

A cluster of methylations in the domain IV of 25S rRNA is required for ribosome stability

ANDRIANA GIGOVA,¹ SUJITHA DUGGIMPUDI,^{1,4} TIM POLLEX,^{2,3,5} MATTHIAS SCHAEFER,^{2,3,6} and MARTIN KOŠ¹

¹Biochemistry Center and Cluster of Excellence CellNetworks, University of Heidelberg, 69120 Heidelberg, Germany

²Division of Epigenetics, DKFZ-ZMBH Alliance, German Cancer Research Center, 69120 Heidelberg, Germany

ABSTRACT

In all three domains of life ribosomal RNAs are extensively modified at functionally important sites of the ribosome. These modifications are believed to fine-tune the ribosome structure for optimal translation. However, the precise mechanistic effect of modifications on ribosome function remains largely unknown. Here we show that a cluster of methylated nucleotides in domain IV of 25S rRNA is critical for integrity of the large ribosomal subunit. We identified the elusive cytosine-5 methyltransferase for C2278 in yeast as Rcm1 and found that a combined loss of cytosine-5 methylation at C2278 and ribose methylation at G2288 caused dramatic ribosome instability, resulting in loss of 60S ribosomal subunits. Structural and biochemical analyses revealed that this instability was caused by changes in the structure of 25S rRNA and a consequent loss of multiple ribosomal proteins from the large ribosomal subunit. Our data demonstrate that individual RNA modifications can strongly affect structure of large ribonucleoprotein complexes.

Keywords: ribosomal RNA; RNA modification; ribosome stability; translation; yeast

INTRODUCTION

In all organisms ribosomal RNAs (rRNAs) undergo extensive post-transcriptional modification at numerous sites by methylation or pseudouridylation. The sites of modification are conserved from prokaryotes to eukaryotes and cluster in functionally important regions of the ribosome, which interestingly, are largely devoid of ribosomal proteins. The number of modified residues has increased during evolution from prokaryotes to eukaryotes (33 in *Escherichia coli*, 108 in yeast, and >200 in human) (Decatur and Fournier 2002). While individual modifications are not essential, collectively they are required for cell viability. Deletion of either the pseudouridylylase *CBF5* (Dyskerin) or the 2'-O-ribose methyltransferase *NOPI* (Fibrillarin) is lethal (Tollervey et al. 1991, 1993; Lafontaine et al. 1998). Interestingly, while the catalytic activity of Nop1p is essential (Tollervey et al. 1993), the catalytically inactive Cbf5p supports viability; however, the loss

of rRNA pseudouridylation results in strongly reduced growth and diminished translational fidelity (Zebarjadian et al. 1999; Jack et al. 2011). Effects of individual modifications can have far-reaching consequences in multicellular organisms. In a recent report, removal of a single methylation in the 28S rRNA lead to developmental defects in zebrafish (Higa-Nakamine et al. 2012). Nonetheless, our understanding of the precise function of rRNA modification remains largely incomplete.

In this study, we investigated the role of a conserved cluster of methylations in the helices H70 and H71 of domain IV in 25S rRNA in yeast. Helix 70 is a highly conserved noncanonical structure formed by four systems of stacking interactions of mostly unpaired bases between helices 69, 71, and 67 in 25S rRNA (Fig. 1A; Noller 2005). Mutations in this region of *E. coli* 23S rRNA impaired subunit-subunit interactions (Levieu et al. 1995). Due to its location within the ribosome, it might also influence interactions of tRNAs at the A- and P-sites and activity of the peptidyl transferase center (Noller 2005). Helix 71 together with helix 44 of the 18S rRNA form a purely RNA-based intersubunit bridge B3,

³These authors contributed equally to this work.

⁴Present address: Heinrich-Heine-Universitätsklinikum, Klinik für Kinder-Onkologie, Hämatologie und Klinische Immunologie, 40225 Düsseldorf, Germany

⁵Present address: Mammalian Developmental Epigenetics Group, Institut Curie, CNRS UMR3215, INSERM U934, F-75005 Paris, France

⁶Present address: Department of Biochemistry & Biophysics, Max F. Perutz Laboratories, 1030 Vienna, Austria

Corresponding author: martin.kos@bzh.uni-heidelberg.de

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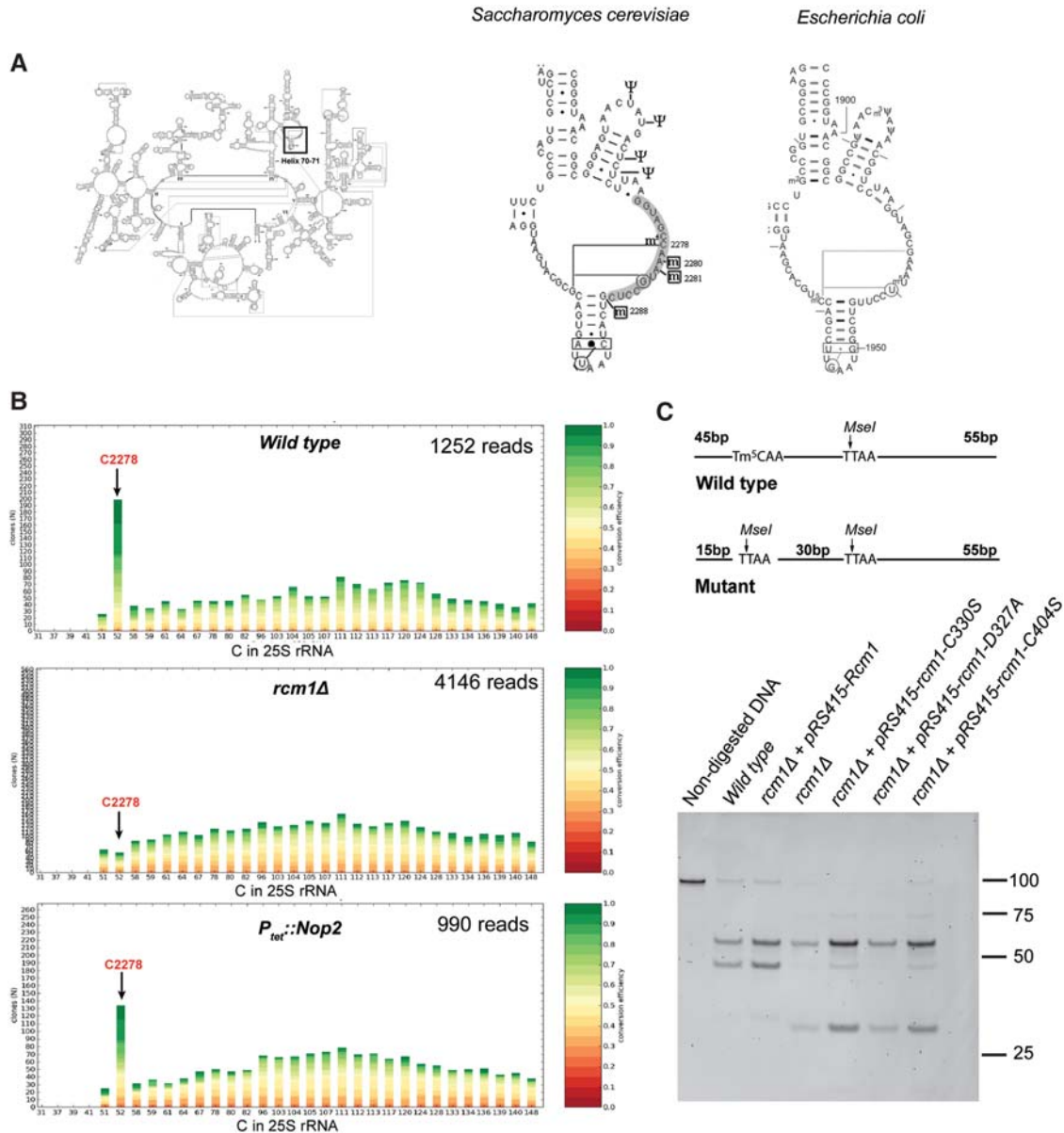


FIGURE 1. Rcm1p methylates C2278 of 25S rRNA. (A) Secondary structure of the here-studied region of the domain IV in yeast 25S rRNA (left) and *E. coli* 23S rRNA (right). The conserved tertiary cross-strand interactions are indicated by lines. The nucleotides forming the noncanonical helix 70 are shaded. (B) Bisulfite sequencing analysis of 25S rRNA methylation patterns from wild-type, *rcm1Δ*, and *P_{tet}::Nop2* strains. Fractions of nondeaminated cytosines are plotted against the position of the cytosine in individual rRNA amplicons, thus revealing the pattern of m⁵C modifications in individual rRNAs. Position of C2278 is indicated. (C) COBRA assay; the scheme on the top shows the expected digestion patterns in the presence or absence of m⁵C2278. The MseI digestion of RT-PCR products of bisulfite-treated 25S rRNA resolved in a 4% polyacrylamide gel is shown at the bottom. Fragment sizes are indicated to the right of the gel.

which may serve as an anchoring point during the ratcheting movement in translation (Ben-Shem et al. 2010, 2011). There are two types of RNA methylations present in this region: 2'-O-ribose methylations at residues A2280, A2281, and G2288, and a single base methylation, cytosine-5 methylation of C2278. The m⁵C2278 forms a universally conserved tertiary stacking interaction with G2283 and G2305 in yeast. Interestingly, while the nucleotide identity is not fully conserved, the base methylation of one of the residues

involved in this stacking interaction is present in both prokaryotes and eukaryotes. Additional nucleotides between H70 and H71 are methylated in all organisms. In yeast, the three ribose methylations in H70/71 are guided by two snoRNAs, snR13 (A2280m and A2281m), and snR75 (G2288m) (Lowe and Eddy 1999; Piekna-Przybylska et al. 2007). The cytosine-5 methyltransferase for C2278 in yeast was unknown prior to this study. During the writing of this manuscript, the identification of Rcm1p as a

methyltransferase for C2278 has been independently reported (Sharma et al. 2013).

The biophysical properties of modified RNA nucleotides and their effects on RNA structure and stability have been extensively investigated (Davis 1995, 1998; Agris 1996; Helm 2006). Pseudouridines have been shown to stabilize RNA structure due to improved stacking or additional hydrogen bonding (Charette and Gray 2000), whereas base methylations increase hydrophobicity leading to improved stacking and/or induction of structural changes due to a reduced hydrogen-bonding capacity. Finally, 2'-O-ribose methylation augments the thermal stability of RNA by shifting the preferred ribose conformation toward the 3' endo pucker (Davis 1998). Ribose methylation also changes the hydration of the sugar edge affecting interactions with other RNAs or proteins and it can protect RNA from hydrolysis (Helm 2006). It is important to note that, due to technical limitations, most studies on the effects of RNA modifications were performed on single nucleosides, oligonucleotides, or small RNAs such as tRNAs. Consequently, while considerable progress has been made in our understanding of how nucleotide modifications affect conformation and function of small RNAs, it is currently impossible to predict their global impact on larger, more complex RNAs, such as rRNA. The currently accepted hypothesis is that rRNA modifications enhance the general stability of ribosomes and fine-tune their structure to promote efficient and precise translation (Decatur and Fournier 2002; Chow et al. 2007; McMahon et al. 2013). However, to date only a handful of studies demonstrating the importance of rRNA modifications for correct ribosome function have been reported. For example, a cluster of pseudouridines in the A-site finger of the large ribosomal subunit in yeast positively affects translation efficiency and fidelity (Piekna-Przybylska et al. 2008; Baudin-Baillieu et al. 2009). Also, simultaneous removal of several modifications in the 18S rRNA decoding center results in lower amino acid incorporation rates, reduced translational fidelity, and delayed 18S rRNA synthesis (Baudin-Baillieu et al. 2009; Liang et al. 2009). In addition, function of the peptidyl-transferase center (PTC) of the large ribosomal subunit seems also to be optimized by rRNA modifications. Elimination of multiple pseudouridines and base methylations in the PTC of yeast 25S rRNA changed the sensitivity of ribosomes to inhibitors of translation and caused specific defects in translational fidelity (Baxter-Roshek et al. 2007). Additionally, in vitro probing of the RNA structure revealed minor changes in the conformation of 25S rRNA (Baxter-Roshek et al. 2007). To date the strongest reported phenotype caused by a lack of rRNA modifications has been observed for a cluster of four pseudouridines and one 2'-O-ribose methylation in helix 69 of 25S rRNA in yeast (Yusupov et al. 2001). Removal of three or more of the modifications resulted in a temperature-sensitive growth phenotype, significantly reduced protein synthesis, and an increased sensitivity to antibiotics (Liang et al. 2007). While all of the above studies demonstrate a clear role of

rRNA modifications for ribosome functions, detailed examples of their effect on structural stability of the ribosome and how they modulate interaction between rRNAs and ribosomal proteins are unknown.

Here we show that the m⁵C2278 together with G2288m are critical for stable incorporation of several large ribosomal proteins into the 60S subunit. We found that a previously uncharacterized open reading frame *YNL022C* encodes a cytosine-5 methyltransferase that modifies C2278. A lack of methylation at C2278 and G2288 results in a dramatic instability of the large ribosomal subunit, caused by labile association of Rpl23p with 25S rRNA. Loss of Rpl23p leads to subsequent loss of other adjacent large ribosomal proteins. These findings demonstrate that individual rRNA methylations can critically affect RNA conformation and the overall structure and protein composition of large ribonucleoprotein complexes.

RESULTS

RCM1 (YNL022C) methylates the cytosine 2278 in 25S rRNA

It has previously been suggested that a putative methyltransferase Nop2p methylates C2278; however, it has never been formally proved (King et al. 1999; Hong et al. 2001). Several putative methyltransferases were recently identified in the yeast genome (Petrossian and Clarke 2009). Among those was an uncharacterized open reading frame *YNL022C*, which has been predicted to be involved in ribosome biogenesis (Wade et al. 2006). To test whether Nop2p or Ynl022cp catalyze the m⁵C2278 formation we used RNA bisulfite sequencing (Schaefer et al. 2009). *YNL022C* is a nonessential gene, thus we created a strain with the complete open reading frame of *YNL022C* deleted (*ynl022cΔ*). As *NOP2* is an essential gene, known to be involved in ribosome biogenesis, we created a strain expressing *NOP2* under the control of a tetracycline repressible promoter (*Ptet-NOP2*). The 25S rRNA was isolated from wild-type, *ynl022cΔ*, and *Ptet-NOP2* strains, treated by bisulfite, and sequenced (Schaefer et al. 2009). While deletion of *YNL022C* completely abolished methylation of C2278, the depletion of Nop2p resulted in only a mild decrease in m⁵C2278, most likely a secondary effect due to its essential role in ribosome biogenesis (Fig. 1B). The lack of methylation in the *ynl022cΔ* strain does not exclude the possibility that Ynl022cp is involved in ribosome biogenesis but not directly in the C2278 methylation. We therefore aligned the protein sequences of Ynl022cp, Nop2p, and the well-characterized tRNA cytosine-5 methyltransferase Trm4p, and identified three conserved amino acids likely required for the putative catalytic activity of Ynl022cp (Supplemental Fig. 1). The Asp327, Cys330, and Cys404 in *YNL022C* are highly conserved among RNA methyltransferases and required for formation of m⁵C. Mutations of the equivalent residues were shown to abolish the catalytic activity of Trm4p and other methyltransferases without negative effects on the protein

stability (King and Redman 2002; Bujnicki et al. 2004). The *ynl022cΔ* strain was transformed with plasmids expressing either wild-type or single-point mutant Ynl022cp (D327A, C330S, or C404S). All three mutated proteins failed to rescue the C2278 methylation, whereas wild-type Ynl022cp fully restored the levels of m⁵C2278 (Fig. 1C). In conclusion, YNL022C is the cytosine-5-methyltransferase for C2278 and we propose to call it *RCM1* (Ribosomal RNA Cytosine Methyltransferase 1).

Competitive fitness and translational fidelity are diminished in strains lacking methylations in the H70 and H71

Identification of the m⁵C2278 methyltransferase allowed us to address in detail the role of the cluster of rRNA methylations in helices H70 and H71. Strains lacking one or more modifications were created by deletion of either *Rcm1* or snoRNAs *snR13* and *snR75* or their combinations (Table 1) and assayed for their growth phenotype in different condi-

tions, ribosome biogenesis defects, antibiotics sensitivity, and translational fidelity (Supplemental Table S1). The growth of all strains was not substantially affected at any temperature tested. The *rcm1Δsnr75Δsnr13Δ* strain grew only slightly slower than wild type in the minimal media, with a doubling time of 3.8 and 3.4 h, respectively. However, mutant strains, especially *snr75Δ*, *rcm1Δsnr75Δ*, and *rcm1Δsnr75Δsnr13Δ*, were efficiently outcompeted by the parental wild-type strain in growth competition assays, demonstrating importance of methylations in this region for cellular fitness (Fig. 2A). An aberrant ribosome biogenesis is unlikely the cause of the observed decrease in fitness, as analysis of the ribosomal RNA processing did not reveal any significant defect in the mutant strains (Supplemental Fig. 2). Therefore, the function of mature ribosomes is likely to be affected by these rRNA methylations.

As each of the modifications investigated here are in the proximity of tRNAs in the A-site, we also examined the sensitivity of the mutant strains to anisomycin, puromycin, and paromomycin, which are known to affect A-site function

TABLE 1. Strains used in this study

Strain	Genotype	Reference	Effect on methylations
YMK118 (Parental strain)	<i>MATa; his3 Δ1; leu2-3,112; trp1-289; ura3-52; MAL2-8C; SUC2, lys2:: tTA, ura3::PCMVtetR'-SSN6 URA3-K.I.</i>	Alexander et al. 2010	None
<i>rcm1Δ</i> (YMK305)	<i>rcm1::KanMX6</i>	This study	Δ5mC2278
<i>snr13Δ</i> (YMK347)	<i>snr13::NatMX4</i>	This study	ΔA2280m, ΔA2281m
<i>rcm1Δsnr13Δ</i> (YMK348)	<i>rcm1::KanMX6,snr13::NatMX6</i>	This study	Δ5mC2278, ΔA2280m, ΔA2281m
<i>snr75Δ</i> (YMK565)	<i>snr75::HIS</i>	This study	ΔG2288m
<i>rcm1Δsnr75Δ</i> (YMK566)	<i>snr75::HIS,rcm1::KanMX6</i>	This study	ΔG2288m, Δ5mC2278
<i>rcm1Δsnr75Δsnr13Δ</i> (YMK567)	<i>snr75::HIS,rcm1::KanMX6,snr13::NatMX4</i>	This study	ΔG2288m, ΔA2280m, ΔA2281m, Δ5mC2278
<i>Ptet-NOP2</i> (YMK194)	<i>tet::3HA-Nop2 (NatMX4)</i>	This study	Unknown
<i>rcm1Δ, pRS415-Rcm1</i> (YMK310)	<i>rcm1::KanMX6, pRS415-Rcm1 (LEU2)</i>	This study	None
<i>rcm1Δ, pRS415-rcm1-C330S</i> (YMK338)	<i>rcm1::KanMX6, pRS415-C330S::rcm1 (LEU2)</i>	This study	Δ5mC2278
<i>rcm1Δ, pRS415-rcm1-D327A</i> (YMK339)	<i>rcm1::KanMX6, pRS415-D327A::rcm1 (LEU2)</i>	This study	Δ5mC2278
<i>rcm1Δ, pRS415-rcm1-C404S</i> (YMK340)	<i>rcm1::KanMX6, pRS415-C404S::rcm1 (LEU2)</i>	This study	Δ5mC2278
YMK118+TQ (A4)	pAC/PGK-TQ in YMK118	This study	None
YMK118+EST (A6)	pAC/PGK-EST in YMK118	This study	None
YMK118+IBV (A5)	pAC/PGK-IBV in YMK118	This study	None
YMK118+TAG/TGA/TAA (A1/A2/A3)	pAC/PGK-TAG/TGA/TAA in YMK118	This study	None
YMK567+TQ (A28)	pAC/PGK-TQ in <i>rcm1Δsnr75Δsnr13Δ</i>	This study	ΔG2288m, ΔA2280m, ΔA2281m, Δ5mC2278
YMK567+EST (A30)	pAC/PGK-EST in <i>rcm1Δsnr75Δsnr13Δ</i>	This study	ΔG2288m, ΔA2280m, ΔA2281m, Δ5mC2278
YMK567+IBV (A29)	pAC/PGK-IBV in <i>rcm1Δsnr75Δsnr13Δ</i>	This study	ΔG2288m, ΔA2280m, ΔA2281m, Δ5mC2278
YMK567+TAG/TGA/TAA (A25/A26/A27)	pAC/PGK-TAG/TGA/TAA in <i>rcm1Δsnr75Δsnr13Δ</i>	This study	ΔG2288m, ΔA2280m, ΔA2281m, Δ5mC2278
YMK520	<i>argΔ::HIS</i> in YMK118	This study	None

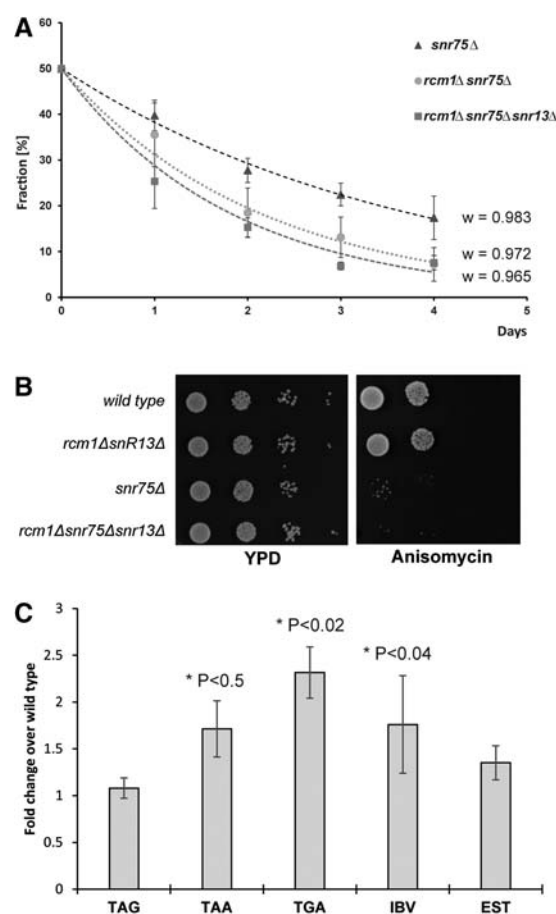


FIGURE 2. Phenotype of strains lacking methylations in Helix 70–71. (A) Competition fitness assay: Changes in the fractions of wild-type and mutant strains co-cultured in YPD at 30°C are plotted against time. Average of triplicates, error bars represent standard deviation. Exponential functions curves were fitted to the data. The calculated fitness “w” of the strains is shown next to the curves. (B) Strains lacking G2288 methylation are sensitive to anisomycin: Cells were spotted in a series of 10-fold dilutions on YPD agar plates with and without 5 μg/mL of anisomycin and grown at 30°C for 2 d. (C) Translational fidelity assay: fold change over wild-type levels of stop codon read-through, –1 frame-shift (IBV) or +1 frame-shift (EST3) in the *rcm1Δ snr75Δ snr13Δ* strain. Error bars, SEM of four experiments. An asterisk indicates a statistically significant result. The *P* values were estimated using unpaired one-tailed Student’s *t*-test.

(Fourmy et al. 1998; Hansen et al. 2003). Surprisingly, the absence of a single ribose methylation of G2288 (*snr75Δ*) was sufficient to render the cells hypersensitive to anisomycin (Fig. 2B). None of the strains showed changed sensitivity to paromomycin or puromycin.

It has been previously observed that rRNA modifications can influence translational fidelity. We used dual reporter vectors (Baudin-Baillieu et al. 2009) to determine whether the lack of methylations in H70 and H71 of 25S rRNA affect programmed frame shifting or stop codon recognition. In the *rcm1Δ snr75Δ snr13Δ* strain the read-through of the UAA and UGA termination codons was significantly increased ~1.6-fold and 2.4-fold, respectively. Also the –1 frame shifting fre-

quency was significantly increased, ~1.6-fold (unpaired, one-tailed Student’s *t*-test) (Fig. 2C).

In summary, the cluster of methylations in H70 and H71 affects translational fidelity of the ribosome and is important for strain fitness.

Methylation of C2278 and G2288 is critical for stability of 60S ribosomal subunits

The m⁵C2278 is involved in a highly conserved stacking interaction with G2305, at the base of helix 71. Interestingly, the 2′-O-ribose methylated G2288 is also located at the base of H71. H71 forms a major intersubunit bridge B3 (Ben-Shem et al. 2010). We therefore hypothesized that both modifications might stabilize the B3 bridge and consequently the 80S ribosome. We compared polysome profiles from the wild-type and strains lacking methylations under standard low-salt conditions (10 mM MgCl₂, 50 mM NaCl). While the wild-type profile showed symmetrical peaks for 80S, 60S, and 40S ribosomes as expected, the profiles from strains lacking m⁵C2278 (*rcm1Δ*) or multiple methylations have a reproducible “shoulder” between the 60S and 80S peaks, and reduced levels of 80S ribosomes (Fig. 3). A profile identical to *rcm1Δ* was also obtained from the *rcm1Δ* strain expressing catalytically inactive Rcm1p-C404S, confirming that the lack of C2278 methylation and not the absence of Rcm1p is responsible for the phenotype (data not shown). These results suggest that methylations influence the stable association of ribosomal subunits in 80S monosomes. In the absence of the methylations the 80S monosomes are more likely to dissociate during the centrifugation and create the observed “shoulder” on the left side of the 80S peak.

To further assess the stability of 80S ribosomes, ribosomes were isolated in low-salt conditions as above and then exposed for 15–30 min to higher NaCl and/or lower MgCl₂ concentrations, and subsequently loaded on standard low-salt sucrose gradients. Surprisingly, even a mild increase in the salt concentration (300 mM NaCl, 5 mM MgCl₂), well below the concentrations used for purification of ribosomal subunits (Mašek et al. 2011), led not only to further destabilization of 80S monosomes but also to appearance of smaller sub-60S peaks in the strains lacking methylations, but not the wild-type strain (Fig. 4). In particular, the simultaneous lack of methylations at C2278 and G2288 caused a dramatic instability of 60S subunits. In *rcm1Δ snr75Δ* and *rcm1Δ snr75Δ snr13Δ* strains both 80S monosomes and, importantly, 60S subunits were almost completely lost (Fig. 4). We wished to purify pure 60S subunits from the mutant strains for further detailed in vitro characterization. However, we were not able to obtain sufficient amounts of the 60S subunits, as they disintegrated during multiple centrifugation steps even under low-salt conditions (50 mM NaCl/KCl, 10 mM MgCl₂) likely due to their inherent instability. These observations show that rRNA methylation in the helices H70 and H71 greatly enhances the stability of large ribosomal subunit.

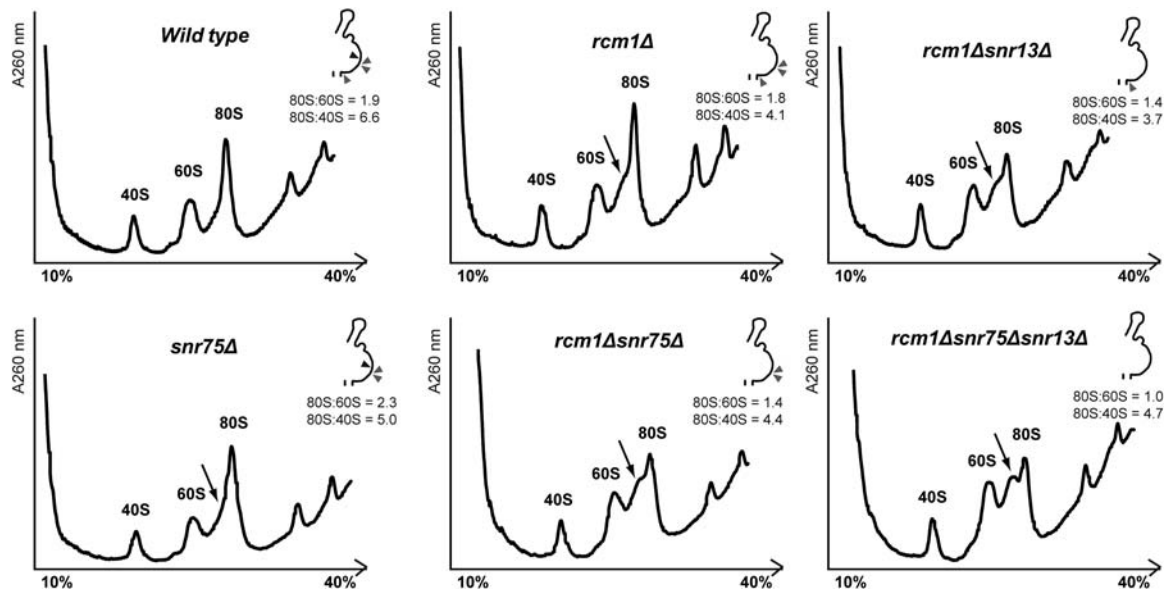


FIGURE 3. 80S stability in low salt in strains lacking methylations. Polysome profiles in low-salt conditions (50 mM NaCl, 10 mM MgCl₂). A reproducible shoulder indicative of 80S instability is indicated by an arrow. Schemes of rRNA methylations present in individual strains are shown as insets. All experiments were performed at least five times with identical results. Profiles shown are representative for each strain. Ratio of 80S to 40S or 60S subunits were calculated from the areas below the corresponding peaks and are shown at top right in each chart.

Methylations stabilize the conformation of the H70 and H71 of 25S rRNA

In the polysome profiles from strains lacking two or more methylations, the 80S and 60S peaks are greatly reduced; however, one to two lighter peaks appear. These lighter peaks most likely represent the large ribosomal subunits that partially lost some rRNA and/or ribosomal proteins. We have

estimated their sedimentation coefficients as 46S and 50S, respectively, by means of nonlinear regression and the known sedimentation distances of 40S, 60S, and 80S particles in the polysome profiles.

We first assessed whether the rRNA content of large ribosomal subunit is reduced in the strains missing the methylations. To test this possibility, RNA was isolated from the polysome profile fractions and probed by Northern blotting.

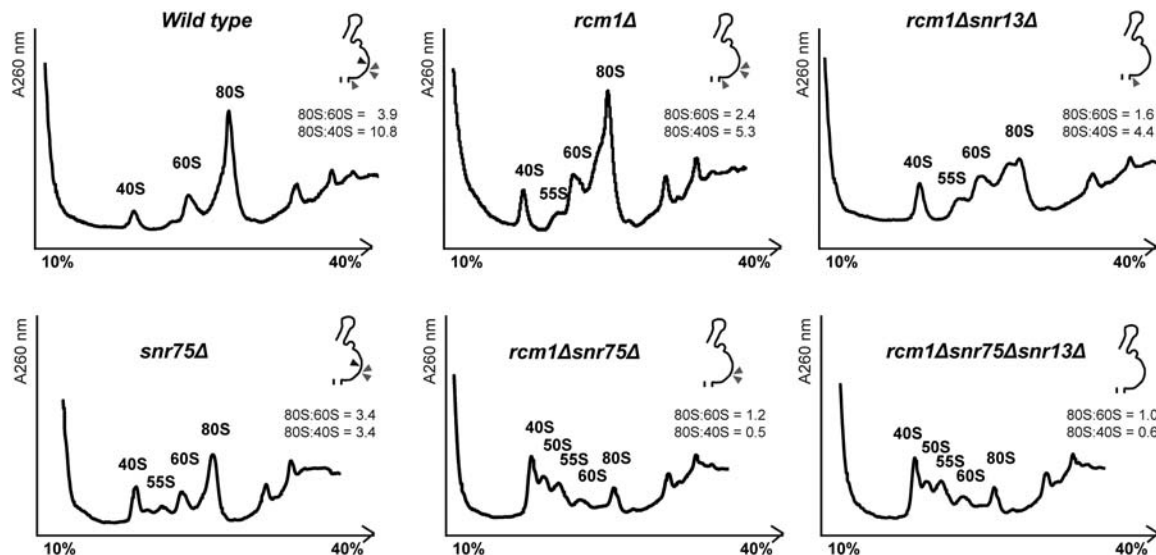


FIGURE 4. 60S subunits lacking methylations are salt sensitive. Polysome profiles after exposure to 300 mM NaCl, 5 mM MgCl₂. Schemes of rRNA methylations present in individual strains are shown as insets. All experiments were performed at least five times with identical results. Profiles shown are representative for each strain. Ratio of 80S to 40S or 60S subunits were calculated from the areas below the corresponding peaks and are shown at top right in each chart.

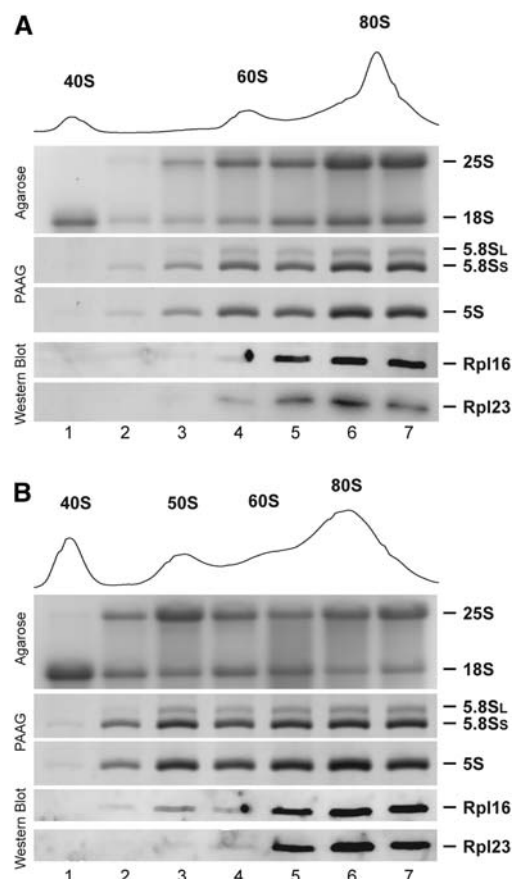


FIGURE 5. RNA and protein composition of the polysome fractions. Polysome fractions from the wild-type (A) and *rcm1Δsnr75Δsnr13Δ* (B) strains were analyzed for their RNA and protein composition. (Top) Total RNA from polysome fractions corresponding to 40S, 60S, and 80S peaks was separated by agarose or polyacrylamide gel electrophoresis and detected by SYBR Green II staining directly in gel. (Bottom) Western blot using antibodies recognizing Rpl16p or Rpl23p.

In neither the wild-type nor mutant strains were the 25S rRNA steady-state levels affected. Intact 25S, 5.8SL/S, and 5S rRNAs were detected in fractions corresponding to the both sub60S peaks (Fig. 5). Also, their relative abundance ratios were unchanged in all gradient fractions. Therefore, the absence of rRNA methylations did not appear to affect the rRNA composition of ribosomal subunits.

To determine changes in the rRNA conformation in the presence or absence of methylations we probed the RNA structure with 1-methyl-7-nitroisatoic acid (1M7), which acylates 2′hydroxyl groups of ribose in “flexible” nucleotides, unconstrained by base-pairing or tertiary interactions (Mortimer and Weeks 2007). The gradient fractions of the 50S, 60S, 80S ribosomes and polysomes were treated with 1M7 and the modified nucleotides identified by a primer extension across the H70, H71 regions (Fig. 6A). In both wild-type and *rcm1Δsnr75Δsnr13Δ* intact 60S/80S subunits the pattern of 1M7 modifications is in a good agreement with the RNA conformation in the ribosome crystal structure. However, in the

50S particles from the *rcm1Δsnr75Δsnr13Δ* strain multiple residues showed higher 1M7 modification, indicating that these residues are not anymore engaged in base-pairing, tertiary RNA–RNA interactions, or protein interactions. The increased 1M7 modification of U2268, U2269, U2274, and A2275 indicate loss of tertiary interactions responsible for forming/stabilizing the H70. In addition, the residues U2268 and U2269 are in the vicinity of the helix 69 stem. This region, including U2268, interacts with tRNAs in the A- and P-site and plays an important role in translational fidelity (Hirabayashi et al. 2006; Liang et al. 2007). Changes in the conformation of this region are consistent with the significantly increased read-through stop codons and –1 frame-shifting frequency in the *rcm1Δsnr75Δsnr13Δ* strain. The increase in modification of U2294, U2295, U2298 and A2299 indicated that also the loop of the helix H71 is more flexible in the absence of methylations. Moreover, the nucleotides U2294 and U2295 interact with Rpl23p suggesting a possible loss of this RNA-protein interaction. Interestingly, no position seems to show decrease of 1M7 modification, indicating that no new base-pairing occurs. Therefore, it seems that the RNA secondary structure of the H69/H71 region is overall more flexible and/or some of the RPLs interactions are lost in the ribosome lacking the studied methylations. It also suggests that no new alternative conformation is formed. These findings demonstrate that the conformation of domain IV of 25S rRNA is influenced by methylations in H70 and H71.

Lack of methylations leads to a loss of ribosomal proteins from the large subunit

The other plausible cause for the lower sedimentation of the 46S and 50S particles is a reduced protein content. To thoroughly investigate this possibility, the protein compositions of all peaks were analyzed by SILAC and spike-in SILAC techniques (Lanucara and Eysers 2011). For the SILAC analysis, the cultures of the wild-type strain grown in heavy amino acids was mixed with a *rcm1Δsnr75Δsnr13Δ* strain grown in light amino acids in a 1:1 ratio, the cells were lysed, treated by salt, and loaded on the sucrose gradient. The fractions corresponding to 80S, 60S, and 50S peaks were collected and analyzed by mass spectroscopy. We then quantified individual proteins in each fraction and calculated their mutant vs. wild-type ratio (Fig. 6B). If the 50S peak in the mutant strain corresponds to a large ribosomal subunit that lost some ribosomal proteins, we would expect to see only RPLs to be detected in this fraction. This was indeed the case. Out of 320 quantified proteins, only large ribosomal proteins (RPLs) were significantly enriched (two- to fivefold) and only in the 50S fraction (Fig. 6B, red bars). The small ribosomal subunits proteins (RPSs) levels were unchanged between the wild-type and mutant strain, indicating that small ribosomal subunits are not present in the 50S peak. Furthermore, the intact 60S subunits from the *rcm1Δsnr75Δsnr13Δ* did not show

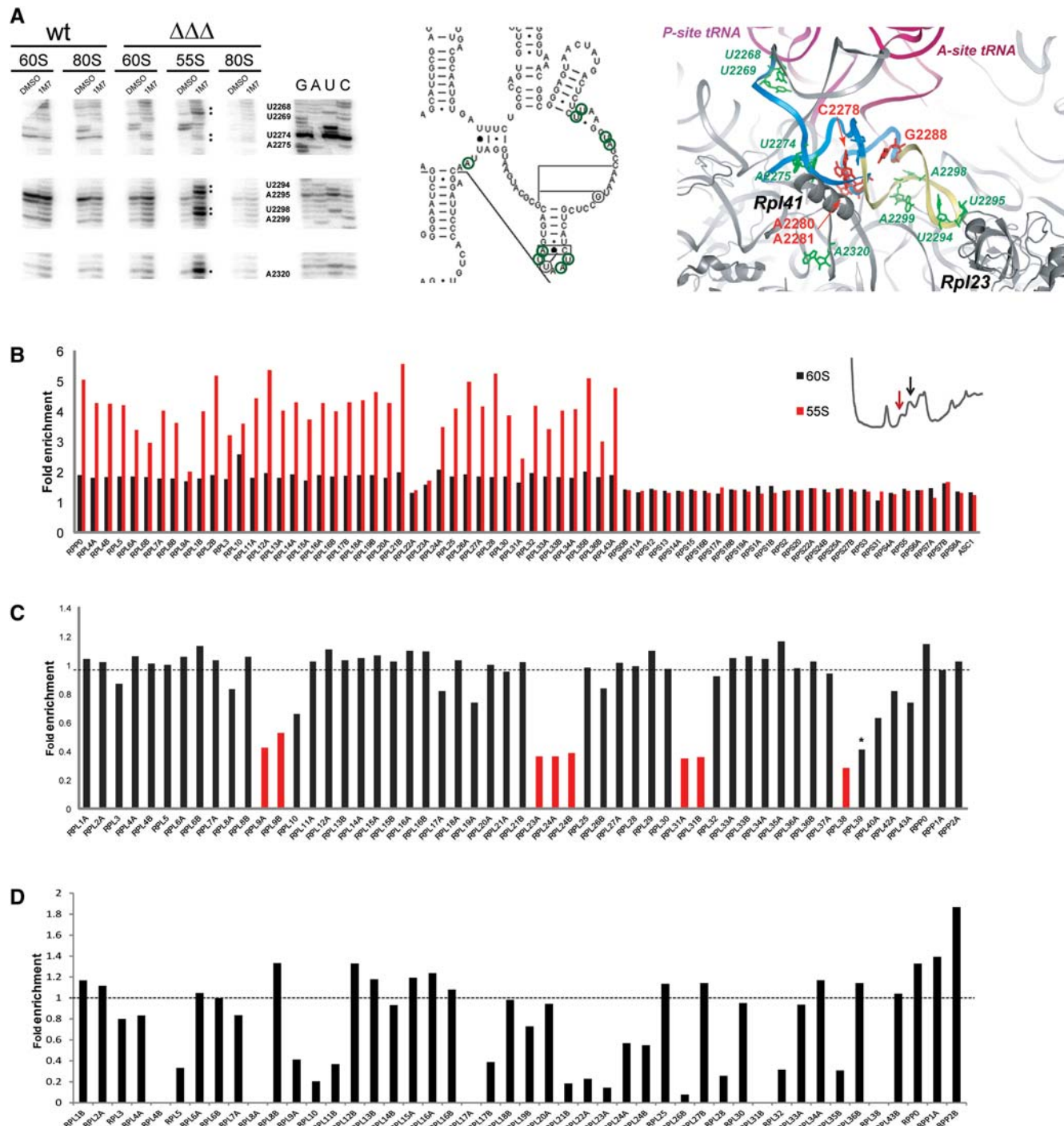


FIGURE 6. RNA structure probing and SILAC analysis. (A) SHAPE analysis; primer extensions across the domain IV of 25S rRNA (left), position of nucleotides with increased 1M7 modification (green) is shown in the secondary structure of 25S rRNA (middle), and in the 3D structure of yeast ribosome (Ben-Shem et al. 2011). Helices 70 and 71 are in blue and yellow, respectively. The methylated residues are shown as sticks in red. (B) SILAC analysis of protein composition of 60S and 50S gradient fractions (arrows) from wild-type and *rcm1 Δ snr75 Δ snr13 Δ* strains. Protein abundance is expressed as relative ratios mutant:wild type. (C,D) Spike-in SILAC analysis of protein composition changes in the 50S (C) and 46S (D) gradient fractions from the *rcm1 Δ snr75 Δ snr13 Δ* strain. Relative ratios 50S:60S or 46S:60S were normalized to the median of all RPLs. Proteins severely affected in two independent experiments are shown in red. Rpl39 indicated by an asterisk was identified only in one of two independent spike-in experiments.

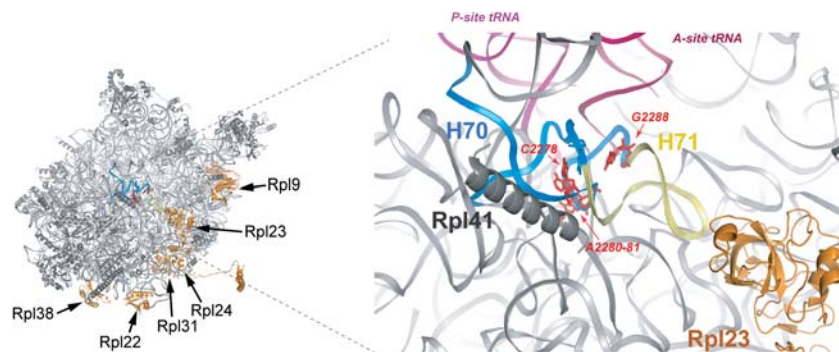


FIGURE 7. Location of affected RPLs in the crystal structure of ribosome. Intersubunit interface view of the crystal structure of the yeast 60S ribosomal subunit (Ben-Shem et al. 2011). Proteins destabilized in the *rcm1Δsnr75Δsnr13Δ* strain are highlighted in orange. The methylated residues C2278, A2280, A2281, and G2288 are shown as sticks in red. Helices 70 and 71 are in blue and yellow, respectively.

significant changes in levels of any of the proteins (Fig. 6B, black bars). Therefore, we can conclude that the 50S peak in the strains lacking methylations corresponds to 60S ribosomal subunits, which are sedimenting slower. Importantly, not all RPLs were equally enriched in the 50S peak. As all RPLs are considered to be stoichiometric components of the large ribosomal subunit, their relative mutant vs. wild-type enrichment ratios should also be approximately equal. However, some RPLs were not enriched in the 50S fraction. It indicated that these RPLs were more labile and partially lost from the 50S ribosomal particle, with the most affected being Rpl9p, Rpl22p, Rpl23p, Rpl24p, Rpl31p, and Rpl38p.

To confirm this observation, we repeated the experiment but used the spike-in SILAC approach that allowed us to directly compare protein abundance in different fractions from the same gradient. The cultures were not mixed; instead, to the gradient fractions of 60S, 50S, and 46S peaks from the *rcm1Δsnr75Δsnr13Δ* strain, the same amount of heavy labeled 60S fraction from the wild-type strain was added. The spiked-in wild-type fraction thus serves as a loading and normalization control. The proteins were quantified and their levels were expressed as ratios 50S:60S or 46S:60S (Fig. 6C,D). The results revealed that the same RPLs as in the previous experiment were depleted from the 50S particle (Fig. 6C). The proteins Rpl23p, Rpl24p, Rpl9p, Rpl31p, and Rpl38p were lost from the 50S particle. Moreover, analysis of the 46S particle showed further depletion of additional RPLs (Fig. 6D).

To further validate the SILAC data, we also performed a Western blot analysis of different gradient fractions using antibody against Rpl23p that should be absent from the 50S particle based on the results from the SILAC experiments. As a control, we also used antibody recognizing Rpl16p, which should not be lost from the 50S particle. The results showed that while Rpl16p is readily detectable in both 60S and 50S fractions in the mutant strain, the Rpl23p protein is detected only in the 60S peak, thus independently confirming the results of the SILAC experiments (Fig. 5).

Interestingly, when the missing proteins are localized in the crystal structure of the yeast ribosome (Ben-Shem et al. 2011) the proteins most affected are clustered together at the bottom of the 60S subunit (Fig. 7). Importantly, the loop of H71 interacts directly with Rpl23p and therefore might influence its association with the ribosome. On the other side, Rpl23p interacts directly with Rpl24p. We propose that the initial loss of Rpl23p probably leads to further destabilization of the region and subsequent loss of the other RPLs. We conclude that, in particular, the methylated residues m⁵C2278 and G2288m, and A2280m, A2281m stabilize the structure of 25S rRNA and conse-

quently the association of ribosomal proteins with the large ribosomal subunit.

DISCUSSION

In all three domains of life ribosomal RNAs are extensively post-transcriptionally modified in functionally important regions of the ribosome. The rRNA modifications are assumed to enhance the conformational stability and to fine-tune translation efficiency and fidelity of the mature ribosome. However, our understanding of the precise role of individual modifications or their clusters remains limited. In this study we analyzed in detail the function of a universally conserved cluster of rRNA methylations in the helices H70 and H71 in yeast 25S rRNA. Our data show that methylations in this region of rRNA not only affect the fidelity of translation, but also greatly affect the stability of the 60S ribosomal subunit.

Removal of individual methylations in domain IV had only a minor effect on the growth of yeast. However, all strains lacking more than one methylation were fast outcompeted by a wild-type strain in co-cultures. This is not unusual as similar minor effects were observed for modifications in other regions of rRNA (Decatur et al. 2007). As modifications generally appear to be clustered, functions of the clusters of modifications rather than of individual modified nucleotides need to be studied. In agreement with reports for other rRNA modifications (Baxter-Roshek et al. 2007; Liang et al. 2007; Piekna-Przybylska et al. 2008), our results show that individual methylations can have synergistic effects on the ribosome structure and function. The competition assays clearly demonstrate that, no matter how small, the benefit of individual modifications for the fitness of a strain ensures their preservation during evolution. In the case presented here, the diminished competitiveness of the strains lacking m⁵C2278 and/or G2288m is likely a consequence of both the large ribosomal subunits instability and the reduction in translational fidelity, which may lead to harmful accumulation of aberrant proteins.

The observed hypersensitivity of the *snR75Δ* strain to anisomycin points to a defect in the A-site (Dinman and Wickner 1994). This is corroborated by the increase of –1 frame-shifting frequency observed in the mutant strain as has been previously observed (Dinman et al. 1997, 1998). The positions of the studied rRNA methylations within the ribosome are in a good agreement with their potential role for the correct A-site conformation. The anisomycin hypersensitivity was in our hands limited to strains lacking *snR75*. We did not observe anisomycin sensitivity in our *rcm1Δ* strain. Interestingly, Sharma et al. (2013) reported recently that yeast strains lacking Rcm1p were also anisomycin sensitive. This discrepancy could be due to different genetic backgrounds of the parental strains used in each study and/or different experimental setups used to assay the sensitivity. Moreover, according to the Materials and Methods section, Sharma et al. (2013) used higher concentrations of anisomycin (20 μg/mL) than were used in this study (5 μg/mL).

We observed that 80S ribosomes in *rcm1Δ* lacking the single cytosine methylation at C2278 were unstable and their polysome profiles had an obvious “shoulder” to the left of the 80S peak. In a recent report (Sharma et al. 2013), polysome profiling of yeast lacking Rcm1p did not produce such an obvious “shoulder.” However, Sharma et al. (2013) used higher sucrose concentrations in their gradient centrifugation that probably resulted in a more compressed profile with such a shoulder far less obvious. In addition, we used shorter time centrifugation conditions that exert larger hydrodynamic forces on the 80S monosomes that could enhance their dissociation into subunits as has been previously described (Infante and Baierlein 1971).

We found that 80S and 60S ribosomes lacking the studied methylations largely disintegrated during a short treatment with 300 mM NaCl. Such a dramatic destabilization of 60S subunits was unexpected, as ribosomal subunits are known to remain stable and functional for extended periods of time under high ionic strength, such as 0.5–0.8 M KCl/NaCl (Falvey and Staehelin 1970; Martin and Hartwell 1970; Zylber and Penman 1970). Also, standard salt concentrations used to dissociate 80S ribosomes into subunits for their purification are in the same high range (Mašek et al. 2011). Our data strongly suggest that the m⁵C2278 and G2288m affect the equilibrium of conformations that helices H70 and H71 can assume. It seems that only individual 60S subunits and the nontranslating 80S monosomes were affected, as the fraction of ribosomes in the polysomes was not changed in our experiments. It is possible that binding of the 40S subunit and mRNA forces the 25S rRNA and RPLs into a stable structure. Such translating ribosomes are then insensitive to salt treatment.

How can the lack of rRNA methylations result in a loss of ribosomal proteins from the mature ribosome? The end loop of H71 interacts directly with the Rpl23p. In the absence of methylation, the association of Rpl23p with ribosome is likely weakened due to changes in H71 conformation, and

this in turn leads to further destabilization of the other affected RPLs. These findings imply that local changes in structure can be propagated, via rRNA, to more distant regions of the ribosome, as has been suggested before (Rhodin and Dinman 2011).

The change in 25S rRNA conformation could impair the correct RPLs assembly during ribosome biogenesis. However, the SILAC data show that 80S ribosomes in the strain lacking all methylations have a full set of RPLs. We also did not detect any defects in ribosome biogenesis. Thus ribosomes appear to be made normally. For the same reasons it is also improbable that the lack of base-pairing between pre-rRNA and snoRNAs *snR13* and *snR75* had a significant negative impact on the ribosome biogenesis. Similarly, Liang and colleagues found no difference in phenotypes of strains where snoRNAs were deleted and strains expressing snoRNAs capable of base-pairing but not methylation (Liang et al. 2007). The phenotypes observed here are therefore likely to be direct outcomes of the lack of rRNA methylations.

RNA structure probing showed an increase in the modification of U2294 and U2295, which are located in the H71 loop interacting with Rpl23p. We cannot distinguish whether the increased accessibility of these two nucleotides to 1M7 is due to the change in the conformation of the RNA or due to the lack of Rpl23p binding, which could protect the sites from 1M7 modification. However, both residues are more accessible to 1M7 also in the 60S particle in which no obvious loss of Rpl23p was observed in the SILAC experiments. Therefore, it seems likely that the change in rRNA conformation is the primary cause of the increased accessibility of these two residues.

Taken together our results show that individual RNA methylations modulate not only local conformation but have far reaching effects on the overall structure and stability of the ribosome.

MATERIALS AND METHODS

Yeast strains and media

All yeast strains used in this study are described in Table 1. Strains lacking single or multiple gene deletions of *RCM1*, *SNR13*, or *SNR75* genes were constructed by PCR based disruption as previously described (Baudin et al. 1993; Wach et al. 1994; Longtine et al. 1998; Van Driessche et al. 2005). Oligonucleotides used for creation of the strains are listed in Supplemental Table 2. The parental yeast strain YMK118, allows simple creation of strains for depletion of essential genes using tetracycline promoters (Alexander et al. 2010). All yeast work was done using standard yeast techniques (Guthrie and Fink 1991).

RNA extraction and RNA bisulfite sequencing

Nop2p was depleted for 4 h by addition of doxycycline (2 μg/mL) and cells were harvested one doubling time before the growth rate change to avoid secondary effects from depletion of this essential

gene. Total RNA was isolated from wild-type, *rcm1Δ* and depleted *Ptet-NOP2* strains using a small scale version of the guanidium/phenol extraction method as previously described (Tollervey and Mattaj 1987). RNA was treated with DNase I (Ambion), subjected to bisulfite-mediated deamination using the EpiTect kit (Qiagen), and region-specific PCR amplicons containing bar-coded 454 adapter sequences were prepared as described before (Schaefer et al. 2009). Sequencing was performed on a 454 machine (Roche); obtained reads were aligned and methylation was quantified using a custom-made python script.

Combined bisulfite restriction analysis (COBRA) assay

cDNA derived from the bisulfite treated RNA was used to generate a 100-bp PCR product surrounding C2278 of 25S rRNA with forward primer 5'-GGGAGTAATTATGATTTTTCTAAGGTAG-3' and reverse primer 5'-ATAATAAATAAAACAATAAAATCTCACTAA TCCATTTCATACAC-3'. The reverse primer removes one of the naturally occurring MseI restriction sites in the 25S rRNA gene. The PCR product was digested with MseI restriction enzyme (New England Biolabs) resulting in two digestion products of 45 and 55 bp in size if C2278 is methylated and three digestion products of sizes 15, 30, and 55 bp if C2278 is nonmethylated and thus converted to T2278 by the bisulfite treatment (Fig. 1C).

Competition assay and antibiotic sensitivity tests

Wild-type and mutant strains were grown in liquid culture to exponential phase and then mixed in equal ratios. The combined liquid culture (wild type + mutant) was grown at 30°C and maintained in exponential phase by dilution when needed. Each day an equal volume of culture was plated on a YPD agar plate and on a plate containing an antibiotic selective for the mutant strain. The plates were incubated at 30°C and after 2 d the colonies were counted to estimate wild-type:mutant ratio. The fitness “w” of the mutant strains was calculated as previously described (Hartl 1981).

To measure antibiotic sensitivity, YPD plates containing 100–300 µg/mL paramomycin, 5 µg/mL puromycin, and 5 µg/mL anisomycin were prepared. Strains were grown until exponential phase, then dot-spotted at 10-fold decreasing concentrations from 0.1 OD to 0.0001 OD and incubated at 30°C for 2 d.

Translational fidelity assay

Wild type and *rcm1Δsnr75Δsnr13Δ* were transformed with pAC/PGK dual reporter vectors, consisting of a β-galactosidase gene, followed by a recoding window containing one of each TAA, TAG, and TGA stop codons (pAC/PGK-TAA/TAG/TGA), a –1 frame shift mutation (pAC/PGK-IBV), a +1 frameshift mutation (pAC/PGK-EST), or a control vector with no mutation (pAC/PGK-TQ), followed by the luciferase gene (Stahl et al. 1995; Baudin-Baillieu et al. 2009). In total, 30 OD units of exponential cultures were lysed with glass beads in a lysis buffer (10 mM MgCl₂, 50 mM NaCl, 50 mM Tris at pH 7.4). To measure the β-galactosidase activity, half of the cell lysate was mixed with an equal volume of lysis buffer and 1 mM chlorophenolred-β-D-galactopyranoside (CPRG, Roche), incubated at 37°C for 30 min and absorbance was measured

at 575 nm on Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek). The remaining lysate was evaluated for luciferase activity, using the Pierce Firefly Luciferase Glow Assay Kit (Thermo Fischer Scientific) as per the manufacturer's instruction. The luminescence of samples was measured at 597 nm after 10 min incubation at room temperature.

Polysome profiling

Yeast was grown in 100 mL YPD to A₆₀₀ between 0.4 and 0.7, cyclohexamide (100 µg/mL final concentration) was added and cells were chilled in an ice-water bath for 10 min. Cells were pelleted by centrifugation and washed once with a lysis buffer (10 mM MgCl₂, 50 mM NaCl, 50 mM Tris at pH 7.4, 100 µg/mL cyclohexamide). The pellet was resuspended in 100 µL/40 ODs of lysis buffer and transferred to an Eppendorf tube with 100 µL of pre-chilled 0.5 mm zirconia-silica beads (BioSpec Products). Cells were lysed by vortexing five times for 30 sec, with a 30-sec break on ice between each vortexing. Beads and cellular debris were removed by centrifugation (10,000g, 5 min). If salt-treated, lysate was then incubated with an equal volume of 2× salt buffer (final concentration 5 mM MgCl₂, 300 mM NaCl, 50 mM Tris at pH 7.4, 100 µg/mL cyclohexamide) for 30 min at 4°C. The lysates were loaded on a 10%–40% sucrose gradient in lysis buffer and centrifuged in SW40 Ti rotor, at 400,000g for 3.5 h. Fractions were collected using Foxy Jr. Fraction Collector (Teledyne ISCO).

RNA structure probing (SHAPE)

The selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) was used as previously described (Wilkinson et al. 2006). Briefly, 40 ODs of exponentially growing cells were lysed and polysomal profiles were generated as described above. Sub-60S, 60S, and 80S fractions from wild-type and mutant strains were collected, sucrose was removed with PD10 Columns (GE Healthcare), re-concentrated with Amicon Ultra Centrifugal Filter Units (Millipore) and treated with 1-methyl-7-nitroisatoic anhydride (1M7) (Prime Organics, Woburn, US) or DMSO. RNA was extracted using a standard phenol:chloroform extraction. Primer extension was performed with a ³²P 5'-end labeled primer (5'-AAAAGTAGTGA TTCTGCCAAGCCCG-3') complementary to 25S rRNA 95 nucleotides downstream from C2278. Samples were resolved on a 6% urea-polyacrylamide gel at 20W for 2.5 h. The gel was dried for 1 h at 80°C using a gel dryer (BioRad), exposed to imaging plate (Fujifilm), and scanned on the Fujifilm FLA7000 PhosphorImager.

Protein quantification using SILAC

The stable isotope labeling with amino acids in cell culture (SILAC) technique for protein quantification was used as previously described (Ong et al. 2002). The wild-type strain YMK520 was grown in a “heavy” synthetic complete media containing ¹³C₆, ¹⁵N₄-L-Arginine (Arg-10) and ¹³C₆, ¹⁵N₂-L-Lysine (Lys-8) (Silantes). The mutants were grown in “light” media with standard amino acids. For standard SILAC experiments, 40 OD units of wild-type and a mutant strain were mixed, processed for polysome profiles as described above. The fractions corresponding to sub-60S, 60S, and 80S peaks were TCA precipitated, run shortly into polyacrylamide gel, trypsin digested, and peptide masses were analyzed

by nLC-MS/MS (in-house core facility or at FingerPrints Proteomics Facility, University of Dundee, Scotland). Protein quantification was done using MaxQuant software (Cox et al. 2009).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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