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Journal

Biochemical and Biophysical Research Communications, 470(3)

ISSN

0006-291X

Authors

Al-Hadid, Qais
White, Jonelle
Clarke, Steven

Publication Date

2016-02-01

DOI

10.1016/j.bbrc.2016.01.107

Peer reviewed

HHS Public Access

Author manuscript

Biochem Biophys Res Commun. Author manuscript; available in PMC 2017 February 12.

Published in final edited form as:

Biochem Biophys Res Commun. 2016 February 12; 470(3): 552–557. doi:10.1016/j.bbrc.2016.01.107.

Ribosomal Protein Methyltransferases in the Yeast *Saccharomyces cerevisiae*: Roles in Ribosome Biogenesis and Translation

Qais Al-Hadid[a], **Jonelle White**[a], and **Steven Clarke**[a,b]

[a]Department of Chemistry and Biochemistry and the Molecular Biology Institute, University of California, Los Angeles, Los Angeles, CA 90095, USA

Abstract

A significant percentage of the methyltransferasome in *Saccharomyces cerevisiae* and higher eukaryotes is devoted to methylation of the translational machinery. Methylation of the RNA components of the translational machinery has been studied extensively and is important for structure stability, ribosome biogenesis, and translational fidelity. However, the functional effects of ribosomal protein methylation by their cognate methyltransferases are still largely unknown. Previous work has shown that the ribosomal protein Rpl3 methyltransferase, histidine protein methyltransferase 1 (Hpm1), is important for ribosome biogenesis and translation elongation fidelity. In this study, yeast strains deficient in each of the ten ribosomal protein methyltransferases in *S. cerevisiae* were examined for potential defects in ribosome biogenesis and translation. Like Hpm1-deficient cells, loss of four of the nine other ribosomal protein methyltransferases resulted in defects in ribosomal subunit synthesis. All of the mutant strains exhibited resistance to the ribosome inhibitors anisomycin and/or cycloheximide in plate assays, but not in liquid culture. Translational fidelity assays measuring stop codon readthrough, amino acid misincorporation, and programmed −1 ribosomal frameshifting, revealed that eight of the ten enzymes are important for translation elongation fidelity and the remaining two are necessary for translation termination efficiency. Altogether, these results demonstrate that ribosomal protein methyltransferases in *S. cerevisiae* play important roles in ribosome biogenesis and translation.

Keywords

ribosomal protein; ribosome biogenesis; translation elongation; translation fidelity; aminoacyl-tRNA; peptidyl transferase

[b]To whom correspondence should be addressed: Dept. of Chemistry and Biochemistry and the Molecular Biology Institute, University of California, Los Angeles, 607 Charles E. Young Dr. E., Los Angeles, CA 90095-1569. Tel.: 310-825-8754; Fax: 310-825-1968; clarke@mbi.ucla.edu.

*This work was supported, in whole or in part, by National Institutes of Health Grant GM026020 (to S. G. C.) and an NSF LSAMP Bridge to the Doctorate Fellowship (to J. W.).

INTRODUCTION

Translational components are modified by the addition of methyl groups in all domains of life. These modifications occur on ribosomal RNA (rRNA), transfer RNA (tRNA), messenger RNA (mRNA), translation factors, and ribosomal proteins [1–7]. More than half of the known methyltransferases in the yeast *Saccharomyces cerevisiae* modify these ribosomal components, suggesting that methylation of translational components is important for cellular function [8]. Methylation of the RNA components of the ribosome is important for ribosome synthesis, structure stability, and translational fidelity [3,4,9,10]. Similarly, methylation of elongation and release factors has been demonstrated to be important for translational fidelity and termination efficiency [5,11]. However, the functions of methyltransferases responsible for ribosomal protein methylation are not well characterized and little has been done to uncover their biological activities.

We previously showed that the yeast methyltransferase, Hpm1, plays an important role in ribosome biogenesis and translation [12,13]. Cells deficient in Hpm1 exhibited defects in 60S large ribosomal subunit synthesis and decreased translation elongation fidelity [12,13]. To determine if the nine other known ribosomal protein methyltransferases in *S. cerevisiae* are playing similar roles as Hpm1, we investigated the consequences of depleting each ribosomal protein methyltransferase on ribosome biogenesis and translation. Using a variety of assays, we show that the loss of each of these enzymes results in one or more alterations in ribosomal biogenesis (altered levels of ribosomal subunits), resistance to ribosome-binding antibiotics, readthrough of stop codons, amino acid misincorporation, and programmed −1 ribosomal frameshifting (−1 PRF). These results suggest that all the ribosomal protein methyltransferases in *S. cerevisiae* are necessary for ribosome biogenesis and accurate translation elongation or termination.

MATERIALS AND METHODS

Strains and growth media

All strains used are in the BY4742 background (*MATα, his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0*) obtained from the Open Biosystems yeast knockout collection (Thermo Scientific). All strains contain a kanamycin resistance marker in place of the open reading frame of each ribosomal protein methyltransferase gene. Yeast strains were grown in 1% yeast extract, 2% peptone, and 2% dextrose (YPD, Difco) or minimal synthetic defined media lacking uracil (SD –ura) containing 0.17% yeast nitrogen base without ammonium sulfate and amino acids (BD Biosciences), 0.077% complete supplement mixture without uracil (MP Biomedicals; 114511212), 0.5% ammonium sulfate, and 2% dextrose.

RESULTS

Loss of yeast ribosomal protein methyltransferases results in abnormal ribosomal subunit levels and increased polysomes

In prior work, we showed that the protein histidine methyltransferase, Hpm1, in *S. cerevisiae*, is needed to promote normal ribosome biogenesis [12]. We sought to determine if loss of the other yeast ribosomal protein methyltransferases results in defects in ribosomal

subunit levels and/or translation by polysome profile analysis. Lysates were prepared from wild type and each of the ten mutant strains and ribosomal components separated by sucrose density ultracentrifugation. We examined the levels of small (40S) and large (60S) ribosomal subunits, intact ribosomes (80S), and polyribosomes (polysomes) to indicate possible defects in ribosome biogenesis and/or translation. Like Hpm1-deficient cells, loss of Rkm1, Ntm1, Rmt1, or Rmt2 resulted in a deficit of 60S subunits (Fig. 1A). This defect in 60S biogenesis is highlighted by a significant decrease in the free 60S/free 40S subunit ratio in these strains, compared to wild type (Fig. 1B). Ntm1 was previously shown to be important for 60S biogenesis [14]. Loss of Rkm2, Rkm3, Rkm4, Rkm5, or Sfm1 had little or no impact on the levels of ribosomal components, suggesting they are not required for ribosomal subunit synthesis (Fig. 1A, 1B). Little or no change in the polysome/80S ratio was seen for the ten methyltransferase mutants, suggesting no significant change in translational output and cellular proliferation [15,16]. This result is consistent with similar growth of these mutant strains to the wild type strain in the absence of antibiotics on agar plates (Fig. 2) or in liquid medium (Fig. 3).

Cells deficient in ribosomal protein methyltransferases are resistant to the A-site and E-site ribosome-binding drugs, anisomycin and cycloheximide, respectively on agar plates

Next, we tested if the defects in ribosomal subunit biogenesis and/or the elevated levels of polysomes in the ribosomal protein methyltransferase mutants correlate with altered sensitivities to ribosome-binding drugs. Altered sensitivities to these drugs may indicate structural and/or functional distortions to the regions that these drugs bind. Drugs that bind to different functional centers of the ribosome were used including: anisomycin, paramomycin, and cycloheximide. Anisomycin binds to the A-site of the ribosome and acts as a competitive inhibitor of aminoacyl-tRNAs [17]. Paramomycin binds to the decoding center of the small subunit and induces translational errors [18]. Cycloheximide binds to the E-site of the large subunit and inhibits translation elongation [19]. Previously, we showed that Hpm1-deficient cells have enhanced resistance to cycloheximide in plate assays, suggesting alterations to the A and E-sites of the large ribosomal subunit of *hpm1*Δ cells (12). Remarkably, we were able to now show that all of the mutant strains demonstrated increased resistance to cycloheximide in plate assays with the exception of *rkm4*Δ (Fig. 2). Increased sensitivity of *rkm4*Δ strains to cycloheximide has previously been reported [20,21]. No differences in sensitivity of the mutants were seen on paramomycin-containing agar plates, compared to wild type (data not shown). In the presence of anisomycin, we also observed increased resistance in the ribosomal methyltransferase mutants with the exception of *hpm1*Δ cells (Fig. 2). The latter result is in accord with a previous study (10). The similar resistance phenotype of many of these strains to anisomycin and cycloheximide indicates a defect at a common functional step, likely in translation elongation or termination, rather than a common structural distortion at the A-site and E-site of the large subunit. We also measured growth rates in liquid culture of wild type and mutant strains in the presence or absence of cycloheximide (Fig. 3). Because the 500 ng/ml concentration used in the plate assays severely limited the growth of the cells in liquid culture, we used a lower concentration of 50 ng/ml that resulted in about a two-fold increase in doubling time. To our surprise, we found little or no differences in the growth rates of the wild type and mutant

cells. Cells thus respond differently to the antibiotics in a colony on agar plates (Fig. 2) compared to individual cells in liquid media (Fig. 3).

Loss of ribosomal protein methyltransferases results in defects in the fidelity of translation elongation or termination

Previously, we demonstrated that loss of Hpm1 results in reduced fidelity in translation elongation [12]. To determine if the other ribosomal protein methyltransferases are important for translational fidelity, we performed assays measuring stop codon suppression, amino acid misincorporation, and programmed −1 ribosomal frameshifting (−1 PRF). These assays utilize dual-luciferase reporter genes: *Renilla* followed by firefly luciferase, separated by a linker region, under the control of a constitutive promoter. The amount of firefly luciferase luminescence correlates with translational errors in all three assays and the amount of *Renilla* luciferase luminescence is used to correct for differences in translation initiation and mRNA levels of the dual reporters. To measure stop codon suppression, vectors containing stop codons (UAA and UAG) in the linker region between the *Renilla* and firefly luciferase genes were used and the amount of reporter luminescence was measured. Increased readthrough of the stop codons, as a result of defects in elongation or termination, would result in increased firefly luciferase luminescence. Loss of each of the ten ribosomal protein methyltransferases resulted in increased readthrough of the UAA and UAG stop codons, compared to wild type (Fig. 4A, 4B). This result suggests that all ten ribosomal protein methyltransferases in *S. cerevisiae* are important for translation elongation or termination fidelity. To determine if the translational fidelity defects in all ten strains is occurring at the elongation or termination step, amino acid misincorporation was measured, which measures elongation fidelity defects. Amino acid misincorporation levels were determined using a dual-luciferase reporter vector with a point mutation in the firefly gene at a catalytically-important residue (K529) to a near-cognate asparagine residue [22]. This mutation renders firefly luciferase catalytically-deficient. High fidelity translation would result in incorporation of the asparagine residue at position 529 and synthesis of an inactive firefly luciferase. However, reduced translational accuracy would result in increased frequencies of near-cognate and non-cognate aminoacyl-tRNA accommodation and increases the chances of misincorporating the wild type lysine reside, resulting in the synthesis of an active firefly luciferase enzyme. Hence, reduced translation elongation accuracy would result in the production of more active firefly luciferase enzymes and as a consequence, greater firefly luciferase luminescence. All ribosomal protein methyltransferase-deficient strains exhibited significantly higher frequencies of amino acid misincorporation (> 2-fold), except for *rkm2*Δ and *ntm1*Δ, which had similar levels of misincorporation as wild type (Fig. 4C). This suggests that most ribosomal protein methyltransferases are important for translation elongation fidelity, whereas Rkm2 and Ntm1 are important in translation termination efficiency. Finally, to corroborate that these strains have defects in translation, we measured the frequencies of programmed −1 ribosomal frameshifting (−1 PRF), which has previously been shown to positively correlate with translation elongation defects [23]. To measure −1 PRF efficiency, a dual-luciferase reporter vector was used that contained a viral L-A direct −1 PRF signal between the *Renilla* and firefly genes [24]. Firefly luciferase synthesis depends on the −1 PRF event as it is out of frame of the *Renilla* open reading frame. Defects in translation elongation or termination

increases the transit times of ribosomes on mRNAs and consequently, increases the probability of a −1 PRF event occurring [15,16,25]. Therefore, defects in translation elongation or termination should result in more firefly luciferase production. Loss of each of the ten ribosomal protein methyltransferases resulted in enhanced −1 PRF efficiency (Fig. 4D). Notably, loss of Rkm5 or Ntm1 resulted in > 2-fold increase in −1 PRF efficiency (Fig. 4D). These results suggest that ribosomal protein methyltransferases in *S. cerevisiae* are important for translation elongation and/or termination fidelity.

DISCUSSION

In this study, we showed that most ribosomal protein methyltransferases in *S. cerevisiae* are playing roles in ribosome biogenesis. These enzymes (except Ntm1) can localize or are predominantly localized in the nucleus [26,27], where the bulk of ribosome assembly takes place [28–30]. This suggests that these methyltransferases (Hpm1, Rkm1, Rmt1, and Rmt2) are likely active participants during the assembly process of the ribosome. Ntm1 may not be actively involved in the assembly process of the ribosome but instead methylation of its ribosomal protein substrate, Rpl12, may be important for ribosomal assembly, as Rpl12 is known to be imported into the nucleus and assemble with pre-ribosomes along with most of the ribosomal proteins [28,30]. Ntm1 may also have unknown substrates whose methylation is important for the assembly process.

Translational fidelity assays measuring stop codon readthrough, amino acid misincorporation, and programmed −1 ribosomal frameshifting demonstrated that all of the ribosomal protein methyltransferases in *S. cerevisiae* are important for translational accuracy. Loss of Ntm1 or Rkm2 resulted in increased readthrough of stop codons but had no major effect on amino acid misincorporation, suggesting that these enzymes are important for translation termination but not necessary for elongation, unlike the other eight enzymes. Importantly, Ntm1 and Rkm2 methylate the same ribosomal substrate, Rpl12, at the N-terminus of the protein [20,31] that is exposed to the cytoplasm and a component of the GTPase-associated center (GAC), which is known to interact with translation factors and couples GTP hydrolysis with translation elongation or termination [32–34]. It is therefore possible that methylation of Rpl12 by Ntm1 and Rkm2 is important for recruitment of release factors to the GAC and/or coupling GTP hydrolysis to translation termination. Moreover, previous work has shown that these two enzymes may be functionally linked as cells deficient in Ntm1 not only lose methylation at the Ntm1 target site (P1) but also at the Rkm2 target site (K3) [31]. The other eight mutant strains all had increased levels of amino acid misincorporation and stop codon suppression, suggesting these enzymes are important for elongation accuracy. The stop codon readthrough phenotype in these cells is likely a consequence of an elongation rather than a termination defect as has previously been reported [22]. It is unclear if these translational fidelity phenotypes are caused by the ribosome biogenesis defects in the methyltransferase mutants. It is possible that aberrant ribosome biogenesis results in ribosomes with altered structures and/or functionality that diminishes fidelity of protein synthesis. Alternatively, these enzymes may be multifunctional with separate roles in ribosome biogenesis and translation and methylation of their ribosomal protein substrates may be important for maintaining proper ribosome conformations during the decoding, peptidyl transfer, and translocation steps of elongation.

To address this concern, similar analyses need to be done with ribosomes lacking methylation at each of the sites targeted by these ribosomal protein methyltransferases. Also rRNA structure, biochemical, and biophysical characterization of ribosomes isolated from each of these ribosomal protein methyltransferase-deficient strains needs to be done to get a clear understating as to how or if methylation of ribosomal proteins in *S. cerevisiae* promotes ribosome biogenesis and accurate protein production.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank William Munroe for his help with the polysome profile analysis, Ming Du and David Bedwell for providing the stop codon readthrough and amino acid misincorporation plasmids, and Jonathan Dinman for providing the frameshifting plasmids.

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- Ribosomal protein methylation is prevalent in the three domain of life
- Ten cognate methyltransferases have been identified in *Saccharomyces cerevisiae*
- Half of the enzymes are important for ribosome biogenesis
- All of the methyltransferases are important for translational fidelity

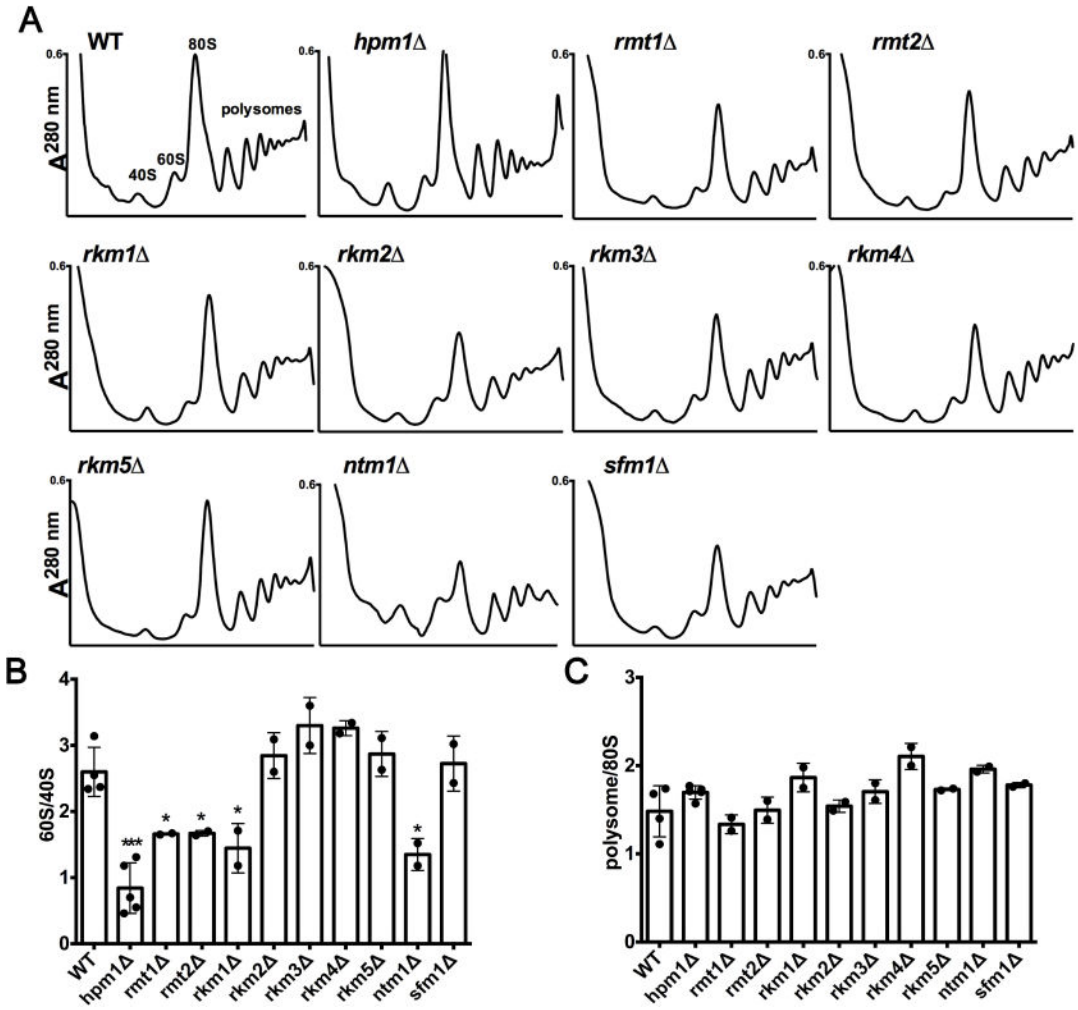

FIG. 1. Cells lacking ribosomal protein methyltransferases in *S. cerevisiae* have altered levels of ribosomal subunits

(A) Polysome profile analyses of wild type and cells deficient in each of the ribosomal protein methyltransferases in *S. cerevisiae* was done as described previously [12]. A total of four independent profiles were obtained for wild type cells, five profiles for *hpm1*Δ cells, and two profiles for each of the other strains. The profiles for wild type and *hpm1*Δ cells were previously reported [13]. A representative profile for each strain is shown here. (B) Ribosomal subunit levels were determined by directly weighing cutouts of half peak areas (assuming Gaussian-like distribution of ribosome particles) from printer paper using an analytical balance. Error bars represent standard deviation of two or more independent profiles. Unpaired t-test two-tailed p-values were calculated to determine significant differences between wild type and each of the ribosomal protein methyltransferase mutants. *hpm1*Δ $p = 0.0002$, *rmt1*Δ $p = 0.028$, *rmt2*Δ $p = 0.0292$, *rkm1*Δ $p = 0.0232$, *ntm1*Δ $p = 0.0137$. (C) Levels of intact ribosomes (80S) was determined by directly weighing half the peak area, assuming Gaussian-like distribution, and polysome levels were determined by weighting the peaks after 80S. Error bars represent standard deviation of two or more independent profiles. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

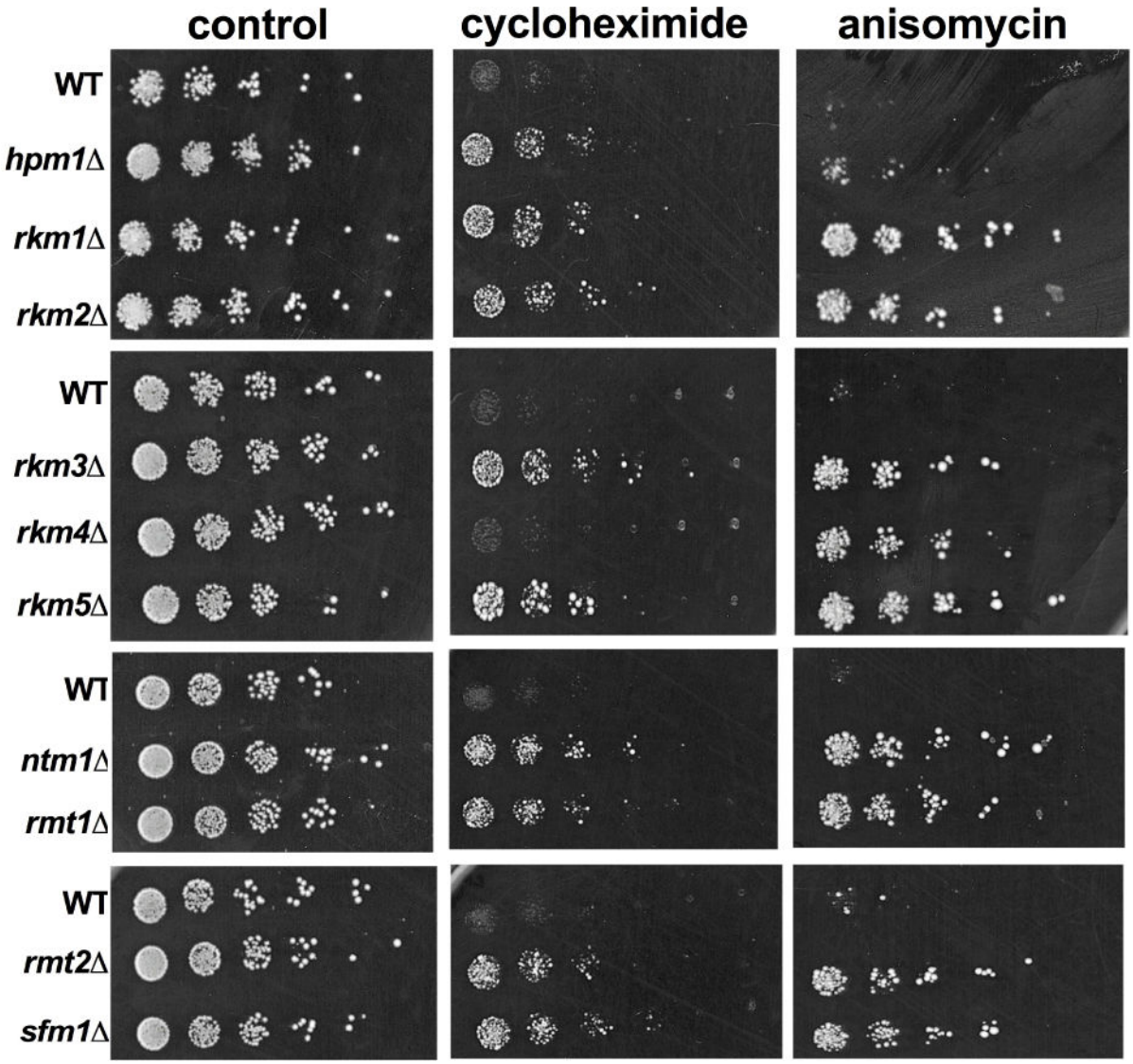

FIG. 2. Cells deficient in ribosomal protein methyltransferases show altered sensitivities to the ribosome-targeting drugs anisomycin and cycloheximide in plate assays

Exponentially growing wild type and ribosomal protein methyltransferase knockouts were inoculated overnight in 20 ml of YPD at 30 °C with shaking at 250 rpm at a dilution to ensure a starting OD_{600nm} around 0.5 the following morning. Cells were pelleted by centrifugation for 5 min at 5,000 × g and washed three times in sterile water. Cells were resuspended in water to a final $OD_{600\ nm}$ of 0.5 and serially diluted in 5-fold steps with water. Three μl of each dilution starting at an OD of 0.02 was spotted on YPD agar plates in the presence or absence of anisomycin (10 μg/ml; CalBiochem #176880) and cycloheximide (500 ng/ml; Sigma #C7968). Plates were then incubated for 4 days at 30°C. Each panel shown is from a single plate of one of two biological replicates.

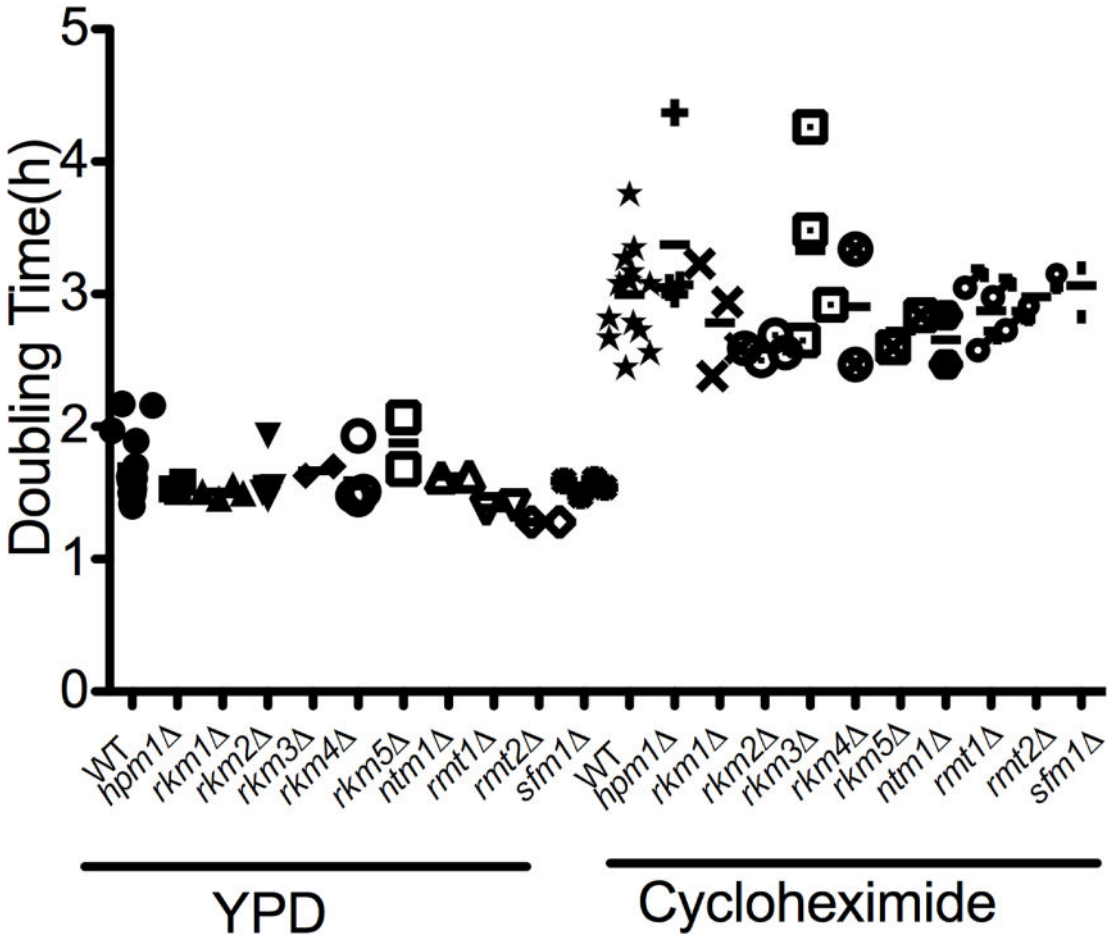

FIG. 3. Cells deficient in ribosomal protein methyltransferases show similar growth rates in liquid culture to wild type cells in the presence or absence of cycloheximide

Exponentially growing wild type and mutant strains were inoculated overnight in 25 ml of YPD at 30 °C with shaking at 250 rpm at a dilution to ensure a starting OD_{600nm} around 0.1 the following morning. At that time, either no drug or cycloheximide (50 ng/ml final concentration) was added and cell growth at 30 °C was determined by the increase in OD_{600nm} at 90 min intervals for 9 h. When needed, 5-fold or 10-fold dilutions were done to insure that the measured OD was below 1. Doubling times were calculated from the slope of a log OD versus time plot. The data shown are from five separate experiments done in duplicate.

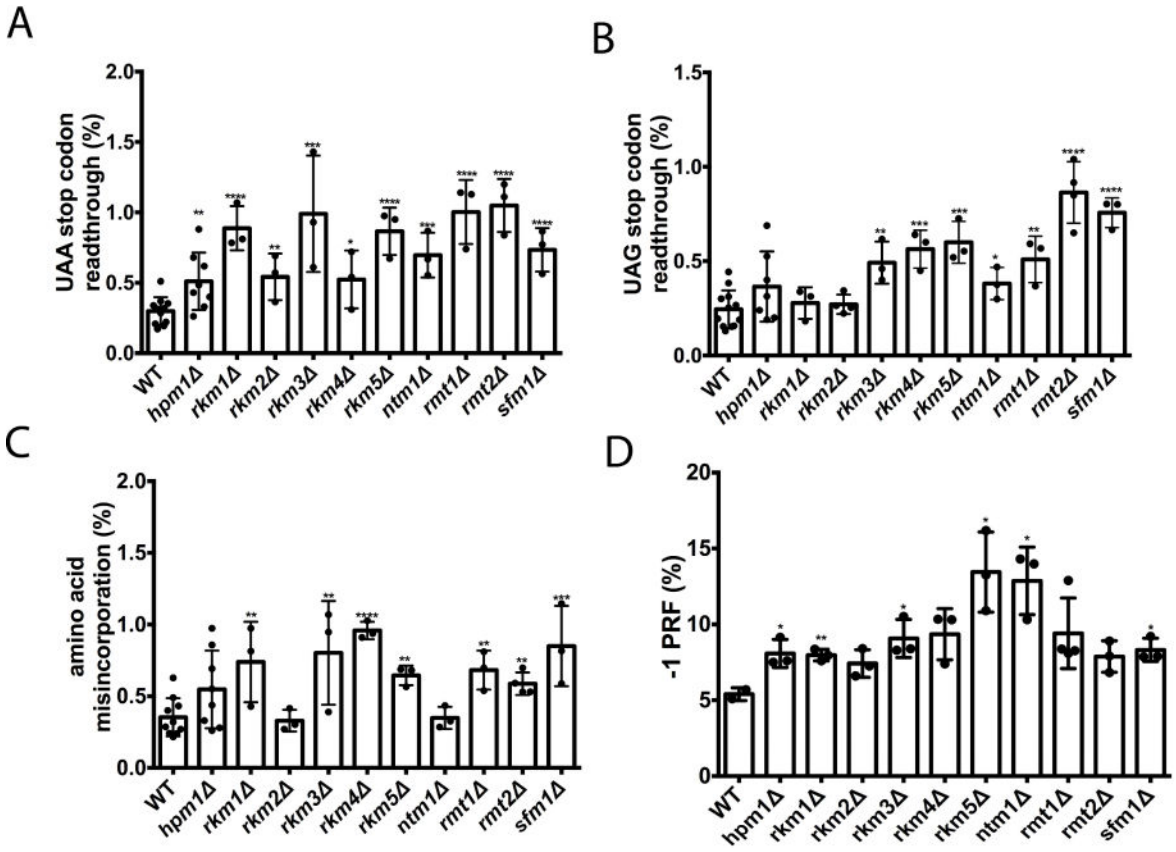

FIG. 4. Cells deficient in ribosomal protein methyltransferases have reduced translational fidelity

Translation elongation and termination accuracy were measured in cells lacking each of the ten ribosomal protein methyltransferases. A dual-luciferase reporter assay consisting of a *Renilla* luciferase gene fused C-terminally to a firefly luciferase gene separated by a linker region was used to measure stop codon readthrough, amino acid misincorporation, and programmed −1 ribosomal frameshifting (−1 PRF), as described previously [12,22,24]. (A) Percent readthrough was calculated by taking the firefly/*Renilla* luminescence ratio of the UAA-containing vector divided by the same ratio in the respective control. Error bars represent standard deviation. *hpm1*Δ p = 0.0077, *rkm1*Δ p < 0.0001, *rkm2*Δ p = 0.006, *rkm3*Δ p = 0.0001, *rkm4*Δ p = 0.0158, *rkm5*Δ p < 0.0001, *ntm1*Δ p = 0.0001, *rmt1*Δ p < 0.0001, *rmt2*Δ p < 0.0001, *sfm1*Δ p < 0.0001. Wild type was assayed 11 independent times, *hpm1*Δ 8 times and mutants were assayed three independent times. Data for the wild type and *hpm1*Δ cells were previously reported [13]. (B) Same as in (A) except a UAG stop codon was used. Wild type was assayed for a total of 12 biological replicates, *hpm1*Δ was assayed 7 times, and mutants 3–4 times. Data for wild type and *hpm1*Δ cells were previously reported [13]. *hpm1*Δ p = 0.0811, *rkm1*Δ p = 0.6114, *rkm2*Δ p = 0.626, *rkm3*Δ p = 0.0024, *rkm4*Δ p = 0.0003, *rkm5*Δ p = 0.0001, *ntm1*Δ p = 0.05, *rmt1*Δ p = 0.0017, *rmt2*Δ p < 0.0001, *sfm1*Δ p < 0.0001. (C) Percent amino acid misincorporation was measured as in (A). Wild type was assayed 10 independent times, *hpm1*Δ was assayed 8 times, and mutants were assayed 3–4 independent time. Data for wild type and *hpm1*Δ cells were previously reported [13]. *hpm1*Δ p = 0.0621, *rkm1*Δ p = 0.0054, *rkm2*Δ p = 0.7818, *rkm3*Δ p = 0.0051, *rkm4*Δ p

< 0.0001, *rkm5*Δ p = 0.0043, *ntm1*Δ p = 0.9623, *rmt1*Δ p = 0.0032, *rmt2*Δ p = 0.0069, *sfm1*Δ p = 0.001. (D) Percent −1 PRF was calculated by taking the firefly/*Renilla* luminescence ratio of cells containing pJD376 (L-A virus gag-pol frameshift signal) divided by the same ratio of cells containing the no frameshift control (pJD375). Error bars represent standard deviation of at least two independent experiments. Strains were assayed 2–4 independent times as indicated by the number of data points. *hpm1*Δ p = 0.03, *rkm1*Δ p = 0.006, *rkm3*Δ p = 0.03, *rkm5*Δ p = 0.03, *ntm1*Δ p = 0.02, *sfm1*Δ p = 0.02. Frameshift vectors were generously provided by Jonathan Dinman at the University of Maryland, MD and described [24]. p < 0.05 (*), p < 0.01 (**), p < 0.001 (***), p < 0.0001 (****).