed along with nucleotides at sites of deletions. At the ends of rRNA molecules, ~20 nt are only queried from one end due to the gel purification of 20–40 nt library fragments.

The RMS score used throughout this paper describes the fraction of molecules methylated at the queried position and is calculated by comparing the number of read-ends at the queried position to six flanking positions on either side, as previously described ('Score C' in (18)). In addition to the data set used here, we generated another triplicate data set from HeLa cells that yielded very similar results. The primary data and analyses of all data sets are deposited to the NCBI Gene Expression Omnibus (GEO) at GSE76393.

rRNA fragment isolation and MS

Fragments for MS were isolated as previously described (18,20). Briefly, an oligo (Supplementary Table S2) spanning the queried site was annealed to rRNA followed by degradation of unprotected RNA with Mung Bean nuclease and RNase A. The protected fragment was then isolated from a 10% denaturing (urea) polyacrylamide gel and further digested with RNase A or RNase T1. These fragments were then analyzed by MALDI-TOF MS on an Autoflex Speed (Bruker Daltonics, Bremen) instrument. Spectra were recorded in reflector, positive ion mode.

Array analysis of SNORD expression

SnoRNA expression was measured in HeLa and HCT116 cells, using custom designed arrays (Nimblegene HD2-12 platform; 135K 60mer probes) as previously described

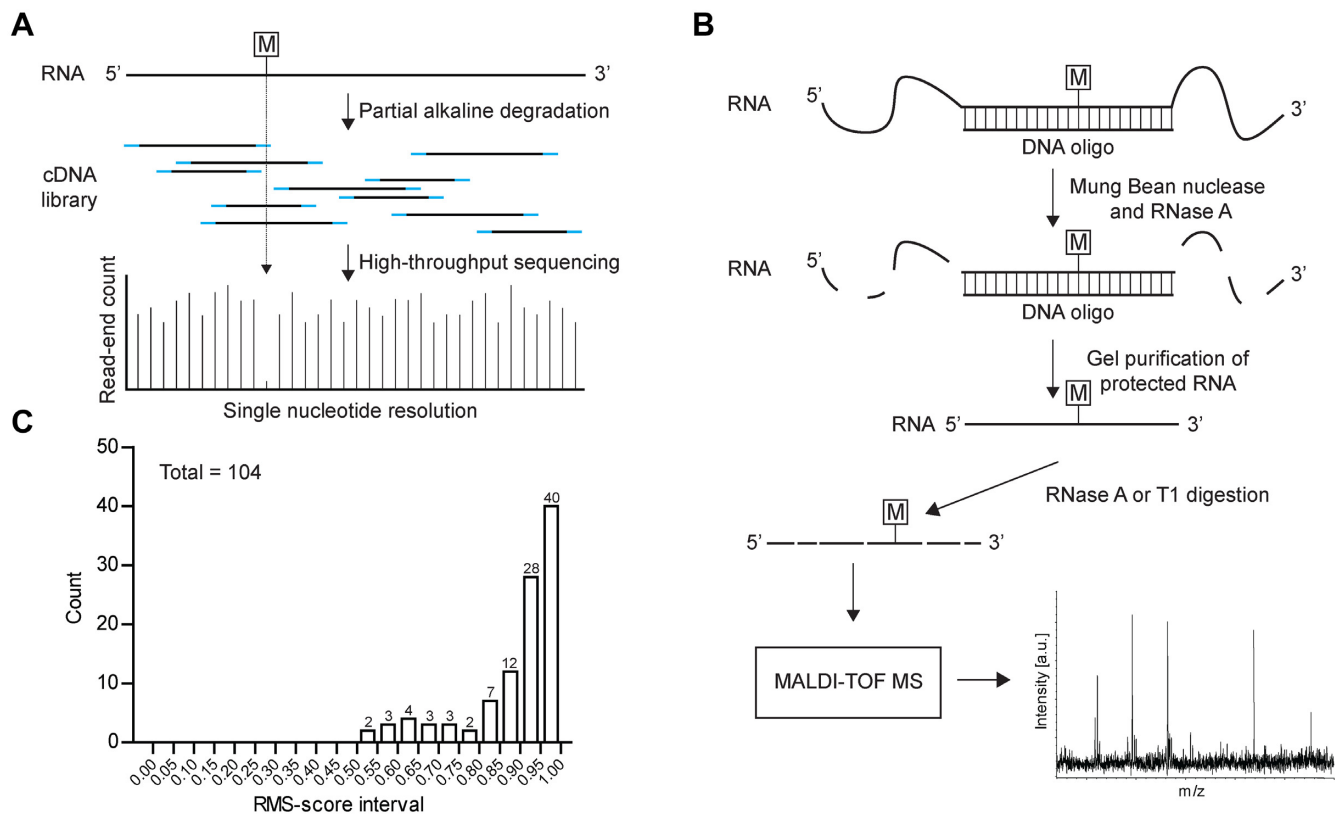


Figure 1. Outline of experimental strategies. (A) In RiboMeth-seq (RMS) analysis, library fragments obtained by alkaline degradation were ligated to adapters (blue), sequenced and 5' and 3' read-ends recorded. Ends at cleavable bonds will be highly represented whereas ends at bonds protected by 2'-*O*-methylation will be under-represented proportionally to the fraction of molecules modified at the position. (B) For mass spectrometry (MS) validation, a fragment encompassing the queried positions was isolated by annealing of a complementary oligo followed by digestion of unprotected RNA with RNases. The protected fragment was subjected to RNase A or T1 digestion followed by MALDI-TOF MS. (C) Distribution of RMS scores in HeLa rRNA. The score expresses the fraction of molecules methylated at the queried position. The figure excludes the two methylations in 5.8S rRNA.

(doi:10.1261/rna.038927.113). Array data were analyzed in R (www.r-project.org). Arrays were normalized using the RMA implementation of the oligo software package (doi:10.1093/nar/gng015).

RESULTS

Application of RiboMeth-seq to human rRNA

RMS was applied to small subunit (SSU) and large subunit (LSU) rRNA from HeLa cells. This strategy excluded 5.8S rRNA, previously shown to be methylated at two sites, from the analysis. For completeness, these two positions were incorporated in figures and tables. The RMS results are expressed as an RMS score corresponding to the fraction of molecules methylated at the given position (see Materials and Methods). An RMS score was tabulated for all positions with the lower scores giving higher standard deviations due to inherent high background in the method (18). For detection of sites, we applied different thresholds to the RMS score (Supplementary Table S1). The snoRNABase lists a total of 108 2'-*O*-Me sites with 42 in SSU, 64 in LSU and 2 in 5.8S rRNA. At the 0.75 threshold, RMS detects 34/42 sites in SSU and 55/64 in LSU (Supplementary Table S1), corresponding to 84% of previously annotated sites. In order to arrive at a conservative high confidence set of

2'-*O*-Me sites in human SSU and LSU rRNA, this threshold was used to give a compromise between a low number of undetected positions and a manageable number of new candidates for independent MS validation. In regard to the known methylation sites falling below the 0.75 threshold, we suspect that these sites simply are methylated to a lesser degree. The nine candidates for new sites were reduced to five by closer examination of the RMS data (see legend to Supplementary Table S1).

Validation of 2'-*O*-Me sites by MS

We used MALDI-TOF MS to complement and validate the results from the RMS analyses (Figure 1B) at 28 sites (Supplementary Figure S1, Tables S2–S3). Two new sites were detected by RMS analysis, one with a low RMS score (0.63 at LSU-C1868; Figure 2) and one with a high RMS score (1.00 at LSU-G3771; Figure 3). The former site is misannotated in snoRNABase, where snoRD48 is suggested to modify LSU-2279. The interaction has a mismatch at pos. 2 upstream of the D box followed by 11 consecutive base pairs. The methylation is supported by a partially methylated GC_mUG-fragment in RNA fingerprinting (15), but there is no evidence of methylation in our RMS data. Analysis of interactions using Snoscan (21) offers an alternative target for snoRD48 at LSU-1868, with nine consecutive

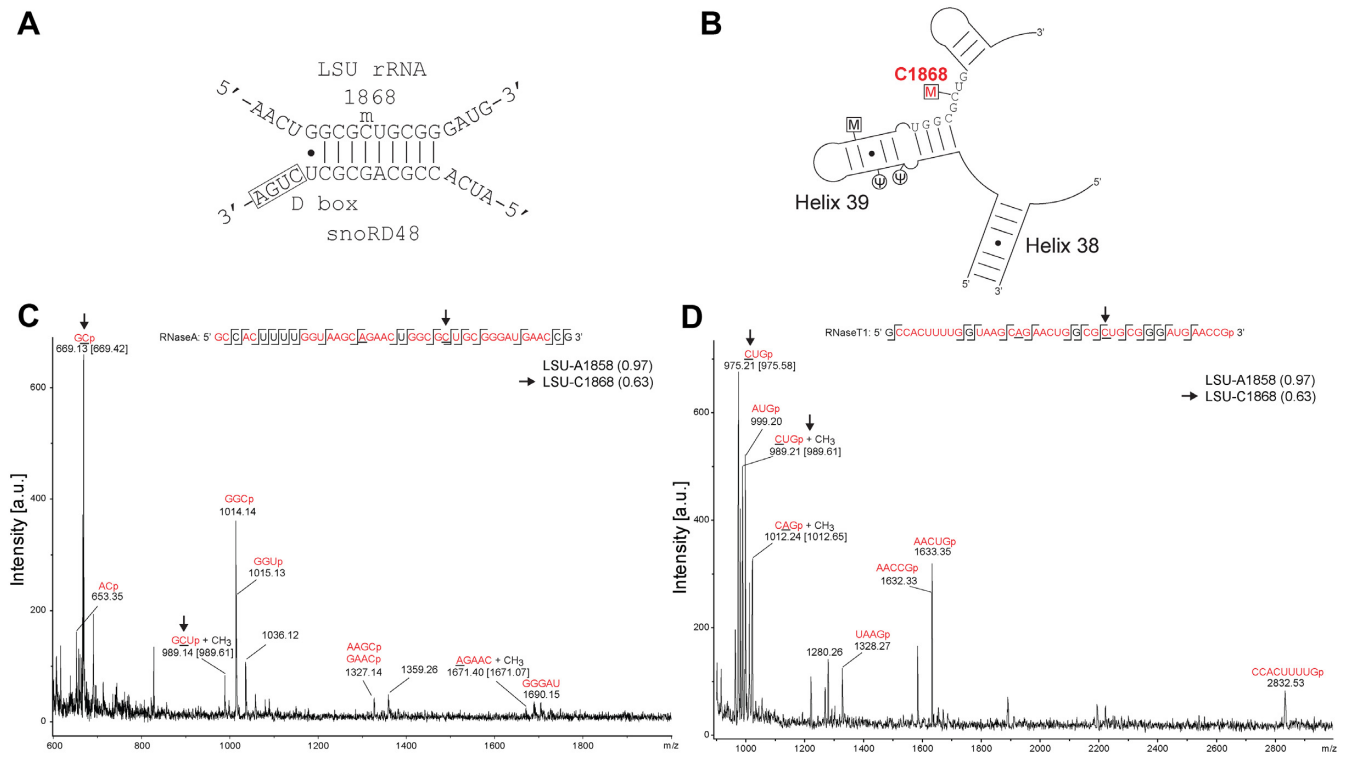


Figure 2. A new methylation at LSU-C1868. **(A)** Base pairing of snoRD48 and target. **(B and C)** MS analysis. Both methylated and unmethylated fragments are present in the RNase A and T1 spectra, as expected from the RMS score. The sequence of the fragment is shown above the spectra, with an indication of the expected cleavage sites by RNases. All identified fragments are labeled in red. Arrows mark C1868 positions and spectra peaks with indications of observed and theoretical (in brackets) masses. **(D)** LSU-C1868 is located at the base of H39 that harbors two 2'-O-Me (M) and one pseudouridine (Ψ) and close to H38, the 'A-site finger'.

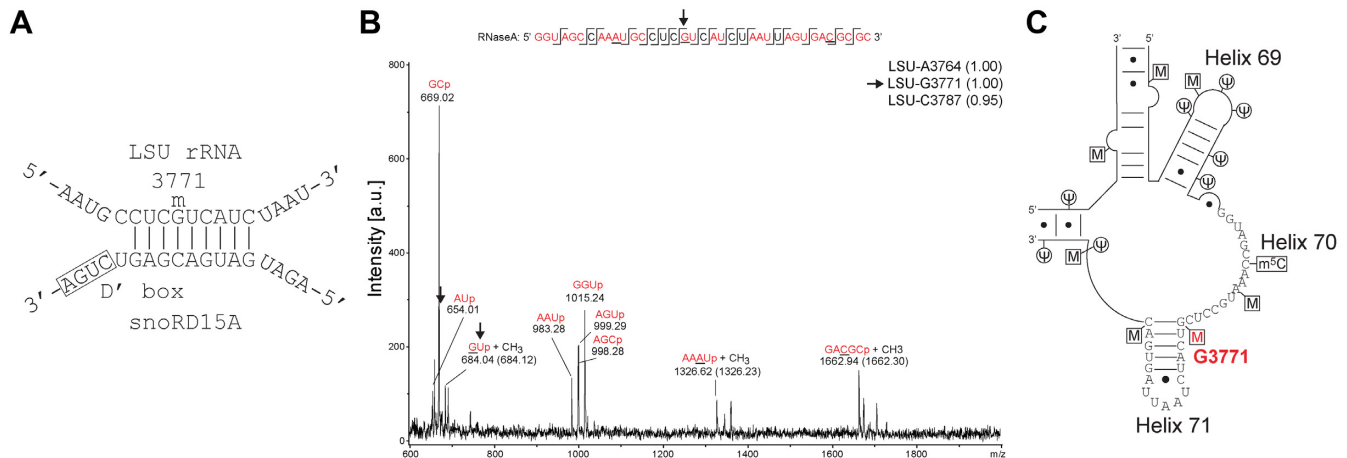


Figure 3. A new methylation at LSU-G3771. **(A)** Base pairing of snoRD15A and target. **(B)** MS analysis. Only the methylated fragment is observed, as expected from the high RMS score. The sequence of the fragment is shown above the spectrum, with an indication of the expected cleavage sites by RNase A. All identified fragments are labeled in red. Arrows mark the G3771 position and spectrum peak for the methylated fragment with indications of observed and theoretical (in brackets) masses as well as the expected position of the unmethylated fragment that was not observed. **(C)** LSU-G3771 is located at the base of H71 and base paired to the 2'-O-methylated residue LSU-C3787.

base pairs starting at pos. 2 upstream of the D box (Figure 2A). This is equally supported by the GC_mUG-fragment. Although this position consistently scores slightly below the threshold level of 0.75 in HeLa, MS supports this assignment and its fractional modification (Figure 2C and D). Thus, we have re-assigned the methylation guided by snoRD48 to LSU-1868.

The new site we find at LSU-3771 is supported by re-interpretation of the original data from Maden (15) and by our MS analysis. SnoRD15A can form a 9 bp interaction upstream of box D' with the target, conforming to the consensus snoRNA-target interaction rules (Figure 3A). SnoRD15A is also predicted to use nucleotides upstream of box D to target the nearby LSU-3764. Supporting the RMS data, in which LSU-3771 is fully methylated, only the methylated fragment is observed in MS analysis (Figure 3B).

In addition to the two validated novel 2'-O-Me sites, we have preliminary evidence for two further methylations. One is LSU-3606 guided by snoRD31 and with a RMS score of 0.80. MS demonstrated a fragment comprising this modification but the spectrum could not be fully explained from the theoretical masses (data not shown). The second is SSU-1232, which is likely the correct assignment for the 5'-GCUU_mGC assigned to SSU-1668 by Maden (14). We note that the description of this methylation was ambiguous, that the primary data were not shown, and that the modification was not found in a previous paper using similar methodology (22). SnoRD103 has been proposed as a guide for this site, but the interaction has two centrally located mismatches. Furthermore, the RMS score is consistently very low at SSU-1668, but 0.90 and unaccounted for at SSU-1232. We suggest that the sequence of the modified fragment is 5'-GCCU_mGC and that the modification at SSU-1232 is Ψ_m as suggested by the potential targeting of this position by box H/ACA guide snoRA70. The double modification may have caused aberrant migration in chromatography analysis and resulted in misinterpretation of the sequence.

Fourteen previously annotated sites, predicted on the basis of snoRD-target alignments were examined in our MS validation experiments. Eleven of these are listed in snoRNABase and eight of these were validated here, excluding SSU-1536, SSU-1668 and LSU-2402. The remaining three sites (SSU-1517, SSU-1533, SSU-1549) were proposed in more recent literature (12,16) but could not be validated by MS. Of note, the sites that we were unable to validate by MS all have RMS scores <0.75. Thus, we provide the first experimental evidence for methylation at eight previously predicted sites.

Finally, 12 sites from snoRNABase, both with previous experimental evidence and supported by snoRD interactions were confirmed by MS (Supplementary Table S3).

Reinterpretation of the human rRNA methylation landscape

Table 1 summarizes the changes we observe in relation to snoRNABase, and Supplementary Table S4 contains the full updated list. In the Maden papers (14,15), 11–13 mols of methylated nucleotides could not be unambiguously mapped to the rRNA sequence. A fragment with the

sequence 5'-(G)CC_mCG could not be placed on the map because the SSU rRNA has five instances of this sequence, but only the sequence in which SSU-1272 corresponds to the methylated position is consistent with base pairing to a known snoRD (snoRD66) as well as with our RMS and MS data. We find that the reported 2–3 mols of GA_mG are consistent with methylation at LSU-389, LSU-391 and LSU-2388 (fractional modification). A total of 7 mols of GG_mG can be placed at LSU-1612, LSU-1747, LSU-2863 (as G_mGG), LSU-3878, LSU-4166 and LSU-4362. Less than 1 mol of GC_mUG is consistent with fractional modification of the new position LSU-1868 reported here, and finally, that 1 mol of G_mU support modification at the other new site at position LSU-3771, reported in this study. The consistency of the data from RNA fingerprinting and RMS data serves as a further validation of our approach. Importantly, these assignments leave only two positions (SSU-1536 and SSU-1602) in the database without experimental validation. These two positions are guided by snoRD12C and may represent a special case of a snoRD whose binding does not elicit methylation. In addition, SSU-1668 is not supported by RMS or MS analysis of HeLa cells and is most likely a mis-assignment (see Discussion). In summary, the combined evidence from RMS, the resulting re-interpretation of the original RNA fingerprinting data, and MS analysis provide new experimental evidence at the 14 sites listed in Table 1.

Table 2 lists the update of the assigned snoRDs compared to snoRNABase. Only predictions that are experimentally supported are included. The contributions are from studies based on the conservation of snoRNA:target interactions (12,17), one study that combines experimental (PAR-CLIP) and bioinformatics approaches (16), and from our own observations. These assignments leave only three positions without a corresponding snoRD guide. Intriguingly, these occur at the only three instances of neighboring methylation sites in human rRNA.

The update of snoRNABase results in a data set comprising 106 experimentally validated ribose methylations in HeLa cell rRNA of which 103 are assigned with a snoRD. This data set excludes SSU-1536, SSU-1602, SSU-1668 and LSU-2279 annotated in snoRNABase, and includes new sites at LSU-1868 and LSU-3771.

Ribosome heterogeneity at the level of RNA 2'-O-Methylation

In addition to mapping of 2'-O-Me sites, RMS provides a measure of the fraction of molecules modified at a particular site thus providing a new layer of information to the description of human ribosomes. Although no rigorous testing of these data can be performed due to the absence of comparable ribosome-wide methods, several observations support this claim. Firstly, the modifications noted by Maden (14,15) to be fractional, have low scores in RMS. Secondly, the seven sites with lowest RMS scores (0.60–0.84) that were subjected to MS, all displayed a methylated as well as an unmethylated fragment in contrast to the 15 high scoring positions (>0.95) that only displayed a methylated fragment (Supplementary Table S3). Thirdly, RMS scores in yeast were found to be consistent with the liter-

Table 1. Update of 2'-O-Me sites in HeLa cells in relation to snoRNABase

New experimental evidence for 2'-O-Me in HeLa cells					
Subunit	Position	Maden (14,15)	RiboMeth-seq	MS	Comment
SSU	C1272	+ (fractional)	<0.75	+	
LSU	A389	+	+	+	
	A391	+	+	+	
	G1303		<0.75	+	
	G1612	+	+	+	
	G1747	+	+	NA	
	A1868	+ (fractional)	<0.75	+	New site
	A2388	+ (fractional)	<0.75	+	
	G2863	+	+	NA	
	G3771	+	+	+	New site
	A3846		<0.75	+	
	G3878	+	+	NA	
	G4166	+	+	+	
	G4362	+	+	NA	
	Absence of experimental evidence for 2'-O-Me in HeLa cells				
Subunit	Position	Maden (14,15)	RiboMeth-seq	MS	Comment
SSU	G1536	-	-	-	snoRD12C/ D
SSU	U1602	-	-	NA	snoRD12C/ D'
	U1668	(+) ^a	-	-	snoRD103/ D

^aThis methylation is likely to be a misassignment and should be placed at SSU-1232; see Discussion. NA: not analyzed.

Table 2. Update of snoRD assignments in relation to snoRNABase

Newly assigned snoRDs at experimentally validated positions				
Subunit	Position	Guide	Alias	Reference/Comment
SSU	C174		SNORD45C	(12,17)
	A468	68	SNORD83A	(12)
	C797	ZL107	-	(12,16)
		GGgCD20		(12)
LSU	A1383	30	SNORD30	(12)
	C1868		SNORD48	This study/ reassignment
	U2402	ZL5/6	SNORD143/144	(16)
	G3771		SNORD15A	This study
	U4468	62	SNORD62A/B	(12)
Positions lacking assigned snoRD				
Subunit	Position	Comment		
LSU	G2351	Strong experimental evidence for 2'-O-Me		
	U4197	Strong experimental evidence for 2'-O-Me		
	G4469	Strong experimental evidence for 2'-O-Me		

ature overall and matched well at the few positions that have been analyzed by other methods (18). The distribution of RMS scores in the present analysis of HeLa cells is displayed in Figure 1C and the scores at all methylated sites listed in Supplementary Table S5. Approximately two-thirds of the positions are found to be fully or close to fully methylated, and one-third to be fractionally methylated. The high number of positions that are not fully methylated implies that the pool of ribosomes is heterogeneous in composition either within or between cells and supports the notion of specialized ribosomes (23). Importantly, RMS analysis paves the way for grouping of sites according to their level of modification and enables correlation of the RMS score with other properties.

The differences in RMS scores are not explained by variation in snoRD structure or binding affinity

We speculated that our comprehensive set of experimentally validated ribose methylations could be used to derive a more uniform consensus on the properties of snoRNA guided ribose methylation and to establish correlations between the fraction score and features of the guiding system. First, we extracted information on all snoRD sequences from the UCSC Genome Browser that have the potential to target the validated rRNA positions. This set was manually curated to exclude snoRD family members that presented bulges or substantial mismatches in the pairing of the antisense elements to the putative targets. The subset consists of 152 snoRDs with 50, 88 and 14 members that use box D, box D', and both boxes, respectively (Supplementary Table

S6). Using this subset, we created an alignment of the conserved sequence elements. Boxes C and D form a terminal kink-turn motif that binds the 15.5K protein that is essential for processing and assembly of the snoRNP (24). Boxes C' and D' are thought to form an internal kink-loop that does not bind stably to the 15.5K protein (25). We find that 61/64 snoRDs, which use the D box for guiding methylations, strictly abide by the 5'-CUGA box D consensus sequence and that the remaining three (snoRDs 14/5'-CUAU, 14D/5'-UUGA and 33/5'-UCAG) all have paralogs with a 5'-CUGA box D (Figure 4A). Thus, all of the positions guided by a D box could be assigned with a 5'-CUGA box D. Importantly, this observation could be extended to include the box D in snoRDs that use box D' for methylation guiding, suggesting strong selection for this sequence in functional methylation guiding snoRDs, most likely related to the biogenesis of the snoRD rather than interaction with the target site. Box D' showed considerable variation (Figure 4B). Box C (5'-RUGAUGA) was highly conserved at the five central nucleotides that form base pairing interactions in the kink-turn motif, but showed slight variation in the flanking nucleotides. Thus, it appears that the two sequence elements forming a kink-turn, serving both as a binding site for the protein 15.5K and to stabilize the snoRD during processing and assembly, are highly conserved. In contrast, the D' and C' boxes are less conserved, as previously noted (26), and in many cases it is questionable whether they can interact to form a kink-turn motif (27). We found no correlation between the type of D box (D or D') and the RMS score (Supplementary Figure S2).

Human snoRDs use an antisense element associated with a D' box more frequently than with a D box (Figure 4C). It has been suggested that the use of both boxes by a snoRD could bring otherwise distant parts of the rRNA together (28), similar to what is known for U3 snoRNA (29,30), or result in sequential modifications related to the order of folding of rRNA (31). However, only 14 potential cases of snoRDs using both D and D' boxes are found in our data set and only 3 of these are clear cases where no paralogs or other snoRDs can target the positions in question. These are: snoRD24 that targets LSU-2352 (D') and LSU-2338 (D) in domain II; snoRD30 that targets LSU-3804 (D') and SSU-1383 (D); and snoRD68 that targets LSU-2388 (D') and SSU-428 (D). The two latter snoRDs guide modifications that are far apart in the mature ribosome (19) and occur in separate subunits that supposedly mature independently (10). Thus, we find no support for a role of snoRDs in coordinating folding or assembly of the ribosome by using linked D and D' boxes.

Next, we examined the interaction between the antisense elements located upstream of boxes D and D' and the target sequences in rRNA. In predictions based on snoRD sequences, a rule that requires 7–20 base pairs and allows GU pairs and a few mismatches (excluding bulges) is frequently applied (12). Figure 4D shows an analysis of base pairs in relation to the distance upstream of the D (or D') box. The first position is considered not to be base paired (32). There are eight instances of mismatches in position 2, and five instances at three internal positions. Two involve A–C mismatches (snoRD62A/B and snoRD79), and one (snoRD49A/B) is a U–U mismatch. All of these in-

teractions involve fully methylated positions for which no other snoRDs are known. Thus, it appears that internal mismatches are rare, although they are tolerated in specific contexts that are not fully understood.

It has been shown in yeast that additional base pairing between the guide RNA and target stimulate methylation (33). Thus, it would be expected that the strength of the interaction would influence the RMS score. The lengths of the putative interactions in our data set are distributed around 12 bp with 8 bp being the shortest and 19 bp the longest (Figure 4E). Only the primary interaction site immediately upstream of the D box was considered. We also calculated the minimal free energy of the interaction using RNAduplex (34) (Figure 4F). Neither of the two interaction measures showed correlation with the RMS score (data not shown).

Comparison of 2'-O-Me signatures in HeLa and HCT116 cancer cell lines

RMS allows for profiling of several samples in parallel making comparisons of methylation profiles feasible. This is illustrated in Figure 5 that depicts a position-by-position comparison of SSU and LSU rRNA from HeLa and HCT116 colorectal carcinoma cells (Figure 5A and B). The analysis of HCT116 cells did not reveal new modifications and the two cell lines show similar methylation at most sites, underlining that the repertoire of rRNA 2'-O-me sites is probably not much larger than documented here. At approximately 20% of the positions, the difference in average RMS scores appears significant (unpaired t-test; P value < 0.05) and differs by ≥ 0.05 . A total of 2/20 of these positions are conserved in yeast, whereas 38/84 of the invariant positions are conserved in yeast. Thus, there is considerable enrichment of human-specific positions among the variable sites. Many of the variable sites are clustered, e.g. in the 5' domain of SSU and domain III in LSU, although the functional implications of this are presently unclear.

We next compared the RMS scores of HeLa and HCT116 to expression data derived from microarray analysis of a set of 125/152 of the snoRDs responsible for guiding the methylations at 93/103 sites (35). Only a very weak positive correlation was found for either of the two cell lines (Figure 5C and Supplementary Figure S3). Many 2'-O-Me sites can be targeted by several snoRDs. In most cases these are paralogs, but there are several examples of snoRDs belonging to different families that target the same site. There is a clear tendency for sites targeted by multiple snoRDs to have a high RMS score (Figure 5D), suggesting that gene duplication and convergence in targeting among snoRD families play a role in ensuring a high level of modification at several sites. Finally, we compared the differences in RMS scores between the two data sets with the differences observed in snoRD expression levels (Figure 5E). Here, no correlation was observed. This suggests that the overall modification profile of rRNA is not a direct consequence of the snoRD expression profile. Our analyses do not rule out that individual sites are regulated by snoRD availability but suggest that other factors regulate the modification at most sites.

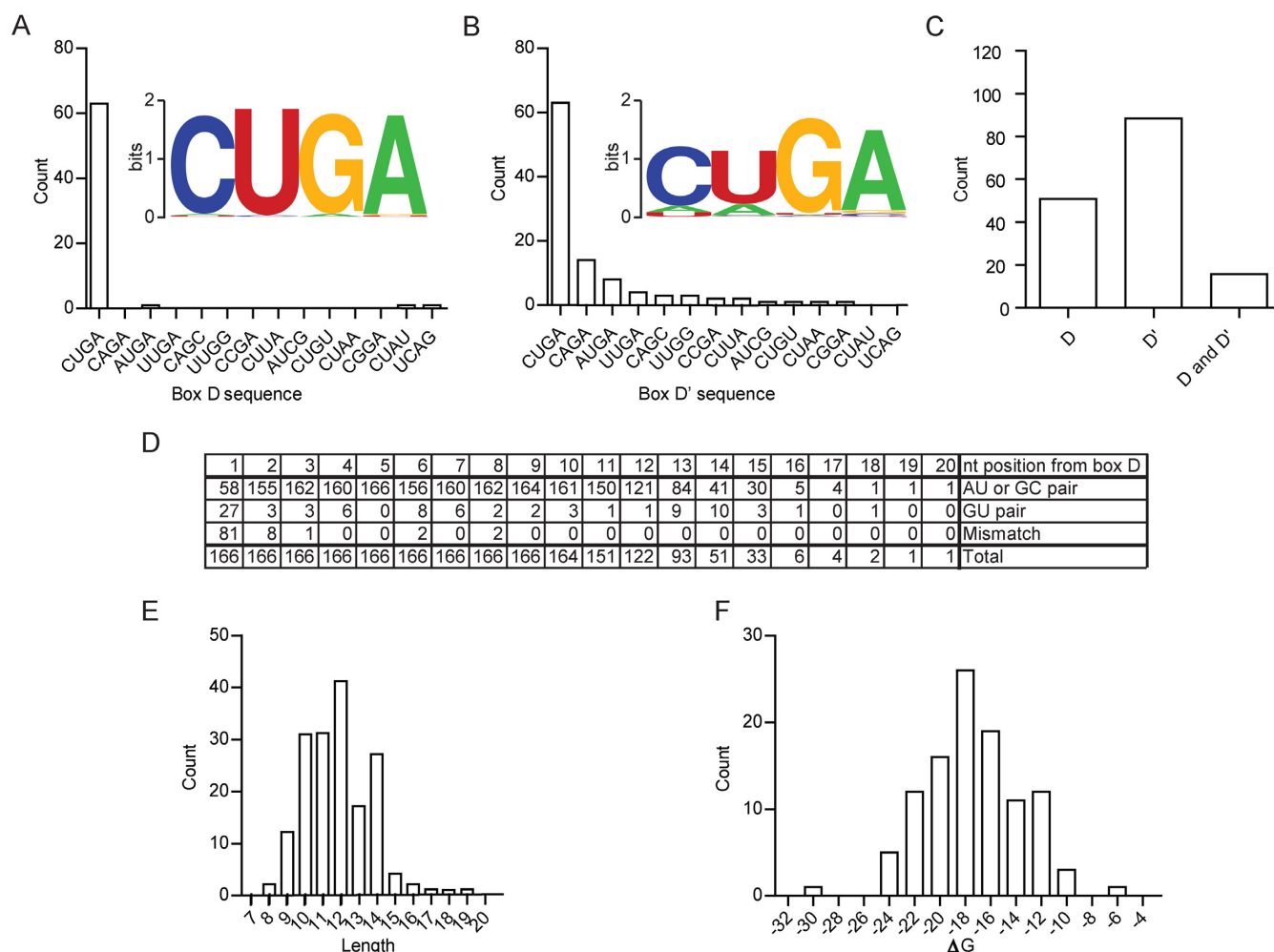


Figure 4. Sequence analysis of snoRDs guiding 2'-O-methylation of rRNA. The analysis was applied to the minimal set of snoRDs defined in this study. (A) Distribution of box D types and consensus sequence represented as a sequence logo for box D generated by WebLogo (65). (B) Same for box D'. (C) Distribution of snoRDs using box D, box D' and both. (D) Analysis of base pair type in relation to the distance from box D. The numbers show the type of base pair (or mismatch) at the indicated distance from box D cumulated for all proposed interactions between the selected set of snoRDs in Supplementary Table S6 and their target. (E and F) Distributions of duplex length and free energy of the interaction between snoRD and target, respectively.

DISCUSSION

We have used RMS to profile rRNA isolated from HeLa and HCT116 cancer cell lines with the principal aim of arriving at a conservative description of 2'-O-Me and corresponding snoRD guides in human cells. We report a revised reference sequence based on high coverage sequencing of rRNA, a comprehensive map of experimentally validated 2'-O-Me sites, a fractional score for each position, and a list comprising a subset of snoRDs that comply with consensus rules and are sufficient to explain the observed rRNA methylations. Our results are consistent with data obtained by isotope incorporation and RNA fingerprinting by Maden (14,15), but inconsistent with some of the reports based on primer extension at low dNTP followed by qRT-PCR (36,37). As previously pointed out, primer extension based methods are error-prone, in particular for quantitative applications (38). Alternative methods for quantitation, such as the RNase H (39,40) or deoxyribozyme methods (41,42) have not yet been applied on a large scale,

and ribosome-wide MS analysis using standards based on heavy-isotope labeling have so far only been applied to *E. coli* (43) and *S. pombe* (44). In the absence of independent, comparable and recent data sets, it is important to keep some of the limitations of the present study in mind. First, the RMS score has a relatively high background and putative sites with low scores were not considered. However, our list accounts for all of the moles of methyl groups detected by isotope incorporation in HeLa rRNA by Maden (14,15) and it is unlikely that there will be additional highly modified sites discovered in the cell lines studied here. Second, the assignment of snoRDs is hypothetical and not proven by genetic experiments. This type of validation was achieved on a large scale in yeast (21) and the predictions applied in humans follow the same general rules. Finally, the extent of base pairing at individual sites was not addressed, but the general rules for this have previously been the scope of studies in yeast (33) and mammals (45).

The overall picture that emerges from our study is that the 2'-O-Me sites in different cells are highly similar, and that

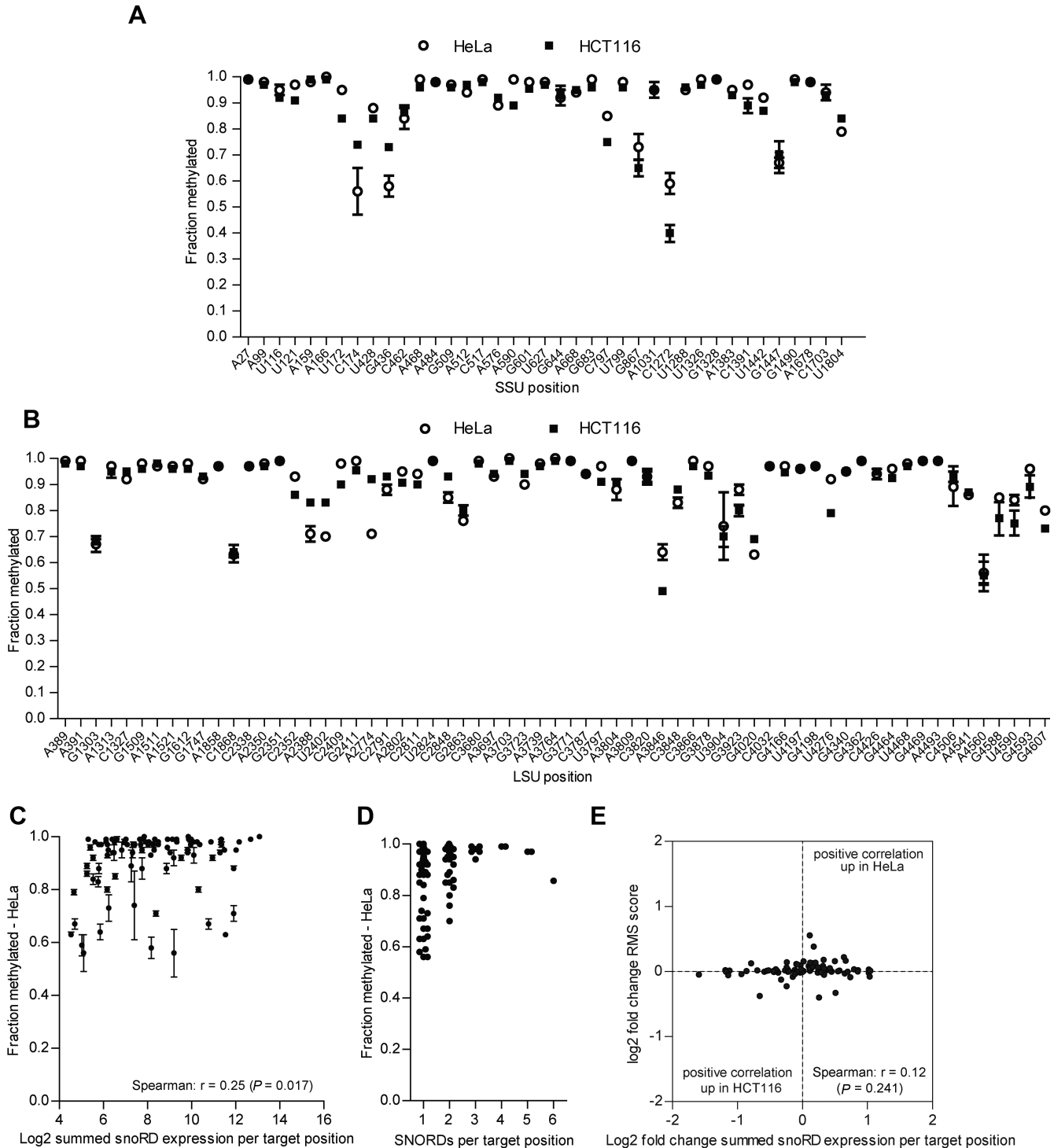


Figure 5. RMS and snoRD expression analyses of HeLa and HCT116 cells. (A and B) RMS analysis of SSU and LSU rRNA, respectively. RMS score data plotted as the average \pm s.d. for biological triplicates (C) RMS score for individual HeLa 2'-O-Me rRNA positions plotted against the corresponding summed average expression from two experiments of all snoRDs targeting the site. Bars indicate standard deviation in RMS score for triplicate experiments. (D) RMS score as a function of the number of genes encoding snoRDs targeting the position. (E) Log2 fold-change of RMS score for individual 2'-O-Me rRNA positions between HeLa and HCT116 cells plotted against the log2 expression fold change for the respective snoRD guides.

most sites are fully methylated with variable sites highly enriched at human-specific (in comparison to yeast) sites. Few, if any, snoRDs use both D and D' box in a single binding event suggesting that the methylation mechanism differ from Archaea (31). Some snoRDs may not elicit methylation, but function only through binding to their target. Conversely, the three sites for which no snoRD has been found may use so far unidentified snoRDs, an unconventional mechanism for methylation, or be methylated by a stand-alone enzyme. The high RMS score at all of these positions argues against a simple 'slippage' mechanism. Finally, the degree of methylation is not a simple function of the sequence of boxes C or D, the strength of interaction with the target, or the snoRD expression level.

New 2'-O-Me sites in human rRNA

The two new modifications that were uncovered add to the picture of 2'-O-Me sites being concentrated in conserved and functionally important parts of rRNA. Position LSU-1868 is located at the base of Helix 39 in domain II close to three other modifications (Figure 2B), two of which (Am1858 and Ψ 1849) are also found in yeast (5). Helix 39 is close to both the 5S RNP (46) and the A-site finger, Helix 38 (Supplementary Figure S4A). It will be of interest to investigate if this modification influences the association of 5S RNP with the ribosome, e.g. during nucleolar stress (47).

G3771 forms the first base pair of Helix 71 in domain IV together with C3787 that also is fully methylated (guided by snoRD10) (Figure 3C). Helix 71 is involved in the formation of the RNA-based intersubunit bridge B3 together with helix 44 in SSU rRNA (Supplementary Figure S4B) (19). B3 may serve as an anchoring point during the ratcheting movement in translation (48,49), and the two 2'-O-Me may be required for increased structural stabilization. In yeast, loss of the modification corresponding to LSU-3771 (yeast LSU-2288) in combination with loss of methylation of the nearby conserved m⁵C at position LSU-2278 results in dramatic ribosome instability (50).

In conclusion, we find 106 validated 2'-O-Me sites and have preliminary evidence for two additional methylations at SSU-1232 and LSU-3606. It is possible that more 2'-O-Me sites will be found, likely guided by some of the snoRDs not directing methylation in HeLa cells. However, based on the conservation of sites in HTC116 cells and the relatively few predictions not validated in this study, we suspect that the number of additional new sites in rRNA will be low.

Among the sites found to be unmethylated, SSU-G1536 (evidence from RMS and MS) and LSU-U1602 (evidence from RMS), were of particular interest. Intriguingly, both sites are targeted by snoRD12C. The interactions between the snoRD and the target sites conform to the consensus, yet neither of these positions appears to be methylated in any of several cell lines (unpublished observations). The target sites flank helix 41 in the 3' major domain of SSU rRNA and it is possible that snoRD12C functions in the folding of this domain without eliciting methylation of target residues. It cannot be ruled out that the targets become methylated in other cell types, but it is important to note that snoRD12C is expressed in HeLa cells (unpublished observation) and

that other snoRDs (e.g. U3) base pairs with ribosomal targets without eliciting methylation (29).

Canonical versus non-canonical functions of snoRDs

Surprisingly, we found no strict correlation between cumulated expression levels of snoRDs targeting the same sites and the RMS score. It is possible that a descriptor, integrating several features, including the length and strength of the snoRD to target interaction and the expression levels will correlate better with the RMS score.

SnoRDs are highly deregulated in cancer (51,52). However, the frequently observed vast upregulation of many snoRDs is unlikely to be paralleled by upregulation of their cognate 2'-O-Me sites since most sites are already close to fully methylated. This suggests the possibility that aberrant snoRD expression could be a consequence of host gene dysregulation, although there is evidence that expression of intron-encoded snoRDs can be uncoupled from expression of flanking exons (53). Alternatively, this observation could hint at non-canonical functions for snoRDs (54,55). Non-canonical functions are a common theme for ribosomal constituents. There are multiple examples of non-canonical functions of ribosomal proteins (56), and 5S rRNA has non-ribosomal functions along with rpL5/rpL11 during the nucleolar stress response (47,57). In the case of the box C/D snoRNPs, the RNA component has been suggested to give rise to small fragments with other functions, e.g. as microRNAs (58), and the central protein component, fibrillar, has been shown also to modify histones (59). Hence, both the lack of a global correlation between snoRD levels and their corresponding ribosomal ribose methylations, together with the relative abundance of orphan snoRDs in humans suggests that additional non-ribosomal targets and functions may be revealed. Taken together, our study advises caution in relating snoRD expression levels to their function in rRNA modification.

Fractional scores suggest ribosome heterogeneity

Determination of the stoichiometry of modification at all sites may hold the key to discriminate between the two main functions that have been proposed for snoRD guided ribose methylations, namely to assist in folding of ribosomal RNA and to impact the fidelity of translation (3). Folding of ribosomal RNA and assembly of ribosomes is believed to be mostly a constitutive process with some redundancy in the assembly line (10,60). In contrast, the recruitment of mRNA and several aspects of translation, e.g. cap-dependent versus cap-independent translation, are regulated processes. Thus, we speculate that the two-thirds of methylation events resulting in highly methylated positions, which appear largely invariant, are mostly involved in rRNA folding functions. Some of the remaining one-third of methylation events may similarly be involved in folding. For a methylation to occur, the guide RNA has to base pair with its target. Alternative folding pathways may preclude a snoRD from association with the target in a fraction of the molecules. Viewed in this way, the fractional scores at many sites may reflect the partitioning of rRNA into different pathways during folding. Finally, we suggest that changes

in 2'-O-Me stoichiometry at a subset of sites, the majority of which are fractionally modified, affect the function of the mature ribosome, i.e. translation.

Our observation of several sites with low 2'-O-Me stoichiometry further implies that the pool of mature ribosomes is heterogeneous at the level of RNA modifications, although technically, we cannot rule out that ribosomes could vary between cells in a population. The comparison of HeLa and HCT116 cells reveals that different cell types may have different profiles with respect to the 2'-O-Me stoichiometry at certain sites. Presently, it is unclear to what extent this heterogeneity has functional implications, but it is consistent with the notion of specialized ribosomes (23).

RMS analyses can contribute, together with transcriptome-wide methods for detection of Ψ sites (61,62) and dedicated studies for discovery of new base modifications (63), to update the annotation of the human rRNA reference, an undertaking we hope to achieve in the near future when results from more diverse cell lines are analyzed. RMS offers a flexible format that allows for many RNA libraries representing different cell types or different experimental situations to be profiled. Observed differences will likely reflect regulated processes and differentially modified sites thus constitute candidates for exploration of the functionality of individual methylations. Specialized ribosomes are an important theme in cancer research (37,64). It is hypothesized that ribosome composition plays a direct role in shaping the cancer proteome (64) and it will be of interest to assess whether individual ribose methylations play a role in constituting such functional ribosome diversity.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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