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The human mitochondrial translation release factor HMRF1L is methylated in the GGQ motif by the methyltransferase HMPrmC

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ABSTRACT

We have recently identified the human mitochondrial release factor, HMRF1L, which is responsible for decoding of UAA/UAG termination codons. Here, we identified human mitochondrial methyltransferase, HMPrmC, which methylates the glutamine residue in the GGQ tripeptide motif of HMRF1L. We demonstrate that HMPrmC is targeted to mitochondria and the glutamine residue in the GGQ motif of HMRF1L is methylated *in vivo*. HMPrmC depletion in HeLa cells leads to decreased mitochondrial translation activity in the presence of the translation fidelity antibiotic streptomycin in galactose containing medium. These results suggest that the methylation of HMRF1L by HMPrmC in human mitochondria is involved in the control of the translation termination process, probably by preventing the undesired suppression of termination codons and/or abortive termination events at sense codons under such conditions, as observed in prokaryotes and eukaryotes systems.

Termination codons in mRNA are recognized on the ribosome by class 1 release factors (RFs) [1,2]. RFs bind to the ribosome when a stop codon is present in the A-site and promote hydrolysis by the ribosomal peptidyl transferase center of the ester bond between the polypeptide and the tRNA of the peptidyl-tRNA bound to the P-site. Two class 1 RFs are used in eubacterial cells to recognize the three stop codons, UAG, UAA, and UGA. RF1 recognizes UAG and UAA, while RF2 recognizes UAA and UGA. In contrast, a single RF is sufficient for termination at all three stop codons in eukaryotic and archaebacterial cells. The region of RFs that interacts with the peptidyl transferase center has been identified and it contains a universally conserved tripeptide motif, GGQ [3,4]. The RF-peptidyl transferase center interaction has recently been confirmed by X-ray crystallography of RFs bound to the *Thermus thermophilus* 70S ribosome [3]. The GGQ motif is the only part of the RFs that is conserved between the eubacterial, eukaryotic, and archaebacterial factors. The GGQ motif is essential for the function of both prokaryotic and eukaryotic RFs, and most mutations within that motif in *Escherichia coli* are lethal [5].

The side-chain amide group of the glutamine residue in the GGQ tripeptide is post-translationally modified by methylation. This methylation was first demonstrated in bacteria [6], where both RF1 and RF2 are modified by the methyltransferase (MTase) PrmC [7,8]. RF methylation was subsequently demonstrated to occur in the yeast *Saccharomyces cerevisiae* [9,10]. Methylation of the yeast cytoplasmic RF, eRF1 (also designated Sup45p), and the mitochondrial RF, Mrf1p, depends on Mtq2p and Mtq1p, respectively. The roles of RF methylation in translation have been thoroughly studied in *E. coli*, both *in vitro* and *in vivo*. In *E. coli*, an *in vitro* study showed that methylation of both RF1 and RF2 correlates with an increased efficiency of peptide chain release [6,11]. PrmC inactivation has no effect on cell growth in rich media, but reduces growth considerably on poor carbon sources [7]. These observations suggest that the expression of genes needed for optimal growth under the latter conditions can become growth limiting as a result of inefficient translation termination. Limited information is available for RF methylation in eukaryotes. In yeast, the lack of eRF1 methylation causes multiple phenotypes, including sensitivity to the translation fidelity drugs paromomycin and geneticin [10]. Although the lack of methylation of the yeast mitochondrial RF Mrf1p in the mtq1-Δ strain produces only moderate growth defects in cells grown on non-fermentable carbon sources, the lack of Mrf1p methylation increases the readthrough in a strain containing COX2 with an early-stop ochre codon [10]. To date, methylation of mammalian RFs has not yet been demonstrated.

We and another group have recently identified the human mitochondrial translation release factor HMRF1L, which is responsible for decoding of the mitochondrial UAA and UAG termination codons [12,13]. HMRF1L has sequence similarities to bacterial RFs, including the potential for glutamine methylation in the GGQ mo-

Abbreviations: AdoMet, *S*-adenosylmethionine; bEF-Tumt, bovine mitochondrial translation elongation factor Tu; MALDI-TOF, matrix-assisted laser desorption/ionization mass spectrometry time-of-flight analysis; MS, mass spectrometry; MTase, methyltransferase; RF, release factor; SM, streptomycin.

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tif. In the present work, we demonstrate that HMRF1L is methylated at its GGQ motif *in vivo*, and have identified the mitochondrial protein HMPrmC as the MTase that is responsible for HMRF1L methylation. We further demonstrate that HMPrmC depletion leads to decreased mitochondrial translation activity, especially in the presence of the translation fidelity antibiotic streptomycin (SM) in galactose containing medium, in which cells are forced to depend on oxidative phosphorylation. These results suggest that the methylation of HMRF1L by HMPrmC may be advantageous for the cell, probably by preventing the undesired suppression of termination codons and/or abortive termination events at sense codons by HMRF1L in the mammalian mitochondrial translation system under such conditions.

Materials and methods

Construction of plasmids. To generate HMPrmC/pcDNA3.1 myc-His B, the mammalian expression vector for C-terminal myc-His-tagged HMPrmC, the HMPrmC coding sequence was amplified by RT-PCR using total RNA from 293T cells, using an oligo-dT primer and the specific primers 5′- CCCAAGCTTCCGCCACCATGGAGCT TTGGGGCCGAATGCTGTG-3′ and 5′-GCTCTAGAGGCCCAGACCTCCG GATATGCAGGA-3′. The amplified fragment was cloned into pcDNA3.1 myc-HisB (Invitrogen) between the HindIII and XbaI sites.

Confocal fluorescence microscopy. HeLa cells were transfected with HMPrmC/pcDNA3.1 myc-His B, using Lipofectamine2000 (Invitrogen). At 24 h after transfection, cells were stained with a final concentration of 100 nM MitoTracker CMXRos dye (Molecular Probes) for 15 min to visualize mitochondria. Subsequently, cells were fixed in PBS with 4% (v/v) formaldehyde and stained with mouse anti-myc (Sigma) and FITC conjugated anti-mouse IgG (Sigma) diluted 1:1000 and 1:2000, respectively, in PBS containing 0.1% Triton X-100, 3% (w/v) bovine serum albumin, and 0.5% (v/v) whole goat serum (ICN). The cells were observed using confocal fluorescence microscopy (Leica TCS SP2).

Cellular fractionation and Western analysis. Cellular fractionation was performed as described [14]. Mitochondrial and cytoplasmic proteins (20 μg each for HMPrmC-myc and bEF-Tumt, and 2.5 μg for β-actin) were separated on SDS–polyacrylamide gels and transferred onto nitrocellulose membranes. Subsequently, the proteins were detected using specific antibodies against myc-tag (1:1000), bEF-Tumt (1:1000), and β-actin (Sigma, 1:5000). The polyclonal antisera for bEF-Tumt were generated in our laboratory by injecting rabbits with purified recombinant bEF-Tumt.

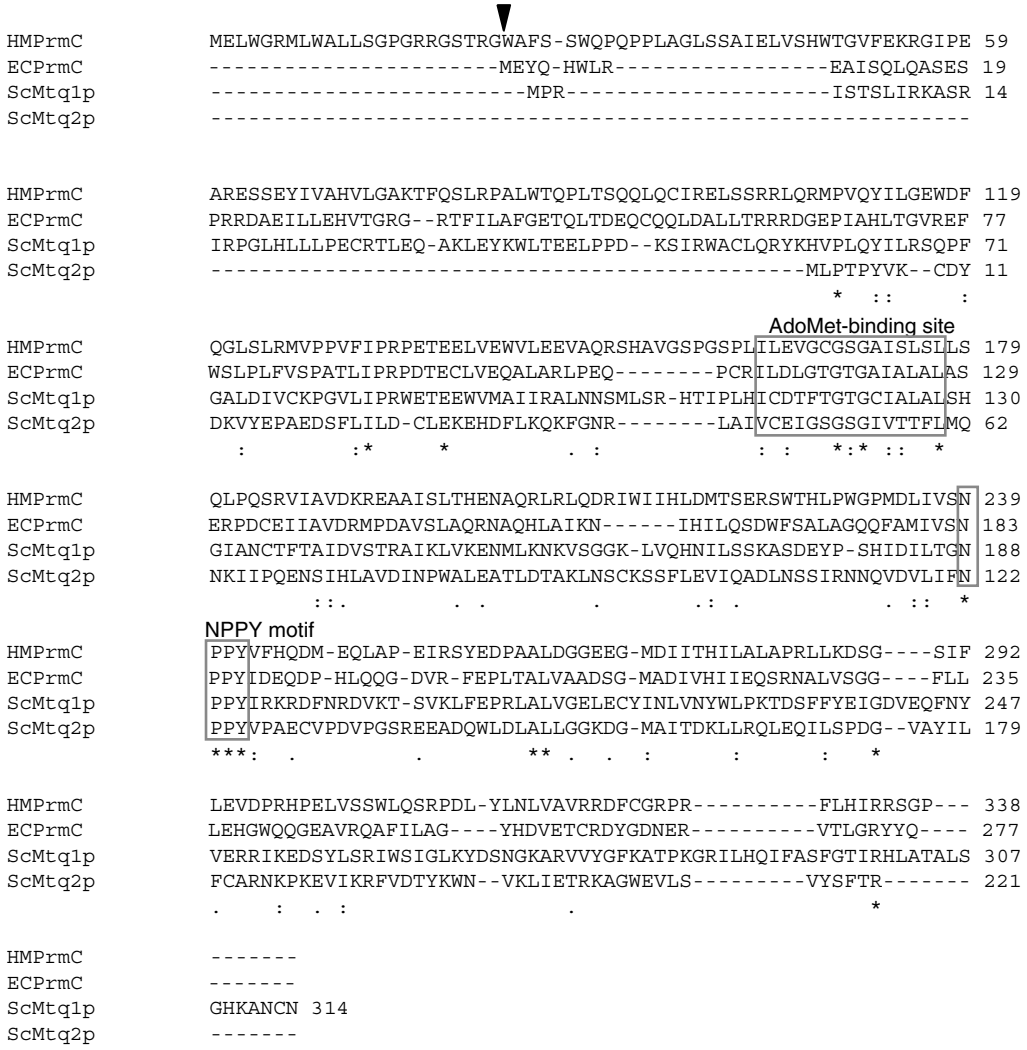

Fig. 1. Alignment of the sequences of a translation release factor methyltransferase from various organisms. The sequences of human mitochondrial PrmC (HMPrmC, Accession No. NP_057257), *E. coli* PrmC/HemK (ECPrmC, Accession No. NP_415730), *S. cerevisiae* mitochondrial Mtq1p (ScMtq1p, Accession No. NP_014336) and cytoplasmic Mtq2p (ScMtq2p, Accession No. NP_010424), were aligned using the ClustalW program. The arrows indicate the cleavage site for removal of the mitochondrial import signal, as predicted by PSORT II (http://psort.nibb.ac.jp/). Boxes indicate the signature sequences for a methyltransferase, including the highly conserved Ado-Met binding motif and the catalytic NPPY motif [16].

Cell culture and RNA interference. HeLa cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 4 mM L-glutamine, 1% penicillin/streptomycin solution. HeLa derivatives that stably over-expressed HMRF1L-3xFLAG were maintained as above, but in the presence of Zeocin (0.3 mg/ml). HeLa/HMRF1L-3xFLAG were established by transfection of HeLa cells with the HMRF1L-3xFLAG-pcDNA3.1/Zeo(+) plasmid.

The sequences of small interfering RNAs (siRNAs) targeting HMPrmC were designed using the RNA interference activity-predicting algorithm.2 software (iGENE Therapeutics, Inc.). The sequence of the sense strand of the HMRF1L siRNA is: 5′-CUGGUAGUAUCUUCUUAGAAGUGGA-3′. As a control, siRNA-scramble (iGENE Therapeutics, Inc.) was also used. After passaging the cells (5×10^5 cells/15 cm plate), the HeLa/HMRF1L-3xFLAG cells were transfected with siRNA (final concentration 50 nM) using the Lipofectamine2000 reagent (Invitrogen) and incubated in 5% CO_2 at 37 °C without penicillin or streptomycin. After 24 h, the medium was changed to DMEM with 10% fetal bovine serum. At 48 h after the siRNA transfection, total RNA was extracted from cells using ISOGEN (Nippon Gene) according to the manufacturer's instructions. RT- PCR was carried out to measure HMPrmC mRNA using SuperscriptIII (Invitrogen). The primers used for RT-PCR were 5′-CAGAAAGGAGCTGGACACACC-3′ and 5′-TGTCCTGGTGGAAGACGTAGG-3′, for HMPrmC; 5′-ACTGCCACCCAGAAGACTGTG-3′ and 5′-CCAGTGAGCTTCCCGTTCAG-3′ for GAPDH. For analysis of *in vivo* methylation of HMRF1L, mitochondrial extracts were prepared 72 h after transfection, according to a previously published procedure [14].

Mass spectrometry analysis of in vivo methylation in the GGQ motif of HMRF1L. The 3xFLAG-tagged HMRF1L was immunoprecipitated with an anti-FLAG antibody (Sigma) from a mitochondrial extract from HeLa/HMRF1L-3xFLAG cells, and fractionated on SDS–PAGE. A gel fragment containing the corresponding protein bands were excised, washed, dehydrated, and reswollen in trypsin solution. The peptides were further purified using a Nutip C-18 column (Glygen Corp.) before MALDI-TOF MS analysis in a Voyager-DE STR mass spectrometer. Proteins and peptides were identified by searching a protein database with the MS-Fit/Prospector program (University of California, San Francisco, prospector.ucsf.edu/ucsfhtml4.0/msfit.htm).

Pulse labeling of mitochondrial translation products. After HeLa cells were transfected with siRNAs targeting HMPrmC, cells were cultured in DMEM or galactose containing medium. Where indicated, 600 μg/ml SM was included in the medium. At 72 h after transfection, mitochondrial translation products were labeled, as described in [15], with slight modifications. Briefly, cells were labeled for 120 min at 37 °C in methionine-free DMEM containing 100 μCi/ml [^{35}S]methionine and 100 μg/ml emetine. Emetine specifically inhibits the translation in cytoplasm, only mitochondrial translation products are labeled with [^{35}S]methionine. Where indicated, 600 μg/ml SM was also included in the medium.

Results and discussion

Identification of a candidate methyltransferase that methylates HMRF1L

If HMRF1L is methylated at the N^5-glutamine of the GGQ motif, as are the class 1 RFs from other organisms, one would predict that a mitochondrial homologue of the PrmC methyltransferase would exist in the genome. We initially searched for such a homologue in the human genome database. Using a BLAST search with the amino acid sequence of Mtq1p, which methylates yeast mitochondrial RF-1 [10], NP_057257 (GenBank) was retrieved (BLAST, nr database, http://www.ncbi.nlm.nih.gov/BLAST/). NP_057257 has conserved functional motifs responsible for binding the AdoMet cofactor and for the catalysis, which are characteristic of methyltransferase [16] (Fig. 1). Moreover, NP_057257 is predicted to localize to mitochondria, according to an algorithm predicting protein localization sites in cells (Target P, http://www.cbs.dtu.dk/services/TargetP/; mitochondrial localization score, 0.8). Thus, we propose that NP_057257 is a candidate methyltransferase for HMRF1L, and we refer to this protein as the human mitochondrial PrmC (HMPrmC).

HMPrmC is targeted to mitochondria

We examined whether HMPrmC is indeed a mitochondrial protein. HeLa cells were transiently transfected with a construct expressing C-terminal myc-tagged HMPrmC. After staining the cells to visualize mitochondria (MitoTracker CMX-Ros), HMPrmC was detected using an anti-myc antibody. Mitochondrial co-localization was confirmed by the superimposition of the HMPrmC (Fig. 2A, left panel) and mitochondrial (Fig. 2A, middle panel) signals, as shown in the right panel of Fig. 2A.

To independently verify the mitochondrial localization of myc-tagged HMPrmC, cellular fractionation was performed. After transfection with the expression construct for myc-tagged HMPrmC, lysates prepared from cells were separated into mitochondrial and cytoplasmic fractions and subjected to Western blot analysis with anti-myc, anti-bovine mitochondrial translation elongation factor Tu (bEF-Tumt) (control mitochondrial protein [17]) and anti-β-actin (control cytoplasmic protein). Myc-tagged HMPrmC was specif-

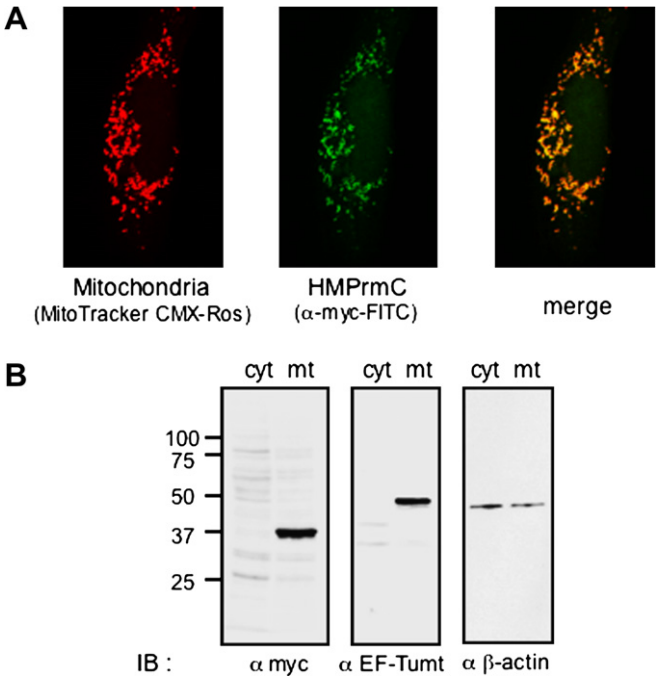

Fig. 2. Exogenously expressed HMPrmC is targeted to mitochondria. (A) HeLa cells were transiently transfected (24 h) with an expression construct for C-terminal myc-tagged HMPrmC. After staining of the cells to visualize mitochondria (MitoTracker CMX-Ros), the cells were fixed and stained with mouse anti-myc and FITC conjugated anti-mouse IgG. Fluorescence images were captured and mitochondrial colocalization of the myc-tagged HMPrmC was confirmed by superimposition of the mitochondrial (left panel) and HMPrmC (middle panel) signals, as shown in the right panel. (B) HeLa cells were transiently transfected (24 h) with an expression construct for C-terminal myc-tagged HMPrmC. Mitochondrial and cytoplasmic fractions were prepared and subjected to Western blot analysis using anti-bEF-Tumt (mitochondrial protein), anti-β-actin (cytoplasmic protein), and anti-myc antibodies. The myc-tagged HMPrmC was specifically found in the mitochondrial fraction.

ically present in the mitochondrial fraction, confirming its mitochondrial localization (Fig. 2B, α-myc).

HMRF1L is methylated in vivo by HMPrmC

Next, we examined whether HMPrmC is responsible for methylation of HMRF1L *in vivo*. HeLa cells stably overexpressing HMRF1L-3XFLAG were treated with an siRNA for HMPrmC or with the control siRNA-scramble. HMPrmC mRNA levels in siRNA-treated cells were reproducibly reduced to approximately 30% of levels in siRNA-scramble-treated cells, at 48 h after siRNA treatment (Fig. 3A). Subsequently, 72 h after siRNA treatment, FLAG-tagged HMRF1L was purified by immunoprecipitation with FLAG-specific antibodies, and its methylation status was analyzed by MALDI-TOF mass spectrometry. A complete tryptic digest of HMRF1L is predicted to produce a 1627 Da peak, corresponding to an unmodified peptide with the amino acid sequence ASGAGGQHVNTTDSAVR, in which Q is the putative methylation site. Two peaks at 1627 Da and 1641 Da were observed in samples prepared from siRNA-scramble treated cells (Fig. 3B), whereas only one peak, at 1627 Da, was detected in HMRF1L samples from cells treated with the HMPrmC siRNA (Fig. 3C). The 14 Da increase in the molecular mass of the second peptide, 1641 Da, corresponds to the molecular mass of a methyl group. This result clearly indicates that HMPrmC methylates HMRF1L at the N^5-glutamine of the GGQ motif *in vivo*. Approximately 90% of exogenously expressed HMRF1L was methylated by HMPrmC.

HMPrmC depletion leads to a decrease in mitochondrial translation activity

Finally, to investigate the effect of HMRF1L methylation on mitochondrial translation, we analyzed *de novo* mitochondrial translation activity by pulse-labeling. After HeLa cells were transfected with siRNA-scramble or with the siRNA targeting HMPrmC, cells were cultured in standard glucose-free medium supplemented with galactose (galactose medium) for 72 h, and mitochondrial translation products were pulse-labeled. The galactose medium was used because it forces the cells to depend on mitochondrial oxidative phosphorylation, and the effects of HMRF1L methylation on mitochondrial translation are predicted to be more pronounced under such conditions. Cells were lysed and the total lysates were resolved on Tricine–SDS–PAGE. Equal loading of the proteins was confirmed by CBB staining of the gel (Fig. 4A, left). *De novo* mitochondrial trans-

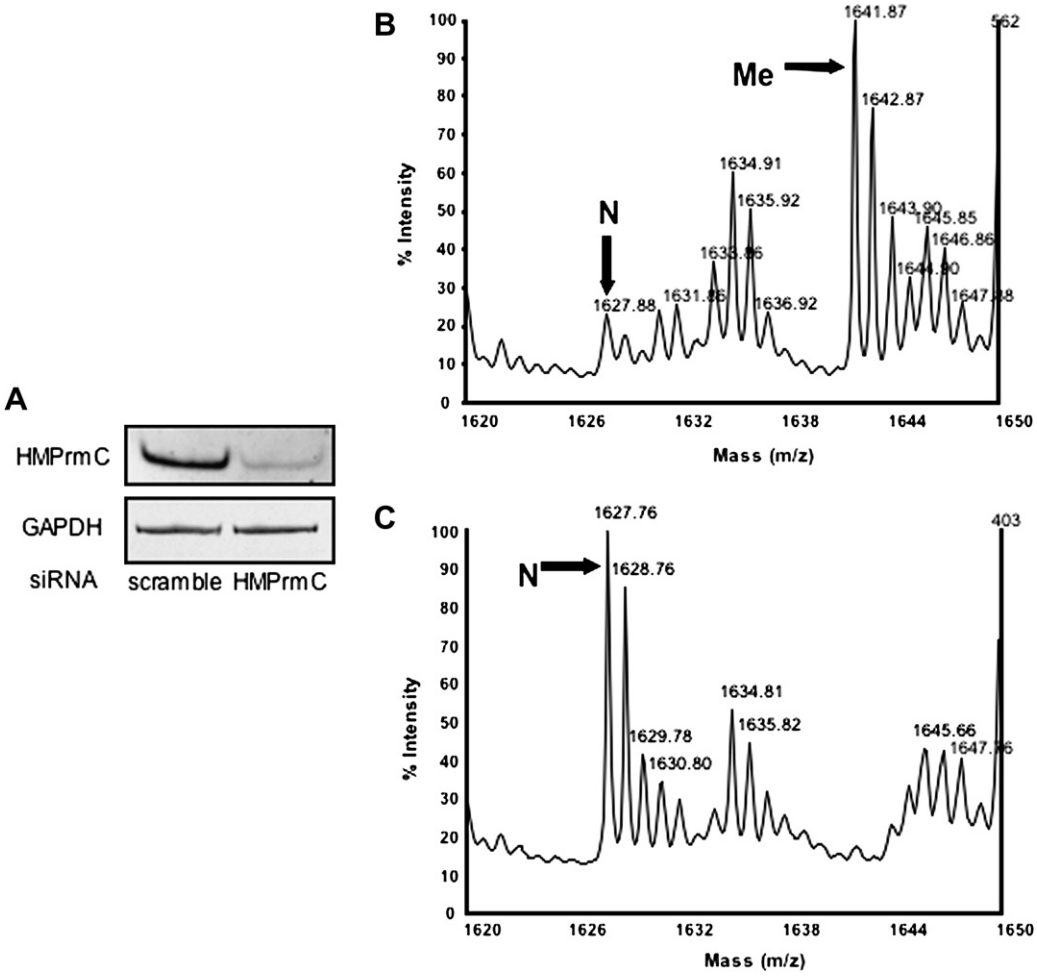

Fig. 3. HMRF1L is methylated by HMPrmC *in vivo*. (A) After transfection (48 h) of HeLa cells with a HMPrmC siRNA, or with siRNA-scramble, total RNA was extracted for semi-quantitative RT-PCR to assess the mRNA levels for HMPrmC and GAPDH (internal control gene). PCR products were separated on a 10% native polyacrylamide gel and stained with EtBr. *Upper*, HMPrmC; *lower*, GAPDH. The mRNA level of HMPrmC in siRNA treated cells reduced to less than 30% of that in control cells. (B, C) At 72 h after transfection with siRNA, 3xFLAG-tagged HMRF1L was purified from the cells, and subjected to MALDI-TOF MS analysis. A complete tryptic digest of HMRF1L is predicted to produce a 1627 Da peak, corresponding to an unmodified peptide with the amino acid sequence ASGAGGQHVNTTDSAVR, in which Q is the proposed methylation site. Two peaks (1627 and 1641 Da) were observed in the sample prepared from cells treated with an siRNA-scramble (B). The 14 Da increase in the molecular mass of the second peptide, 1641 Da, corresponds to the molecular mass of a methyl group. In the HMRF1L sample from cells treated with the HMPrmC siRNA, only one peak (1627 Da), corresponding to the unmodified peptide, was detected (C). The peaks of 1642, 1643, and 1644 Da in (B) are the isotope peaks of 1641 Da. The peaks of 1628, 1629, and 1630 Da in (C) are the isotope peaks of 1627 Da.

lation products in cells cultured in galactose medium were visualized by phosphor-imaging and quantified (Fig. 4A, right, a and b, and Fig. 4B, a and b). Mitochondrial translation activity was decreased in HMPrmC-depleted cells compared to siRNA-scramble treated cells (Fig. 4A, right, a and b, and Fig. 4B, a and b). However, the effect was very moderate. The effect of the translation fidelity antibiotic SM in the galactose medium was also examined. Mitochondrial translation products were labeled, as described above, in the presence of SM. Mitochondrial translation activity in HMPrmC-depleted, SM-treated cells was significantly reduced, to approximately 60% of that in siRNA-scramble treated cells, under these conditions (Fig. 4A, right, c and d, Fig. 4B, c and d).

These results are consistent with previous *in vivo* studies on RF-methyltransferases from other organisms as below: (i) PrmC inactivation in *E. coli* has no effect on cell growth in rich media but reduces growth considerably on poor carbon sources [7], (ii) the lack of eRF1 methylation in yeast causes multiple phenotypes, including sensitivity to the translation fidelity drugs paromomycin and geneticin [10], and (iii) the lack of methylation of the yeast mitochondrial RF Mrf1p in the mtq1-Δ strain produces moderate growth defects in cells cultured on non-fermentable carbon sources, and the lack of Mrf1p methylation increases readthrough in a mtq1-Δ strain containing COX2 with an early-stop ochre codon [10]. In addition, considering the *in vitro* study which showed that methylation of bacterial RFs increases the efficiency of peptide chain release [6,11] together with our results suggests that methylation of HMRF1L by HMPrmC plays a role in mitochondrial translation, possibly by preventing the undesired suppression of termination codons and/or abortive termination events at sense codons by HMRF1L. Methylation of HMRF1L may be advantageous for the cell, especially when cells are exposed to stress, such that cells are forced to depend on oxidative phosphorylation, or when translation errors are induced by translation fidelity antibiotics.

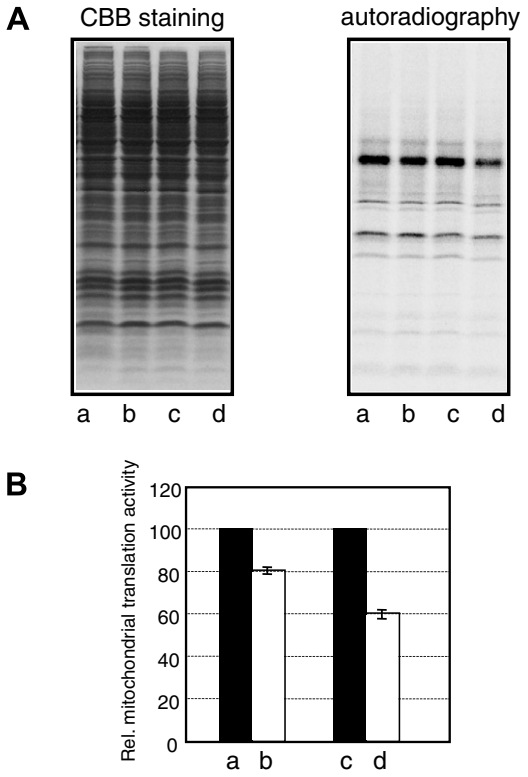

Fig. 4. HMPrmC depletion leads to a decrease in mitochondrial translation activity. (A) At 72 h after transfection with siRNA, mitochondrial gene products were labeled by pulse-labeling, in the absence (a and b) or presence (c and d) of SM in galactose containing medium. Total lysates were subjected to Tricine–SDS–PAGE, and equal protein loading was confirmed by CBB staining of the gel (*left*). Mitochondrial translation products were then visualized using a BAS5000 instrument with an Imaging Plate (Fujifilm) (*right*). (a and c) siRNA-scramble treated cells; (b and d) HMPrmC siRNA treated cells. (B) The total amount of radio-labeled proteins was quantified (A, right), and the mitochondrial translation activity of siRNA-scramble treated cells (a and c) was normalized to 100%. The relative mitochondrial translation activity of HMPrmC-depleted cells (b versus a, and d versus c) was measured. HMPrmC depletion led to a decrease in mitochondrial translation activity to approximately 60% of that in siRNA-scramble treated cells in the presence of SM. Results represent the average of three independent experiments, and the error bars indicate the SD.

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