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Multiple lysine methylation of PCAF by Set9 methyltransferase

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ABSTRACT

The molecular functions of several non-histone proteins are regulated through lysine modification by histone methyltransferases. The p300/CBP-associated factor (PCAF) is an acetyltransferase that has been implicated in many cellular processes. Here, we report that PCAF is a novel substrate of Set9 methyltransferase. *In vitro* mapping experiments revealed six lysine residues could be methylated by Set9. A comparison of amino acid sequences of target sites revealed the novel consensus motif which differs from previously identified Set9-consensus sequence. Further methyltransferase assays focusing on the six lysine residues showed that K78 and K89 are preferentially methylated in full-length PCAF *in vitro*. Using specific antibodies recognizing mono-methylated K89, *in vivo* PCAF methylation and its nuclear localization were demonstrated. Our data may lead to a new insight into PCAF functions and provide additional information to identify unknown targets of Set9.

Various covalent histone modifications, such as phosphorylation, acetylation, methylation and ubiquitination are involved in the ability of higher-order chromatin structure to affect many fundamental biological processes [1,2]. Histone methyltransferases (HMTase) specific to H3-K4, -K9, -K27, -K36, -K79 and H4-K20 have been identified and well characterized in terms of their functional roles in transcriptional activation or repression [1,2]. Recently, several groups reported that HMTases including Set9, Smyd2 and G9a can also target non-histone proteins, modulating the function of target proteins through lysine methylation [3–7]. Therefore, interest in the molecular mechanism of lysine methylation goes beyond histones.

Among the SET domain-containing HMTases, Set9 possesses characteristic features on substrate recognition. Set9 was originally identified as a H3-K4-specific HMTase, however, this HMTase is highly active upon free histone H3-K4 but not nucleosomal histone H3-K4 [8,9]. On the other hand, it was shown that Set9 could stimulate activator-induced transcription *in vivo* [9], suggesting that Set9 regulates function of non-histone proteins which plays a role of transcription activation. Subsequent studies revealed that Set9 functions as a transcriptional regulator through lysine methylation for p53, TAF10 and Estrogen receptor α (ER) [3,4,7]. Methylation of p53 and ER results in the stabilization of these proteins and the transcriptional activation. The methylated TAF10 has an increased affinity for RNA polymerase II, suggesting a direct role in pre-initiation complex formation. These results support the idea that Set9 controls transcription by methylating non-histone transcription regulators. In view of diverse transcription network, however, substrates for Set9 are not yet fully understood.

Here, we demonstrate that Set9 methylates p300/CBP-associated factor (PCAF) at multiple lysine residues *in vitro* and *in vivo*. The amino acid sequences around new target sites appeared to be distinct from the known consensus sequence. Our data suggest that Set9 may control PCAF functions through lysine methylation and may have more additional targets.

Materials and methods

Cell cultures and constructs. HEK293 cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids and penicillin–streptomycin. U2OS cells were cultured in McCoy's 5A supplemented with 10% FBS and penicillin–streptomycin. Human Set9, PCAF and GCN5L2 cDNAs were cloned into the pGEX-2T (GE Healthcare, preparation of GST-fusion proteins from *E. coli*) and pCMV-Myc or -HA (Clontech, expression in mammalian cells) vectors. The substitution mutations were generated by PCR-directed mutagenesis and then subcloned into vectors as described. Details of individual plasmid constructs, which were all verified by sequencing, are available upon request.

Immunoprecipitation and Western blotting. To prepare whole cell extracts, cells were lysed in RIPA buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate) supplemented with protease inhibitors. Nuclear extracts were prepared by the Dignam's method [10]. For immunoprecipitation, cell extracts were incubated with 10 μl of the anti-Myc beads or the indicated antibody-coupled protein G beads at 4 °C for 1 h.

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The immunoprecipitates were analyzed by Western blotting as described previously [11].

RNA interference experiments. HEK293 cells were transfected with 10 nM of siRNA using Lipofectamine RNAiMax (Invitrogen). The control (siGL3) and Set9 (siSet9) siRNAs were purchased from B-Bridge International, Inc.: siGL3 (S20C-0200) and siSet9 (SHF27A-1403).

Peptides. The following peptides were chemically synthesized for *in vitro* methyltransferase (MTase) assays and dot blot assays. PCAF 71–85: NH2-GSARIAVKKAQLRSA-COOH; PCAF 71–85 mono-methyl-K78: NH2-GSARIAVK(Kme1)AQLRSA-COOH; PCAF 82–96: NH2-LRSAPRAKKLEKLGV-COOH; PCAF 82–96 mono-methyl-K89: NH2-LRSAPRAK(Kme1)LEKLGV-COOH; PCAF 72–95: NH2-SARIAVKKAQLRSAPRAKKLEKLG-COOH; PCAF 72–95 mono-methyl-K78 and mono-methyl-K89: NH2-SARIAVK(Kme1)AQLRSAPRAK(Kme1)LEKLG-COOH; PCAF 631–645: NH2-TKYVGYIKDYEGATL-COOH; PCAF 631–645 mono-methyl-K638: NH2-TKYVGYI(Kme1)DYEGATL-COOH; PCAF 656–670: NH2-YTEFSVIIKKQKEII-COOH; PCAF 670–684: NH2-IRRLIERRQAQIRRV-COOH; PCAF 685–699: NH2-YPGLSCFKDGVRQIP-COOH;

Antibodies. Polyclonal antibodies specific to PCAF-K89me1 and PCAF-K638me1 were generated in rabbits using PCAF 82–96 mono-methyl-K89 and PCAF 631–645 mono-methyl-K638 peptides as antigens, respectively. The sera were absorbed with the corresponding non-methylated peptide and this was followed by affinity purification using a methylated peptide column. The following antibodies were used in this study: anti-PCAF (Abcam, ab12188 and Santa Cruz, sc-13124), anti-Set9 (Upstate, 07-314), and anti-β-Actin (Abcam, ab8226).

In vitro MTase assays. In vitro MTase assays were performed as described previously [9]. Briefly, 2 μg of recombinant Set9 and 2 μg of recombinant truncated or full-length PCAF proteins or synthetic peptides were incubated with 0.075 μCi of *S*-adenosyl-L-[methyl-^{14}C] methionine (GE Healthcare) in a 30 μl reaction buffer containing 50 mM Tris–HCl (pH 8.5), 5 mM $MgCl_2$, and 4 mM DTT at 30 °C for 1 h. The reaction mixtures were analyzed by SDS–PAGE followed by autoradiography. Radioactivity was visualized using Bioimaging analyzer BAS-2500 (Fujifilm).

Immunofluorescence microscopy. U2OS cells were transfected with the indicated plasmid constructs. Cells were fixed 24 h after transfection in 4% paraformaldehyde in phosphate-buffered saline followed by permeabilization with 1% Triton-X. After blocking with 20% FBS, cells were doubly stained with anti-Myc (Upstate, 05-724) or anti-PCAF-K89me1, followed by incubation with secondary antibodies, Alexa Fluor 546- or 488-conjugated anti-mouse or anti-rabbit IgG (Invitrogen) in Tris-buffered saline containing 5 μg/ml of DAPI. The cellular localization of Set9 and K89-methylated PCAF was visualized using an Olympus IX71.

Results and discussion

Set9 methylates PCAF at multiple lysines in vitro

While searching for non-histone substrates for Set9, we found that Set9 methylated GST-PCAF *in vitro*. As shown in Fig. 1A, three positive bands including expected size of full-length PCAF (indicated by black triangle) and additional smaller size of degradated products were observed. In order to identify the PCAF residues methylated by Set9, the initial analysis was performed using four PCAF constructs composed of the PCAF functional domains: the N-terminus containing the ubiquitin E3 domain (residues 1–352), the middle portion (residues 353–497), the acetyltransferase domain (AT domain, residues 498–660), and the C-terminus containing the Bromo domain (residues 661–832) (Fig. 1B). It was observed that Set9 methylated three expected size of truncated PCAF *in vitro* (Fig. 1C).

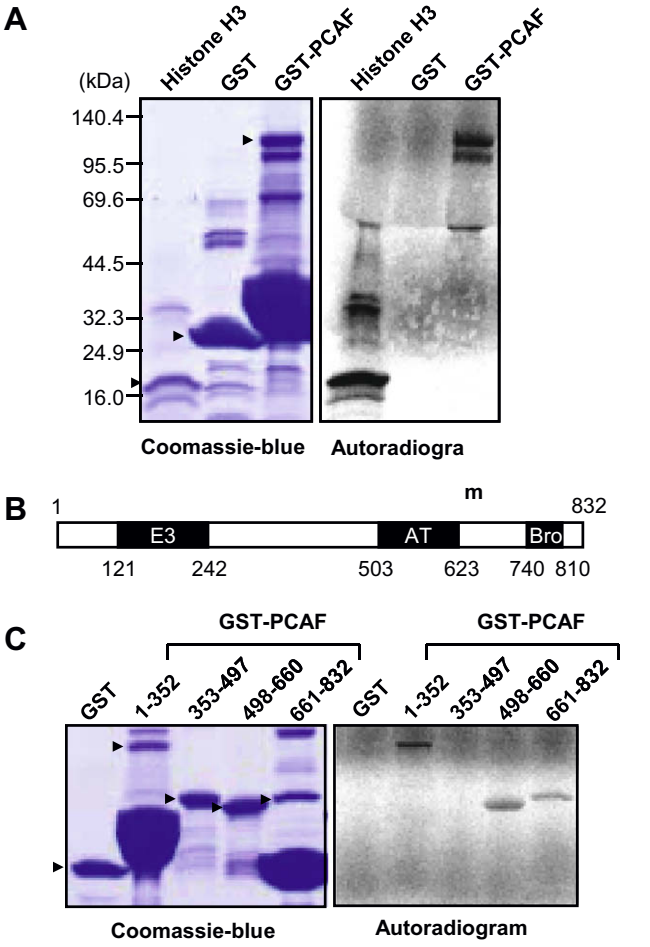

Fig. 1. Set9 methylates PCAF at multiple sites *in vitro*. (A) Coomassie-blue staining and autoradiogram of MTase assay with Set9 and histone H3, GST and GST-PCAF. The black triangles indicate each target protein that was expected by molecular size. (B) Schematic representation of functional domains of human PCAF. E3, ubiquitin E3 ligase domain; AT, acetyltransferase domain; Bro, Bromo domain. (C) Coomassie-blue staining and autoradiogram of MTase assay with Set9 and GST-PCAF constructs. The black triangles indicate each target protein that was expected by molecular size.

The N-terminus was further divided into three protein fragments which were tested for methylation. The most N-terminal region, corresponding to residues 1–84, was appeared to be a substrate, as two proteins corresponding to residues 85–252 and 209–352 were not methylated (Fig. S1A, lanes 1–3). This region contained two lysines, K78 and K79, which were substituted individually, or in combination with arginine in the 1–252 construct. Unexpectedly, the methylation signal was not completely lost, even in the proteins with the K78R and K79R mutations (Fig. S1A, lanes 4–6), suggesting that other lysines, which were missed in the initial test (Fig. S1A, lanes 1–3), could be methylated by Set9. Therefore, K89 and K90, which are located near the N-terminal edge of construct (85–252), were substituted with arginine and tested for methylation. We found that Set9 methylated PCAF at K78 and K89 *in vitro* (Fig. S1A, lanes 7–12).

Next, the AT domain was divided into two protein fragments and tested for methylation. The C-terminal region, corresponding to residues 622–660, appeared to be a substrate, because a protein corresponding to residues 498–621 was not methylated (Fig. S1B, lanes 1 and 2). This region contained five lysines, and these residues were substituted individually with arginine to test for methylation. We found that K638 is a target of Set9 (Fig. S1B, lanes 3–7).

The C-terminus (residues 661–832) was also examined. The region corresponding to residues 673–704 was initially hypothesized to be a substrate because a protein containing residues 673–832, but not 705–832, was methylated by Set9 (Fig. S1C, lanes 2 and 3). This region contains three lysines, which were individually mutated to arginine residue. We found that K692 is a target of Set9 (Fig. S1C, lanes 4–6). Nonetheless, the K692R mutant PCAF protein (661–832) was still methylated by Set9 (data not shown). This finding indicated that a more N-terminal region, corresponding to residues 661–672, could be a substrate. This narrow region contains five lysines, and we therefore, generated two peptides. The peptide corresponding to residues 670–684, but not 656–670, was methylated by Set9 (Fig. S1C, lanes 7 and 8). This peptide contains four lysines, which were simultaneously substituted with arginine. As only the peptides containing wild type K671 and/or K672 could be methylated, we concluded that K671 and K672 are also target of Set9 (Fig. S1C, lanes 9–14). Collectively, *in vitro* mapping experiments indicated that Set9 could methylate PCAF at six lysine residues *in vitro*; K78, K89, K638, K671, K672 and K692 (Fig. 2A).

Sequences around methylation-target lysines

Various studies on the structure of Set9 bound to H3, p53, TAF10 or an ER peptide clarified the molecular mechanism of lysine methylation by the conserved SET domain [7,12–14]. Couture et al. proposed that Set9 recognizes a conserved K/R-S/T/A motif preceding the lysine substrate, and has a propensity to bind aspartate (D) and asparagine (N) on the C-terminal side of the lysine target [14]. Indeed, a motif-based search succeeded in identifying TAF7 as a novel substrate for Set9, suggesting that this motif is sufficient for recognition by Set9. In contrast, the sequences surrounding the six newly identified methylation sites are not fully explained by this motif. The featured difference is that neutral non-polar residues such as alanine (A), phenylalanine (F), isoleucine (I) or valine (V) precede the target lysine in five of the six sites (Fig. 2B). At the C-terminal side, aspartate (D) or lysine (K) is observed in five of the six sites, similar to p53, TAF10, and ER. Therefore, our data suggest that more target proteins exist which possess sequence motifs such as A/F/I/V-K-D/K (where K is the methylation site). Our results provide additional information to clarify the consensus sequence and identify additional substrates of Set9.

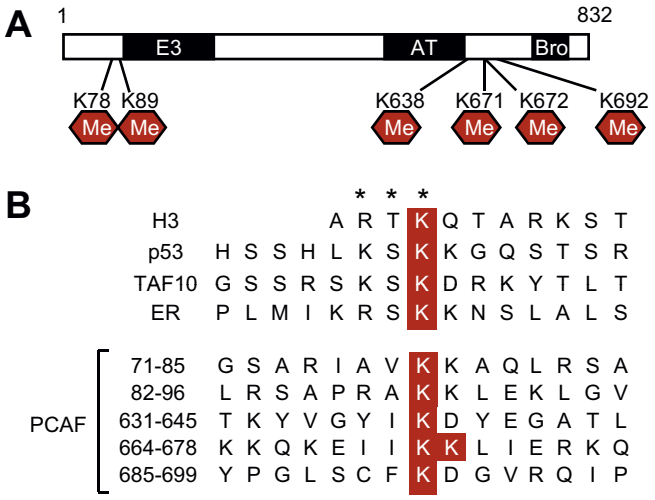

Fig. 2. Sequences around methylation-targets. (A) Schematic representation of methylation-targets in PCAF. (B) Sequence alignment of the regions surrounding human PCAF, H3, p53, TAF10 and ER methylation sites. Methylated lysines are highlighted in red, and the asterisks represent putative consensus sites for substrate recognition by Set9 reported by Chuikov et al. [4].

K78 and K89 are mainly methylated in full-length PCAF

In order to test whether the six lysines were methylated by Set9 in the full-length form of PCAF, we generated GST-full-length PCAF where five of the six lysines were substituted with arginine. The two N-terminal lysines, K78 and K89, could be methylated even if the other lysines were mutated (Fig. S2, lanes 2 and 3). Consistent with this result, methylation of the K78, 89R mutant was substantially reduced compared with that of wild type (Fig. 3A). Therefore, it was suggested that K78 and K89 are the main methylation-targets in full-length PCAF.

PCAF is mono-methylated by Set9

Previous studies showed that Set9 functions as a mono-methyltransferase [3,4,7,15]. Therefore, it was predicted that Set9 monomethylates the target lysines of PCAF. In order to determine the methylation state, we selected two main methylation-target (K78 and K89) and one of four weaker methylation-target (K638), and unmodified or mono-methylated K78, K89 and K638 peptides (residues 71–85, 82–96 and 631–645, respectively) were generated to test for methylation. Set9 could methylate all of the unmodified peptides. However, it could no longer methylate the mono-methylated K78, K89 and K638 peptides (Fig. 3B). Therefore, the methylation status mediated by Set9 is mono-methylation. Next, polyclonal antibodies that specifically recognize mono-methylated K89 (PCAF-K89me1) and mono-methylated K638 (PCAF-K638me1) were generated in rabbits. Because methylation-targets locate in N-terminal (K78 and K89) and C-terminal (K638, K671, K672 and K692) regions of PCAF, we selected K89 and K638 as a representative of these regions for producing antibody. Dot blot analysis showed that anti-PCAF-K89me1 and -K638me1 antibodies recognized specific, mono-methylated lysine residues (Fig. S3). K78-mono-methylation did not interfere with the recognition of anti-PCAF-K89me1 for K89-mono-methylation on the same peptide (Fig. S3, lane 6). Therefore, these antibodies were used for further experiments.

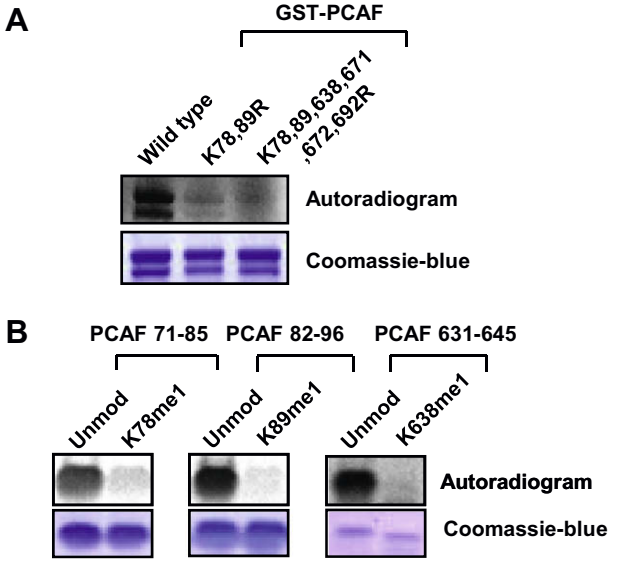

Fig. 3. K78 and K89 are main methylation-targets in full-length PCAF and methylation status of K78, K89 and K638 are mono-methylation. (A) Autoradiogram of MTase assay with Set9 and full-length wild type and multi-site mutated (lysine to arginine) GST-PCAF proteins, and Coomassie-blue staining. (B) Autoradiogram of MTase assays with Set9 and the PCAF peptides 71–85 unmodified (unmod) or K78 mono-methylated (K78me1), peptides 82–96 unmod or K89me1, and peptides 631–645 unmod or K638me1. Coomassie-blue stainings are shown below the autoradiogram.

PCAF is methylated in vivo

We then examined whether PCAF can be methylated by Set9 in cells. HEK293 cells were transiently transfected with Myc-PCAF or Myc-PCAF (K89R), either alone or with HA-Set9 or HA-Set9 (H297A). Whole cell extracts were subjected to Western blot analysis using the anti-PCAF-K89me1 antibody. Myc-PCAF, but not the K89R mutant, was detected by the anti-PCAF-K89me1 only when co-transfected with HA-Set9 (Fig. 4A, compare lanes 2–5). K89 methylation was not detected when co-transfected with the catalytically inactive mutant HA-Set9 (H297A). We also examined K638 methylation; however, this methylation event was difficult to detect in whole cell extracts. Using immunoprecipitates with anti-Myc-beads for Western blot analysis, K638 methylation was detected in the sample transfected with wild type PCAF and Set9 (Fig. S4, lane 2). These data suggested that K638 may be methylated in a small fraction of PCAF. GCN5L2 and PCAF, which both belong to the GCN5-related *N*-acetyltransferase family, are highly homologous [16,17]. Therefore, we tested whether the anti-PCAF-K89me1 antibody was able to specifically detect PCAF-K89 methylation *in vivo*. Western blot analysis using extracts from HEK293 cells expressing Myc-GCN5L2 with HA-Set9 or HA-Set9 (H297A) showed no positive signal (Fig. S5). This result indicated that the anti-PCAF-K89me1 antibody does not recognize GCN5L2.

To address whether endogenous PCAF can be methylated by Set9, we initially analyzed HEK293 cells transiently expressing HA-Set9 or HA-Set9 (H297A). The Western blot analysis detected a weak signal of methylated PCAF in extracts obtained from cells expressing Set9, but not Set9 (H297A) (Fig. S6, indicated by black triangle). We confirmed the methylation by immunoprecipitation using anti-PCAF-K89me1 antibody (Fig. S6, bottom panel). Next, we tested the ability of endogenous Set9 to methylate endogenous PCAF. Knockdown using a Set9-specific siRNA showed that the amount of PCAF-K89me1 was reduced when cells were treated with Set9 but not a control siRNA, although the amount of PCAF was almost same in both cells (Fig. 4B). Taken together, these data indicates that PCAF can be endogenously methylated by Set9.

We next investigated the localization of methylated PCAF. U2OS cells expressing Myc-PCAF with HA-Set9 or HA-Set9 (H297) were fractionated into nuclear and cytosolic fractions and subjected to Western blot analysis. The results showed that methylated PCAF was localized to the nucleus, although transfected PCAF and Set9 were both distributed in the nuclear and cytosolic fractions (Fig. 4C). We further analyzed PCAF-K89 methylation by immunofluorescence in U2OS cells expressing Myc-Set9 or Myc-Set9 (H297A). Cells transfected with Myc-Set9, but not Myc-Set9 (H297A), were stained with the anti-PCAF-K89me1 antibody. It was observed that the positive signal was localized in the nucleus (Fig. 4D).

The methylation-targets in PCAF are not located in any domains and are concentrated in two distinct regions. Previous structural analyses of PCAF and GCN5 have focused on the AT domain or Bromo domain, which do not include the methylation-targets, except for K638. Based on the crystal structure, K638 is located at the alpha5-beta6 loop, which is involved in a conformational change of AT domain during substrate binding, catalytic turnover and product release [18,19]. Further studies focusing on the N-terminal region at the structural level would be required. Since it has been demonstrated that human PCAF and GCN5 exist as components of large 2 MDa and 700 kDa multi-subunit complexes [17,20,21], our results cannot be generalized to PCAF in complexes, and require further verification. However, the data showing that TAF10, a recently identified substrate of Set9, is also a component

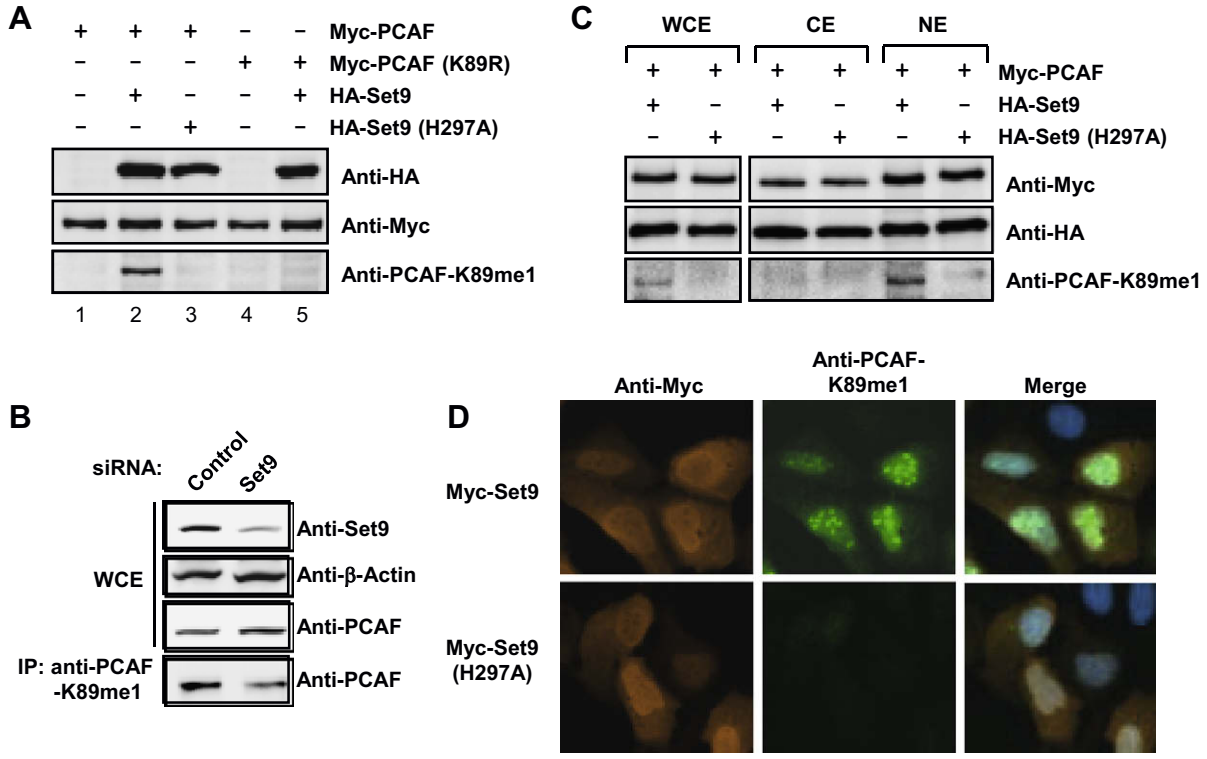

Fig. 4. Set9 methylates PCAF *in vivo*. (A) Western blot analyses of whole cell extracts from HEK293 cells transfected with Myc-PCAF (lanes 1–3) or Myc-PCAF (K89R) (lanes 4 and 5), either alone (lanes 1 and 4), or with HA-Set9 (lanes 2 and 5) or HA-Set9 (H297A) (lane 3). (B) U2OS cells were transfected with 10 nM of control or Set9 siRNA. Forty-eight hours after transfection, Western blot analyses of whole cell extracts (WCE) or immunoprecipitates with anti-PCAF-K89me1 (IP) were performed. (C) Western blot analyses of whole cell extracts (WCE), cytoplasmic extracts (CE), and nuclear extracts (NE) from U2OS cells co-transfected with Myc-PCAF with HA-Set9 or HA-Set9 (H297A). (D) Immunofluorescence staining for K89-methylated PCAF. U2OS cells were transiently transfected with Myc-Set9 or Myc-Set9 (H297A) for 24 h and then stained with the indicated antibodies. The nuclei were stained by DAPI (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

of the 2 MDa complex, and that PCAF or GCN5 and TAF10 co-localize in domain III, suggest a functional link exists between Set9 and the 2 MDa complexes through methylation of these proteins in domain III [17,22,23].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.01.185.

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