A Conserved SET Domain Methyltransferase, Set11, Modifies Ribosomal Protein Rpl12 in Fission Yeast*\(^\text{\textregistered}\)

Mahito Sadaie\(^1\), Kaori Shinmyozu\(^5\), and Jun-ichi Nakayama\(^1\)\(^2\)

From the \(^1\)Laboratory for Chromatin Dynamics and the \(^5\)Proteomics Support Unit, Center for Developmental Biology, RIKEN, Kobe, Hyogo 650-0047, Japan

The post-translational modification of proteins is a ubiquitous mechanism by which cells expand the function of proteins. The SET domain-containing methyltransferases post-translationally modify a variety of cellular proteins, such as histones, cytochrome c, ribulose-bisphosphate carboxylase/oxygenase, and ribosomal proteins. In the fission yeast Schizosaccharomyces pombe, at least 13 SET domain-containing proteins have been identified in the genome, four of which are involved in transcriptional regulation through their modification of histone tails. However, the roles played by the other SET domain proteins in cellular processes and their physiological substrates remain unresolved. We show here that S. pombe Set11, a SET domain-containing protein encoded by SPCC1223.04c, specifically modifies Rpl12 (ribosomal protein L12). Recombinant Set11 prepared from Escherichia coli had catalytic activity and methylated a 17-kDa polypeptide in cellular extracts of set11 mutant cells. The methylated protein was isolated by two-dimensional gel electrophoresis or by reverse-phase chromatography and was identified as Rpl12 by mass spectrometry. In vitro methyltransferase experiments using wild-type and mutant Rpl12 proteins verified that Set11 modified recombinant Rpl12 and suggested that its potential target site was lysine 3. The methylation site modified by Set11 was also confirmed by mass spectrometric analysis, which also revealed other unique methylation sites of Rpl12. Finally, we found that Set11 predominantly localized to the nucleolus and that the overproduction of Set11 caused a severe growth defect. These results suggest that Rpl12 methylation occurs during the ribosomal assembly processes and that control of the Set11 expression level is important for its cellular function.

SET domain-containing methyltransferases post-translationally modify a variety of cellular proteins, such as histones, cytochrome c, ribulose-bisphosphate carboxylase/oxygenase, and ribosomal proteins. In the fission yeast Schizosaccharomyces pombe, at least 13 SET domain-containing proteins have been identified in the genome, four of which are involved in transcriptional regulation through their modification of histone tails. However, the roles played by the other SET domain proteins in cellular processes and their physiological substrates remain unresolved. We show here that S. pombe Set11, a SET domain-containing protein encoded by SPCC1223.04c, specifically modifies Rpl12 (ribosomal protein L12). Recombinant Set11 prepared from Escherichia coli had catalytic activity and methylated a 17-kDa polypeptide in cellular extracts of set11 mutant cells. The methylated protein was isolated by two-dimensional gel electrophoresis or by reverse-phase chromatography and was identified as Rpl12 by mass spectrometry. In vitro methyltransferase experiments using wild-type and mutant Rpl12 proteins verified that Set11 modified recombinant Rpl12 and suggested that its potential target site was lysine 3. The methylation site modified by Set11 was also confirmed by mass spectrometric analysis, which also revealed other unique methylation sites of Rpl12. Finally, we found that Set11 predominantly localized to the nucleolus and that the overproduction of Set11 caused a severe growth defect. These results suggest that Rpl12 methylation occurs during the ribosomal assembly processes and that control of the Set11 expression level is important for its cellular function.

Acknowledged as substrates for the SET domain-containing methyltransferases (4), this domain has also been found in a subset of methyltransferases that modify non-histone proteins, such as ribulose-bisphosphate carboxylase/oxygenase (5), cytochrome c (6), p53 (7, 8), and ribosomal proteins (9).

The methylation of ribosomal protein has been identified in a diverse range of species, including bacteria, yeast, and humans. Escherichia coli L11, one of the most well-characterized ribosomal proteins, is α-N-trimethylated at Ala\(^3\) and ε-N-trimethylated at Lys\(^3\) and Lys\(^39\) (10). These methyl groups are added by a single methyltransferase called PrmA (11). The methylation positions and PrmA are highly conserved among bacterial clades, suggesting an important function, although PrmA is dispensable for normal growth (12, 13). In the budding yeast Saccharomyces cerevisiae, direct mass spectrometric analysis of the large ribosomal proteins revealed that six of them, Rpl1, Rpl3, Rpl12, Rpl23, Rpl42, and Rpl43, are post-translationally modified by the addition of methyl groups (14). Rpl23 is specifically ε-N-dimethylated at two residues, Lys\(^105\) and Lys\(^109\), and these modifications are catalyzed by the SET domain-containing methyltransferase Rkm1 (15, 16). S. cerevisiae Rpl12, the counterpart of bacterial L11, is also modified; by ε-N-dimethylation at Lys\(^3\), ε-N-trimethylation at Lys\(^10\), and δ-N-monomethylation at Arg\(^66\) (17, 18). Rkm2, another SET domain-containing protein, and Rmt2, a protein arginine methyltransferase, catalyze the methylation at Lys\(^10\) and Arg\(^66\) of Rpl12, respectively (17, 18). The enzyme(s) responsible for the ε-N-dimethylation of Lys\(^10\) in Rpl12 has yet to be identified. In mammals, several mass spectrometric studies have identified methyl modifications on ribosomal proteins (19–22). In most cases, however, the precise methylation sites and responsible enzyme(s) have yet to be explored. Currently, only one mammalian ribosomal methyltransferase (Pmmt3) has been shown to modify a ribosomal protein, S2 (23). Although ribosomal protein methylation appears to be conserved among different organisms, the physiological role(s) of the methyl modification is poorly understood.

In the fission yeast Schizosaccharomyces pombe, at least 13 SET domain-containing proteins have been identified in the genome. Our previous studies showed that the fission yeast Clr4, a homolog of Su(var)3-9, is a histone H3 Lys\(^7\)-specific methyltransferase that is required for heterochromatin assembly (24). Although several other SET domain-containing proteins in fission yeast add methyl groups to specific lysine residues of histone tails (25–27), the roles played by other SET domain proteins in cellular processes and their physiological substrates remain unresolved. Furthermore, although S. pombe...
Rmt3, a protein-arginine methyltransferase, has been shown to modify the small ribosomal protein S2 (Rps2) and to be involved in stability of the small subunit (28, 29), little attention has been paid to the involvement of SET domain-containing proteins in ribosomal biogenesis.

In this study, we analyzed S. pombe Set11 and found that it is responsible for the methylation of ribosomal protein L12 (Rpl12). Interestingly, although S. pombe Set11 shows a higher sequence similarity to S. cerevisiae Rkm2, which is responsible for modifying Rpl12 at Lys10 (18), it preferentially catalyzed the S. pombe Rpl12 specifically in ribosome assembly.

### EXPERIMENTAL PROCEDURES

**Strains and Plasmids**—The strains used in this study are listed in supplemental Table S1. All of the yeast strains were grown at 30 °C in YEA (0.5% yeast extract, 3% glucose, 75 μg/ml adenine) or minimal medium EMM (MPP Biomedicals) supplemented with amino acids (75 μg/ml adenine, leucine, and histidine and 150 μg/ml uracil). The deletion and tagging of endogenous genes were conducted using a PCR-based gene-targeting protocol (30) and YEA medium containing antibiotics (200 μg/ml G418 or hygromycin). To construct plasmids for producing recombinant Set11 or Rpl12 proteins in E. coli, the coding sequence for set11 + or rpl12 + was amplified by PCR and cloned into the pSET (Invitrogen) and pTriEX-4 Hygro (Novagen) vectors, respectively. To obtain plasmids for expressing mutant recombinant Rpl12 protein in E. coli, the above plasmids were subjected to site-directed mutagenesis as described (31). To express Set11 and EGFP-fused3 Set11 in S. pombe, the set11 + coding sequence was cloned into the multi-ple cloning site of pREP1 (a multicopy episomal plasmid carrying the nmt1 promoter) or a pREP1 derivative containing the EGFP-coding sequence (pREP1-EGFP), and the resulting plasmids were designated pREP1-set11 + and pREP1-EGFP-set11 + , respectively. To obtain S. pombe strains expressing Set11 with short internal deletions (∆NHSPI89–192 and ∆GEQFLCY216–224), the set11 + -coding sequence was first cloned into the pCR2.1-TOPO plasmid (Invitrogen), and each deletion was introduced into E. coli strain BL21(DE3) by transformation. To express the wild-type SET domain with the mutated domain, strains that lost the ura4 + gene by internal homologous recombination were isolated using counter-selective medium containing 5-fluoroorotic acid. The deletions of the set11 + coding region were confirmed by PCR. All other strains were constructed using standard genetic crosses.

**Expression and Purification of Recombinant Proteins**—Expression vectors for N-terminal His-tagged Set11 (His-Set11) and C-terminal His-tagged wild-type and mutant Rpl12 (Rpl12-His, Rpl12ΔC-His, Rpl12ΔC-HisK43A, Rpl12ΔC-HisK10A, Rpl12ΔC-HisK10A, Rpl12ΔC-HisK39,40A, and Rpl12ΔC-HisK39,40A) were introduced into E. coli strain BL21(DE3) or BL21(DE3)-pLysS. Protein expression was induced by adding 0.5–1.0 mM isopropyl-β-D-thiogalactopyranoside. The culture was incubated for 2 h more at 25 °C (for His-Set11) or 37 °C (for Rpl12-His and its derivatives) before harvesting, and the cells were then lysed by sonication (for His-Set11) or with buffer containing guanidine hydrochloride (for Rpl12-His). The His-tagged proteins were purified using TALON metal affinity resin, according to the manufacturer’s instructions (Invitrogen). The eluted materials were dialyzed against phosphate-buffered saline alone or phosphate-buffered saline with 10% glycerol, divided into aliquots, and stored at −80 °C before use.

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### TABLE 1

| Product a | Open reading frame b | Substrate | Localization c | S. cerevisiae (open reading frame) d | M. musculus e | Other domains | Reference |
|-----------|---------------------|-----------|----------------|------------------------------------|---------------|--------------|-----------|
| Set1      | SPCC306.04c         | Histone H3K4 | Nucleus        | Set1 (YHR119w)                     | MIL2          | RRM          | Ref. 25    |
| Set2      | SPAC29B12.02c       | Histone H3K36 | Nucleus        | Set2 (YIL168c)                     | Whsc11        | SRI          | Ref. 26    |
| Set3      | SPAC22E12.11c       | Unknown    | Nucleus        | Set3 (YKR029c)                     | M115          | PHI          |           |
|           |                     |           |                | Set4 (YIL105w)                     |               |              |           |
| Cbr4      | SPBC428.08c         | Histone H3K9 | Nucleus        | None                               | Swc39h1.2     | Chromo       | Ref. 24    |
| Set5      | SPCC1739.05         | Unknown    | Nucleus         | None                               | Hkm-B         |              |           |
| Set6      | SPBP897.07c         | Unknown    | Nucleus         | None                               | Zym1          |              |           |
| Set7      | SPCC297.04c         | Unknown    | Cytoplasm       | None                               | G9a, Glp1     |              |           |
| Set8      | SPAC3C7.09          | Unknown    | Nucleus/cytosol | None                               | XP_134310     | Similar to Set10 | Ref. 15 |
| Set9      | SPCC4R3.12          | Histone H4k20 | Nucleus        | None                               | Su4–20h1,2    |              |           |
| Set10     | SPBC1709.13c        | Unknown    | Nucleus         | None                               | NP_082338     | Similar to Set8 | Ref. 27 |
|           |                     |           |                | Rkm1 (YLP268w)                     |               |              |           |
|           |                     |           |                | YHL039w (YHL039w)                  |               |              |           |
| Set11     | SPCC1223.04c        | Unknown    | Nucleus        | None                               | Rkm2 (YDR198c) |              |           |
| Set12     | SPBC16C6.01c        | Unknown    | Nucleus/cytosol | None                               | C21orf18      |              |           |
|           |                     |           |                | Set7 (YDR257c)                     |               |              |           |
| Set13     | SPAC688.14          | Unknown    | Nucleus        | Set7 (YDR257c)                     |               |              |           |

* The naming of the S. pombe SET domain proteins is defined as published previously (25, 35).
* a S. pombe GeneDB (http://www.genedb.org/genedb/pombe/).
* b Saccharomyces Genome Database (http://www.yeastgenome.org/).
* c The most related proteins in S. cerevisiae and M. musculus are listed with reference to Refs. 27 and 35. No apparent ortholog for S. cerevisiae Set6 (YPL165c) or YBR030w has been identified in the S. pombe genome.

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3 The abbreviations used are: EGFP, enhanced green fluorescent protein; [3H]AddMet, S-adenosyl-L-[methyl-3H]methionine; PAGE, polyacrylamide gel electrophoresis; LC, nano-liquid chromatography; MS/MS, tandem mass spectrometry; AUT, acetic acid-urea-Trition X-100; AUC, acetic acid-urea-cetyltrimethylammonium bromide.
Antibodies—To obtain anti-Set11 antibodies, recombinant His-Set11 was used to immunize rabbits. Antibodies in crude antisera were used for Western blot analysis. Other antibodies used in this study were: anti-GFP (clones 7.1 and 13.1; Roche Applied Science, 11814460001) and anti-RPL19 (clone 3H4; Abnova, H00006143-M01).

Preparation of HeLa and Yeast Cell Extracts—To prepare histone-enriched HeLa nuclear extracts, HeLa cells grown to ~80–90% confluence in 100-mm dishes were washed twice with phosphate-buffered saline and then lysed in 1 ml (per dish) of nuclear lysis buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 0.65% Nonidet P-40, 10 mM butyrate, pH 8.0, 1 mM phenylmethylsulfonyl fluoride) supplemented with proteinase inhibitor mixture (Complete™ EDTA-free; Roche Applied Science). The nuclei were harvested by scraping into a 1.5-ml microcentrifuge tube and collected by centrifugation at 22,000 × g for 5 min at 4 °C. After removal of the supernatant, the nuclei were resuspended in 200 μl of 0.4 × H₂SO₄, and the suspension was further incubated with rotation for 2 h at 4 °C. The insoluble fraction was removed by centrifugation at 5,500 × g for 5 min at 4 °C. The acid-soluble supernatant was collected into a new centrifuge tube, and the proteins were precipitated with 50 μl of 100% trichloroacetic acid (final concentration, 20%). The suspension was placed on ice for 1 h and spun at 22,000 × g for 15 min at 4 °C. The protein pellet was washed once with acidified acetone (0.1–0.3% HCl) and twice with acetone, air-dried, and resuspended in 100 μl of deionized H₂O by vortexing. After the recovery was estimated, the extracted proteins were stored at ~80 °C until use. *S. pombe* nuclear extracts of the wild-type or Δset11 mutant cells were prepared as described previously (32).

In Vitro Methyltransferase Assay—Several micrograms of HeLa or *S. pombe* nuclear extract were incubated with 1 μCi of S-adenosyl-l-[methyl-³H]methionine ([³H]AdoMet; 85 Ci/mmol) and 2 μg of recombinant His-Set11 or His-Clr4 protein in 25 μl of MTase buffer (50 mM Tris-HCl, pH 8.0, 10% (v/v) glycerol) for 1 h at 30 °C. The reaction was terminated by adding 6 × SDS loading buffer (300 mM Tris-HCl, pH 6.8, 12% SDS, 30% (v/v) glycerol, 600 mM 2-mercaptoethanol, 0.3% bromphenol blue), and the proteins were resolved on 15% SDS-polyacrylamide gel electrophoresis (PAGE) gels. After fixation with sodium solution (50% methanol, 10% acetic acid) for 30 min, the gels were soaked with gentle shaking in radio-sensitizing reagent (Amplify fluorographic reagent; GE Healthcare) for 30 min, dried, and exposed to x-ray film (Hyperfilm MP; GE Healthcare).

Two-dimensional Electrophoretic Analysis of Proteins—The two-dimensional gel analysis of methylated proteins was performed as described previously (33).

Chromatographic Fractionation of Proteins—*S. pombe* cell extracts prepared as described above were loaded on a SOURCE 15RPC ST 4.6/100 column (GE Healthcare) that had been equilibrated with Eluent A (0.065% trifluoroacetic acid in water) using a liquid chromatography system (AKT/Aexplore 105; GE Healthcare). The bound proteins were eluted with a 2–100% linear gradient of Eluent B (0.055% trifluoroacetic acid in acetonitrile). The proteins in each fraction were dried under a vacuum, dissolved in deionized H₂O, and subjected to the in vitro methyltransferase assay.

Analysis of Methylated Peptide by Nano-liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)—The proteins in each gel slice were subjected to reduction with 10 mM dithiothreitol, alkylation with 55 mM iodoacetamide, and digestion with 10 μg/ml modified trypsin (Promega), 20 μg/ml ArgC (Sigma), or 20 μg/ml GluC (Sigma) at 37 °C for 16 h. After the in-gel digestion, the peptides were extracted with 5% formic acid and 50% acetonitrile, dried under a vacuum, and dissolved in 2% acetonitrile and 0.1% formic acid. The multiple digested peptides were then fractionated by C18 reverse-phase chromatography (Paradigm MS4; Microm BioResources) and applied directly into a quadrupole ion trap mass spectrometer (Finni-
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Fractionation of Ribosomes—
Fractionation of ribosomes from the wild-type and Δset11 mutant S. pombe strains was performed as described previously (34).

Microscopy Analysis—To analyze the localization of EGFP-fused Set11, wild-type and Δset11 mutant S. pombe cells were transformed with the pREP1-EGFP-set11+ plasmid. The transformed cells were cultured on plates with minimal medium lacking leucine (AA-leu). Single colonies were picked and patched onto new plates. The cells were grown to early log phase in liquid medium and washed twice with deionized H2O, and the DNA was visualized by incubation with 1 μg/ml Hoechst 33342. Microscopic images were captured on a Zeiss Axioplan 2 imaging microscope and an ORCA-ER camera (Hamamatsu).

Spotting Assay—Wild-type and mutant (Δset11 and Δrpl1202) cells were grown in YEA medium. 5-fold serial dilutions were made (1 × 10^7–1.6 × 10^4 cells/ml), and 5–10 μl was spotted on plates with YEA alone or YEA containing 10–30 μg/ml cycloheximide. The plates were then incubated at 30°C for 2.5–4 days. For cells harboring the pREP1-set11+ plasmid, minimal medium lacking leucine (AA-leu) was used for the culture and spotting.

RESULTS

S. pombe set11+ Encodes an Active Methyltransferase—In the fission yeast S. pombe, at least 13 genes that potentially encode SET domain-containing proteins (SET proteins) have been identified in the genome (Table 1). Among these genes, four (set1+; set2+, clr4+, and set9+) encode histone lysine methyltransferases (24–27). Although several of the encoded SET proteins have homologous counterparts in other organisms (35) (Table 1), the function of the remaining nine SET proteins is unclear. To elucidate the roles of the SET proteins in S. pombe, we started by analyzing the enzymatic activity of these uncharacterized proteins using an in vitro methyltransferase assay. Based on a previous genome-wide protein localization analysis (36), we initially examined Set3, Set11, and Set13, because they are localized predominantly to the nucleus (Table 1). We prepared recombinant His-tagged
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The set11 mutant strains were fractionated by reverse-phase chromatography, and the eluted proteins were tested in the in vitro methyltransferase assay. As shown in Fig. 2B, the target protein(s) was eluted in several fractions with a peak at fraction 22. The protein band showing the same elution profile in the chromatography was excised and subjected to LC-MS/MS analysis. From both the two-dimensional gel and chromatographic approaches, we obtained a series of peptides that matched perfectly with the deduced amino acid sequence of S. pombe ribosomal protein L12 (Rpl12) (Fig. 2C). Rpl12, a counterpart of bacterial L11 protein, is a highly conserved protein from yeast to humans (Fig. 2C). It was recently shown that Rpl12 in S. cerevisiae is methylated at Lys\(^{10}\) by a SET domain-containing protein, Rkm2 (18).

Recombinant Rpl12 Is Methylated in Vitro by Set11—To confirm that Rpl12 is a physiological substrate for Set11, an in vitro methyltransferase assay was performed using recombinant full-length and C-terminal-deleted Rpl12 proteins (Fig. 3A, Rpl12-His and Rpl12AC-His). We found that full-length Rpl12-His was clearly methylated by Set11 and that Rpl12AC-His was a better substrate for the Set11 methyltransferase activity (Fig. 3B). These results indicated that Rpl12 is a physiological substrate for Set11 and demonstrate that its methylation site(s) may have been modified already by endogenous Set11 in the wild-type cells. In addition, these results imply that p17 and its target site(s) are evolutionarily conserved from fission yeast to humans.

Set11 Modifies the Ribosomal Protein Rpl12 in S. pombe—To identify the target protein(s) of the Set11 methyltransferase, we first fractionated the methylated product(s) by two-dimensional acetic acid-urea-Triton X-100 (AUT) and acetic acid-urea-cetyltrimethylammonium bromide (AUC) gel analysis, which is generally used to analyze histone species (33). After separation on the first (AUT) and second (AUC) gels, two discrete signals were detected in the autoradiograph (Fig. 2A). These two protein species migrated to almost the same level in the first AUT gel but showed a different migration pattern in the second AUC gel. The difference might be attributable to the presence or absence of modifications on proteins for full-length Set11 (His-Set11) and Set13 (His-Set13), and the N-terminal half of Set3 (His-Set3N) (Fig. 1A and data not shown), and incubated each of these recombinant proteins with histone-enriched HeLa nuclear extract in the presence of a \(^{3}H\)-labeled methyl donor ([\(^{3}H\)]AdoMet). Recombinant His-tagged Clr4 (His-Clr4) was also tested as a control for histone H3-K9 methyltransferase (24). Although no activity was detected for His-Set13 and His-Set3N under these conditions, we found that His-Set11 possessed an intrinsic methyltransferase activity and that it specifically methylated a protein(s) with a molecular mass of \(~17\) kDa on SDS-PAGE (Fig. 1B, p17me). Because no methylated band was detected in the mock experiments (Fig. 1B, mock), it is unlikely that the methylation of p17 was catalyzed by endogenous methyltransferases. These results led us to focus on the physiological roles and substrate(s) of the Set11 methyltransferase.

Because the methylated protein(s) appeared to migrate slightly more slowly than that of histone H3 methylated by His-Clr4 (Fig. 1B), it was likely that Set11 target protein(s) was a minor histone species or other nuclear protein. To characterize this substrate of Set11, we performed the same in vitro methyltransferase assay using histone-enriched nuclear extracts prepared from wild-type or Δset11 mutant S. pombe strains. Although specific methylation signals were not detected in the assay using wild-type cellular extract, a strongly methylated band with a similar molecular mass as in the HeLa nuclear extract (\(~17\) kDa) was observed in the assay using the Δset11 cellular extract (Fig. 1C, p17me). These results suggested that p17 is a physiological target of the Set11 methyltransferase, and that its methylation site(s) may have been modified already by endogenous Set11 in the wild-type cells. In addition, these results imply that p17 and its target site(s) are evolutionarily conserved from fission yeast to humans.

**FIGURE 3. Recombinant Rpl12 is methylated in vitro by His-Set11.** A, schematic drawing of full-length and C-terminal-deleted Rpl12 (Rpl12-His and Rpl12AC-His). The position of a lysine residue is indicated by a K. Potentially methylated residues deduced from mass spectrometry analysis are marked in red. The same residues replaced with alanine in substitution experiments (C). The initial methionine (M), amino acid numbers, and expected molecular mass are also shown. B and C, in vitro methyltransferase assay using recombinant Rpl12-His, Rpl12AC-His, and alanine-substituted mutants of Rpl12 were incubated with His-Set11 and [\(^{3}H\)]AdoMet. The proteins were resolved by 15% SDS-PAGE and visualized by Coomassie staining (B, left panel; C, bottom panel). Proteins methylated by Set11 were detected by autoradiography (B, right panel; C, top panel).
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strated that the methylated residue(s) resides in the N-terminal 1–106 residues of Rpl12. The role played by the C-terminal region of Rpl12 in the methyltransferase reaction is currently unclear, although it is possible that steric interactions between the N- and C-terminal domains of Rpl12 affect the ability of Set11 to methylate its target site. Because we failed to produce an N-terminally deleted recombinant Rpl12 mutant in E. coli, we cannot exclude the possibility that a target residue(s) of Set11 is also present in the C-terminal region of Rpl12. However, previous studies on E. coli L11 (10) and S. cerevisiae L12 (18) and our mass spectrometric analysis support the idea that a specific lysine residue in the N-terminal region of Rpl12 is the preferred target site of Set11 (see below).

To characterize further the site of Rpl12 methylation, we introduced a series of Ala substitutions for the candidate Lys residues in Rpl12ΔC-His (Fig. 3A, indicated by red) and used these mutant proteins in the in vitro methyltransferase assay (Fig. 3C). We found that several combinations of the Ala substitution greatly affected the Set11 methyltransferase activity (see Rpl12ΔC-HisK3, 10, 82, 85, 92, 93, 95A and Rpl12ΔC-HisK39, 40, 82, 85, 92, 93, 95A). Further detailed mapping revealed that the Ala substitution of Lys3 completely abolished the Set11 methylation activity (Rpl12ΔC-HisK3A). In addition, the combined Ala substitution of Lys39 and Lys40 severely reduced the Set11 methyltransferase activity (Rpl12ΔC-HisK39, 40A). These results suggested that the three residues Lys3, Lys39, and Lys40 of Rpl12 are critical to the in vitro methyltransferase activity of Set11. It has been reported that Lys10 of Rpl12 is the target of S. cerevisiae Rkm2 (18), which is closely related to S. pombe Set11 (Table 1). However, this site does not appear to be an exclusive target of Set11, because the mutant Rpl12ΔC-His with an Ala substitution at Lys10 (Rpl12ΔC-HisK10A)
was clearly methylated by Set11 (Fig. 3C). Taken together, these results suggest that Rpl12 is a physiological substrate of Set11 and that the Lys3, and potentially Lys39 and Lys40, of Rpl12 are the candidate target residues for Set11 activity.

**Determination of the Methylation Sites of Rpl12 by MS/MS—**To determine the in vivo methylation sites of Rpl12, the endogenous Rpl12 in wild-type (set11−) or Δset11 mutant cells was isolated by reverse-phase chromatography (as shown in Fig. 2B). The corresponding band was excised from an SDS-PAGE gel and digested with ArgC or GluC peptidase, which cleaves preferentially on the C-terminal side of arginine or glutamate residues. The digested peptide fragments were then analyzed using LC-MS/MS. The overall elution profiles of ArgC-digested Rpl12 peptides in the nano-LC spectra were superimposed, and the masses of representative peaks were matched between the wild-type and Δset11 mutant cells (Fig. 4, A and D). However, a relatively abundant peptide was eluted in a prominent peak at 13.20 min in the Δset11 mutant (Fig. 4D, asterisk) that was not observed in the wild-type cells. MS/MS analysis revealed that the amino acid sequence of this fragment matched the N-terminal residues 1–15 of Rpl12 with the initial Met removed (Fig. 4F). The experimental mass of this peptide calculated from the mean m/z of MH3+ was 1848.59 ± 0.32 (MH+) (Table 2). The corresponding 1–15 peptide for Rpl12 in wild-type cells was eluted at 13.34 min (Fig. 4, A, asterisk, and C), and its experimental mass was 1890.37 ± 0.49 (MH+) (Table 2). Of particular interest was that the mass difference between these wild-type and Δset11 mutant peptides was ~41.78 Da, which corresponds to the mass of three methyl groups. Although there are two potentially methylated lysine residues in this peptide at Lys3 and Lys30, the MS/MS results (Fig. 4, C and F) and parallel experiments using GluC, in which a similar ~42-Da difference was observed for the N-terminal 1–8 peptide (supplemental Fig. S1 and Table 2), strongly suggest that the Rpl12 in wild-type cells is trimethylated at Lys3, and this methylation is absent in the Δset11 mutant cells. This is quite consistent with our observation obtained from the in vitro methyltransferase assay (Fig. 3C).

Of note, the mass of the N-terminal 1–8 peptide even in the Δset11 mutant was still 28–29 Da larger than that of the theoretical mass of the corresponding peptide (Table 2; experimental mass, 972.04 (MH+) and theoretical mass, 943.45). Although the margin of mass error needs to be considered, the additional mass appears to correspond to that of two methyl groups. In addition, MS/MS results using ArgC or GluC suggested that the additional mass could be localized to the first three amino acids, aside from the e-N-trimethylation at Lys3. These observations support the idea that the N-terminal Proline 1 (Pro1) is modified by Set11—To investigate the enzymatic function of Set11 in vivo, we introduced short internal deletions (ΔNHSP189–192; ΔNHSP and ΔGEQFLCY 216–224; ΔGE-Y) into the two conserved regions of the core SET domain in Set11 (2). Nuclear extracts prepared from wild-type or mutant strains (Δset11, Δset11NHSP, and Δset11GE-Y) were then subjected to the in vitro methyltransferase assay (Fig. 5A). Rpl12 from the Δset11NHSP and Δset11GE-Y strains was clearly methylated by recombinant His-Set11 to the same extent as that from Δset11, indicating that these deletions abolished the in vivo enzymatic activity of the endogenous Set11.

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**TABLE 2**

| Peptide          | Peptidase | Strain   | Observed mass | Experimental mass | Theoretical mass | Difference |
|------------------|-----------|----------|---------------|------------------|-----------------|------------|
| PPKFDPNEVKTFIMR  | ArgC      | Wild type| 630.79 (MH3+) | 1890.37 (MH+)    | 1818.96 (MH+)   | +71.41     |
|                  |           | Δset11   | 616.86 (MH3+) | 1848.59 (MH+)    | 1818.96 (MH+)   | +29.63     |
| PPKFDPNE         | GluC      | Wild type| 507.63 (MH3+) | 1014.26 (MH+)    | 943.45 (MH+)    | +70.81     |
|                  |           | Δset11   | 486.52 (MH3+) | 972.04 (MH+)     | 943.45 (MH+)    | +28.59     |
| VAGGSTAPKIGPLGLSPKVEGE | GluC | Wild type | 731.32 (MH3+) | 2191.96 (MH+)    | 2176.27 (MH+)   | +15.69     |
|                  |           | Δset11   | 1103.13 (MH3+) | 2205.04 (MH+)  | 2176.27 (MH+)   | +28.77     |
|                  |           |          | 735.91 (MH3+) | 2205.72 (MH+)    | 2176.27 (MH+)   | +39.45     |
|                  |           |          | 754.74 (MH3+) | 2262.21 (MH+)    | 2176.27 (MH+)   | +85.94     |
|                  |           | Δset11   | 731.30 (MH3+) | 2191.98 (MH+)    | 2176.27 (MH+)   | +15.69     |
|                  |           |          | 1103.02 (MH3+) | 2205.06 (MH+)  | 2176.27 (MH+)   | +28.79     |
|                  |           |          | 735.90 (MH3+) | 2205.72 (MH+)    | 2176.27 (MH+)   | +29.45     |
|                  |           |          | 754.71 (MH3+) | 2262.21 (MH+)    | 2176.27 (MH+)   | +85.94     |
| LTIQNBQAAVSVPVSALVPE (61–82 amino acids) | Trypsin | Wild type | 1140.57 (MH3+) | 2279.12 (MH+)    | 2265.33 (MH+)   | +13.79     |

* ^a Average of at least three independently observed masses.
* ^b Representative masses.
proteins. These results demonstrate that the two conserved regions of the SET domain core are important for the catalytic site for Set11 methyltransferase activity.

To obtain further insight into the physiological role of Set11, we examined its localization by expressing EGFP-fused Set11 protein in wild-type or Δset11 mutant cells. Although the previous study described a nuclear localization of Set11 (36), EGFP-Set11 predominantly localized to the nucleolus, which coincides with the nuclear hemisphere that stained weakly with Hoechst 33342, in both wild-type and Δset11 mutant cells (Fig. 5B). These results suggest that the Lys3 methylation of Rpl12 occurs during the ribosome assembly process in the nucleolus and support the idea that, if Set11 has substrates other than Rpl12, they are probably also involved in ribosomal assembly or function.

S. pombe cells in which the set11 gene was completely deleted or that expressed catalytically inactivated Set11 protein were viable and showed no growth defects under a variety of conditions (data not shown). Bacterial ribosomal protein L11, the counterpart of eukaryotic Rpl12, binds a highly conserved domain of 23 S rRNA and is thought to be involved in the ribosomal GTPase activity of the dynamic decoding process (38). Although little is known about the roles played by L11 methylation in this process, the methyl modifications of Rpl12 could play a role in its association with the ribosome or in ribosomal function. To test these possibilities, we first examined whether the Rpl12 methylation affects its association with the ribosome. Mature ribosomes were isolated from wild-type and Δset11 mutant cells by sucrose density centrifugation, and the level of Rpl12 associated with the ribosomes was assayed by immunoblotting. We found, however, that the level of Rpl12 was not affected by the Δset11 mutation (Fig. 5C).

Next, to examine the potential role of Rpl12 methylation in ribosomal function, we analyzed the sensitivity to cycloheximide of Δset11 mutant cells. Cycloheximide is a widely used compound that inhibits protein synthesis by blocking transla-
S. pombe Set11 Modifies Ribosomal Protein Rpl12

Summary of post-translational methyl modifications in E. coli L11 and in Rpl12 of eukaryotic cells. Schematic representations of E. coli L11, S. cerevisiae Rpl12, S. pombe Rpl12, and human Rpl12 are shown. The methylation sites and corresponding enzymes are depicted (10, 17, 18). The red lollipops represent methyl modifications.

FIGURE 6. Summary of post-translational methyl modifications in E. coli L11 and in Rpl12 of eukaryotic cells. Schematic representations of E. coli L11, S. cerevisiae Rpl12, S. pombe Rpl12, and human Rpl12 are shown. The methylation sites and corresponding enzymes are depicted (10, 17, 18). The red lollipops represent methyl modifications.

In this study, we used an *in vitro* methyltransferase assay and identified Rpl12 as a physiological substrate of Set11, which is the first SET domain-containing methyltransferase in *S. pombe* discovered to modify a non-histone protein. Although the targets of *S. cerevisiae* Rkm1 and Rkm2 were identified by a combination of *in vitro* labeling of mutant strains for potential SET methyltransferases and mass spectrometric analysis (15, 18), our *in vitro* methyltransferase assay appears to be useful for identifying physiological substrate(s) of uncharacterized SET domain methyltransferases. Indeed, by applying this approach, the growth defect was simply caused by a change in Rpl12 stability. Although it remains to be tested whether this effect is attributable to the disturbance in Rpl12 modification, these results indicate that set11+ expression is tightly regulated, and its level is critical for proper cellular function.

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we have identified the substrate of Set13 methyltransferase as another ribosomal protein.4 Considering that S. pombe Set8 and Set10 show similarity with Rkm1 (Table 1), it is highly likely that they also target and modify ribosomal proteins. If this is the case, at least four S. pombe SET methyltransferases, Set8, Set10, Set11, and Set13, are involved in the modification of ribosomal proteins. In this respect, one hypothesis to explain this is that a subfamily of SET methyltransferases may have evolved to target the lysine residues of highly basic proteins, such as histones and ribosomal proteins, that stably associate with DNAs and RNAs, respectively (16).

Although our results demonstrate that Rpl12 is a physiological substrate of Set11 methyltransferase, it is also possible that Set11 has other substrate proteins. We have searched for potential target proteins by using different fractions of cellular lysate. However, we have yet to identify any other target proteins for Set11 (data not shown). Considering the nucleolar localization of Set11, it is likely that its other substrate proteins would also be involved in ribosomal biogenesis. Our results indicate that Rpl12 is methylated at five residues: α-N-dimethylation at Pro3, ε-N-trimethylation at Lys39 and/or Lys40, and 6-N-monomethylation at Arg66 and thus underscore the evolutionary conservation of Rpl12 methylation (Fig. 6). Although we showed that Set11 modifies Lys39 and it is likely that an Rmt2 homolog (SPAC26A3.17c) catalyzes Arg66 methylation, the responsible enzymes for the other methyl modifications remain unclear. Detailed mass spectrometric analyses of Rpl12 treated with candidate SET methyltransferases and their mutants will help identify the responsible enzyme(s) for the Lys39/Lys40 methylation.

N-terminal methylation has been described for only a small number of proteins (41). Of ribosomal proteins, S. cerevisiae Rps25 was recently shown to be dimethylated at the α-N-terminal proline after cleavage of the initial methionine (37). Interestingly, in the N-terminal sequence of S. cerevisiae Rps25, [M]PPKQ0, the first three residues are the same as those of Rpl12 ([M]PPKFD-). In addition, human RCC1 has also been shown to have α-N-terminal methylation (42). Although the methylating enzyme has yet to be determined, mutational analysis of human RCC1 revealed that [M]-[S/P/A]-P-K serves as a substrate recognition motif for N-terminal methylation, which is consistent with yeast Rpl12 and Rps25, implying that there are conserved mechanisms for N-terminal methylation. In most cases, the physiological function of N-terminal methylation is unclear. However, intriguingly, methylation-defective mutants of RCC1 bind less effectively to chromatin during mitosis, which causes a spindle pole defect (42). Therefore, it is possible that the N-terminal methylation of Rpl12 regulates its interaction with ribosomal RNA.

The physiological function of the Set11 methyltransferase is unclear. Yeast cells lacking the enzyme are viable, and no particular defect in ribosomal function was observed in Set11 cells. The same is true for the Δrkm2 mutant budding yeast cells (18) and ΔprmA mutant E. coli cells (12). Because Rpl12 possesses multiple methyl modifications, it is possible that these modifications act cooperatively, and the roles played by the methyl modifications may become clear when set11 is combined with other mutations in the relevant SET domain methyltransferases. We have shown that Set11 predominantly localizes to the nucleolus, suggesting that the methylation of Rpl12 at Lys3 occurs during the ribosome assembly processes. The dynamics and stability of methyl methylation are not known. Because Rpl12 prepared from wild-type cells was not a good substrate for recombinant Set11 in vitro, it is likely that Rpl12 is predominantly methylated at Lys3 in wild-type cells. Interestingly, we found that the overproduction of Set11 caused a severe growth defect (Fig. 5E). In Caenorhabditis elegans, Rpl12 regulates its own splicing, and the overproduction of Rpl12 increases the proportion of unproductively spliced mRNAs (43). A similar alternative splicing mechanism has been suggested for the production of mammalian Rpl12 (44). Although the molecular mechanisms underlying the effect of Set11 overproduction are currently unclear, it is possible that Rpl12 or another Set11 target protein(s) is involved in controlling the levels of ribosomal proteins through its RNA binding properties, and methyl modification may modulate this regulatory function. It is also possible that the autoregulation system of ribosomal biogenesis as observed for C. elegans Rpl12 may suppress the effect of the Δset11 mutation. These hypotheses will be tested in future studies that take ribosome homeostasis into consideration.

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