Partial Methylation at Am100 in 18S rRNA of Baker’s Yeast Reveals Ribosome Heterogeneity on the Level of Eukaryotic rRNA Modification

Markus Buchhaupt¹*, Sunny Sharma²*, Stefanie Kellner³, Stefanie Oswald², Melanie Paetzold¹, Christian Peifer², Peter Watzinger², Jens Schrader¹, Mark Helm³, Karl-Dieter Entian²*

¹ DEHEMA Research Institute, Biochemical Engineering, Frankfurt am Main, Germany, ² Institute for Molecular Biosciences, Johann-Wolfgang Goethe University, Frankfurt am Main, Germany, ³ Institute of Pharmacy and Biochemistry, Johannes Gutenberg University, Mainz, Germany

Abstract

Ribosome heterogeneity is of increasing biological significance and several examples have been described for multicellular and single cells organisms. In here we show for the first time a variation in ribose methylation within the 18S rRNA of Saccharomyces cerevisiae. Using RNA-cleaving DNAzymes, we could specifically demonstrate that a significant amount of S. cerevisiae ribosomes are not methylated at 2'-O-ribose of A100 residue in the 18S rRNA. Furthermore, using LC-UV-MS/MS of a respective 18S rRNA fragment, we could not only corroborate the partial methylation at A100, but could also quantify the methylated versus non-methylated A100 residue. Here, we exhibit that only 68% of A100 in the 18S rRNA of S. cerevisiae are methylated at 2'-O ribose sugar. Polysomes also contain a similar heterogeneity for methylated Am100, which shows that 40S ribosome subunits with and without Am100 participate in translation. Introduction of a multicopy plasmid containing the corresponding methylation guide snoRNA gene SNR51 led to an increased A100 methylation, suggesting the cellular snR51 level to limit the extent of this modification. Partial rRNA modification demonstrates a new level of ribosome heterogeneity in eukaryotic cells that might have substantial impact on regulation and fine-tuning of the translation process.

Introduction

Ribosomes are cellular organelles essential to all known forms of life on earth. In all organisms these macromolecular machines achieve the translation of the genetic code into proteins. All ribosomes are composed of two subunits, each of them consisting of at least one rRNA molecule and several ribosomal proteins. Although function and overall structure of these complexes are highly conserved during evolution, their composition varies from species to species. Whereas the small subunit of Escherichia coli contains 21 ribosomal proteins, the human counterpart consists of 33 proteins [1]. And although most cell biology textbooks still give the impression that an organism contains only one type of ribosome that is composed of clearly defined parts, the concept of ribosome heterogeneity has been discussed for quite a long time. The first review on this topic, prepared by S. Ramagopal [2], summarized the first reports describing non-uniformity of ribosomes in one species. The earliest hints for the occurrence of slightly different ribosomal proteins in different organs of rabbit were published by Deluany et al. [3]. Also using two-dimensional gel electrophoresis Lambertsson [4] could detect developmental stage-specific ribosomal proteins in Drosophila melanogaster cells.

Following reports, also discussed by Ramagopal [2], showed other examples of ribosomal protein heterogeneity but also ribosome heterogeneity at the level of tRNA sequence. The most exciting finding was the rather specific synthesis of a distinct type of 18S rRNA in different life cycle stages of the protozoan parasite Plasmodium berghei [5]. Two recently published reviews summarize many more examples for ribosome heterogeneity on the level of ribosomal proteins and rRNA sequence and also discuss physiological functions of changes in ribosome composition [6,7]. Both reviews also speculate about post-transcriptional modifications as a source of ribosomal diversity for more specialized functions in eukaryotic cells. S. cerevisiae rRNAs contain 44 pseudouridine (Ψ), 54 2'-O-ribose methyl groups and nine base modifications [8,9]. Whereas base modifications are introduced by specific enzymes, the pseudouridinylations and 2'-O-ribose methylations are carried out by small nucleolar ribonucleoprotein particles (snoRNPs), each containing a modification site-specifying snoRNA. Interestingly, Gilbert [6] as well as Xue and Barna [7] reviewed data which might contradict the prevailing idea of snoRNAs playing constitutive roles in ribosome biogenesis, and that all rRNA target sites are fully modified under all conditions. Heterogeneous snoRNA expression levels in different tissues [10] and oscillation of the expression of some snoRNAs in a circadian manner [11] further supported their notion. Additionally, knockdown studies using morpholino oligomers showed that depletion of different snoRNAs in zebrafish causes specific developmental phenotypes.
Candida glabrata strain. All these microorganisms 
unmethylated A100 18S rRNA was the 
to A100 is marked with an 
versus 
that lack the 2
D
A) Detection of 18S rRNA molecules lacking the 
M
S. cerevisiae
S. cerevisiae
9
Escherichia coli
9
Sulfolobus acidocaldarius
cell culture lacks a specific snoRNA-
s
9
S.cerevisiae
9
contains partial 2
4
c
s
(i
58x178
the first report of ribosome heterogeneity regarding snoRNA-
mediated modification of the 18S rRNA. To our knowledge this is 
unambiguously demonstrates the existence of a significant amount 
ribose methylation at residue A100 (data not shown). This 
phenomenon is unique to the CEN.PK strain we also tested the 
strain CEN.PK2-1C (figure 1). To rule out the possibility that this 
only detect the cleavage product with an RNA sample from the 
23-snR51-Am100, specific for the Am100 was used, we could not 
the 18S rRNA of 
DNAzymes for the investigation of several modified nucleotides in 
S. cerevisiae
DNAzymes can be used to detect 2’-O-ribose methylated nucleotides in RNA 
molecules in a very simple and straightforward way [18]. 
DNAzyme-mediated site-specific scission of a RNA molecule at 
a given position is impeded by a 2’-O-ribose methyl group at the 
nucleotide 5’ to the cleavage site. During our analysis of different 
DNAzymes for the investigation of several modified nucleotides in 
S. cerevisiae
rRNAs, we observed an unexpected result for one 
ribose methylation, catalysed by snR51 snoRNP. snR51 catalyzes 
the 2’-O ribose methylation of adenosine (Am) at position 100 in the 
18S rRNA of S.cerevisiae. Intriguingly, when the DNAzyme 10– 
23-snR51-Am100, specific for the Am100 was used, we could not 
only detect the cleavage product with an RNA sample from the 
Δsnr51 strain, but also with RNA from the respective wild type 
strain GEN.PK2-1C (figure 1). To rule out the possibility that this 
phenomenon is unique to the GEN.PK strain we also tested the 
lab strain BY4741, a baker’s yeast strain isolated from cider (SC- 
F3-1) and a Candida glabrata strain. All these microorganisms 
contained a comparable amount of 18S rRNA devoid of 2’-O 
ribose methylation at residue Am100 (data not shown). This 
unambiguously demonstrates the existence of a significant amount 
of ribosomes in yeast cells that do not contain this snoRNA-
mediated modification of the 18S rRNA. To our knowledge this is 
the first report of ribosome heterogeneity regarding snoRNA-
mediated rRNA modifications.

Quantification of the amount of Am100 methylation in 
wild type 18S rRNA

To further augment the partial ribose methylation at Am100 and 
quantify the exact amount of Am100 ribose methylation in 18S 
rRNA of the wild type, we isolated the fragment containing the 
Am100 residue from wild type 18S rRNA by Mung bean nuclease 
protection method [9] (figure 2A).

The isolated rRNA fragment was then subjected to LC-UV-
MS/MS analysis. For quantification of the 2’-O-methyladenosine

Results and Discussion

18S rRNA of S. cerevisiae contains partial 2’-O-ribose 
methylation at A100

We previously demonstrated that RNA-cleaving DNAzymes 
can be used to detect 2’-O-ribose methylated nucleotides in RNA 
molecules in a very simple and straightforward way [18]. 
DNAzyme-mediated site-specific scission of a RNA molecule at 
a given position is impeded by a 2’-O-ribose methyl group at the 
nucleotide 5’ to the cleavage site. During our analysis of different 
DNAzymes for the investigation of several modified nucleotides in 
S. cerevisiae
rRNAs, we observed an unexpected result for one 
ribose methylation, catalysed by snR51 snoRNP. snR51 catalyzes 
the 2’-O ribose methylation of adenosine (Am) at position 100 in the 
18S rRNA of S.cerevisiae. Intriguingly, when the DNAzyme 10– 
23-snR51-Am100, specific for the Am100 was used, we could not 
only detect the cleavage product with an RNA sample from the 
Δsnr51 strain, but also with RNA from the respective wild type 
strain GEN.PK2-1C (figure 1). To rule out the possibility that this 
phenomenon is unique to the GEN.PK strain we also tested the 
lab strain BY4741, a baker’s yeast strain isolated from cider (SC- 
F3-1) and a Candida glabrata strain. All these microorganisms 
contained a comparable amount of 18S rRNA devoid of 2’-O 
ribose methylation at residue Am100 (data not shown). This 
unambiguously demonstrates the existence of a significant amount 
of ribosomes in yeast cells that do not contain this snoRNA-
mediated modification of the 18S rRNA. To our knowledge this is 
the first report of ribosome heterogeneity regarding snoRNA-
mediated rRNA modifications.

Quantification of the amount of Am100 methylation in 
wild type 18S rRNA

To further augment the partial ribose methylation at Am100 and 
quantify the exact amount of Am100 ribose methylation in 18S 
rRNA of the wild type, we isolated the fragment containing the 
Am100 residue from wild type 18S rRNA by Mung bean nuclease 
protection method [9] (figure 2A).

The isolated rRNA fragment was then subjected to LC-UV-
MS/MS analysis. For quantification of the 2’-O-methyladenosine

level in the sample, two approaches were used as described in 
Materials and Methods. Figure 2 shows the results of the sample 
measurement in comparison to three calibration measurements. 
The calibration samples contained the same amount of guanosine 
as the analyzed RNA fragment, observed as similar peak areas 
(figure 2B and C). However, the amount of 2’-O-methyladenosine 
varies in the calibration samples from 10% to 100% compared 
with the guanosine level. As evident in figure 2C, the peak area of 
the RNA fragment was in between the 50% and 100% calibration 
sample. With the help of an internal standard, the amount of 2’-O-
methyladenosine was found to be 68% (raw data and applied 
calculations can be found in the supplementary information).

Ribosomes lacking the Am100 modification in the 18S 
rRNA participate in translation

Although it can be assumed that in cells from the logarithmic 
growth phase used here, most 18S rRNA molecules are part of 
actively translating ribosomes, the possibility exists that a certain 
population are a constituent of immature or inactive ribosome 
forms. In order to find out if ribosomes without the 2’-O-ribose 
methyl group at Am100 in the 18S rRNA are such nonfunctional 
specimens, we analyzed RNA from purified polysomes. As evident 
from figure 3, our results clearly show that the ratio of 18S rRNA 
containing Am100 versus unmethylated A100 18S rRNA was the 
same as found in total RNA. This experiment proved that

![Figure 1. Identification of 18S rRNA molecules in wild type (WT) and Δsnr51 that lack the 2’-O-ribose methyl group at nucleotide A100. A) Detection of 18S rRNA molecules lacking the Am100 modification by DNAzyme cleavage. Total RNA from wild type and Δsnr51 cells grown in YEPD medium to exponential phase was incubated with or without the DNAzyme 10-23-snR51-A100 and afterwards analyzed by gel analysis. The RNA band marked with an asterisk represents the large 18S rRNA fragment after cleavage at nucleotide A100. The 100 nucleotide fragment ran out of the gel. B) Schematic illustration of DNAzyme 10–23-snR51-A100 binding to its target site in 18S rRNA. The cleavage site 3’ to A100 is marked with an arrow. The resulting fragments have lengths of 100 nt and 1700 nt. doi:10.1371/journal.pone.0089640.g001](image)
ribosomes without the methylation at A100 are actively engaged in protein synthesis.

Extent of A100 modification is probably limited by the amount of snR51

After demonstrating ribosome heterogeneity in *S. cerevisiae* on the level of rRNA modification, we addressed possible reasons for the phenomenon. First we hypothesized that two snoRNA-mediated modifications (Ψ106 and Ψ120) in close proximity to A100 might lead to spatial hindrance and impair the efficiency of 2'-O-ribosome methylation at A100 during the maturation process. A very similar situation regarding the spatial clustering of rRNA modifications exists in case of the snR40-mediated 2'-O-ribose methylation of G1271. However, despite the adjacent modifications at U1269 and U1290, no molecules lacking the methylation at position 1271 could be detected with a highly efficient DNAzyme using our method (data not shown). This finding also excludes that snoRNAs responsible for modifications at several target sites always lead to only partial modification of rRNAs in the cell, as both snR51 and snR40 target one nucleotide in the 18S rRNA and one nucleotide in the 25S rRNA. This observation supported our view that the lack of A100 methylation in some ribosomes is no inevitable consequence of the target nucleotide position but a fine-tuned state evolved during evolution.

The intention of our next experiment was therefore to test the possibility of achieving a more complete modification at this position. For that purpose a multicopy plasmid containing the snoRNA gene cluster 3 encoding snR51, snR70 and snR41 (pRS426-Cluster3) was constructed and transformed into wild type and Dsnr51 strain. The results obtained after DNAzyme analysis of RNA samples from the resulting strains are shown in figure 4. Introduction of the vector into the Dsnr51 strain led to a strong recurrence of modified and hence non-cleavable 18S RNA, demonstrating the functionality of the construct. The presence of pRS426-Cluster3 in wild type indeed resulted in a clear increase of 2'-O-ribose methylation at A100. This finding suggests that it is apparently the amount of snR51 that limits the extent of A100 modification in the 18S rRNA of bakers yeast.

Given the heterogeneity of cellular yeast ribosomes with regard to this specific rRNA modification to be a precisely adjusted state, the question arises what special function do the ribosomes lacking this modification might have. Up to now no significant growth disadvantage of the Dsnr51 strain or the SNR51-overexpression strain compared to the wild type could be detected. This is however not surprising as single modification guide snoRNA gene deletion mutants exhibit in most cases only marginal growth phenotypes under standard growth conditions but show altered growth dynamics under a wide array of environmental perturbations, including ribosomal antibiotics [14]. Besides transcriptome, proteome or metabolome analysis other promising approaches to uncover functional necessities of this type of ribosome heterogeneity are the use of high-resolution phenotyping [14] as well as genetic arrays [19]. For all approaches in this direction it is extremely important to consider that observed phenotypes displayed by a snoRNA gene deletion or overexpression mutant.

**Figure 2.** LC-UV-MS/MS analysis of the isolated rRNA fragment and calibration samples. (A) Schematic diagram for the mung bean nuclease method, used here to isolate the 18S rRNA fragment containing Am100 residue for subsequent mass spectrometry analysis. (B) UV chromatograms of all 4 samples and peak areas. In red, the sample containing 100% guanosine and 10% 2'-O-methyladenosine is shown. In green and blue the respective 50% and 100% Am turnover samples are shown. The black chromatogram shows the guanosine peak of the rRNA fragment. (C) Overlay of MS/MS chromatograms for 2'-O-methyladenosine. The methylation extent of the rRNA fragment was found to be 68% as could be seen in comparison to the calibration samples.

doi:10.1371/journal.pone.0089640.g002

**Figure 3.** Comparison of 18S rRNA molecules lacking the 2'-O-ribose methyl group at nucleotide A100 in polysomal RNA and total RNA. Polysomal RNA and total RNA isolated from wild type cells that were grown in YEPD medium to exponential phase was incubated with or without the DNAzyme 10–23-snR51-A100 and afterwards analyzed by gel analysis. The RNA band marked with an asterisk represents the large 18S rRNA fragment after cleavage at nucleotide A100.

doi:10.1371/journal.pone.0089640.g003
can not only be due to the failure or a higher degree of modification but also due to the lack of the specific snoRNA itself which might function in some other biochemical context, e.g. as rRNA chaperone [20].

In vitro approaches able to find functional differences for specialized ribosomes but circumventing the intricacy of in vivo experiments are translation assays dedicated to test efficiency and accuracy of the synthesis of different polypeptides.

In addition it would be highly interesting to find environmental conditions that lead to changes in the ratio of unmodified and modified rRNA. In first experiments we could not detect significant differences in the extent of A100 modification in cells growing in a synthetic or a complex medium.

To enable a fast identification and quantification of eukaryotic rRNA modification heterogeneities in future, sensitive analytical tools will be necessary. Currently the most common way to detect 2'-O-ribose methylations but circumventing the intricacy of in vivo experiments is translation assays dedicated to test efficiency and accuracy of the synthesis of different polypeptides.

In this type of analysis the band appearing on the autoradiogram represents the methylated RNA molecules, making the sensitive detection of small amounts of unmodified RNA difficult. In this regard the advantage of the DNAzyme approach [18] and the RNAse H cleavage assay [22] becomes evident, as they directly visualize the non-methylated RNA molecules. Although the DNAzyme method used here bears many advantages, it has still limited applicability with regard to different sites of analysis. This is reflected by our failure to identify a DNAzyme for nucleotide U2729 in the 25S rRNA, whose modification is also guided by snR51. After identification of SNR18 overexpression to be able to suppress a translation termination defect, Hatin et al. [23] speculated about hypo-methylation of the target sites (A647 and C648 of the 25S rRNA) and the possibility that ribosomes carrying the respective modifications might be more efficient in translation termination. Unfortunately all six different DNAzymes we tested for cutting of rRNA isolated from a Δsnr18 strain (GEN.MB793-6B) did not yield visible cleavage products, which made investigation of the proposed hypo-methylation hypothesis impossible up to now. Esquerra [13] who postulated in his PhD thesis the existence of differentially regulated rRNA modification patterns due to the different resistance phenotypes of snoRNA gene deletion mutants under different stress conditions, tried to detect rRNA molecules lacking the 2'-O-ribose methylations at nucleotide A796 (18S rRNA) or A1133 (25S rRNA) by the use of DNAzymes. However, although he could find the expected cleavage products with RNA samples from the respective snoRNA gene deletion mutants, they were not identified in RNA samples from wild type. Our own experiments ([18] and unpublished results) with mid to high efficiency DNAzymes showed also no significant cleavage of wild type 18S rRNA at nucleotides A794, G1126, G1271 and wild type 25S rRNA at nucleotides G1450, G2815 (numbering according to the rRNA sequence numbering used at the Saccharomyces Genome Database, www.yeastgenome.org). The absence of a visible cleavage band in the analytical gel does not prove, though, the respective nucleotide to be modified in all ribosomes of the cell culture, as the sensitivity of the DNAzyme approach is clearly limited by DNAzyme efficiency and the detection limit of ethidium bromide staining.

In the present study, we provided the highly sensitive LC-UV-MS/MS analysis as an important tool for the accurate quantification of rRNA modification. Using this approach we could demonstrate the hitherto unknown heterogeneity in the 18S rRNA, showing that approximately one third of the 18S rRNA population lacks Am100. Application of this approach for other sites might help to elucidate even more heterogeneous population of ribosomes with respect to rRNA modifications.

Mutations in snoRNA genes or other RNA modification factors have been associated with Prader-Willi syndrome [24], Dyskeratosis congenita [25], Hoyeraal-Hreidarsson syndrome [26] and different forms of cancer [17,27–31]. In a recent work the yeast homolog of the DKC1 gene, which is affected in many cases of Dyskeratosis congenita and Hoyeraal-Hreidarsson syndrome, was mutated, resulting in ribosomes with an overall decrease in the amount of pseudouridine residues [32]. These ribosomes were impaired in translational fidelity and IRES-dependent translational initiation in the same way as ribosomes from DKC1-deficient human cells, demonstrating ribosome-associated disease phenotypes to be the consequence of changes in rRNA modification.

Figure 4. Investigation of changes in Am100 modification extent in 18S rRNA after introduction of a multicopy plasmid containing SNR51. Total RNA isolated from wild type and Δsnr51 cells grown in synthetic medium to exponential phase containing no plasmid, the plasmid pRS426 or the plasmid pRS426-Cluster3 was incubated with the DNAzyme 10–23-snR51-A100 and afterwards analyzed by gel analysis. The RNA band marked with an asterisk represents the large 18S rRNA fragment after cleavage at nucleotide A100.
Our findings of heterogeneity in snoRNA-mediated RNA modifications might also have some relevance in human diseases.

**Materials and Methods**

**Yeast strains and plasmids**

*S. cerevisiae* strains CEN.PK2, CEN.PK2-1C and BY4741 were obtained from EUROSCARF [http://web.uni-frankfurt.de/fb15/mikro/euroscarf/]. SC-F3-1 is a baker’s yeast strain isolated from cider (P. Kotter, Frankfurt, unpublished). *Candida glabrata* ATCC 2001 was obtained from ATCC. For PCR-mediated deletion of the snoRNA cluster 3 which contains *SNR18, SNR70* and *SNR51*, the loxP-KanMX-loxP gene disruption cassette was amplified with primers Clu3-F1 (TGATGTTCTCTTTTTTTTTCTTTTCTTGTTGACACGGCTGAAGCTTCGTACGC) and Clu3-R1 (CTACATAGGGTGCAAGATTAGTTAGGTTGTAGAAGCATTGGATAGCCACTAGTGGATCTG) and plasmid pUT6 [33] as template and used for yeast transformation [34]. After selection on YEPD plates containing 0.2 mg mL\(^{-1}\) G418, isolation of single colonies, verification of correct gene replacement by diagnostic PCR and tetrad dissection, the haploid deletion strain CEN.S02-1A (referred to as Δsnr3) was received. For deletion of the *SNR18* gene the same procedure was followed with primers SNR18-F1 (GTTAACTAATAATGATTACTTTTGATATCG) and SNR18-R1 (ATAATGATACTCTGCTCTGTGCTATCG). For deletion of the *SNR18-R1* (ATAATGATACTCTGCTCTGTGCTATCG) was received.

For construction of plasmid pRS426-Cluster3 the snoRNA gene cluster 3 was amplified by PCR with oligonucleotides Cluster3-F1 (ACGTGTCTCAAGTAAACGGATCTATCCGTCTGATCATGAC) and Cluster3-R3 (ACGTGAAATTCTGCTACTTTTCCTTGCTTTCTG) and ligated into pRS426-Cluster3 3 DNA as template, digested with XhoI and EcoRI and ligated into pRS426 [35] digested with the same enzymes.

**Cultivation, RNA isolation and polysome preparation**

For isolation of RNA, yeast strains were cultivated in 5 mL medium at 30 °C to an OD600 of 0.6–1.0. YEPD medium (1% yeast extract, 2% bactopeptone, 4% glucose) or synthetic complete medium containing 4% glucose (SCD) was used. To select for maintenance of plasmids pRS426 or pRS426-Cluster3 3 medium lacking uracil was used. After harvesting the cells grown to exponential growth phase by centrifugation, total RNA was isolated by phenol/chloroform extraction [36] or by use of the RNeasy Mini Kit (QIAGEN).

To isolate polysomes, sucrose gradient centrifugation was performed on a 20% to 50% gradient in a buffer (20 mM HEPES pH 7.5, 10 mM KCl, 2.5 mM MgCl\(_2\), 1 mM EGTA and 1 mM DTT). Yeast cell lysates were prepared in the same buffer by vortexing with glass beads for 2 min at 4 °C. The amount of RNA was determined at 254 nm and 20 OD254 units were loaded on the gradients. Ultracentrifugation was performed in an SW40 Ti rotor (Beckman Coulter, Inc.) for 17 h at 24,300 rpm and 4 °C. Polysome fractions were pooled and precipitated with TCA, followed by RNA isolation.

**DNAAzyme reactions and gel analysis**

The DNAAzymes used are listed in Table 1. The DNAAzyme 10–23-snR18-A649-1, 10–23-snR18-A649-2 and 10–23-snR18-A649-3 were designed for cleavage of 25S rRNA at nucleotide A649. DNAAzymes 9–17-snR18-C650-1, 8–17-snR18-C650-2 and 0–17-snR18-C650-3 were designed for cleavage of 25S rRNA at nucleotide A650. The DNAAzymes were purchased from Sigma-Genosys as deprotected and desalted oligonucleotides. DNAAzyme-catalyzed RNA cleavage and gel analyses were performed as described previously [18].

**Mung bean nuclease protection method**

The mung bean nuclease protection method was performed exactly as described previously [37]. The specific sequence of the 18S rRNA was isolated by hybridization to complementary oligo 18S-Am100 (ATCAATAAACGATAACTTATTAGCCATTGCGACTTTACGTAT). 1,000 pmol of the synthetic deoxyligonucleotide complementary to A76–T125 of yeast 18S rRNA were incubated with 100 pmol of total rRNA and 1.5 µl of DMSO in 0.5 volumes of hybridization buffer (250 mM HEPES, 500 mM KCl, pH 7.0). After hybridization mung bean nuclease and 0.02 µg/µl RNase A (Sigma-Aldrich) was added to start the digestion. Prior to the separation of the samples on a 13% polyacrylamide gel containing 7 M urea, RNA was extracted once with phenol/chloroform and subsequent ethanol precipitation. Bands were extracted using the D-Tube™ Dialyzers according to the manufacturer’s protocol for electroelution (Novagen®).

**LC-UV-MS/MS quantification of 2′-O-methyladenosine**

The RNA fragment (final concentration 0.1 nmol/µL) was dissolved in 20 mM NH\(_4\)OAc pH 5.3 and digested as described previously [38]. In addition, commercially available guanosine and 2′-O-methyladenosine (Sigma-Aldrich, Munich, Germany) were mixed in such ratios to simulate a 10%, 50% and 100% adenosine methylation turnover. An internal standard containing \(^{13}\)C labelled 2′-O-methyladenosine was added (10% of sample volume) to the samples for absolute quantification of adenosine ribose methylation turnover. This internal standard was received by digestion of total RNA from *E. coli* grown in a M9 media with \(^{13}\)C-glucose as sole carbon source.

The samples were analyzed on an Agilent 1260 series equipped with a diode array detector (DAD) and Triple Quadrupole mass spectrometer Agilent 6460. A Synergy Fusion RP column (4 µm particle size, 80 Å pore size, 250 mm length, 2 mm inner diameter) from Phenomenex (Aschaffenburg, Germany) was used at 35 °C. The solvents consisted of 5 mM ammonium acetate with a diode array detector (DAD) and Triple Quadrupole mass spectrometer Agilent 6460. A Synergy Fusion RP column (4 µm particle size, 80 Å pore size, 250 mm length, 2 mm inner diameter) from Phenomenex (Aschaffenburg, Germany) was used at 35 °C. The solvents consisted of 5 mM ammonium acetate buffer adjusted to pH 5.3 using acetic acid (solvent A) and pure acetonitrile (solvent B). The elution started with 100% solvent A followed by a linear gradient to 8% solvent B at 10 min and 40% solvent B after 20 min. Initial conditions were regenerated by rinsing with 100% solvent A for 10 minutes. The flow rate was 0.35 mL/min.

The effluent from the column was first measured photometrically at 254 nm by the DAD followed by the mass spectrometer equipped with an electrospray ion source (Agilent Jet Stream), ESI parameters were as follows: gas temperature 300 °C, gas flow 5 L/min, nebulizer pressure 35 psi, sheath gas temperature 350 °C, sheath gas flow 12 L/min, capillary voltage 3500 V. Nitrogen was used as source and collision gas, the cell accelerator voltage was set to 2 V and the collision was induced for all analyzed with a collision energy of 15 eV. The MS was operated in positive ion mode to monitor selectively the transitions for 2′-O-methyladenosine (m/z: 282→136) and \(^{13}\)C-2′-O-methyladenosine (m/z: 293→141) in dynamic SRM mode with a time window of 1 minute for each analyte. For the quantification of 2′-O-methyladenosine, all samples were measured as described above. The data was then
Table 1. DNAzyme sequences.

| DNAzyme        | Sequence                                                                 |
|----------------|--------------------------------------------------------------------------|
| 10–23-snR51-A100 | ATAAAGCTAACTGATTATAAGGCTATGACAACAGGAGACCATCGC                              |
| 10–23-snR18-A649-1 | GACTCTTGGTCGGGCTAGCTACAACAGGTTCAAGACGCC                                  |
| 10–23-snR18-A649-2 | CTTGTGCGGGCTAGCTACAACAGGTTCAAGACGCC                                    |
| 8–17-snR18-C650-1 | AGACCTCTTGGTCCCGGAGCCGGAGTGTTTCAAGACGG                                  |
| 8–17-snR18-C650-2 | CTTGTGCCCCGAGCCGCGAGATTTTCAAGACGG                                    |
| 8–17-snR18-C650-3 | CTTGTGCCCCGAGCCGCGAGATTTTCAAGACGG                                    |

Processed using the UV data of guanosine for quantification of injected rRNA and MS/MS data for quantification of 2'-O-methyladenosine.

The injected rRNA fragment was quantified using UV 254 nm area of the main nucleoside guanosine (9 guanosine residues per RNA fragment). Here, calibration measurements of guanosine dilutions were applied for exact quantification.

For both 2'-O-methyladenosine and its 13C-labeled derivative, the MS/MS peaks were integrated. The peaks were correlated by a previously determined response factor (RFN) (Kellner S and Helm M unpublished), which allows the calculation of the total amount of 2'-O-methyladenosine per sample. The raw data and calculation is shown in the supporting data S1.

References

1. Anger AM, Armache JP, Berninghausen O, Hable M, Subklewe M, et al. (2013) Structures of the human and Drosophila 28S ribosome. Nature 497: 80–85.
2. Ramagopal S (1992) Are eukaryotic ribosomes heterogeneous? Affirmations on the horizon. Biochem Cell Biol 70: 269–272.
3. Delamuy J, Mathieu C, Schapira G (1972) Eukaryotic ribosomal proteins. Interspecific and intraspecific comparisons by two-dimensional polyacrylamide-gel electrophoresis. Eur J Biochem 51: 361–364.
4. Lamberton SG (1975) The ribosomal proteins of Drosophila melanogaster. In: Charlebois RL, Magee RE (eds) Ribosomes. Academic, New York, pp 23–37.
5. Gunderson JH, Sogin ML, Wollett G, Hollingdale M, de la Cruz VF, et al. (1987) Structural and functional genomics of the human and Drosophila genomes: implications for human biology and disease. Trends Biochem Sci 12: 335–369.
6. Gilbert VV (2011) Functional specialization of ribosomes? Trends Biochem Sci 36: 127–132.
7. Xue S, Barra M (2012) Specialized ribosomes: a new frontier in gene regulation and organismal biology. Nat Rev Mol Cell Biol 13: 135–139.
8. Machnicka MA, Milanowska K, Osman Ogou O, Purta E, Kurkowski M, et al. (2013) MODOMICS: a database of RNA modification pathways 2013 update. Nucleic Acids Res 41: D262–267.
9. Sharma S, Yang J, Watzinger P, Kotter P, Entian KD (2013) Yeast Nop2 and Rim1 methylate C2870 and C2278 of the 25S rRNA, respectively. Nucleic Acids Res 41: 9062–9076.
10. Castle JC, Armour CD, Lower M, Haynor D, Bieri M, et al. (2010) Digital genome-wide ncRNA expression, including snoRNAs, across 11 human tissues using polA/nuclear amplification. PLoS One 5: e11779.
11. Hughes ME, Grant GR, Paquin G, Qian J, Nitabach MN (2012) Deep sequencing the circadian and diurnal transcriptome of Drosophila brain. Genome Res 22: 1266–1281.
12. Higa-Nakamine S, Suzuki T, Uechi T, Chakraborty A, Nakajima Y, et al. (2012) Loss of ribosomal RNA modification causes developmental defects in zebras. Nucleic Acids Res 40: 391–398.
13. Esquerra J (2008) High-resolution phenotypic profiling of a eukaryotic ribosome [PhD]. Gothenburg: University of Gothenburg.
14. Esquerra J, Warrington J, Blomberg A (2008) Functional importance of individual rRNA 2'-O-methylribosyl modifications revealed by high-resolution phenotyping. RNA 14: 649–656.
15. Andersen TE, Porse BT, Kirpekar F (2004) A novel partial modification at C2501 in Escherichia coli 23S ribosomal RNA: 907–913.
16. Hansen MA, Kirpekar F, Ritterbusch W, Vester B (2002) Posttranscriptional modifications in the A-loop of 23S rRNAs from selected archaea and eubacteria. RNA 8: 202–213.
17. Belin S, Beighin A, Solano-Gonzalez E, Bezin L, Brunet-Mangaat S, et al. (2009) Dysregulation of ribosome biogenesis and translational capacity is associated with tumor progression of human breast cancer cells. PLoS One 4: e7147.
18. Buchbauer M, Peifer C, Entian KD (2007) Analysis of 2'-O-methylated pseudouridines and pseudouridinyls in ribosomal RNAs using DNAsymes. Anal Biochem 361: 100–108.
19. Tong AH, Evangelista M, Parsons AB, Xu H, Bader GD, et al. (2001) Systematic genetic analysis with ordered arrays of yeast deletion mutants. Science 294: 2364–2368.
20. Schoemaker RJ, Gulayev AP (2006) Computer simulation of shaperone effects of anarchy C/D box rRNA binding on rRNA folding. Nucleic Acids Res 34: 3143–3150.
21. Maden BE, Corbett ME, Henney PA, Pugh K, Ajih PM (1995) Classical and novel approaches to the detection and localization of the numerous modified nucleotides in eukaryotic ribosomal RNA. Biochimie 77: 22–29.
22. Yu YT, Shu MD, Steitz JA (1997) A new method for detecting sites of 2'-O-methylation in RNA molecules. RNA 3: 324–331.
23. Hinai I, Fahren S, Nacy O, Decatur WA, Rosett JP (2007) Fine-tuning of translation termination efficiency in Saccharomyces cerevisiae involves two factors in close proximity to the exit tunnel of the ribosome. Genetics 177: 1527–1537.
24. Suhoo T, du Gaudio D, German JR, Shioawae M, Peters SU, et al. (2006) Prader-Willi phenotype caused by paternal deficiency for the HBH5 C/D box small nucleolar RNA cluster. Nat Genet 40: 719–721.
25. Heiss NS, Knight SW, Vollmy T, Klauck SM, Wiermann A, et al. (1998) X-linked dyskeratosis congenita is caused by mutations in a highly conserved gene with putative nucleolar functions. Nat Genet 19: 32–38.
26. Knight SW, Heiss NS, Vollmy T, Greschner S, Stavrides G, et al. (1999) X-linked dyskeratosis congenita is predominantly caused by missense mutations in the DKC1 gene. Nat Genet 21: 479–480.
27. Dong NY, Guo P, Boyd J, Sun X, Li Q, et al. (2008) Implication of snoRNA U50 in human breast cancer. J Genet Genomics 35: 44–54.
28. Dong NY, Rodriguez C, Guo P, Sun X, Talbot JT, et al. (2008) SnoRNA U50 is a candidate tumor-suppressor gene at 6q14.3 with a mutation associated with clinically significant prostate cancer. Hum Genet 123: 1031–1045.
29. Liao J, Yu L, Mei Y, Guenauer M, Shen J, et al. (2010) Small nucleolar RNA signatures as biomarkers for non-small-cell lung cancer. Mol Cancer 9: 198.
30. Mei YP, Liao JP, Shen J, Yu L, Liu BL, et al. (2012) Small nucleolar RNA 42 acts as an oncogene in lung tumorigenesis. Oncogene 31: 2794–2804.
31. Tanaka R, Satoh H, Moriyama M, Satoh K, Morishita Y, et al. (2000) Intronic U50 small-nucleolar-RNA (snRNA) host gene of no protein-coding potential is mapped at the chromosome breakpoint t(3;6)(q27.1;q15) of human B-cell lymphoma. Genes Cells 5: 277–287.

Supporting Information

Data S1 Calculation of 2'-O-methyladenosine (Am) content in injected rRNA fragment including raw data. (DOC)

Author Contributions

Conceived and designed the experiments: MB KDE JS MH. Performed the experiments: MB SO MP CP PW SK SS. Analyzed the data: MB SO MP CP PW SK SS. Contributed reagents/materials/analysis tools: SO CP PW SK SS. Wrote the paper: MB SS K D.
32. Jack K, Bellodi C, Landry DM, Niederer RO, Meskauskas A, et al. (2011) rRNA pseudouridylation defects affect ribosomal ligand binding and translational fidelity from yeast to human cells. Mol Cell 44: 660–666.
33. Gu¨ldener U, Heck S, Fielder T, Beinhauer J, Hegemann JH (1996) A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res 24: 2519–2524.
34. Schiestl RH, Gietz RD (1989) High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. Curr Genet 16: 339–346.
35. Sikorski RS, Hieter P (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics 122: 19–27.
36. McEntee CM, Hudson AP (1989) Preparation of RNA from unspheroplasted yeast cells (Saccharomyces cerevisiae). Anal Biochem 176: 303–306.
37. Sharma S, Watzinger P, Kötter P, Entian KD (2013) Identification of a novel methyltransferase, Bmt2, responsible for the N-1-methyl-adenosine base modification of 25S rRNA in Saccharomyces cerevisiae. Nucleic Acids Res 41: 5428–5443.
38. Kellner S, Seidu-Larry S, Burhenn J, Motorin Y, Helm M (2011) A multifunctional bioconjugate module for versatile photoaffinity labeling and click chemistry of RNA. Nucleic Acids Res 39: 7348–7360.