11648–11658 *Nucleic Acids Research, 2012, Vol. 40, No. 22* *Published online 15 October 2012*
doi:10.1093/nar/gks956

Pmt1, a Dnmt2 homolog in *Schizosaccharomyces pombe*, mediates tRNA methylation in response to nutrient signaling

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Received June 6, 2012; Revised September 3, 2012; Accepted September 21, 2012

ABSTRACT

The fission yeast *Schizosaccharomyces pombe* carries a cytosine 5-methyltransferase homolog of the Dnmt2 family (termed *pombe m*ethyl*t*ransferase 1, Pmt1), but contains no detectable DNA methylation. Here, we found that Pmt1, like other Dnmt2 homologs, has *in vitro* methylation activity on cytosine 38 of $tRNA^{Asp}$ and, to a lesser extent, of $tRNA^{Glu}$, despite the fact that it contains a non-consensus residue in catalytic motif IV as compared with its homologs. *In vivo* tRNA methylation also required Pmt1. Unexpectedly, however, its *in vivo* activity showed a strong dependence on the nutritional status of the cell because Pmt1-dependent tRNA methylation was induced in cells grown in the presence of peptone or with glutamate as a nitrogen source. Furthermore, this induction required the serine/threonine kinase Sck2, but not the kinases Sck1, Pka1 or Tor1 and was independent of glucose signaling. Taken together, this work reveals a novel connection between nutrient signaling and tRNA methylation that thus may link tRNA methylation to processes downstream of nutrient signaling like ribosome biogenesis and translation initiation.

INTRODUCTION

The establishment and maintenance of cytosine-5 methylation (m^5C) in the DNA of eukaryotic organisms is mediated by DNA methyltransferases and plays a central role in the epigenetic regulation of gene expression (1). In contrast, although m^5C methylation on RNA molecules is an abundant modification (2,3), much less is known about its function.

DNA methyltransferases of the Dnmt1 and Dnmt3 family are well established as the enzymes responsible for m^5C DNA methylation in metazoa, and these enzymes are essential for proper development in mice (4). Interestingly, while proteins of the Dnmt2 family show strong structural and sequence similarity to DNA methyltransferases, their *in vitro* DNA methylation activity is weak and distributive (5–7), and Dnmt2-dependent *in vivo* DNA methylation seems to be strictly locus-specific and to be regulated in a temporal fashion (8,9). However, in contrast to DNA methylation, *in vitro* and *in vivo* tRNA methylation by Dnmt2 can be readily detected. A seminal report identified Dnmt2 as an RNA methyltransferase for the C38 position within the anticodon loop of $tRNA^{Asp}$ in mouse, Drosophila and Arabidopsis (10), and later work extended this activity to human Dnmt2 (11) as well as to C38 positions in $tRNA^{Gly}$ and $tRNA^{Val}$ in Drosophila (12). Subsequently, detailed *in vitro* enzymatic studies on the structural requirements for Dnmt2 activity were performed (11), yet little is known about its cellular function. Early work indicated that mice, flies and plants lacking Dnmt2 are viable and fertile under laboratory conditions (10), with the notable exception of zebrafish, where the knock-down of Dnmt2 imparts strong developmental defects in retina, liver and brain (13). However, more recent work supports the idea that Dnmt2 function becomes important under certain conditions. For instance, Dnmt2 controls silencing of retrotransposons in flies (9) and Dictyostelium (8,14), and it mediates the integrity of telomeres in flies, thus implying a role in genome stability, although whether this effect is related to DNA or RNA methylation is still unclear (15). Furthermore, the absence of Dnmt2-dependent tRNA methylation in flies causes increased cleavage of tRNAs, decreased viability under stress conditions and a shortened lifespan (12,16). Thus, although Dnmt2 function may not be obvious under standard conditions, it appears to be important under exacerbated circumstances.

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The fission yeast *Schizosaccharomyces pombe* apparently lacks m^5C DNA methylation (17), yet the first Dnmt2 homolog was identified in this organism (18). This protein, termed *pombe m*ethyl*t*ransferase 1 (Pmt1), carries all conserved sequence motifs for an m^5C methyltransferase, with one remarkable exception. In DNA (m^5C) methyltransferases, the methylation reaction is initiated by a conserved cysteine residue in motif IV of the enzyme (6). While this cysteine is embedded in the sequence PPCQ in all other Dnmt2 homologs, Pmt1 carries a non-consensus serine residue in this motif (PSCQ). Pmt1 possesses no *in vitro* DNA methylation activity, which subsequently was attributed to this sequence variation and was in agreement with the absence of detectable DNA methylation in *S. pombe* (18). A later study found that insertion of a serine residue N-terminal to the catalytic cysteine in other DNA methyltransferases abrogated their activity, and, perhaps more surprisingly, that significant DNA methylation activity could be restored to Pmt1 by removal of the serine residue (19), but this result has not been confirmed independently. As for several other organisms, no obvious phenotype was observed when the *pmt1*$^+$ gene was deleted (*pmt1*Δ), despite the fact that the *pmt1*$^+$ gene was expressed in wild-type cells (18).

The discovery of tRNA methylation activity in other Dnmt2 proteins prompted us to reevaluate Pmt1 enzymatic activity in *S. pombe*, which has led us to discover an unexpected link between Pmt1-dependent tRNA methylation and nutritional cues. Nutrient signaling pathways in *S. pombe* include the kinases Pka1, Sck1 and Sck2 (20,21). Pka1, which is the active subunit of the PKA complex, is activated through a signaling cascade by high glucose levels that are sensed by the membrane glucose receptor Git3 (22). Overexpression of *sck1*$^+$ or *sck2*$^+$ rescues defects of *pka1*Δ cells, and the three kinases have partially overlapping functions in cell growth and sexual differentiation (21). The Sck1 and Sck2 proteins are homologous to Sch9 from *Saccharomyces cerevisiae*, which is a major target of target of rapamycin (TOR) signaling and couples nutritional signaling to the cell growth machinery (23). It can therefore be speculated that Sck1 and Sck2 in *S. pombe* also act downstream of TOR signaling, although whether this is the case remains to be determined (22).

In this study, we investigated the enzymatic activity and cellular function of Pmt1. We found that Pmt1 has robust methylation activity on two tRNA substrates, both *in vitro* and *in vivo*. Notably, this activity was controlled within the cell by nutrient signaling, and it depended on the kinase Sck2, but not on Sck1, Pka1 or Tor1, thus providing an unanticipated link between nutrient signaling via Sck2 and Pmt1-dependent tRNA methylation in *S. pombe*.

MATERIALS AND METHODS

S. pombe strains, plasmids and media

The *S. pombe* strains and plasmids used in this study are shown in Supplementary Tables S1 and S2, respectively. *pmt1*$^+$ overexpression was obtained by cloning the intron-free *pmt1*$^+$ gene in REP4X (*ura4*$^+$, *nmt1* promoter) using BamHI and SmaI. Cells were cultured in YES (5 g/l yeast extract, 30 g/l glucose, 250 mg/l adenine, 250 mg/l histidine, 250 mg/l leucine, 250 mg/l uracil, 250 mg/l lysine), YPD (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose), YE (5 g/l yeast extract, 30 g/l glucose) with 2% ethanol and 2% glycerol, EMM (5 g/l ammonium chloride) or EMMG (1 g/l glutamate) (24,25).

Purification of recombinant Pmt1

The intron-less *pmt1*$^+$ gene and a mutant derivative (*pmt1-C81A*) were cloned into the pET15b vector. His_6-Pmt1 and His_6-Pmt1-C81A were expressed in *Escherichia coli* (DE3) Rosetta cells. Protein expression was induced with 1 mM IPTG for 1 h at 37°C. Purification of the recombinant protein was carried out using Profinity IMAC resin (Bio-Rad) and 200 mM imidazole (elution buffer: 30 mM potassium phosphate, 300 mM KCl, 10% glycerol, 0.1 mM DTT, 200 mM imidazole). The protein was then dialysed against dialysis buffer I (30 mM potassium phosphate pH 7, 200 mM KCl, 20% glycerol, 0.1 mM EDTA, 1 mM DTT) and dialysis buffer II (30 mM potassium phosphate pH 7, 100 mM KCl, 50% glycerol, 0.1 mM EDTA, 1 mM DTT).

RNA substrates for *in vitro* methylation

Yeast cells were grown to an optical density (OD600) of 0.5–0.9 in 50 ml cultures. Cells were harvested and the pellets were either stored at −20°C or directly used for RNA preparation. Small sample numbers were prepared using 8 ml TriZol reagent (Invitrogen) per sample and glass beads. After vigorous shaking for 5 min, the samples were centrifuged for 20 min at 12 000*g* to clear them from debris. The RNA was extracted using chloroform and precipitated with isopropyl alcohol.

Larger sample numbers were prepared using 8 ml TriFast reagent (Peqlab) per sample and glass beads. After vigorous shaking for 5 min, the samples were transferred to PhaseTrap tubes (Peqlab), and an equal amount of chloroform was added. Centrifugation at 1500*g* lead to a stable phase separation, and the RNA was precipitated with isopropyl alcohol.

S. pombe tRNA sequences were cloned into pJET1 transcription vector. *In vitro* transcribed tRNAs were obtained using T7 transcription kit (Fermentas) according to the supplier's instructions. Briefly, the vector containing the tRNA gene was linearized with *Nco*I, and 0.5 µg of this template DNA were incubated for 2 h at 37°C with nucleotides and T7-RNA polymerase in the reaction buffer. The transcripts were treated with TURBO DNase (Ambion) for 15 min at 37°C and subjected to phenol/chloroform extraction and gel filtration using Sephadex G50 (GE Healthcare). 500 ng of tRNA was used without further treatment in *in vitro* methylation assays. Under such conditions, tRNA folding is concomitant with transcription and is stabilized by Mg^{2+} in the methylation reaction.

In vitro tRNA methylation assay

The *in vitro* methylation of total RNA extracts with Pmt1, DnmA or human Dnmt2 was performed as previously described (11). Briefly, 2–10 µg of total RNA or 0.5 µg of *in vitro* transcribed tRNA were incubated with 3 µM enzyme for 90 min in 20 µl of methylation buffer (5 mM Tris–HCl pH 7.5, 5 mM NaCl, 0.5 mM $MgCl_2$, 0.1 mM DTT) containing 1.25 nM [methyl-^{3}H]-AdoMet (Hartmann Analytic). The reaction was stopped by the addition of 1.2 u/µl Proteinase K and 1 mM AdoMet and further incubation for 30–60 min. After the reaction, samples were mixed with RNA loading dye (formamide containing 5 mM EDTA and bromophenol blue) and separated on 7 M urea 12% denaturing polyacrylamide gels. Gels were stained with ethidium bromide, fixed with 10% acetic acid/10% methanol, immersed for 1 h in Amplify solution (Amersham), dried and exposed to Hyperfilm MP film (Amersham) at −80°C. For the time course of tRNA methylation, 500 ng of *in vitro*-transcribed $tRNA^{Asp}$ was incubated with 1.5 µM of Pmt1 for the indicated times, and the reactions were stopped in liquid nitrogen. Samples were processed as previously mentioned, and quantification was done by densitometric analysis of films. Data were fitted to a single exponential reaction progress curve as described in (11).

tRNA bisulfite sequencing

Primer design and bisulfite conversion on total RNA were carried out as described (26). Only tRNAs containing a cytosine at position 38 were selected for analysis ($tRNA^{Asp(GUC)}$, $tRNA^{Glu(UUC)}$, $tRNA^{Leu(AAG)}$, $tRNA^{His(GUG)}$ and $tRNA^{Val(UAC)}$). Bisulfite-treated tRNAs were reverse transcribed using a tRNA 3′-specific stem-loop primer and amplified with primers binding only to the deaminated sequences at the 5′ end, followed by standard cloning and sequencing. Primer sequences are given in Supplementary Table S3. Amplicons were subcloned in pGEM-T easy (Promega) and sequenced. The primers for tRNA amplifications contain five random nucleotides, such that clones from individual PCR products can be distinguished by this sequence. Only unique sequences that thus result from independent tRNA molecules were included in the analysis of tRNA methylation levels. For $tRNA^{Leu}$, $tRNA^{His}$ and $tRNA^{Val}$, we only obtained sequences for partial tRNA fragments that did not include the relevant C position. Most likely, this is due to tRNA modifications *in vivo* that prevent efficient reverse transcription past such residues.

RESULTS

In vitro tRNA methylation activity of *S. pombe* Pmt1

Because Dnmt2 homologs from several species have tRNA methylation activity, we sought to determine whether Pmt1 from *S. pombe* displayed the same enzymatic activity as its homologs. To this end, recombinant Pmt1 purified from *E. coli* was used in an *in vitro* tRNA methylation assay. In a first approach, we sought to determine whether Pmt1 was able to methylate $tRNA^{Asp}$ from *Dictyostelium discoideum*, which is methylated by the *D. discoideum* Dnmt2 enzyme DnmA (Figure 1A). Significantly, like human Dnmt2 and DnmA, Pmt1 was able to methylate the *in vitro*-transcribed $tRNA^{Asp}$ (Figure 1A). This activity was abrogated on mutation of cytosine 38 of $tRNA^{Asp}$ to adenine (C38A), demonstrating that Pmt1 activity, like its Dnmt2 homologs, was specific to C38. Furthermore, this showed that no other tRNA modifications were necessary for Pmt1 to be active on its substrate.

We next asked whether Pmt1 was able to methylate $tRNA^{Asp}$ from *S. pombe* itself, rather than a tRNA from a different organism. Indeed, Pmt1 was able to methylate *in vitro*-transcribed *S. pombe* $tRNA^{Asp}$, and the mutation C38A in the tRNA abrogated this activity (Figure 1B). Furthermore, mutation of the putative catalytic cysteine in motif IV of Pmt1 to alanine (Pmt1-C81A, (18)), which lies within the PSCQ sequence of motif IV, caused a loss of Pmt1 activity, although similar amounts of mutant protein were used in the assay (Figure 1B and C), showing that the activity resided in Pmt1 and not, for instance, in a copurifying contaminant from *E. coli*.

To measure the rate of tRNA methylation by Pmt1, a time course of methylation on *in vitro*-transcribed $tRNA^{Asp}$ from *S. pombe* was determined. The data were fitted to a single exponential reaction progress curve (11), which revealed an apparent rate of $tRNA^{Asp}$ methylation of 0.11 per minute under these experimental conditions (Figure 1F).

We also tested the activity of Pmt1 on two other *S. pombe* tRNAs with a cytosine at position 38, $tRNA^{Glu}$ and $tRNA^{Lys}$ (the unspliced form of $tRNA^{Lys}$ carries a C38). Pmt1 was active on $tRNA^{Glu}$, albeit to a lower level than on $tRNA^{Asp}$ (Figure 1D). This methylation required active Pmt1 enzyme, as the mutant protein Pmt1-C81A was unable to methylate $tRNA^{Glu}$ (Figure 1E). In contrast, no methylation was detected on $tRNA^{Lys}$ (Figure 1D). Taken together, these results showed that Pmt1 was able to methylate its cognate tRNAs as well as heterologous tRNA substrates *in vitro* and suggested that it may have a preference for $tRNA^{Asp}$ over other tRNAs.

In vivo methylation of $tRNA^{Asp}$ and $tRNA^{Glu}$ upon *pmt1*$^+$ overexpression

We next asked whether we could detect Pmt1-dependent tRNA methylation *in vivo* using RNA bisulfite sequencing, a method that allows the direct analysis of methylation patterns on native RNA (26). For this purpose, PCR amplicons of $tRNA^{Asp}$ were generated from bisulfite-treated total RNA extracted from *S. pombe* strains, and the sequence of several independent clones was determined. Significantly, we detected 100% methylation of the C38 position of $tRNA^{Asp}$ in cells carrying a plasmid expressing *pmt1*$^+$ from the *nmt1* promoter (*nmt1pr-pmt1*$^+$, Figure 2A, Supplementary Figure S1A and B). This result showed that Pmt1 was capable of mediating tRNA methylation *in vivo*. Other positions in $tRNA^{Asp}$ were also methylated, namely C48 and C49 to 100% and C60, C61 and C62 to a lesser degree, but this methylation was

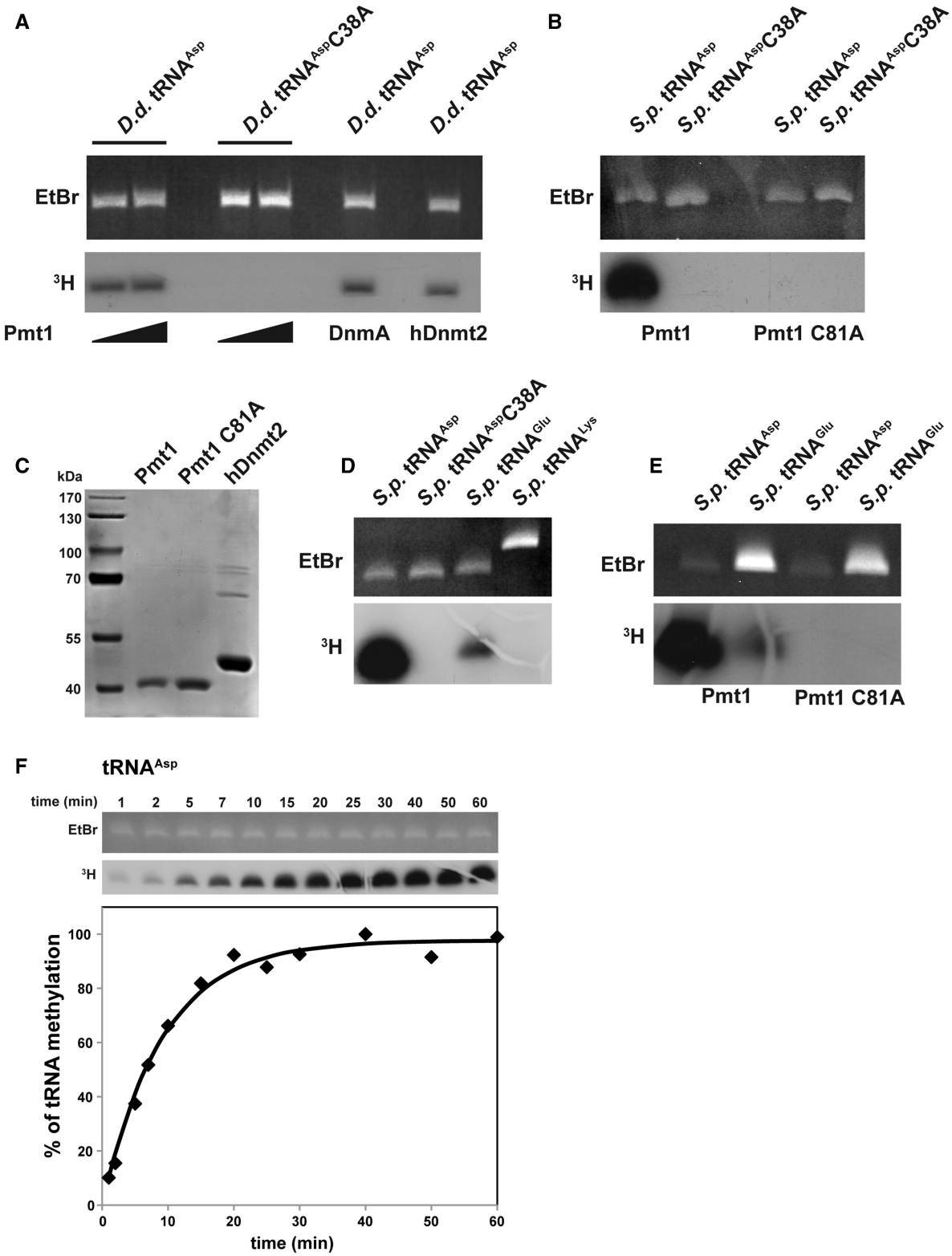

Figure 1. Pmt1 has *in vitro* tRNA methylation activity. (**A**) *in vitro* methylation activity of Pmt1 and its homologs DnmA (*D. discoideum*) and human Dnmt2 on *in vitro*-transcribed $tRNA^{Asp}$ from *D. discoideum (D.d.)*. Methylation assays were performed using ^3H-labeled AdoMet with 3 and 12 μM Pmt1 or 3 μM each for DnmA and hDnmt2 on 500 ng of *in vitro*-transcribed $tRNA^{Asp}$ or $tRNA^{Asp}$C38A from *D. discoideum*. Samples were separated by urea-PAGE, and methylation was detected by autoradiography. (**B**) Pmt1 activity on *in vitro*-transcribed $tRNA^{Asp}$ from *S. pombe*. Pmt1 activity was abrogated by mutation of cysteine 81 within catalytic motif IV (C81A). Assays were performed as in A. (**C**) Coomassie-stained SDS gel of recombinant Dnmt2 homologs used in A and B). (**D**) Pmt1 activity on *in vitro*-transcribed $tRNA^{Glu}$ and $tRNA^{Lys}$. *In vitro*-transcribed tRNAs (500 ng) were incubated with 3 μM Pmt1. Samples were analysed as in A. (**E**) Pmt1 activity on *in vitro*-transcribed $tRNA^{Asp}$ and $tRNA^{Glu}$. *In vitro*-transcribed $tRNA^{Asp}$ (500 ng) and $tRNA^{Glu}$ (5 μg) were incubated with 3 μM Pmt1 or Pmt1C81A. Samples were analysed as in A. (**F**) Time course of $tRNA^{Asp}$ methylation by Pmt1 (1.5 μM). The upper panel shows the time course of incorporation of radioactivity into the tRNA. The radioactive bands were analysed quantitatively and the data fitted to a single exponential reaction progress curve as shown in the lower panel.

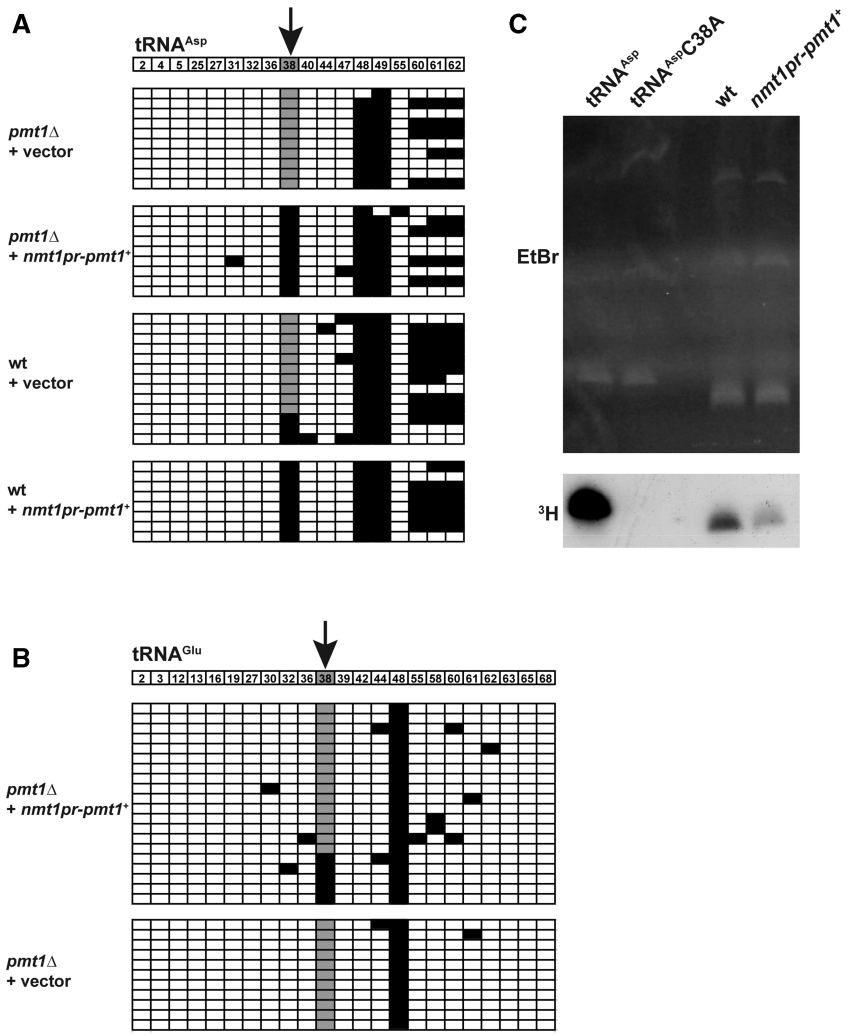

Figure 2. *pmt1*$^+$ overexpression induces methylation of tRNA^{Asp} and tRNA^{Glu} *in vivo* in *S. pombe*. (**A**) *In vivo* RNA bisulfite sequencing of tRNA^{Asp} from total RNA of a *pmt1*Δ (AEP8) and a wt strain (AEP1) carrying an empty vector or a plasmid with *pmt1*$^+$ expressed from the *nmt1* promoter (*nmt1pr-pmt1*$^+$, pAE1462). Cells were grown in supplemented EMM medium. The cytosine residues present in tRNA^{Asp} are indicated in the top row. Each subsequent row represents an independent clone that was sequenced. Black boxes indicated methylated cytosine, and gray or white boxes indicate unmethylated cytosines. The arrow indicates position C38 of tRNA^{Asp}. (**B**) *In vivo* methylation of tRNA^{Glu} on *pmt1*$^+$ overexpression. Representation as in A. (**C**) Right two lanes, *in vitro* methylation of total RNA from a wt and a *pmt1*$^+$ overexpressing strain (*nmt1pr-pmt1*$^+$) by recombinant Pmt1. *pmt1*$^+$ overexpression *in vivo* caused a reduced *in vitro* methylation signal. Left two lanes, methylation of *in vitro* transcribed tRNA^{Asp} and tRNA^{Asp}C38A by Pmt1, representation as in Figure 1B.

independent of Pmt1 (see below). As expected, no *in vivo* tRNA^{Asp} methylation at C38 was detected in this assay in *pmt1*Δ cells, whereas other cytosine methylation sites were unaffected by *pmt1*Δ (Figure 2A). Interestingly, in wild-type (wt) cells carrying a control vector, we detected approximately 23% tRNA^{Asp} methylation at C38 (Figure 2A). This showed that *pmt1*$^+$ expressed under its endogenous promoter provided a lower level of tRNA methylation than when it was expressed from the *nmt1*$^+$ promoter.

We also tested for methylation of other tRNAs that carry a cytosine at position 38 by RNA bisulfite sequencing. We observed approximately 25% methylation of tRNA^{Glu} upon *nmt1pr-pmt1*$^+$ expression, whereas no methylation was detected in a *pmt1*Δ strain (Figure 2B). This was in agreement with our observation of a lower activity of Pmt1 on *in vitro*-transcribed tRNA^{Glu} and suggested that this tRNA was a less efficient substrate for Pmt1 *in vivo*. Other tRNAs with a C38 residue (tRNA^{His}, tRNA^{Val}, tRNA^{Leu}) were not amenable to RNA bisulfite sequencing for technical reasons (see 'Materials and Methods' section).

We further sought to verify the Pmt1-dependent *in vivo* tRNA methylation using an independent assay. For this purpose, total RNA was extracted from *S. pombe* cells with or without *nmt1pr-pmt1*$^+$ expression and used as a substrate for an *in vitro* methylation experiment with recombinant Pmt1, where the incorporation of ^{3}H-marked methyl groups from S-[methyl-^{3}H]-AdoMet into the RNA is detected (11). An *in vitro* methylation signal migrating in the size range of tRNAs was detected in RNA from a wild-type strain without *nmt1pr-pmt1*$^+$ expression, indicating that there were unmethylated C38 residues in this RNA that were subsequently methylated *in vitro* by

recombinant Pmt1 (Figure 2C). Importantly, this signal was strongly decreased on expression of *pmt1*$^+$ from the *nmt1*$^+$ promoter, thus confirming that there was increased *in vivo* methylation under these conditions. This supported the notion that *nmt1pr-pmt1*$^+$ expression mediated tRNA methylation *in vivo*.

Nutrient-dependent tRNA methylation by Pmt1

Our previous results indicated that Pmt1-dependent *in vivo* tRNA methylation was limiting under some conditions because a wild-type strain showed only approximately 23% $tRNA^{Asp}$ C38 methylation. We therefore asked whether nutrient conditions altered the *in vivo* level of tRNA methylation. Surprisingly, wild-type cells grown in standard *S. pombe* complete medium (YES) showed no detectable C38 methylation of $tRNA^{Asp}$ as measured by RNA bisulfite sequencing (Figure 3A), whereas the previously determined 23% level was measured in cells grown in minimal medium (Figure 2A). In agreement with this, there was no difference in the methylation signal by recombinant Pmt1 in RNA extracted from wild-type and *pmt1*Δ cells grown in *S. pombe* complete medium (Figure 3B). In stark contrast, wild-type cells grown in standard complete medium for a different yeast species, *S. cerevisiae* (YPD), showed a striking near 100% level of $tRNA^{Asp}$ C38 methylation, although no $tRNA^{Glu}$ methylation (Figure 3C and D). Accordingly, we detected a pronounced decrease of *in vitro* tRNA methylation by recombinant Pmt1 on total RNA from a wild-type strain grown in *S. cerevisiae* complete medium as compared with the *pmt1*Δ strain (Figure 3B), thus demonstrating a prominent effect of media composition on *in vivo* tRNA methylation by Pmt1.

A simple explanation for the absence of tRNA methylation under certain media conditions would be that *pmt1*$^+$ might be expressed in one condition, but not the other. However, *pmt1*$^+$ RNA was readily detected in standard *S. pombe* medium, and we observed no difference in protein and RNA levels in the two media conditions ((18), Supplementary Figure S1), suggesting that tRNA methylation levels were regulated by a mechanism other than by changing the *pmt1*$^+$ expression level or protein stability.

The above observations begged the question what the difference in media composition between *S. pombe* and *S. cerevisiae* complete media was. To identify the component relevant for tRNA methylation, *in vitro* methylation assays were performed with total RNA from cells grown with or without individual media components. Changes in glucose level (2% versus 3%), amount of yeast extract (1% versus 0.5%), supplements (none versus 225 mg/l adenine, histidine, leucine, uracil and lysine) did not affect tRNA methylation (Supplementary Figure S2A). Intriguingly, however, the addition of peptone in *S. pombe* medium, where it normally is absent, induced tRNA methylation in wild-type, but not *pmt1*Δ cells, whereas omission of peptone from *S. cerevisiae* medium abrogated Pmt1-dependent tRNA methylation (Figure 3B). Importantly, the media composition did not affect the $tRNA^{Asp}$ levels in the cells, as measured by Northern blotting (Supplementary Figure S3A). Peptone is a mixture of nutrients generated by proteolytic digestion of animal protein with pepsin and serves as a nitrogen and amino acid source in culture media. Therefore, it was possible that one or several nutrients in peptone caused tRNA methylation by Pmt1 in *S. pombe*. Interestingly, YPD, but not YES, induces sexual differentiation in *S. pombe* and is considered stressful for the cells (24), and sexual differentiation is also induced by nitrogen starvation. We therefore asked whether other media or stress conditions or nitrogen depletion could induce Pmt1-dependent tRNA methylation *in vivo*. Growth of cells on non-fermentative carbon sources (2% ethanol, 2% glycerol) did not induce tRNA methylation, nor did the treatment of cells with a stressing agent, H_2O_2 (Supplementary Figure S2B). Importantly, however, growth of cells in medium in which the ammonium source (0.5% ammonium chloride, EMM) was replaced by a low level of glutamate (0.1% glutamate, EMMG (24)) caused an increase of Pmt1-dependent tRNA methylation (Figure 3E), suggesting that nitrogen depletion induced tRNA methylation. Again, neither EMM nor EMMG affected cellular $tRNA^{Asp}$ levels (Supplementary Figure S3A). We further asked whether the opposite was the case, namely that increased nitrogen levels were capable of repressing tRNA methylation. For this purpose, conditions were used in which we observed strong Pmt1-dependent *in vivo* methylation, which is on *nmt1pr*-mediated *pmt1*$^+$ expression. Under usual ammonium concentrations (0.5% NH_4Cl in EMM), *nmt1pr-pmt1*$^+$ caused increased *in vivo* tRNA methylation in a *pmt1*Δ strain as compared with the vector control, as indicated by a weak *in vitro* methylation signal (Figure 3E). However, the tRNA methylation decreased in *pmt1*Δ *nmt1pr-pmt1*$^+$ cells grown at a higher ammonium concentration (2.5% NH_4Cl in EMM), indicating that higher ammonium levels inhibited Pmt1 function *in vivo*. Taken together, these results showed that Pmt1-dependent tRNA methylation was controlled by the nitrogen source and indicated that it may be controlled by other nutritional cues.

Pmt1-dependent tRNA methylation depends on the protein kinase Sck2

Nutrient signaling in *S. cerevisiae* is mediated via the kinases Sch9/Tor/PKA (22). In *S. pombe*, the presence of glucose is signaled to Pka1 via the Git3/PKA pathway. Other nutrient sensing is mediated by the Sch9 homologs Sck1 and Sck2 (21), although their link to TOR signaling is less clear than in *S. cerevisiae*, where Sch9 is a major target of Tor1 (23). As we observed that nutrient conditions modulated tRNA methylation, we next asked which of these nutrient-sensing pathways might be involved in this effect. Total RNA from *pka1*Δ, *sck1*Δ or *tor1*Δ cells grown in peptone medium showed a low methylation signal on *in vitro* methylation by recombinant Pmt1, indicating strong *in vivo* methylation as in wild-type cells (Figure 4A and B). Notably, the low methylation signal in these strains was not the result of lower levels of $tRNA^{Asp}$ (Supplementary Figure S3B). Significantly, however, an *in vitro* methylation signal was detected using total RNA

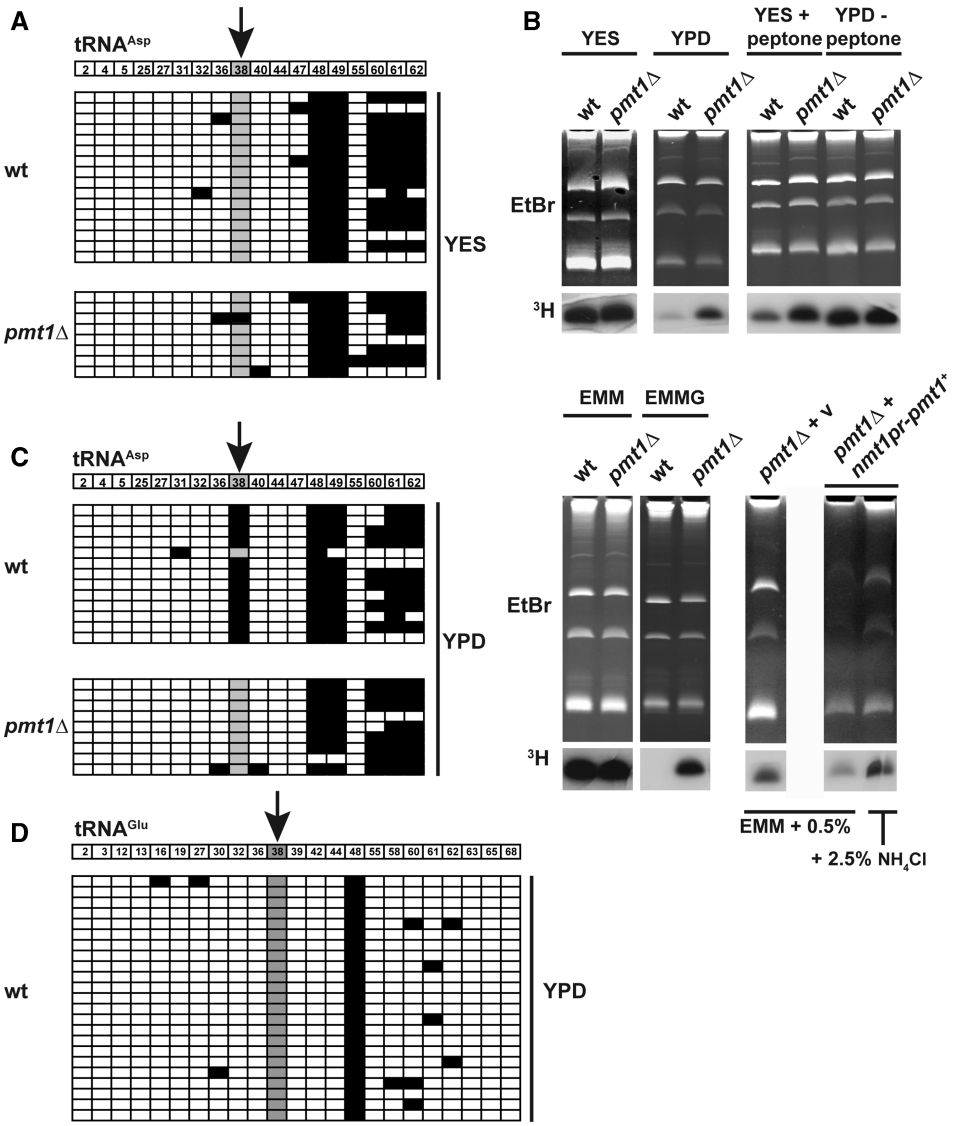

Figure 3. Regulation of Pmt1-dependent *in vivo* tRNA methylation by nutrient conditions. (**A**) RNA bisulfite sequencing of $tRNA^{Asp}$ from wt (AEP1) and *pmt1*Δ (AEP8) cells cultured in *S. pombe* complete growth medium (YES) showed no *in vivo* methylation of $tRNA^{Asp}$ at C38. (**B**) *in vivo* tRNA methylation was induced when cells were cultured in the presence of peptone. Total RNA of cells cultured in YES, YPD, YES with 2% peptone, or YPD in which the peptone had been omitted, was methylated *in vitro* using recombinant Pmt1 as in Figure 2C. (**C**) wt cells showed near 100% methylation of $tRNA^{Asp}$-C38 when cultured in *S. cerevisiae* complete growth medium (YPD), as measured by RNA bisulfite sequencing. Representation as in Figure 2A. (**D**) $tRNA^{Glu}$ was not methylated at the C38 position in wt cells grown in YPD, as measured by RNA bisulfite sequencing. (**E**) Effect of nitrogen levels on Pmt1-dependent tRNA methylation. Cells were grown in ammonium-containing minimal medium (EMM, 0.5% ammonium chloride), 0.1% glutamate (EMMG) or with increased ammonium chloride (EMM + 2.5% NH_4Cl) before RNA extraction and methylation analysis as in Figure 2C.

from an *sck2*Δ strain, indicating that this strain had a reduced *in vivo* tRNA methylation level. The loss of *in vivo* methylation in *sck2*Δ was not 100% because an *sck2*Δ *pmt1*Δ strain showed a stronger *in vitro* methylation signal than *sck2*Δ alone (Figure 4B). These results showed that nutrient-induced tRNA methylation was partially dependent on the serine/threonine kinase Sck2. The absence of an effect of Pka1 was in agreement with the observation that glucose levels did not alter tRNA methylation.

As we had observed previously that *nmt1pr-pmt1*$^+$ overexpression conveyed full *in vivo* tRNA methylation, we next sought to determine whether this was influenced by Sck2. Interestingly, while *in vivo* tRNA methylation was strong upon *nmt1pr-pmt1*$^+$ expression, this methylation was reduced in the absence of Sck2, as indicated by an increased *in vitro* methylation signal (Figure 4C). This showed that even upon expression from a heterologous promoter, full tRNA C38 methylation by Pmt1 required the Sck2 kinase, thus genetically placing Pmt1 in the Sck2-dependent nutrient-signaling pathway.

C48, C49 and C60, C61 and C62 methylation of $tRNA^{Asp}$ depended on two *S. pombe* Trm4 homologs

As we observed methylation positions in $tRNA^{Asp}$ other than C38 that were independent of Pmt1 (Figure 2A) as

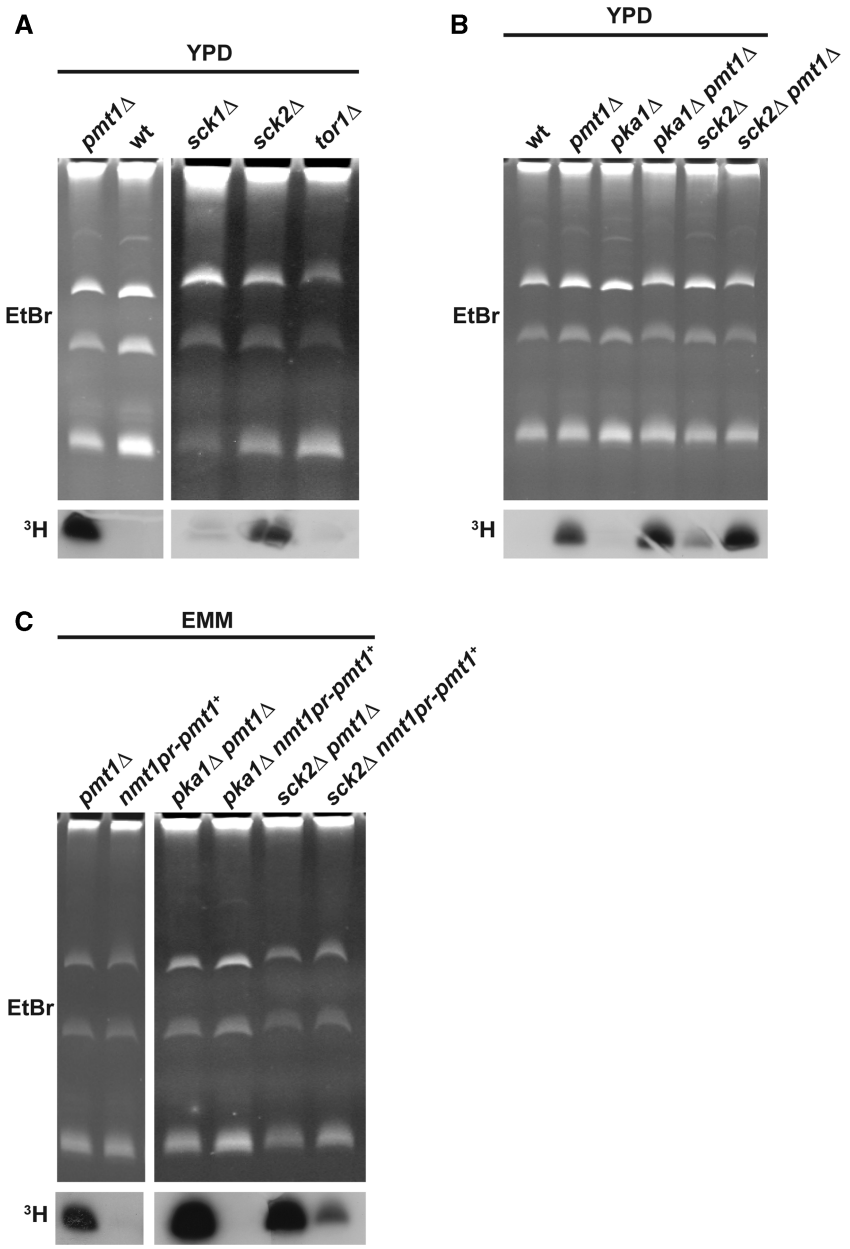

Figure 4. *In vivo* tRNA methylation depended on the kinase Sck2. (**A**) *sck2*Δ, but not *sck1*Δ or *tor1*Δ, caused a reduction of *in vivo* tRNA methylation. The indicated strains (AEP8, AEP1, AEP61, AEP62, AEP120) were grown in rich *S. cerevisiae* medium (YPD), and total RNA was methylated *in vitro* with recombinant Pmt1 as in Figure 2C. (**B**) *sck2*Δ, but not *pka1*Δ, caused a loss of *in vivo* tRNA methylation. Strains used were wt (AEP1), *pmt1*Δ (AEP8), *pka1*Δ (AEP117), *pka1*Δ *pmt1*Δ (AEP125), *sck2*Δ (AEP119) and *sck2*Δ *pmt1*Δ (AEP126). (**C**) *In vivo* tRNA methylation on *pmt1*$^+$ overexpression required Sck2. Cells (AEP8, AEP125 or AEP126 transformed with pAE1429 or pAE1462) were grown in EMM medium for plasmid selection, and *in vitro* methylation by Pmt1 was performed as in Figure 2C.

well as nutrient conditions (Figure 3A and C), we sought to determine which methyltransferase was responsible for these methylation events. In *S. cerevisiae*, C48 and C49 methylation in a number of tRNAs is mediated by the Trm4 methyltransferase (27). Thus, some of the m^5C methylation in $tRNA^{Asp}$ observed here may depend on the two Trm4 homologs present in *S. pombe* (SPAC17D4.04, which we here termed *trm4a*$^+$, and SPAC23C4.17, here termed *trm4b*$^+$), which so far have not been characterized. To test this, we performed bisulfite sequencing of $tRNA^{Asp}$ in either single mutant and in a *trm4a*Δ *trm4b*Δ double mutant. Methylation of residues C48, 49, 60, 61 and 62 was largely unaffected by either single mutant (Figure 5). We note that there is some variability in the methylation level of these residues (compare with Figure 2A). Importantly, however, there was a major decrease in methylation at these residues in the *trm4a*Δ *trm4b*Δ strain. This indicated that the two Trm4 homologs performed redundant functions in $tRNA^{Asp}$ methylation of these residues in *S. pombe*. Of note, we observed an increase in C38 methylation in the *trm4b*Δ strain as well as in the double mutant, which may indicate

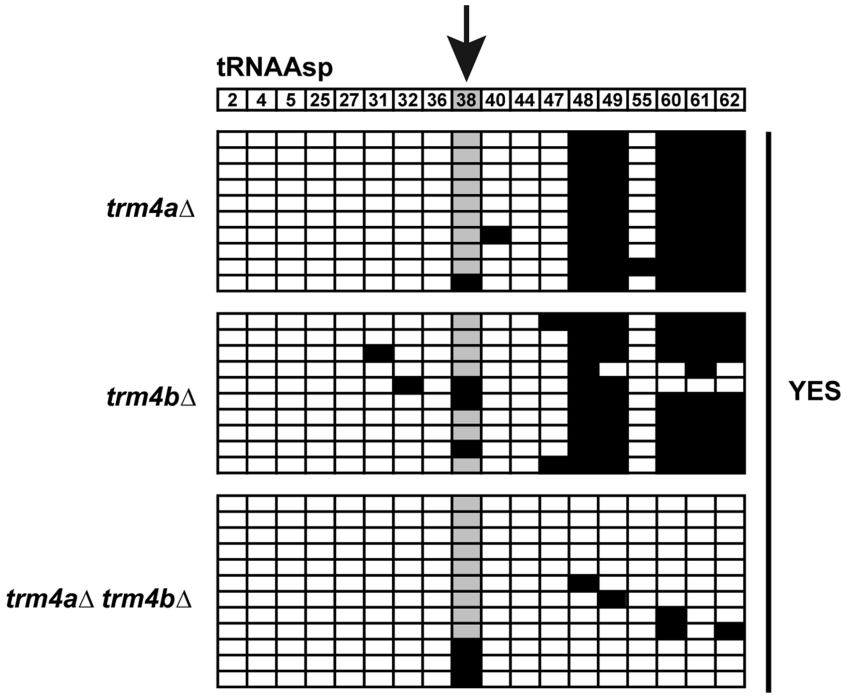

Figure 5. Trm4-dependent methylation of *S. pombe* $tRNA^{Asp}$. RNA bisulfite sequencing of $tRNA^{Asp}$ from *trm4a*Δ (AEP102) and *trm4b*Δ (AEP103) cells cultured in *S. pombe* complete growth medium (YES) showed *in vivo* methylation of positions C48, C49, C60, C61 and C62. The double mutant *trm4a*Δ *trm4b*Δ (AEP162) showed a significant loss of m^5C at these positions. Representation as in Figure 2A.

an induction of Pmt1-dependent tRNA methylation in the absence of Trm4b.

DISCUSSION

The Dnmt2 family of methyltransferases is conserved from unicellular to multicellular organisms (28), but the presence of a homolog in fission yeast has been particularly intriguing because this organism apparently lacks DNA methylation. In this work, we demonstrate that the *S. pombe* Pmt1 protein is a methyltransferase for C38 of $tRNA^{Asp}$ and $tRNA^{Glu}$ whose activity *in vivo* is controlled by nutrient conditions via a signaling pathway involving the serine/threonine kinase Sck2. Thus, Pmt1 is an active enzyme both *in vitro* and *in vivo*, although it carries a non-consensus serine residue in catalytic motif IV as compared with its Dnmt2 homologs.

One of the most surprising discoveries of this study is the observation that tRNA methylation levels *in vivo* are controlled by the nutritional environment of yeast cells (for an overview, see Supplementary Table S4). We propose that growth of *S. pombe* in different nitrogen sources induces a nutritional change in the cells, such that nutrient sensing pathways become activated and induce Pmt1 to perform tRNA methylation. Intriguingly, we found that Pmt1 activity partly depends on the kinase Sck2, but not on Pka1 or Tor1. Thus, the induction of tRNA methylation seems to be selective to the nature of the stimulus (nutrition/nitrogen source) and the activation of the respective signaling pathway(s). It seems to be independent of glucose signaling via the Git3/PKA pathway as well as of non-fermentative growth conditions or stress by H_2O_2, but is activated by low, and repressed by high, levels of nitrogen. Furthermore, our data suggest that Sck2 mediates part of the nutrient sensing that leads to the activation of Pmt1. Intriguingly, the Sck2 homolog in *S. cerevisiae,* Sch9, is stimulated by amino acids and nitrogen sources via direct phosphorylation through TORC1, and it mediates some of the cellular effects of TORC1 signaling on ribosome biosynthesis and cell-size control (23). Notably, we did not observe a Tor1 dependence of Pmt1 activation, which argues that Sck2 in *S. pombe* may not act downstream of Tor1 (22). However, *S. pombe* Tor1 corresponds to the second TOR homolog from *S. cerevisiae*, Tor2, (29), and it therefore is possible that Sck2 acts downstream of Tor2, whose role in tRNA methylation we have not been able to assess because it is essential for viability (30). Thus, our work suggests an intriguing link between the activation of TOR signaling, Sck2 and subsequent Pmt1-dependent tRNA methylation. In light of our work, we hypothesize that Pmt1-dependent tRNA methylation is linked to ribosomal functions in translation initiation because activation of the Sck2 homolog Sch9 in *S. cerevisiae* contributes to translation initiation through phosphorylation of the translation initiation factor eIF2a and the ribosomal protein Rps6 (23). It is intriguing to note that Pmt1 activation could be achieved by using one of the amino acids (glutamate) whose tRNA is methylated by Pmt1, although whether this is related to charging of the $tRNA^{Glu}$ with glutamate remains to be determined. Furthermore, it is counter-intuitive that peptone addition and glutamate as nitrogen source have apparently comparable effects on

Pmt1 activation. One possibility is that the nitrogen in peptone is not bioavailable for *S. pombe* cells, such that they in effect become nitrogen starved in its presence. Alternatively, there may be other components in peptone, for instance amino acids, that activate nutrient signaling.

As Pmt1-dependent *in vivo* tRNA methylation depended on the Sck2 kinase, but Pmt1 protein levels were unaffected by nutrient conditions, it is tempting to speculate that Pmt1 enzymatic activity is regulated by phosphorylation, either by being directly phosphorylated by Sck2, or by another protein kinase that is activated by an Sck2-dependent signaling cascade. In light of the observation that Dnmt2 from *Entamoeba histolytica* is inhibited by interaction with a glycolytic enzyme, enolase, (31), we can further hypothesize that this putative phosphorylation may control interactions of Pmt1 with regulatory proteins, although it is also possible that the enzyme *per se* would be stimulated by phosphorylation. This also reveals an intriguing parallel in nutritional control of Dnmt2 activity between *Entamoeba* and *S. pombe*. It will furthermore be interesting to see whether Dnmt2 homologs in other species are also regulated via kinase signaling pathways.

We demonstrate here that Pmt1 is able to methylate tRNAs in the anticodon loop, raising the question as to the function of this modification. Nucleotide modifications in RNA in some cases lead to structural and metabolic stabilization of the RNA molecules (32). Cytosine methylation can impact on Mg^{2+} binding to tRNA molecules, which in turn influences tRNA conformation (33) and may influence tRNA binding or wobble efficiency at the ribosomes during translation. Furthermore, protection against degradation has been reported for some modified tRNAs (12,34). Similar effects can therefore be envisioned for Pmt1-dependent tRNA methylation. Here, we have not observed any Pmt1-dependent growth defects, neither for *pmt1*Δ, nor for *nmt1pr*-mediated *pmt1*$^+$ expression and neither under standard growth conditions, nor under nitrogen starvation. Also, *pmt1*$^+$ was not required for haploid mating and diploid sporulation, which is induced by nitrogen starvation and thus by Pmt1-inducing conditions (data not shown). Because the absence of the Trm4 homolog in mice, NSun2, has been shown to be lethal in combination with the absence of Dnmt2 (35), we hypothesized that *pmt1*Δ might become essential in *trm4a*Δ *trm4b*Δ double mutants, as we found methylation of cytosines other than C38 to be abrogated in *trm4a*Δ *trm4b*Δ cells. However, *trm4a*Δ *trm4b*Δ *pmt1*Δ triple mutants were viable and displayed no obvious growth defect (data not shown). Thus, the absence of $tRNA^{Asp}$ methylation apparently has more drastic consequences in mice than in *S. pombe*, indicating that there are functional differences for C5 tRNA methylation in the different organisms.

What is the substrate specificity of Pmt1? We found two tRNA moieties to be targets for Pmt1, but it is possible that Pmt1 has other *in vivo* substrates that remain to be identified, perhaps other tRNAs, or other small RNAs with sequence similarity and similar size to tRNAs. All evidence so far speaks against DNA methylation activity by Pmt1 (18). It should, however, be noted that our discovery of a nutritional dependence for Pmt1's tRNA methylation activity suggests that its potential DNA methylation activity might have the same nutritional requirements, and a reevaluation under such conditions may therefore be merited.

In conclusion, we have identified an unexpected link between the nutritional status of the cell and the activity of Pmt1/Dnmt2 in *S. pombe*. It will be interesting to see how Pmt1 activity is regulated, how tRNA methylation affects tRNA function and how this relates to the downstream effects of nutrient signaling pathways in yeast as well as in other organisms.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–4, Supplementary Figures 1–3, and Supplementary References [36].

ACKNOWLEDGEMENTS

We wish to thank Matthias Schäfer for advice on RNA bisulfite sequencing, Kurt Runge for *S. pombe* strains, Karolin Nicklasch and Martina Rübeling for technical assistance, and Jessica Stephan for comments on the manuscript. We furthermore thank the members of the Forschergruppe FOR1082 for many helpful discussions.

FUNDING

Deutsche Forschungsgemeinschaft (DFG) [FOR1082/EH237/8-1 to A.E.-M.]. Funding for open access charge: DFG [EH237/8-1].

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