

Histone Lysine Methyltransferase SETD8 Promotes Carcinogenesis by Deregulating PCNA Expression

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Abstract

Although the physiologic significance of lysine methylation of histones is well known, whether lysine methylation plays a role in the regulation of nonhistone proteins has not yet been examined. The histone lysine methyltransferase SETD8 is overexpressed in various types of cancer and seems to play a crucial role in S-phase progression. Here, we show that SETD8 regulates the function of proliferating cell nuclear antigen (PCNA) protein through lysine methylation. We found that SETD8 methylated PCNA on lysine 248, and either depletion of SETD8 or substitution of lysine 248 destabilized PCNA expression. Mechanistically, lysine methylation significantly enhanced the interaction between PCNA and the flap endonuclease FEN1. Loss of PCNA methylation retarded the maturation of Okazaki fragments, slowed DNA replication, and induced DNA damage, and cells expressing a methylation-inactive PCNA mutant were more susceptible to DNA damage. An increase of methylated PCNA was found in cancer cells, and the expression levels of SETD8 and PCNA were correlated in cancer tissue samples. Together, our findings reveal a function for lysine methylation on a nonhistone protein and suggest that aberrant lysine methylation of PCNA may play a role in human carcinogenesis. *Cancer Res*; 72(13); 3217–27. ©2012 AACR.

Introduction

Protein methylation is recently considered an important posttranslational modification and is predominantly found on lysine and arginine residues. Lysine methylation involves the addition of 1 to 3 methyl groups on the amino acid's ε-amine group, to form mono-, di-, or tri-methyllysine. Its function is best understood in histones (1). With the exception of Dot1/DOT1L, all histone lysine methyltransferases (HKMT) contain a SET domain of about 130 amino acids, and so far nearly 40 SET domain-containing HKMTs or potential HKMTs have

been identified (2). While our knowledge of the physiologic functions of HKMTs is growing, their involvement in human diseases including cancer is still not well understood.

Proliferating cell nuclear antigen (PCNA) is an evolutionally well-conserved protein found in all eukaryotic species from yeast to humans, as well as in archaea. PCNA functions are related to vital cellular processes such as DNA replication, chromatin remodeling, DNA repair, sister chromatid cohesion, and cell-cycle control (3). PCNA was originally reported as an antigen for autoimmune disease in patients with systemic lupus erythematosus, detected only in the proliferating cell populations (4). Thereafter, it was shown that expression levels of PCNA during cell cycle are differential and associated with proliferation and transformation (5, 6). In the following years, a number of experiments have been done to uncover the role of PCNA in DNA replication, and one of the first functions clarified was a sliding clamp for DNA polymerase δ (7, 8). Meanwhile, the progress in the field not only strengthened the importance of PCNA, but also even placed PCNA at the crossroad of many essential pathways. Importantly, PCNA is posttranslationally modified in several ways, which affects its function. So far, it has been reported that PCNA is ubiquitinated, phosphorylated, acetylated, and even SUMOylated (3). One of the well-documented posttranslational modifications of PCNA is ubiquitination. In response to DNA damage, PCNA is monoubiquitinated at the lysine 164 residue by the E2 Ub-conjugated enzyme Rad6 and the E3 Ub ligase Rad18 (Rad6/Rad18 complex; ref. 9). Rad18 not only binds to Rad6 and PCNA, but also to DNA (10). Thus, Rad18 recruits the ubiquitination machinery to the chromatin-bound target, PCNA. In addition

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to ubiquitination, it is estimated that approximately 6% of chromatin-bound PCNA is subjected to phosphorylation on Tyr 211 (11). It has been considered that phosphorylation of Tyr 211 on PCNA may stabilize chromatin-bound PCNA, as opposed to polyubiquitination. Furthermore, acetylation is another modification detected on PCNA (12), and in yeast, a poly-SUMOylation on PCNA has been described (13). However, functions of lysine methylation on PCNA have never been elucidated.

In this study, we showed that the histone methyltransferase SETD8 methylates Lys 248 on PCNA and regulates functions of PCNA in cancer cells. This is the first report to describe the significance of lysine methylation on PCNA.

Materials and Methods

Cell line

MRC-5, CCD-18Co, 5637, SW780, SCaBER, UMUC3, RT4, T24, HT-1376, A549, H2170, HCT116, LoVo, and 293T cells were from American Type Culture Collection in 2001 and 2003 and tested and authenticated by DNA profiling for polymorphic short tandem repeat (STR) markers, except for SW780. The SW780 line was established in 1974 by A. Leibovitz from a grade I transitional cell carcinoma. RERF-LC-AI and SBC5 cells were from Japanese Collection of Research Bioresources (JCRB) in 2001, and tested and authenticated by DNA profiling for polymorphic short tandem repeat (STR) markers. 253J and 253J-BV cells were from Korean Cell Line Bank (KCLB) in 2001, and tested and authenticated by DNA profiling for polymorphic STR markers. EJ28 cells were from Cell Line Service (CLS) in 2003, and tested and authenticated by DNA profiling for polymorphic STR markers. ACC-LC-319 cells were from Aichi Cancer Center in 2003, and tested and authenticated by DNA profiling for single-nucleotide polymorphism, mutation, and deletion analysis.

Tissue samples and RNA preparation

Bladder tissue samples and RNA preparation were described previously (14–17). Uroplakin is a marker of urothelial differentiation and is preserved in up to 90% of epithelially derived tumors (18). Use of tissues for this study was approved by Cambridgeshire Local Research Ethics Committee (Ref 03/018).

Quantitative real-time PCR

Specific primers for all human *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase; housekeeping gene), *SDH* (housekeeping gene), *SETD8*, and *PCNA* were designed (primer sequences in Supplementary Table S1). PCR reactions were conducted with the LightCycler 480 System (Roche Applied Science) following the manufacturer's protocol.

siRNA transfection

siRNA oligonucleotide duplexes were purchased from Sigma-Genosys for targeting the human *SETD8* transcript. siEGFP, siFFLuc, and siNegative control (siNC), which is a mixture of 3 different oligonucleotide duplexes, were used as control siRNAs. The siRNA sequences are described in Sup-

plementary Table S2. siRNA duplexes (100 nmol/L final concentration) were transfected into bladder and lung cancer cell lines with Lipofectamine 2000 (Life Technologies) for 72 hours, and cell viability was examined by Cell Counting Kit-8 (Dojindo).

Results

SETD8 is overexpressed in various types of cancer and regulates the growth of cancer cells

To investigate roles of a HKMT in human carcinogenesis, we had examined expression levels of several HKMTs in a small subset of clinical bladder cancer samples and found a significant difference in expression levels of *SETD8* between normal and cancer cells (data not shown). We then analyzed 124 bladder cancer samples and 28 normal control samples and confirmed the significant elevation of *SETD8* expression in tumor cells compared with normal cells (Supplementary Table S4). Expression levels partly correlated with the grade of malignancy in bladder cancer (Supplementary Fig. S1A). We also found overexpression of *SETD8* in both non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC; Fig. 1A). Subsequent immunohistochemical analysis using anti-SETD8 antibody identified strong SETD8 staining mainly in the nuclei of malignant cells, but no staining in nonneoplastic tissues (Fig. 1B). In addition, our expression profiling analysis indicated the upregulation of *SETD8* in chronic myelogenous leukemia, hepatocellular carcinoma, and pancreatic cancer (Supplementary Fig. S2 and Table S5). Furthermore, a high level of *SETD8* was identified in various cancer cell lines than in a normal lung cell line SAEC (Supplementary Fig. S3).

To investigate the role of SETD8 in the growth of cancer cells, we conducted a knockdown experiment using 2 independent siRNAs against SETD8 (siSETD8#1 and #2) and 2 control siRNAs (siEGFP and siFFLuc). We transfected each of these siRNAs into SW780 bladder cancer cells and found that SETD8 expression was efficiently suppressed by either of the 2 different siRNAs targeting SETD8, compared with control siRNAs (Supplementary Fig. S1B). Using the same siRNAs, we conducted cell growth assays and found significant growth-suppressive effects on 1 bladder cell line (SW780) and 2 lung cancer cell lines (RERF-LC-AI and SBC5), whereas no effect was observed when we used control siRNAs (Fig. 1C). Detailed cell-cycle analysis using flow cytometry indicated that the cell populations of cancer cells lacking SETD8 had a significant increase in the amount of S-phase and sub-G₁ phase cells and a concomitant reduction in the proportion of G₁ cells (Fig. 1D). Furthermore, we showed that in bromodeoxyuridine (BrdUrd) incorporation analysis, the amount of newly incorporated BrdUrd in cancer cells was significantly decreased after treatment with siSETD8 (Fig. 1E), implying that knockdown of SETD8 results in the retardation of DNA replication in cancer cells. These results indicated that SETD8 might play an important role in the regulation of cancer cell growth, especially in S-phase, and knockdown of SETD8 would cause apoptosis of cancer cells.

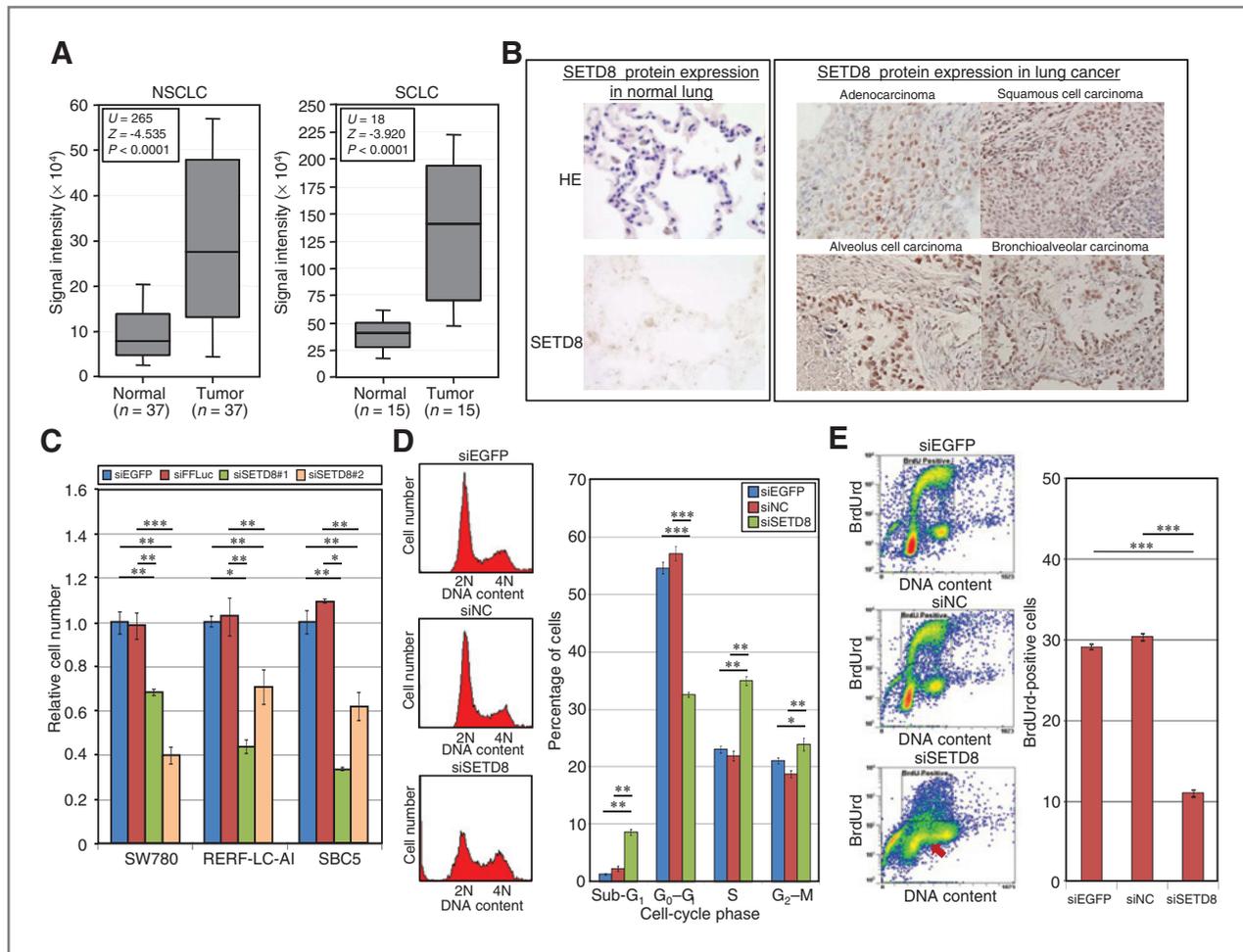


Figure 1. SETD8 is overexpressed in human cancer and regulates the proliferation of cancer cells. **A**, expression of *SETD8* is significantly increased in tumor tissues compared with normal Japanese patients. Signal intensity for each sample was analyzed by cDNA microarray. **B**, immunohistochemical staining of SETD8 in lung tissues. Clinical information for each section is represented above histologic pictures. Original magnification, $\times 100$. HE, hematoxylin and eosin. **C**, effects of *SETD8* siRNA knockdown on the viability of bladder (SW780) and lung (RERF-LC-AI and SBC5) cancer cell lines. Relative cell numbers were normalized to the number of siEGFP-treated cells (siEGFP = 1); results are the mean \pm SD of 3 independent experiments. *P* values were calculated using Student *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). **D**, effect of SETD8 knockdown on cell-cycle kinetics in cancer cells. Cell-cycle distribution was analyzed by flow cytometry after staining with propidium iodide (PI). Left, representative histograms of SBC5 cells stained with PI. Right, numerical analysis of fluorescence-activated cell-sorting (FACS) results in SBC5 cells, classifying cells by cell-cycle status. Results are the mean \pm SD of 3 independent experiments. *P* values were calculated using Student *t* test (**, $P < 0.01$; ***, $P < 0.001$). **E**, detailed cell-cycle kinetics in SBC5 cells after treatment with siSETD8. Cell-cycle distribution was analyzed by flow cytometry after coupled staining with fluorescein isothiocyanate (FITC)-conjugated anti-BrdUrd and 7-amino-actinomycin D (7-AAD) as described in Materials and Methods.

SETD8 methylates lysine 248 of PCNA both *in vitro* and *in vivo*

As PCNA is known to be a key regulator of cell-cycle progression and SETD8 is a component of the PCNA complex (19, 20), we examined the functional relationship between SETD8 and PCNA. Immunoprecipitation assay showed that 3xFLAG-tagged SETD8 bound endogenous PCNA (Fig. 2A). We also confirmed the interaction between endogenous PCNA and SETD8 proteins (Fig. 2B); endogenous SETD8 and PCNA proteins were colocalized in HeLa cells (Fig. 2C). Immunoprecipitation using deletion mutants of SETD8 showed that its N-terminal region of SETD8 is essential for binding to PCNA (Fig. 2D), and this portion contains a PCNA-interacting protein (PIP) box (Supplementary Fig. S4A). Because histone methyl-

transferases have been found to methylate nonhistone substrates, we evaluated a possibility of PCNA to be a substrate of SETD8. First, we conducted an *in vitro* methyltransferase assay and confirmed that PCNA was methylated in a dose-dependent manner (Fig. 2E). The amino acid analysis detected a single lysine methylation site in PCNA following this reaction (Supplementary Fig. S5). To verify *in vivo* SETD8-dependent PCNA methylation, we labeled 293T cells after transfection with FLAG-PCNA (WT) and hemagglutinin (HA)-mock or HA-SETD8 (1-352) expression vectors with L-[methyl-³H] methionine and found that SETD8 could methylate PCNA *in vivo* (Supplementary Fig. S6A). Subsequent liquid chromatography/tandem mass spectrometry (LC/MS-MS) analysis identified monomethylation at lysine 248 on PCNA by SETD8 (Fig. 2F). To

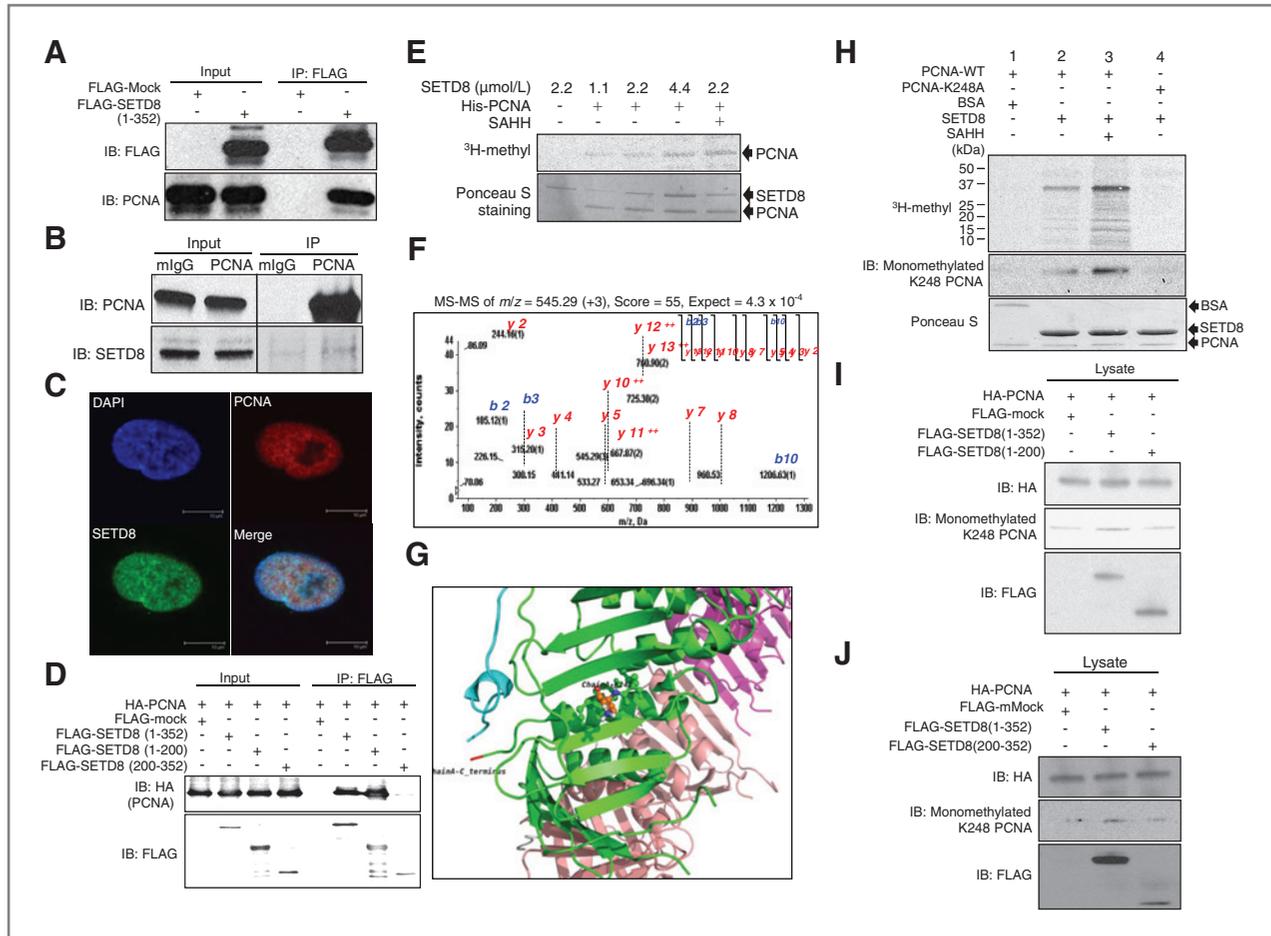


Figure 2. SETD8 methylates lysine 248 of PCNA both *in vitro* and *in vivo*. **A**, FLAG-mock and FLAG-SETD8 expression vectors were transfected into 293T cells. After 48 hours, cells were immunoprecipitated (IP) with anti-FLAG M2 agarose beads, and immunoprecipitants were immunoblotted (IB) with anti-FLAG (F7425; Sigma-Aldrich) and anti-PCNA (PC10, Santa Cruz Biotechnology) antibodies, respectively. **B**, SBC5 cells were lysed and immunoprecipitated with normal mouse IgG (mlgG) and anti-PCNA antibody (PC10). The immunoprecipitates were fractionated by SDS-PAGE and blotted with anti-PCNA (PC10) and anti-SETD8 (ab3798, Abcam) antibodies. **C**, immunocytochemical analysis of HeLa cells. Cells were stained with an anti-PCNA antibody [PC10, Cell Signaling Technology; Alexa Fluor 594 (red)], an anti-SETD8 antibody [C18B7, Cell Signaling Technology; Alexa Fluor 488 (green)], and 4',6'-diamidino-2'-phenylindole dihydrochloride [DAPI (blue)]. **D**, 293T cells were transfected with HA-PCNA and FLAG-mock or indicated FLAG-SETD8 expression vectors containing deletion variants. Cell lysates were immunoprecipitated with anti-FLAG M2 agarose beads. Samples were fractionated by SDS-PAGE and blotted with anti-HA (Y-11, Santa Cruz Biotechnology) and anti-FLAG (F7425) antibodies. **E**, *in vitro* methyltransferase assay of PCNA. Recombinant His-PCNA and ³H-SAM were incubated in the presence or absence of recombinant SETD8, and the reaction products were analyzed by SDS-PAGE followed by fluorography (top). The membrane was stained with Ponceau S (bottom). **F**, the MS-MS spectrum corresponding to the monomethylated PCNA 241–254 peptide. The 14 Da increase of the Lys 248 residue was observed, showing the monomethylated Lys 248. Score and Expect show Mascot Ion Score and Expectation value in Mascot Database search results, respectively. **G**, structure of the methylation site in PCNA protein analyzed by PyMOL. **H**, validation of an anti-monomethylated K248 PCNA antibody. Recombinant PCNA-WT or PCNA-K248A proteins and ³H-SAM were incubated in the presence or absence of recombinant SETD8, and the reaction products were analyzed by SDS-PAGE followed by fluorography (top). The membrane was immunoblotted with an anti-monomethylated K248 antibody (middle) and stained with Ponceau S (bottom). BSA, bovine serum albumin. **I** and **J**, 293T cells were cotransfected with an HA-PCNA vector and an empty vector (FLAG-mock), a FLAG-SETD8 (1–352) vector, (1–200, ΔSET) vector, or a FLAG-SETD8 (200–352, ΔPIP box) vector. The samples were immunoblotted with anti-monomethylated K248 PCNA, anti-FLAG, and anti-HA antibodies. SAHH, S-adenosyl-L-homocysteine hydrolase.

validate this result, we constructed the plasmid (PCNA-K248A) that was designed to substitute lysine 248 of PCNA protein to alanine and conducted *in vitro* methyltransferase assay (Supplementary Fig. S6B). The intensity of the band corresponding to PCNA methylation in PCNA-K248A was significantly diminished compared with that of the wild-type PCNA (PCNA-WT). These data show that lysine 248, which is highly conserved in the PCNA ortholog from green alga to human (Supplementary Fig. S4B), is the primary target of SETD8-dependent methylation (Fig. 2G). On the basis of this result, we generated an

antibody against a methylated K248 synthetic peptide (Supplementary Fig. S7A) that showed high affinity and high specificity by ELISA (Supplementary Fig. S7B). Western blot analysis using this antibody confirmed that it specifically recognizes K248-methylated PCNA (Fig. 2H and Supplementary Fig. S7C and S7D), and this specific signal was dependent on the methyltransferase activity of SETD8 (Fig. 2I). Importantly, the methyltransferase activity of N-terminal-deleted SETD8 protein, which lacks the PIP box domain, was significantly low than that of wild-type SETD8 protein (Fig. 2J). This

result indicates that the N-terminal region of SETD8 containing PIP box domain seems to be important for SETD8-dependent PCNA methylation. This antibody was used to examine the methylation status of PCNA *in vivo* after treatment with siSETD8 (Supplementary Fig. S8). Monomethylation of PCNA at lysine 248 diminished after knockdown of SETD8 in SBC5 cells, implying SETD8-dependent PCNA K248 methylation occurs both *in vitro* and *in vivo*.

SETD8 stabilizes PCNA protein through the methylation of lysine 248

To clarify the physiologic significance of PCNA methylation by SETD8, we examined protein expression levels of PCNA in SW780 cells 48 hours after knockdown of SETD8 using 2 independent siRNAs (Fig. 3A). Knockdown of SETD8 decreased

PCNA protein, suggesting involvement of SETD8 in regulating PCNA stability in cancer cells. To further validate this result, we examined the cell-cycle dependency of SETD8 and PCNA protein expression levels after aphidicolin synchronization (Fig. 3B). Intriguingly, when we treated with SETD8 siRNAs, PCNA protein expression decreased in both G₁ and S-phases according to the levels of SETD8, indicating that SETD8 is likely to be a key regulator of PCNA protein expression at G₁ and S-phases. Because quantitative real-time PCR analysis implied that *PCNA* mRNA level was not affected by treatment with siSETD8 (Fig. 3B), the regulation of PCNA expression by SETD8 was not at the transcriptional level but at the protein level. To examine that this regulation is mediated by SETD8-dependent methylation, we examined PCNA (WT) or PCNA (K248A) protein expression levels in 293T cells transfected with mock

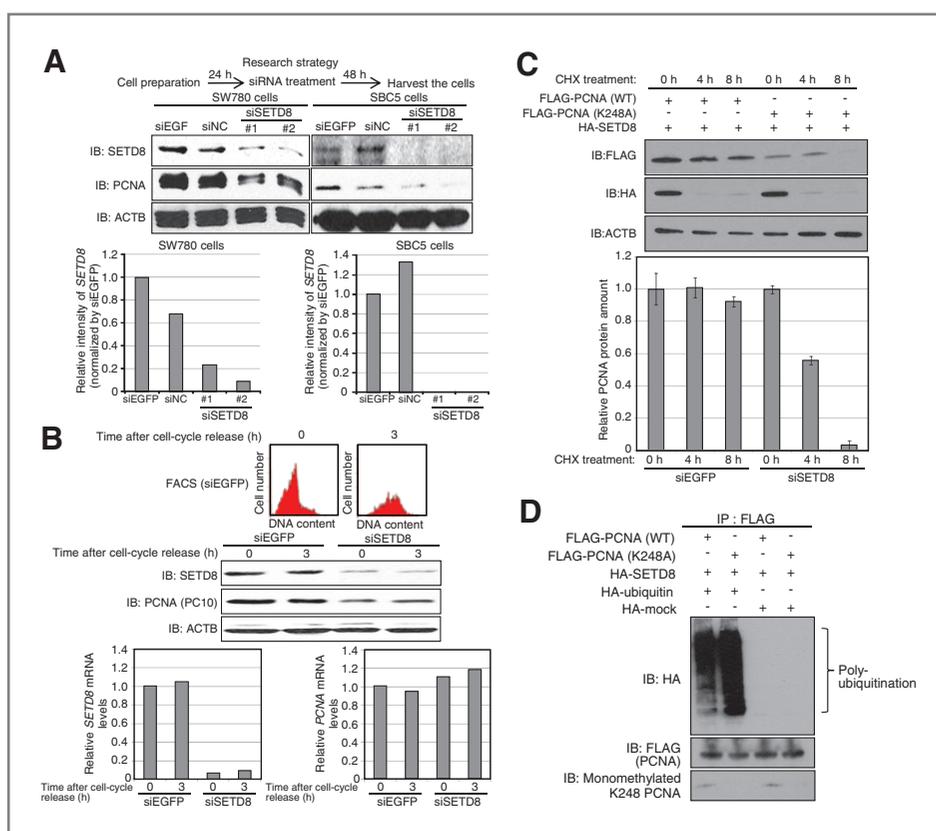


Figure 3. SETD8 stabilizes PCNA protein through the methylation of lysine 248. **A**, top, validation of SETD8 and PCNA expressions at the protein level. Lysates from SW780 and SBC5 cells, 48 hours after treatment with 2 control siRNAs (siEGFP and siNC) and 2 different siRNAs targeting SETD8 (siSETD8), were immunoblotted (IB) with anti-SETD8 (ab3798), anti-PCNA (PC10), and anti-ACTB (I-19, Santa Cruz Biotechnology) antibodies. β -actin (ACTB) served as an internal control. Bottom, the signal intensity corresponding SETD8 protein was quantified by ImageJ (<http://rsb.info.nih.gov/ij/index.html>). **B**, effects of SETD8 knockdown on the stability of PCNA in SW780 cells after synchronizing the cell cycle. SW780 cells were treated with siEGFP and siSETD8#2 for 24 hours and synchronized the cell cycle with 7.5 μ g/mL aphidicolin. After 24 hours of treatment, the culture medium was changed, and the cells were collected at 0 and 3 hours after the release from cell-cycle arrest. Cell-cycle status was analyzed by FACS (top, red), and cell lysates were immunoblotted with anti-SETD8 (ab3798), anti-PCNA (PC10), and anti-ACTB (I-19) antibodies (middle). Expression of ACTB was the internal control. Transcriptional expression levels of *SETD8* and *PCNA* were quantified by real-time PCR (bottom). **C**, SETD8 stabilizes PCNA protein through K248 methylation. 293T cells were transfected with FLAG-PCNA (WT) or FLAG-PCNA (K248A) and HA-SETD8 (1-352) expression vectors. After 24 hours, cells were treated with 100 μ g/mL of cycloheximide (CHX) for 4 and 8 hours, then immunoblotted with anti-HA (Y-11), anti-FLAG (F7425), and anti-ACTB (I-19) antibodies. Signal intensities of PCNA and ACTB proteins were quantitatively analyzed by GS-800 (Bio-Rad), and each PCNA intensity was normalized by ACTB intensity. Relative PCNA protein amount shows the intensity value standardized by the intensity at 0 hour (both siEGFP- and siSETD8-treated samples, 0 h = 1); results are the mean \pm SD of triplicate experiments. **D**, ubiquitin assay of exogenous PCNA. FLAG-PCNA (WT) or FLAG-PCNA (K248A) and HA-SETD8 (1-352) expression vectors were transfected into 293T cells together with a HA-ubiquitin or HA-mock expression vector. Cell lysates were immunoprecipitated (IP) with anti-FLAG M2 agarose beads and immunoblotted with anti-FLAG (F7425), anti-HA (Y-11), and anti-monomethylated K248 PCNA antibodies.

or SETD8 expression vectors after cycloheximide treatment. Although wild-type PCNA was significantly stabilized by SETD8 expression, methylation-inactive mutant PCNA (PCNA-K248A) was unstable (Fig. 3C). Furthermore, we examined the PCNA stability in endogenous level after depletion of SETD8 and found that the degradation rate of PCNA in cells treated with siSETD8 more rapidly than siEGFP (Supplementary Fig. S9). Taken together, SETD8-dependent methylation is crucial for PCNA stabilization. Then, we validated the effect of SETD8-dependent methylation on ubiquitination of PCNA proteins. The PCNA (WT) or PCNA (K248A) expression vector was cotransfected into 293T cells with a vector expressing either the full-length or N-terminal region of SETD8, and ubiquitination and methylation status of PCNA was examined (Fig. 3D). As we expected, the status of ubiquitination and

methylation on PCNA showed the inverse correlation. Hence, we consider that methylation of PCNA inhibited its ubiquitination. We also examined the phosphorylation status of Tyr 211 on PCNA, which is known to influence the stability of PCNA (11), but no significant relationship between methylation and phosphorylation status was observed (data not shown). These data show that PCNA protein is stabilized through inhibition of the ubiquitination by its SETD8-dependent methylation.

Methylation of lysine 248 on PCNA affects its interaction with FEN1

We conducted immunoprecipitation analysis to further investigate the significance of PCNA methylation, using wild-type and methylation-inactive mutant PCNA proteins, and identified a partner protein, FEN1, which interacted with

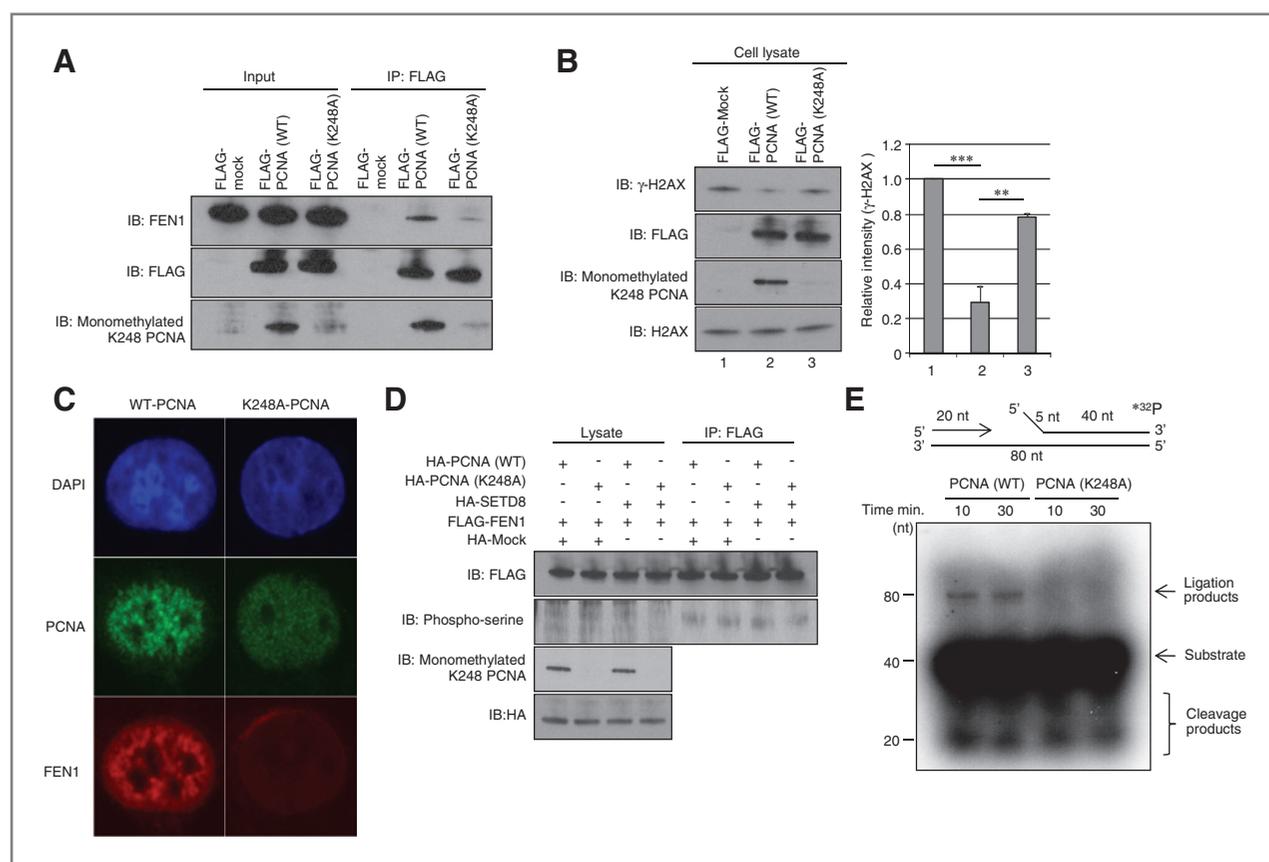


Figure 4. Methylation of PCNA is crucial for the interaction with FEN1. **A**, K248 monomethylation affected the interaction of PCNA with FEN1. 293T cells were transfected with a FLAG-PCNA (WT) vector or a FLAG-PCNA (K248A) vector together with an HA-SETD8 vector. Immunoprecipitation (IP) was conducted using anti-FLAG M2 agarose and samples were immunoblotted (IB) with anti-FLAG (F7425), anti-FEN1 (HPA006748, Sigma-Genosys), and anti-monomethylated K248 PCNA antibodies. **B**, double-strand DNA breaks were detected by Western blotting using an anti- γ H2AX antibody (05-636, Millipore). Lysates from 293T cells transfected with a FLAG-PCNA (WT) or a FLAG-PCNA (K248A) and were immunoblotted with anti-FLAG (F7425), anti-monomethylated K248 PCNA, anti- γ H2AX (05-636), and anti-H2AX (07-627, Millipore) antibodies. Signal intensity was quantified by ImageJ. Results are the mean of 2 independent experiments. **C**, subcellular localization of PCNA and FEN1 in S-phase. HeLa cells transfected with a FLAG-PCNA (WT) vector or a FLAG-PCNA (K248A) vector were synchronized at late G₁ phase by treatment with mimosine (400 μ mol/L for 12 hours). Cell cycle was released by removing mimosine, and cells were costained with anti-FLAG (F7425) and anti-FEN1 (HPA006748) antibodies. Scale bar, 5 μ m. **D**, methylation of PCNA does not alter FEN1 phosphorylation. 293T cells were cotransfected with a HA-PCNA (WT) vector or a HA-PCNA (K248A) together with HA-SETD8 and FLAG-FEN1 expression vectors. Immunoprecipitation was conducted using anti-FLAG M2 agarose, and samples were immunoblotted with anti-FLAG (F7425), anti-HA (Y-11), anti-monomethylated K248 PCNA, and anti-phospho-serine (4A4, Millipore) antibodies. **E**, Okazaki fragment maturation assay. A schematic diagram of the assay (top) showing a gap substrate (20 mer and 40 mer, top; with an 80-mer complementary strand, bottom) with a 5-nt DNA flap (40 mer, top right strand, with or without a ³²P label attached). The gap substrate was incubated with wild-type PCNA [FLAG-PCNA (WT)] and mutant-type PCNA [FLAG-PCNA (K248A)].

PCNA in a methylation-dependent manner. Methylation of PCNA significantly enhanced the interaction between PCNA and FEN1 (Fig. 4A). To validate the effect of PCNA methylation on the interaction with FEN1 in more detail, we conducted an *in vitro* binding assay using methylated PCNA and unmethylated PCNA with FEN1 recombinant protein. SETD8-dependent lysine methylation of PCNA significantly enhanced the interaction between PCNA and FEN1 *in vitro* (Supplementary Fig. S10). FEN1 is a structure-specific nuclease with both 5' flap endonuclease and 5'-3' exonuclease activities (21). During DNA replication, this enzyme is responsible for RNA primer removal during Okazaki fragment processing and was identified as the factor responsible for the completion of replication *in vitro* (22). Yeast cells lacking the *FEN1* gene (also called *RAD27*) are viable

but are unable to grow at high temperatures, indicating defective DNA replication (23). To examine the effect of PCNA methylation on FEN1 function, we measured levels of the phosphorylated form of H2AX histone variant (γ H2AX), an early marker of the cellular response to DNA breaks. In the absence of any exogenous source of DNA damage, basal levels of phosphorylated γ H2AX in 293T cells expressing methylation-inactive mutant PCNA (PCNA-K248A) were higher than those in 293T cells expressing wild-type PCNA (Fig. 4B). This implies the accumulation of DNA double-strand breaks resulting from methylation-inactive mutant PCNA expression. During the S-phase of the cell cycle, FEN1 is recruited to DNA replication loci through the interaction with PCNA. Disruption of the FEN1-PCNA interaction impairs such localization (24).

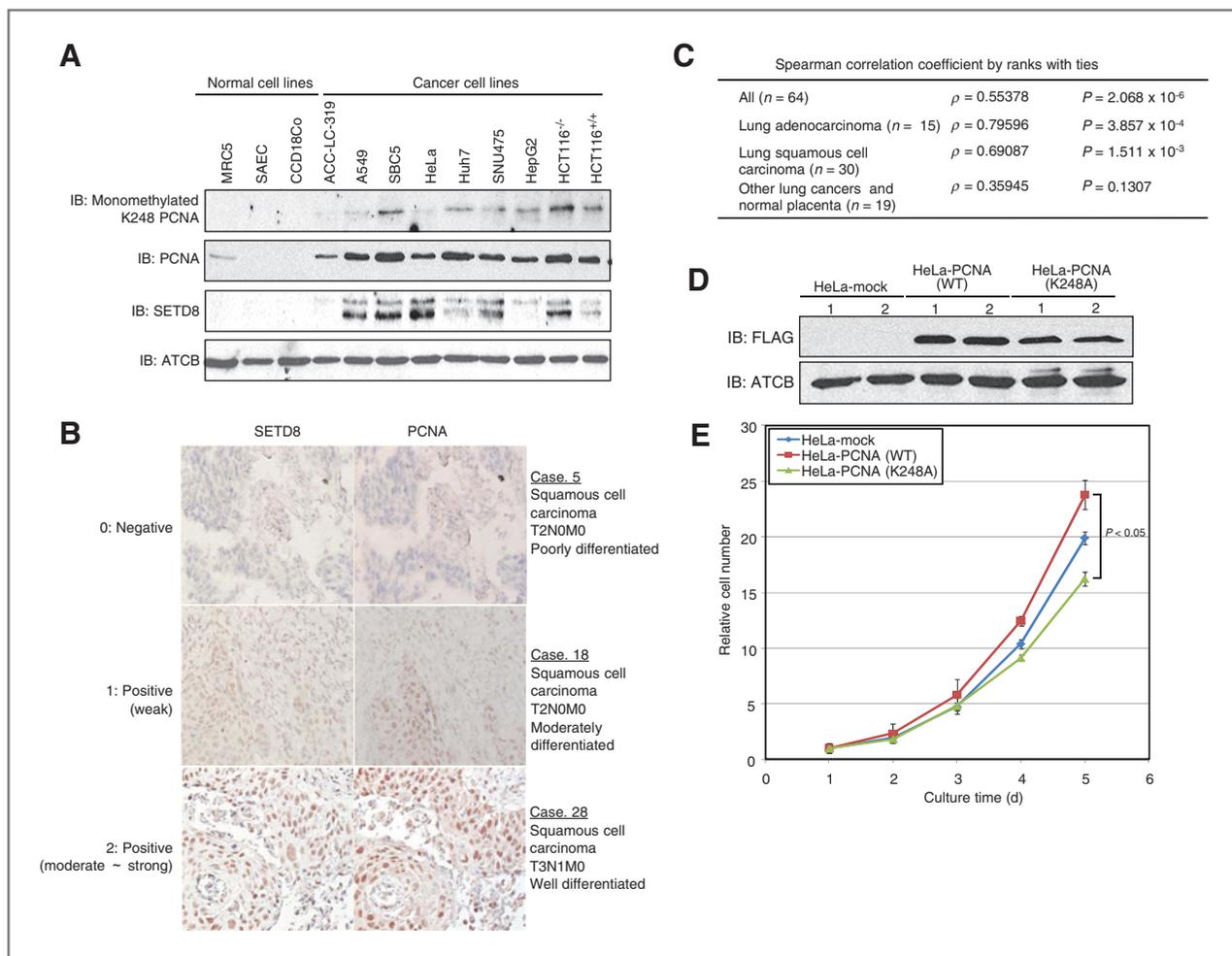


Figure 5. SETD8 and PCNA are coexpressed in lung cancer tissues, and methylation of PCNA promotes the proliferation of cancer cells. **A**, validation of PCNA methylation status in various cell lines. Lysates from normal cell lines and cancer cell lines were immunoblotted (IB) with anti-monomethylated K248 PCNA and anti-ACTB (I-19) antibodies. **B**, immunohistochemical stainings of SETD8 and PCNA in lung cancer tissues. Three typical stained case tissues are shown, with staining intensity of each case categorized into 3 patterns: 0 (no staining), 1 (weak staining), and 2 (moderate or strong staining). Detailed clinical information is described in Supplementary Table S6. **C**, correlation of staining between SETD8 and PCNA was statistically calculated using Spearman correlation coefficient by ranks with ties. **D**, construction of HeLa stable cell lines overexpressing wild-type PCNA [HeLa-PCNA (WT)] and K248-substituted PCNA [HeLa-PCNA (K248A)]. Empty vector-transfected HeLa stable cell lines were used as a control (HeLa-mock). Lysates from each cell line were immunoblotted with anti-FLAG (F7425) and anti-ACTB (I-19) antibodies. **E**, the cell growth assay was conducted using HeLa stable cell lines. Number of cells was measured by Cell Counting Kit-8 (Dojindo) and relative cell number shows the value normalized by the number of cells at day 1 (day 1 = 1). Results are the mean \pm SD in 3 independent experiments. P values were calculated using Student t test.

If methylation of PCNA were important for interacting with FEN1, failure in methylation would lead to a defect in FEN1's localization to replication foci. FEN1 could be colocalized with PCNA at replication foci in cells when PCNA was a wild-type. However, FEN1 was unable to localize to the foci in cells in which methylation-inactive mutant was present (Fig. 4C). These data suggest that PCNA methylation is important for regulation of FEN1's subnuclear localization. Because phosphorylation of FEN1 has been shown to abolish its PCNA interaction (25), we examined FEN1 phosphorylation status in cells expressing wild-type and methylation-inactive mutant PCNA, but found no significant difference in phosphorylation status of FEN1 between wild-type and methylation-inactive mutant PCNA-expressing cells (Fig. 4D). This implies that the different affinity between PCNA and FEN1 seems to be regulated not by phosphorylation status of FEN1 but by methylation status of PCNA (as shown in Fig. 4A and C). Furthermore, an Okazaki fragment maturation assay was conducted using the deoxynucleotide triphosphate mixture containing radiolabeled dCTP and a model substrate containing an RNA-DNA flap, which mimicked the Okazaki fragment maturation intermediate. The assay simulates the sequential reactions of gap filling, RNA primer removal, and DNA ligation during Okazaki fragment maturation. When the assay was conducted *in vitro*, nuclear extracts from PCNA-K248A-

expressing cells showed significant decrease in removing RNA primer flaps and some extent of defect in DNA ligation (Fig. 4E), indicating that the methylation defect of PCNA retarded Okazaki fragment maturation. Defects in the Okazaki fragment maturation process during DNA replication or defects in ligation during DNA repair could lead to accumulation of DNA double-strand breaks (26, 27). To examine the levels of double-strand breaks, 293 cells expressing wild-type and methylation-inactive mutant PCNA were treated with H₂O₂ to determine the survival rate (Supplementary Fig. S11). Consistent with previous data, methylation-inactive mutant PCNA-expressing cells were more sensitive to H₂O₂.

SETD8 and PCNA are coexpressed in lung cancer tissues, and lysine 248 methylation of PCNA promotes the proliferation of cancer cells

We then compared the methylation of endogenous PCNA in normal and cancer cell lines. PCNA was significantly methylated in various types of cancer cell lines, whereas no detectable level of PCNA methylation was found in normal cell lines (Fig. 5A). We subsequently conducted the immunopathologic analysis on clinical lung tissues, analyzing the correlation between SETD8 and PCNA protein expression levels (Fig. 5B). Clinical information and staining patterns of clinical tissues are described in Table 1 and Supplementary Table S6. We found

Table 1. Association between SETD8 and PCNA in lung cancer tissues and patients' characteristics (N = 64)

	Number of cases <i>n</i> = 64	SETD8 expression positive <i>n</i> = 42	SETD8 expression negative <i>n</i> = 22	PCNA expression positive <i>n</i> = 48	PCNA expression negative <i>n</i> = 16
Gender	62	40	22	46	16
Male	40	25	15	32	8
Female	22	15	7	14	8
Age, y	62	40	22	46	16
<65	42	28	14	32	10
≥65	20	12	8	14	6
Histologic type	64	42	22	48	16
ADC	14	10	4	10	4
SCC	30	17	13	20	10
Others ^a	20	15	5	18	2
pT factor	63	42	21	48	15
pT0	4	3	1	3	1
pT1	7	7	0	7	0
pT2	41	25	16	29	12
pT3	11	7	4	9	2
pN factor	59	40	19	44	15
N0	48	33	15	34	14
N1	11	7	4	10	1
M factor	61	41	20	46	15
M0	59	40	19	44	15
M1	2	1	1	2	0

Abbreviations: ADC, adenocarcinoma; ASC, adenosquamous-cell carcinoma; LCC, large cell carcinoma; SCC, squamous cell carcinoma.

^aOthers include SCLC, LCC, and ASC.

a correlation factor (ρ) of 0.55378 with P value of 2.068×10^{-6} (by Spearman correlation coefficient) in a cohort of 64 cases (Fig. 5C); lung adenocarcinoma showed a stronger correlation ($\rho = 0.79596$, $P = 3.857 \times 10^{-4}$), supporting our hypothesis that SETD8 overexpression stabilizes and increases the PCNA protein expression in cancer cells. Finally, we examined the effect of PCNA methylation on the growth of cancer cells (Fig. 5D and E). Methylation-inactive-type PCNA-expressing HeLa cells (HeLa-PCNA-K248A) showed a slower growth rate than those with wild-type PCNA-expressing HeLa cells (HeLa-PCNA-WT). Furthermore, to exclude the effect of endogenous PCNA proteins, we first knocked down PCNA gene expression, and then, conducted a clonogenicity assay of HeLa cells overexpressing wild-type PCNA and methylation-inactive-type PCNA (Supplementary Fig. S12). Consistent with our previous data, wild-type PCNA showed higher growth promoting effects than methylation-inactive type PCNA. Taken together, these results imply that methylation of PCNA is likely to play a crucial role in the growth promotion of cancer cells.

Discussion

Histone lysine methylation plays a central epigenetic role in the organization of chromatin domains and the regulation of gene expression. We previously reported that the HKMT SMYD3 stimulates cell proliferation through its methyltransferase activity and plays a crucial role in human carcinogenesis (28, 29). Of the various posttranslational protein modifications, the role of protein methylation in signal transduction has not been well characterized. While the carboxyl group and arginine methylation have been implicated in several cellular responses, including receptor signaling, protein transport, and transcription (30), lysine methylation has been considered to be histone specific (31). In the present study, we found that the HKMT SETD8 is overexpressed in various types of cancer and regulates PCNA functions through the methylation of lysine 248. This is a new mechanism revealing the importance of lysine methylation in nonhistone proteins in human cancer.

PCNA was originally reported to be a DNA-sliding clamp for replicative DNA polymerases and is an essential component of the eukaryotic chromosomal DNA replisome (32, 33). It interacts with multiple partners including proteins involved in Okazaki fragment processing, DNA repair, DNA synthesis, DNA methylation, chromatin remodeling, and cell-cycle regulation (34). PCNA has been reported to be modified by ubiquitination, SUMOylation, phosphorylation, and acetylation (9, 11, 12, 35, 36) but its lysine methylation has never been. These kinds of protein modifications are vital for a wide variety of PCNA functions. As reported here, PCNA protein is stably overexpressed in various types of cancer cells, together with SETD8 protein, indicating that SETD8-dependent methylation of PCNA enhances its biologic activity. Knockdown of SETD8 significantly suppressed the growth of cancer cells by diminishing PCNA methylation and reduction of its protein levels. It has been recently reported that knockdown of

SETD8 leads to several aberrant phenotypes, including DNA damage, S-phase arrest, and global chromosome condensation (20, 37, 38), consistent with our findings, which suggest that these abnormalities is likely to be caused by dysfunction of the PCNA protein.

PCNA is also considered to be the crucial factor in maintaining the balance between survival and cell death. For instance, PCNA displays an apoptotic activity through interaction with proteins belonging to the Gadd45 family (Gadd45, Myd118, and CR6), which was involved in growth control, apoptosis, and DNA repair (39, 40). Lack of SETD8 induces an increase of the sub-G₁ population of cancer cells (Fig. 1D), so it is possible that apoptosis may be induced by SETD8 depletion through dysfunction of PCNA. Furthermore, we clarified that methylation of PCNA is critical for the interaction with FEN1. It has been reported that FEN1 forms distinct protein complexes for DNA replication and repair. Through its interaction with PCNA, FEN1 is recruited to the replication foci for RNA primer removal and to repair sites for DNA base excision repair (41). Recently, the FEN1-PCNA interaction has been implicated in coordinating the sequential action of polymerase δ (Pol δ), FEN1, and DNA ligase 1 (Lig1) during Okazaki fragment maturation (24). Disruption of PCNA-FEN1 interaction impairs Okazaki fragment ligation (24). We showed that methylation-defective PCNA retards both Okazaki fragment maturation and DNA replication, and induces DNA damages. Cells expressing methylation-inactive mutant PCNA were more sensitive to DNA damage. Because deregulation of FEN1 nuclease has also been reported to be linked to human cancer (42), it is possible that abnormal interactions between FEN1 and PCNA may cause human carcinogenesis. Intriguingly, Guo and colleagues recently showed that methylation of FEN1 suppresses nearby phosphorylation and facilitates PCNA binding (43). Together with our result, this implicates methylation as the crucial player in the interaction between PCNA and FEN1 proteins.

In conclusion, as expression levels of *SETD8* in normal tissues are significantly low (Supplementary Fig. S13), an inhibitor targeting its enzymatic activity might be an effective drug for cancer therapy. Further functional analysis will explore the SETD8-dependent PCNA methylation pathway as a therapeutic target in various types of cancer.

Disclosure of Potential Conflicts of Interest

Y. Yamane, Y. Iwai, and K. Maejima are employed as Researchers in OncoTherapy Science, Inc. R. Hamamoto is a scientific advisor in OncoTherapy Science, Inc. The other authors disclosed no potential conflicts of interest.

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Histone Lysine Methyltransferase SETD8 Promotes Carcinogenesis by Deregulating PCNA Expression

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