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A Conserved SET Domain Methyltransferase, Set11, Modifies Ribosomal Protein Rpl12 in Fission Yeast*[S]

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Mahito Sadaie[‡1], **Kaori Shinmyozu**[§], **and Jun-ichi Nakayama**[‡2]

From the [‡]*Laboratory for Chromatin Dynamics and the* [§]*Proteomics Support Unit, Center for Developmental Biology, RIKEN, Kobe, Hyogo 650-0047, Japan*

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* methylation 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.

The post-translational modification of proteins is a ubiquitous mechanism by which cells expand the function of proteins. The SET domain was originally identified in three *Drosophila* proteins, Su(var)3-9, Enhancer of zest, and Trithorax, which are all involved in epigenetic gene regulation (1) and were later demonstrated to be histone lysine methyltransferases (2, 3). Although specific lysine residues in histone tails are generally 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^1 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*-dimethylated 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^3 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 (Prmt3) 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^9-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*

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1 and S2 and Fig. S1.

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[2] To whom correspondence should be addressed: RIKEN, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe, Hyogo 650-0047, Japan. Tel.: 81-78-306-3205; Fax: 81-78-306-3208; E-mail: jnakayam@cdb.riken.jp.

TABLE 1
SET domain proteins in *S. pombe*

Product[a]	Open reading frame[b]	Substrate	Localization[c]	*S. cerevisiae* (open reading frame)[d,e]	*M. musculus*[e]	Other domains	Reference
Set1	SPCC306.04c	Histone H3K4	Nucleus	Set1 (YHR119w)	Mll2	RRM	Ref. 25
Set2	SPAC29B12.02c	Histone H3K36	Nucleus	Set2 (YJL168c)	Whsc111	SRI	Ref. 26
Set3	SPAC22E12.11c	Unknown	Nucleus	Set3 (YKR029c) Set4 (YJL105w)	M115	PHD	
Clr4	SPBC428.08c	Histone H3K9	Nucleus	None	Suv39h1, 2	Chromo	Ref. 24
Set5	SPCC1739.05	Unknown	Nucleus/cytosol	Set5 (YHR207c)	Hskm-B		
Set6	SPBP8B7.07c	Unknown	Nucleus/cytosol	None	Zym1	zf-MYND	
Set7	SPCC297.04c	Unknown	Cytosol	None	G9a, Glp 1		
Set8	SPAC3C7.09	Unknown	Nucleus/cytosol	YHL039w (YHL039w) Rkm1 (YPL208w)	XP_134310	Similar to Set10	Ref. 15
Set9	SPCC4B3.12	Histone H4K20	Nucleus	None	Suv4–20h1, 2		Ref. 27
Set10	SPBC1709.13c	Unknown	Nucleus	YHL039w (YHL039w) Rkm1 (YPL208w)	NP_082538	Similar to Set8	Ref. 15
Set11	SPCC1223.04c	Unknown	Nucleus	Rkm2 (YDR198c)	C21orf18		Ref. 18
Set12	SPBC16C6.01c	Unknown	Nucleus/cytosol	None	(XP_134310)		
Set13	SPAC688.14	Unknown	Nucleus	Set7 (YDR257c)	(XP_134310)		

[a] The naming of the *S. pombe* SET domain proteins is defined as published previously (25, 35).
[b] *S. pombe* GeneDB (http://www.genedb.org/genedb/pombe/).
[c] Ref. 36.
[d] *Saccharomyces* Genome Database (http://www.yeastgenome.org/).
[e] 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.

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 Lys^{10} (18), it preferentially catalyzed ϵ-*N*-trimethylation at Lys^{3} of Rpl12. We further confirmed the methylation site modified by Set11 by mass spectrometric analysis, which led us to identify other unique methylation sites of Rpl12. Finally, we showed that Set11 predominantly localized to the nucleolus in *S. pombe*, suggesting that it functions 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 pRSET (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-fused[3] Set11 in *S. pombe*, the *set11*$^+$ coding sequence was cloned into the multiple 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 (ΔNHSP189–192 and ΔGEQIFLCY216–224), the *set11*$^+$-coding sequence was first cloned into the pCR2.1-TOPO plasmid (Invitrogen), and each deletion was introduced by *in vitro* site-directed mutagenesis (31). After insertion of a *ura4*$^+$ marker gene, the resultant plasmid was digested with NruI and introduced into the original *set11*$^+$ locus of the SPYB109 strain by isolating *ura4*$^+$-expressing cells. To replace 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-His^{K3A}, Rpl12ΔC-His^{K10A}, Rpl12ΔC-$His^{K3,10A}$, Rpl12ΔC-$His^{K39,40A}$, Rpl12ΔC-$His^{K3,10,82,85,92,93,95A}$, and Rpl12ΔC-$His^{K39,40,82,85,92,93,95A}$) 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.

[3] The abbreviations used are: EGFP, enhanced green fluorescent protein; [^{3}H]AdoMet, *S*-adenosyl-L-[methyl-^{3}H]methionine; PAGE, polyacrylamide gel electrophoresis; LC, nano-liquid chromatography; MS/MS, tandem mass spectrometry; AUT, acetic acid-urea-Triton 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 $MgCl_2$, 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 500 × *g* for 5 min at 4 °C. After removal of the supernatant, the nuclei were resuspended in 200 μl of 0.4 N H_2SO_4, 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 H_2O 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 Δ*set11* 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-^{3}H]methionine ([^{3}H]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-mercaptoetanol, 0.3% bromphenol blue), and the proteins were resolved on 15% SDS-polyacrylamide gel electrophoresis (PAGE) gels. After fixation with fixing solution (50% methanol, 10% acetic acid) for 30 min, the gels were soaked with gentle shaking in radiosensitizing 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 onto 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 (AKTAexplore 10S; 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 H_2O, and subjected to the *in vitro* methyltransferase assay.

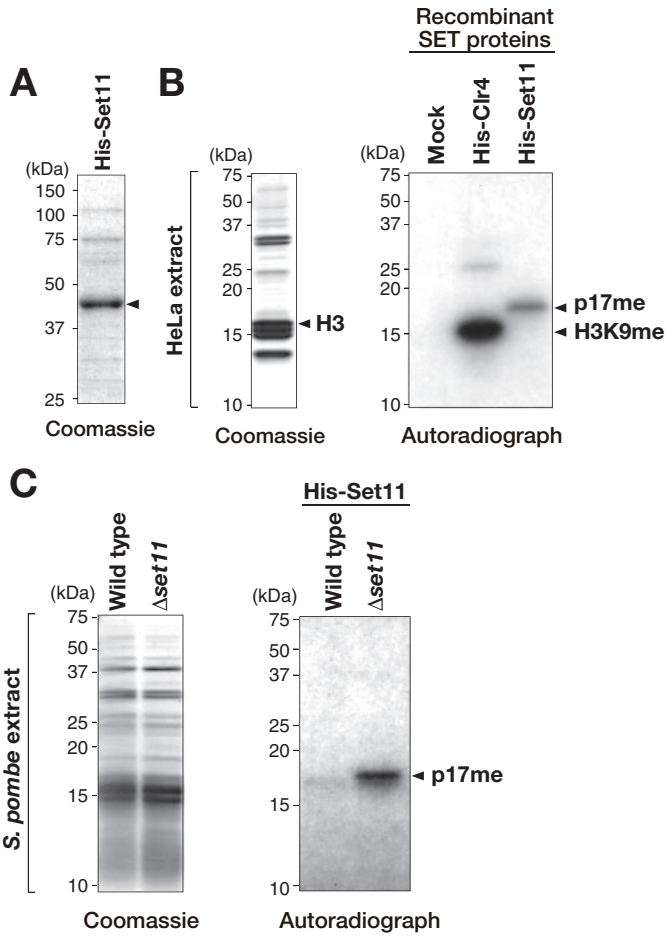
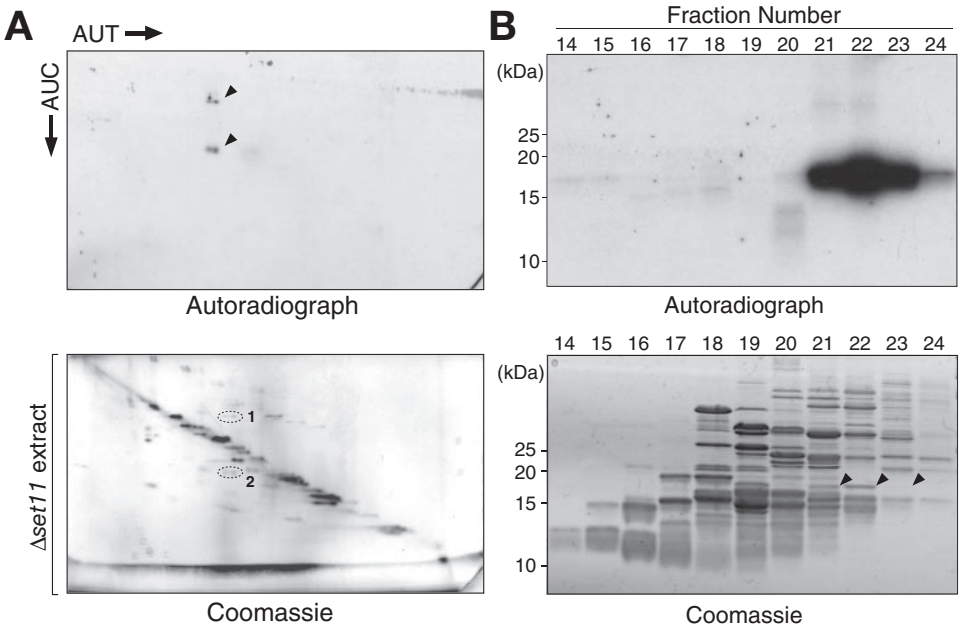


FIGURE 1. ***S. pombe* Set11 specifically methylates a protein in nuclear lysates.** *A*, N-terminal His-tagged Set11 (His-Set11) was expressed in *E. coli* and purified by metal affinity chromatography. The eluted protein was resolved by 10% SDS-PAGE and visualized by Coomassie staining. His-Set11 is indicated by an *arrowhead*. *B*, *in vitro* methyltransferase assay using histone-enriched HeLa nuclear extract. HeLa nuclear extract was resolved on a 15% SDS-PAGE gel and visualized by Coomassie staining (*left*). This extract was incubated with His-Set11 or His-Clr4 (control) in the presence of [^{3}H]AdoMet. After the reaction, the proteins were resolved by 15% SDS-PAGE, and the labeled proteins were detected by autoradiography (*right panel*). The control reaction, lacking recombinant protein, is labeled *Mock*. *C*, *in vitro* methyltransferase assay using *S. pombe* nuclear extract. *S. pombe* nuclear extracts prepared from wild-type or Δ*set11* cells were resolved by 15% SDS-PAGE and visualized by Coomassie staining (*left panel*). These extracts were incubated with His-Set11 and [^{3}H]AdoMet. The proteins were resolved by 15% SDS-PAGE, and labeled proteins were detected by autoradiography (*right panel*). The positions of size markers and labeled proteins are indicated, respectively, to the *left* and *right* of each image.

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 quadrapole ion trap mass spectrometer (Finni-



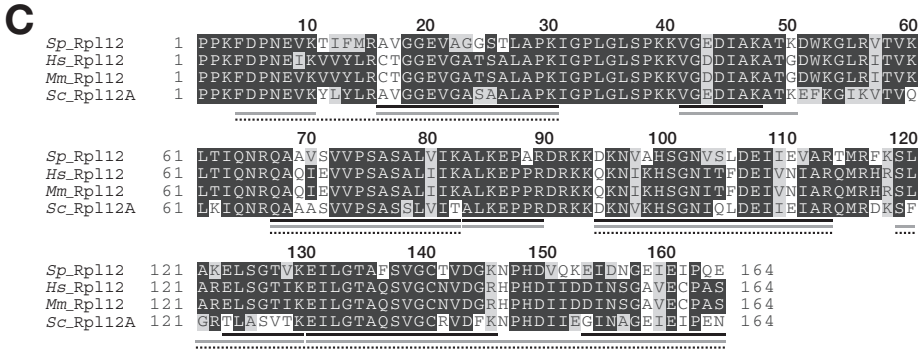


FIGURE 2. **Set11 specifically methylates *S. pombe* ribosomal protein L12.** *A*, two-dimensional gel analysis of methylated proteins. After incubation with His-Set11 and [^{3}H]AdoMet, proteins of the Δ*set11* nuclear extract were separated first in a gel containing 6% (v/v) acetic acid, 8 M urea, and 0.5% Triton X-100 (AUT) and then resolved on a gel containing 6% (v/v) acetic acid and 1.5 M urea, with 0.15% cetyltrimethylammonium bromide in the upper reservoir buffer (33). ^{3}H-Labeled proteins were detected by autoradiography (*top panel*), and the total proteins were detected by Coomassie staining (*bottom panel*). Two spots of ^{3}H-labeled proteins in the autoradiograph and the corresponding spots in the Coomassie-stained gel are indicated by *arrowheads* and *dotted circles*, respectively. *B*, fractionation of Set11 substrate(s) by reverse-phase chromatography. Nuclear extracts prepared from Δ*set11* mutant cells were fractionated by reverse-phase chromatography. The proteins in each fraction were then concentrated and subjected to the *in vitro* methyltransferase assay using His-Set11. After being resolved by 15% SDS-PAGE, ^{3}H-labeled proteins were detected by autoradiography (*top panel*), and the total proteins were detected by Coomassie staining (*bottom panel*). Protein(s) showing a similar migration profile to the ^{3}H-labeled band is indicated by *arrowheads*. *C*, the amino acid sequences of *S. pombe* Rpl12 (SpRpl12), human Rpl12 (HsRpl12), *Mus musculus* Rpl12 (MmRpl12), and *S. cerevisiae* Rpl12 (ScRpl12A) aligned by the ClustalW 1.83 program. Identical amino acids are heavily shaded, and conserved amino acids are shown with light shading. The positions of peptide fragments identified in the LC-MS/MS analysis are indicated by *black* (two-dimensional gel, spot1), *gray* (two-dimensional gel, spot2), and *dotted* (reverse-phase chromatography) *lines* under the alignment.

gan LTQ; Thermo Fisher Scientific) with a Fortis tip mounted on a three-dimensional stage (AMR, Tokyo, Japan). The ion trap was programmed to carry out three successive scans consisting of, first, a full-scan MS over the range 450~2000 *m/z*, and second and third, data-dependent scans of the top two abundant ion obtained in the first scan. Automatic MS/MS spectra were obtained from the highest peak in each scan by setting a relative collision energy of 35% and exclusion time of 15 min for molecules of the same *m/z* value range. The molecular masses of the resulting peptides were searched against the nonredundant NCBI data base using the MASCOT program.

Fractionation of Ribosomes—Fractionation of ribosomes from the wild-type and Δ*set11* mutant *S. pombe* strains was performed as described previously (34).

Microscopy Analysis—To analyze the localization of EGFP-fused Set11, wild-type and Δ*set11* 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 H_2O, 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 (Δ*set11* and Δ*rpl1202*) 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

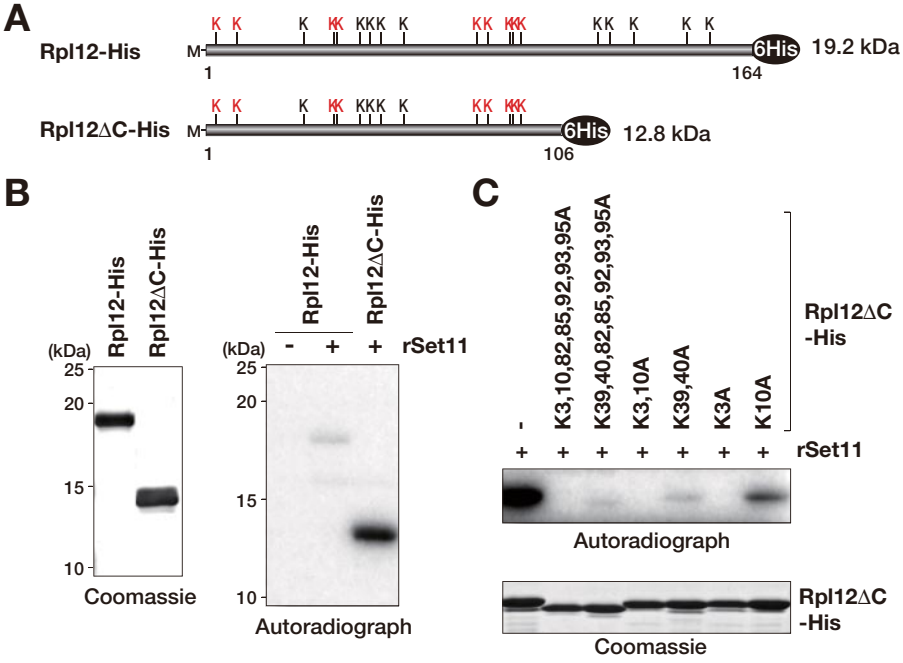


FIGURE 3. **Recombinant Rpl12 is methylated *in vitro* by His-Set11.** *A*, schematic drawing of full-length and C-terminal-deleted Rpl12 (Rpl12-His and Rpl12ΔC-His). The position of a lysine residue is indicated by a *K*. Potentially methylated residues deduced from mass spectrometry analysis are marked 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. Rpl12-His, Rpl12ΔC-His, and alanine-substituted mutants of Rpl12ΔC-His 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*).

proteins for full-length Set11 (His-Set11) and Set13 (His-Set13), and the N-terminal half of Set3 (His-Set3N) (Fig. 1*A* 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 the 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. 1*B*, p17me). Because no methylated band was detected in the mock experiments (Fig. 1*B*, 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. 1*B*), 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 Δ*set11* 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 Δ*set11* cellular extract (Fig. 1*C*, p17me). These results suggested that p17 is a physiological target of 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.

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. 2*A*). 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 modification(s) other than the methylation by Set11. The protein spots corresponding to these signals were excised from the two-dimensional gel and subjected to LC-MS/MS analysis. In parallel with this two-dimensional gel analysis, we also used chromatographic approaches to separate the substrate(s) of Set11 protein. Nuclear extracts prepared from Δ*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. 2*B*, 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. 2*C*). Rpl12, a counterpart of bacterial L11 protein, is a highly conserved protein from yeast to humans (Fig. 2*C*). 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. 3*A*, Rpl12-His and Rpl12ΔC-His). We found that full-length Rpl12-His was clearly methylated by Set11 and that Rpl12ΔC-His was a better substrate for the Set11 methyltransferase activity (Fig. 3*B*). These results indicated that Rpl12 is a physiological substrate for Set11 and demon-

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. 3*A*, indicated by *red*) and used these mutant proteins in the *in vitro* methyltransferase assay (Fig. 3*C*). We found that several combinations of the Ala substitution greatly affected the Set11 methyltransferase activity (see Rpl12ΔC-His$^{K3,\ 10,82,85,92,93,95A}$ and Rpl12ΔC-His^{K39,40,82,85,92,93,95A}). Further detailed mapping revealed that the Ala substitution of Lys^{3} completely abolished the Set11 methylation activity (Rpl12ΔC-His^{K3A}). In addition, the combined Ala substitution of Lys^{39} and Lys^{40} severely reduced the Set11 methyltransferase activity (Rpl12ΔC-His^{K39,40A}). These results suggested that the three residues Lys^{3}, Lys^{39}, and Lys^{40} of Rpl12 are critical to the *in vitro* methyltransferase activity of Set11. It has been reported that Lys^{10} 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 Lys^{10} (Rpl12ΔC-His^{K10A})

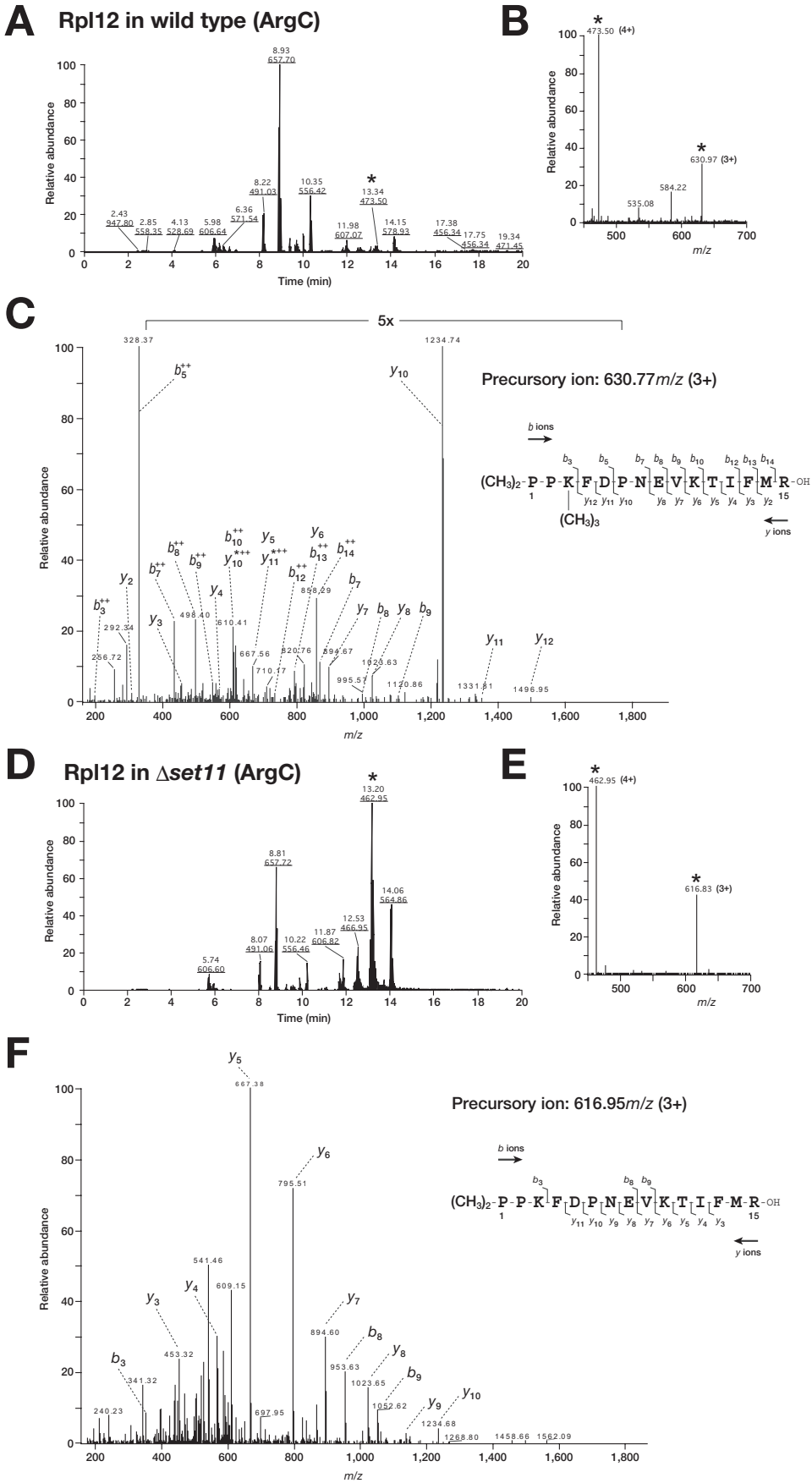


TABLE 2
LC-MS analysis of Rpl12

Peptide	Peptidase	Strain	Observed mass	Experimental mass	Theoretical mass	Difference
PPKFDPNEVKTIFMR (1–15 amino acids)	ArgC	Wild type	630.79 (MH^{3+})[a]	1890.37 (MH^+)	1818.96 (MH^+)	+71.41
		Δ*set11*	616.86 (MH^{3+})[a]	1848.59 (MH^+)		+29.63
PPKFDPNE (1–8 amino acids)	GluC	Wild type	507.63 (MH^{2+})[a]	1014.26 (MH^+)	943.45 (MH^+)	+70.81
		Δ*set11*	486.52 (MH^{2+})[a]	972.04 (MH^+)		+28.59
VAGGSTLAPKIGPLGLSPKKVGE (21–43 amino acids)	GluC	Wild type	731.32 (MH^{3+})[b]	2191.96 (MH^+)	2176.27 (MH^+)	+15.69
			1103.13 (MH^{2+})[a]	2205.04 (MH^+)		+28.77
			735.91 (MH^{3+})[a]	2205.72 (MH^+)		+29.45
			754.74 (MH^{3+})[a]	2262.21 (MH^+)		+85.94
		Δ*set11*	731.30 (MH^{3+})[b]	2191.88 (MH^+)		+15.61
			1103.02 (MH^{2+})[a]	2205.26 (MH^+)		+28.99
			735.90 (MH^{3+})[a]	2205.72 (MH^+)		+29.45
			754.71 (MH^{3+})[a]	2262.11 (MH^+)		+85.86
LTIQNRQAAVSVVPSASALVIK (61–82 amino acids)	Trypsin	Wild type	1140.57 (MH^{2+})[b]	2279.12 (MH^+)	2265.33 (MH^+)	+13.79

[a] Average of at least three independently observed masses.
[b] Representative masses.

was clearly methylated by Set11 (Fig. 3*C*). Taken together, these results suggest that Rpl12 is a physiological substrate of Set11 and that the Lys^3, and potentially Lys^{39} and Lys^{40}, 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 Δ*set11* mutant cells was isolated by reverse-phase chromatography (as shown in Fig. 2*B*). 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 Δ*set11* mutant cells (Fig. 4, *A* and *D*). However, a relatively abundant peptide was eluted in a prominent peak at 13.20 min in the Δ*set11* mutant (Fig. 4*D*, *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. 4*F*). The experimental mass of this peptide calculated from the mean *m/z* of MH^{3+} 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 Δ*set11* 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 Lys^3 and Lys^{10}, 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 that the Rpl12 in wild-type cells is trimethylated at Lys^3, and this methylation is absent in the Δ*set11* mutant cells. This is quite consistent with our observation obtained from the *in vitro* methyltransferase assay (Fig. 3*C*).

Of note, the mass of the N-terminal 1–8 peptide even in the Δ*set11* 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 ϵ-*N*-trimethylation at Lys^3. These observations support the idea that the N-terminal Proline 1 (Pro1) is modified by α-*N*-dimethylation after removal of the initial Met, as previously reported for *S. cerevisiae* Rpl25 (37). By analyzing the MS/MS results, we were also able to identify a monomethylation at Arg^{66} (supplemental Fig. S1*C*) and a methylation and/or another modification(s) at Lys^{39} and Lys^{40} (Table 2). The most frequently observed mass for the latter region was a dimethylation at Lys^{39} (supplemental Table S2). We found that these modifications were the same in the Δ*set11* mutant cells. We could not obtain clear evidence for the methyl modification at Lys^{10}, which is observed in *S. cerevisiae* Rpl12 and is the exclusive target of Rkm2 (18).

Functional Analysis of Set11 in S. pombe—To investigate the enzymatic function of Set11 *in vivo*, we introduced short internal deletions (ΔNHSP189–192; ΔNHSP and ΔGEQIFLCY 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 (Δ*set11*, Δ*set11*NHSP, and Δ*set11*GE-Y) were then subjected to the *in vitro* methyltransferase assay (Fig. 5*A*). Rpl12 from the Δ*set11*NHSP and Δ*set11*GE-Y strains was clearly methylated by recombinant His-Set11 to the same extent as that from Δ*set11*, indicating that these deletions abolished the *in vivo* enzymatic activity of the endogenous Set11

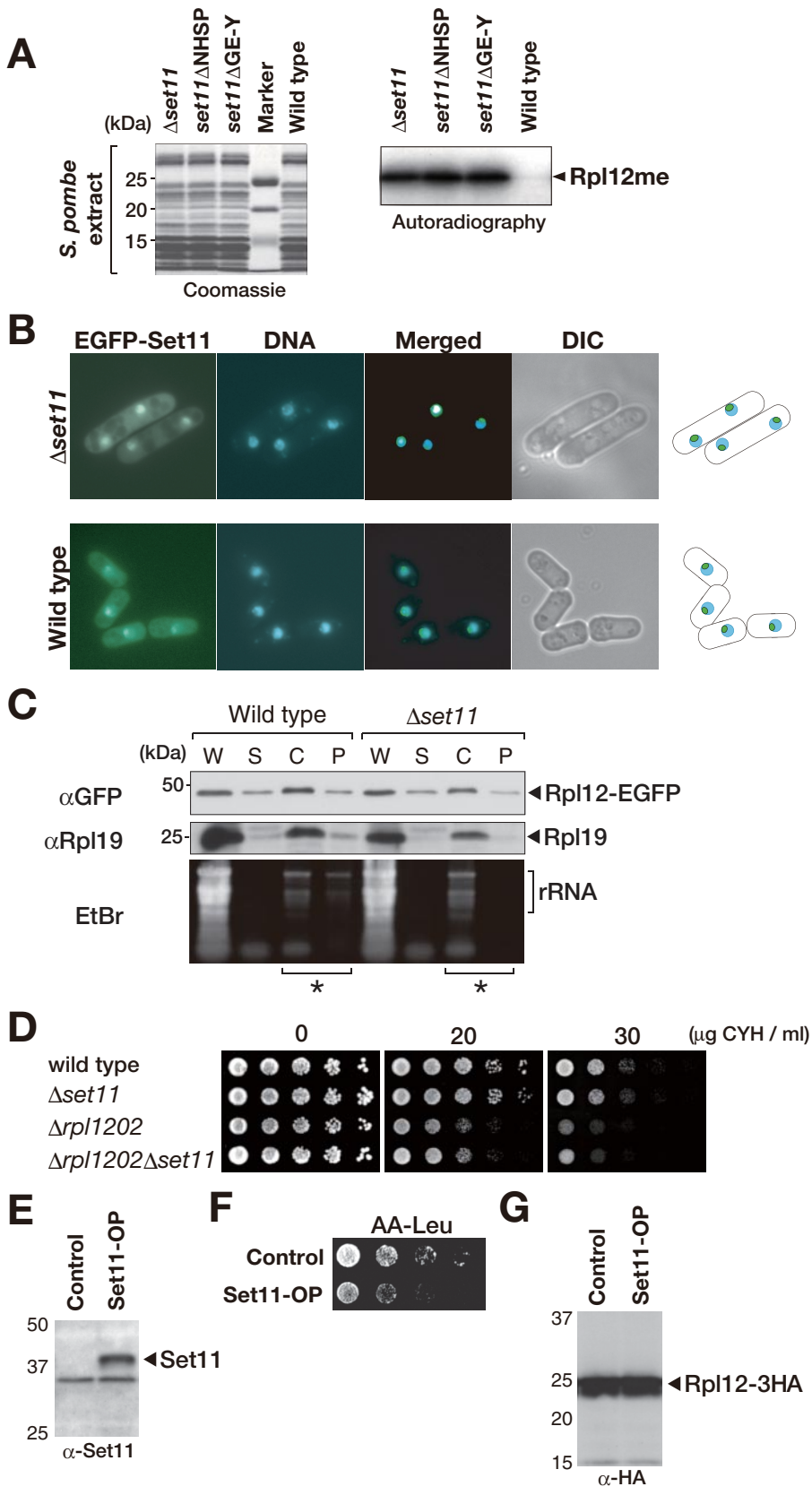
FIGURE 4. **LC-MS/MS analysis of Rpl12 derived from wild-type and Δ*set11* strains.** Rpl12 in wild-type (*set11*$^+$) or Δ*set11* mutant cells was isolated by reverse-phase chromatography, digested with ArgC, and analyzed using a quadrapole ion trap mass spectrometer (Finnigan LTQ; Thermo Fisher Scientific). *A* and *D*, base peak, ion chromatogram for a 20-min separation of digested Rpl12 peptides for the wild-type (*A*) and Δ*set11* strains (*D*). The elution time and detected *m/z* of representative peaks are indicated. The peak for the peptide fragment spanning residues 1–15 is indicated by an *asterisk*. *B* and *E*, MS spectrum of the Rpl12 peptide detected at 13.34 min for wild type (*B*) and at 13.20 min for the Δ*set11* strain (*E*). Prominent peaks for MH^{4+} and MH^{3+} are indicated by an *asterisk*. *C* and *F*, MS/MS spectra of the peptide fragment spanning residues 1–15 for the wild-type (*C*) and Δ*set11* strains (*F*) are depicted. The observed *y* and *b* ions and the fragment map are shown.

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 Δ*set11* 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 Δ*set11* mutant cells (Fig. 5*B*). These results suggest that the Lys^3 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 Δ*set11* 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 Δ*set11* mutation (Fig. 5*C*).

Next, to examine the potential role of Rpl12 methylation in ribosomal function, we analyzed the sensitivity to cycloheximide of Δ*set11* mutant cells. Cycloheximide is a widely used compound that inhibits protein synthesis by blocking transla-



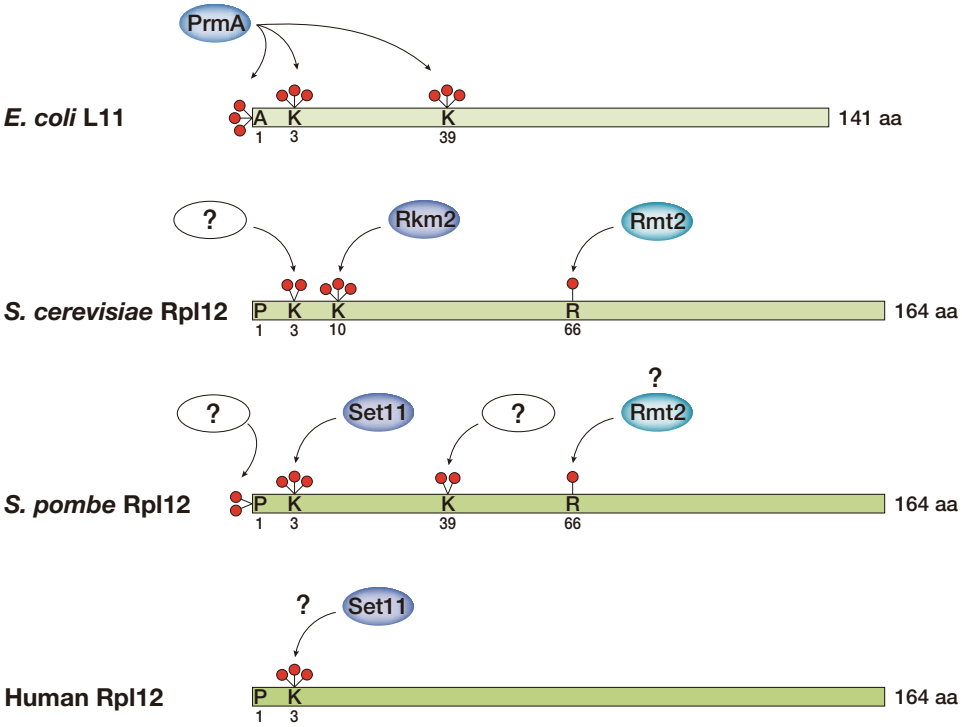


FIGURE 6. **Summary of post-translational methyl modifications in *E. coli* L11 and in Rpl12 of eukaryotic cells.** Schematic representations of *E. coli* L11, *S. cerevisiae* Rpl12, *S. pombe* Rpl12, and human Rpl12 are shown. The methylation sites and corresponding enzymes are depicted (10, 17, 18).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

tional elongation via interference with the peptidyl transferase activity of the 60 S ribosome. In *S. pombe*, two paralogous genes (*rpl1201* and *rpl1202*) encode Rpl12, and mutant cells in which one of these genes was deleted (Δ*rpl1202*) showed higher cycloheximide sensitivity, suggesting that the dosage of Rpl12 is critical to ribosomal function (Fig. 5*D*). However, Δ*set11* cells showed no increased sensitivity to cycloheximide, suggesting that the Rpl12 methylation has little, if any, effect on the ribosomal function. In previous studies, it was shown that the loss of *S. pombe* Rmt3, the arginine methyltransferase for ribosomal protein S2 disrupts the balance of small 40 S and large 60 S subunits because of a deficit in the small subunit level (28, 29). In line with this, we also examined the stability of the 40 S and 60 S subunits by polysome analysis. We observed, however, no clear difference between the wild-type and Δ*set11* mutant strains (data not shown), suggesting that Rpl12 methylation at Lys^3 is not directly involved in ribosomal subunit stability or that it has overlapping functions with other methyl modification(s).

The *set11*$^+$ gene is located immediately downstream of another gene, *spc19*$^+$ (exactly 20 bp away from the *spc19*$^+$ open reading frame), and our Northern blotting and reverse transcription-PCR analyses showed that *set11*$^+$ mRNA is transcribed polycistronically with *spc19*$^+$ mRNA (data not shown). Previous studies have indicated that the expression of *set11*$^+$ is regulated by cell cycle and meiotic processes (39, 40). To examine whether the expression level of *set11*$^+$ is important for its function, we overproduced Set11 in wild-type cells (Fig. 5*E*). Interestingly, we found that the induction of *set11*$^+$ expression by the *nmt1* promoter caused a severe growth defect (Fig. 5*F*, Set11-OP). Because the level of Rpl12 in Set11-overproducing cells was comparable with that in wild-type cells (Fig. 5*G*), it is unlikely that 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.

DISCUSSION

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 vivo* 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,

FIGURE 5. ***In vivo* enzymatic activity and subcellular localization of Set11.** *A*, the SET domain core is essential for the Set11 enzymatic activity. *S. pombe* nuclear extracts prepared from wild-type and *set11* mutant strains (Δ*set11*, Δ*set11*NHSP, and Δ*set11*GE-Y) were resolved by 15% SDS-PAGE and visualized by Coomassie staining (*left panel*). These extracts were incubated with His-Set11 and [^{3}H]AdoMet. The proteins were resolved by 15% SDS-PAGE, and labeled proteins were detected by autoradiography (*right panel*). The positions of size markers and the methylated Rpl12 are indicated. *B*, EGFP-tagged Set11 predominantly localizes to the nucleolus. EGFP-tagged Set11 was expressed from the *nmt1* promoter in wild-type (*bottom row*) or Δ*set11* cells (*top row*). The cells were stained with Hoechst33342 (*DNA*). Merged images of EGFP-Set11 and DNA (*Merged*), differential interference contrast (*DIC*), and schematic drawing of the cells are also shown. *C*, the level of Rpl12-EGFP associated with the ribosome is not affected by *set11*$^+$ deletion. Total cell extracts (*W*) of wild-type and Δ*set11* cells were fractionated by discontinuous sucrose density centrifugation into a post-ribosomal supernatant (*S*), a sucrose cushion (*C*), and pellet (*P*). The fractions were analyzed by immunoblotting using antibodies against green fluorescent protein and Rpl19 (control) proteins. Total RNA was extracted, resolved by 0.8% agarose gel, and stained with ethidium bromide. Most of the ribosomes were fractionated in the sucrose cushion (*C*) and pellet (*P*) fractions as indicated by an *asterisk*. *D*, deletion of *set11*$^+$ does not affect cycloheximide sensitivity. 5-fold dilutions of wild type, Δ*set11*, Δ*rpl1202*, and Δ*rpl1202*Δ*set11* strains were plated onto YEA alone and YEA containing different doses of cycloheximide. *E–G*, overproduction of Set11 causes a growth defect but does not change the level of Rpl12. Wild-type cells were transformed with pREP1 or pREP1-*set11*$^+$, and 5-fold dilutions of the transformed cell cultures were plated onto minimal medium lacking leucine (AA-Leu) (*F*). The level of Set11 (*E*) and Rpl12–3HA (*G*) was analyzed by immunoblotting using antibodies against Set11 and the hemagglutinin tag, respectively.

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 Pro^1, ϵ-*N*-trimethylation at Lys^3, ϵ-*N*-dimethylation at Lys^{39} and/or Lys^{40}, and δ-*N*-monomethylation at Arg^{66} and thus underscore the evolutionary conservation of Rpl12 methylation (Fig. 6). Although we showed that Set11 modifies Lys^3, and it is likely that an Rmt2 homolog (SPAC26A3.17c) catalyzes Arg^{66} 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 Lys^{39}/Lys^{40} 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]PPKQQ-, 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 Δ*set11* cells. The same is true for the Δ*rkm2* mutant budding yeast cells (18) and Δ*prm*A 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 Lys^3 occurs during the ribosome assembly processes. The dynamics and stability of this methyl modification 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 Lys^3 in wild-type cells. Interestingly, we found that the overproduction of Set11 caused a severe growth defect (Fig. 5*E*). 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 Δ*set11* mutation. These hypotheses will be tested in future studies that take ribosome homeostasis into consideration.

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