Histone Lysine Methyltransferase SETD8 Promotes Carcinogenesis by Deregulating PCNA Expression

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Abstract

Although the physiological significance of lysine methylation of histones is well known, whether lysine methylation plays a role in the regulation of non-histone proteins has not yet been examined. The histone lysine methyltransferase SETD8 is overexpressed in various types of cancer and appears to play a crucial role in S-phase progression. Here, we demonstrate 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 non-histone protein and suggest that aberrant lysine methylation of PCNA may play a role in human carcinogenesis.
Introduction

Protein methylation is recently considered an important post-translational modification and is predominantly found on lysine and arginine residues. Lysine methylation involves the addition of one to three 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 (HKMTs) 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 physiological functions of histone lysine methyltransferases 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 SLE (systemic lupus erythematosus) patients, 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 post-translationally 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 post-translational 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) (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 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 demonstrated 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 (ATCC) 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 short tandem repeat (STR) markers. EJ28 cells were from Cell Line Service (CLS) in
2003, and tested and authenticated by DNA profiling for polymorphic short tandem repeat (STR) markers. ACC-LC-319 cells were from Aichi Cancer Center in 2003, and tested and authenticated by DNA profiling for SNP, 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 (housekeeping gene), SDH (housekeeping gene), SETD8 and PCNA were designed (Primer sequences in Supplementary Table S1). PCR reactions were performed using the LightCycler® 480 System (Roche Applied Science) following the manufacture’s protocol.

**siRNA transfection**

siRNA oligonucleotide duplexes were purchased from SIGMA Genosys for targeting the human SETD8 transcript. siEGFP, siFLuc and siNegative control (siNC), which is a mixture of three different oligonucleotide duplexes, were used as control siRNAs. The siRNA sequences are described in Supplementary Table S2. siRNA duplexes (100 nM final concentration) were transfected into bladder and lung cancer cell lines with Lipofectamine 2000 (Life Technologies) for 72 h, 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

In order to investigate roles of a histone lysine methyltransferase 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 in 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 non-neoplastic tissues (Fig. 1B). In addition, our expression profiling analysis indicated the up-regulation of SETD8 in chronic myelogenous leukemia (CML), hepatocellular carcinoma (HCC) and pancreatic cancer (Supplementary Fig. S2 and Table S5). Furthermore, a high level of SETD8 was identified in various cancer cell lines, compared with a normal lung cell line, SAEC (Supplementary Fig. S3).

To investigate the role of SETD8 in the growth of cancer cells, we performed a knockdown experiment using two independent siRNAs against SETD8 (siSETD8#1 and #2) and two 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 two different siRNAs targeting SETD8, compared with control siRNAs (Supplementary Fig. S1B). Using the same siRNAs, we performed cell growth assays and found significant growth suppressive effects on one bladder cell line (SW780) and two lung cancer cell lines (RERF-LC-AI and SBC5) while 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 BrdU incorporation analysis, the amount of newly incorporated BrdU 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.

**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 co-localized in HeLa cells (Fig. 2C). Immunoprecipitation using deletion mutants of SETD8 showed that its N-terminal region of SETD8 is essential for the binding to PCNA (Fig. 2D), and this portion contains a PCNA-interacting protein (PIP) box (Supplementary Fig. S4A). Since histone methyltransferases have been found to methylate non-histone substrates, we evaluated a possibility of PCNA to be a substrate of SETD8. Firstly, we performed 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 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 LC-MS/MS analysis identified mono-methylation at lysine 248 on PCNA by SETD8 (Fig. 2F). To validate this result, we constructed the plasmid (PCNA-K248A) that was designed to substitute lysine 248 of PCNA protein to alanine and performed 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 demonstrate 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 D), 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 compared with 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). Mono-methylation 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 physiological significance of PCNA methylation by SETD8, we
examined protein expression levels of PCNA in SW780 cells 48 h after knockdown of SETD8 using two 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. Since 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 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 co-transfected 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 co-relation. Hence, we consider that methylation of PCNA inhibited its ubiquitination. We also examined the phosphorylation status of tyrosine 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 performed 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 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 un-methylated 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 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 (γH2AX), an early marker of the cellular response to DNA breaks. In the absence of any exogenous source of DNA damage, basal levels of phosphorylated γH2AX 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). If methylation of PCNA is 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. Since 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 appear to be regulated not by phosphorylation status of FEN1 and but by methylation status of PCNA (as shown in Fig. 4A and C). Furthermore, an Okazaki fragment maturation assay was performed, using the dNTP 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 performed 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 \( \text{H}_2\text{O}_2 \).

**SETD8 and PCNA are co-expressed 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 while no detectable level of PCNA methylation was found in normal cell lines (Fig. 5A). We subsequently conducted the immunopathological 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 a correlation factor \( (\rho) \) of 0.55378 with \( P \) value of \( 2.068 \times 10^{-6} \) (by Spearman’s 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 \( \text{PCNA} \) 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 histone lysine methyltransferase 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 histone lysine methyltransferase 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 non-histone 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 biological 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 versus 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-G$_1$ 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 $\delta$ (Pol $\delta$), 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. Since 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 et al. 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.

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Reference


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

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Abbreviations: ADC, adenocarcinoma; SCC, squamous-cell carcinoma
*others, small cell lung cancer (SCLC), large cell carcinoma (LCC), adenosquamous-cell carcinoma (ASC)
Figure legends

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 to normal (Japanese patients). Signal intensity for each sample was analyzed by cDNA microarray. NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer. B, immunohistochemical staining of SETD8 in lung tissues. Clinical information for each section is represented above histological pictures. Original magnification, x100. 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 ±SD of three independent experiments. P values were calculated using Student’s 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 FACS results in SBC5 cells, classifying cells by cell cycle status. Results are the mean ±SD of three independent experiments. P values were calculated using Student’s 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-BrdU and 7-amino-actinomycin D (7-AAD) as described in Methods.

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 h, cells were immunoprecipitated with anti-FLAG® M2 agarose beads, and immunoprecipitants were immunoblotted 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 (mlG) 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’-diamidine-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 (upper panel). The membrane was stained with Ponceau S (lower panel). SAHH: S-adenosyl-L-homocysteine hydrolase. F, the MS/MS spectrum corresponding to the mono-methylated PCNA 241-254 peptide. The 14 Da increase of the Lys 248 residue was observed, demonstrating the mono-methylated 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-mono-methylated 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 (upper panel). The membrane was immunoblotted with an anti-mono-methylated K248 antibody (middle panel) and stained with Ponceau S (lower panel). I and J, 293T cells were co-transfected 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-mono-methylated K248 PCNA, anti-FLAG and anti-HA antibodies.

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 h after treatment with two control siRNAs (siEGFP and siNC) and two different siRNAs targeting SETD8, were immunoblotted with anti-SETD8 (ab3798), anti-PCNA (PC10) and anti-ACTB (I-19, Santa Cruz Biotechnology) antibodies. ACTB served as an internal control. Bottom, the signal intensity corresponding SETD8 protein was quantified by Image J (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 h and synchronized the cell cycle with 7.5 μg/ml aphidicolin. After 24 h of treatment, the culture medium was changed, and the cells were collected at 0 and 3 h 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 (lower). 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 μg/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 intensity of PCNA and ACTB proteins was 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 h (both siEGFP and siSETD8 treated samples, 0 h = 1): results are the mean ±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 an HA-Ubiquitin or an HA-Mock expression vector. Cell lysates were immunoprecipitated with anti-FLAG® M2 agarose beads and immunoblotted with anti-FLAG (F7425), anti-HA (Y-11) and anti-mono-methylated K248 PCNA antibodies.

Figure 4. Methylation of PCNA is crucial for the interaction with FEN1. A, K248 mono-
methylation 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 was performed using anti-FLAG M2 agarose and samples were immunoblotted with anti-FLAG (F7425), anti-FEN1 (HPA006748, Sigma-Genosys) and anti-mono-methylated K248 PCNA antibodies. B, double-strand DNA breaks were detected by western blot using an anti-gamma-H2AX antibody (05-636, Millipore). Lysates from 293T cells transfected with a FLAG-PCNA (WT) or a FLAG-PCNA (K248A) were immunoblotted with anti-FLAG (F7425), anti-mono-methylated K248 PCNA, anti-gamma H2AX (05-636) and anti-H2AX (07-627, Millipore) antibodies. Signal intensity was quantified by Image J. Results are the mean of two 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 µM for 12 h). Cell cycle was released by removing mimosine, and cells were co-stained with anti-FLAG (F7425) and anti-FEN1 (HPA006748) antibodies. Scale bar; 5 µm. D, methylation of PCNA does not alter FEN1 phosphorylation. 293T cells were co-transfected with a HA-PCNA (WT) vector or a HA-PCNA (K248A) together with HA-SETD8 and FLAG-FEN1 expression vectors. Immunoprecipitation was performed using anti-FLAG M2 agarose, and samples were immunoblotted with anti-FLAG (F7425), anti-HA (Y-11), anti-mono-methylated 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, upper, with, an 80 mer complementary strand, lower) with a 5-nt DNA flap (40 mer, upper 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)).

Figure 5. SETD8 and PCNA are co-expressed 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 with anti-
mono-methylated 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 is categorized into three patterns: 0 (no staining), 1 (weak staining), 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’s 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)). An 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 performed 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 ± SD in three independent experiments. P-values were calculated using Student’s t-test.
Figure 1. Takawa et al.
Figure 2. Takawa et al.
Figure 3. Takawa et al.
**Figure 4. Takawa et al.**

A. Diagram showing Western blot results for Input and IP: FLAG conditions, with bands for IB: FEN1, IB: FLAG, and IB: Mono-methylated K248 PCNA.

B. Graph displaying relative intensity of Gamma H2AX over time (1, 2, 3) with bars indicating significant differences.

C. Images of DAPI, PCNA, and FEN1 staining for WT-PCNA and K248A-PCNA samples.

D. Table and diagram illustrating Lysate and IP: FLAG conditions with bands for IB: FLAG, IB: Phospho-Serine, and IB: Mono-methylated K248 PCNA.

E. Diagram showing ligation products and cleavage products for PCNA (WT) and PCNA (K248A) with time (10, 30) and substrate size (20 nt, 5 nt, 40 nt, 80 nt).
Spearman’s correlation coefficient by ranks with ties

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**Figure 5. Takawa et al.**
Histone Lysine Methyltransferase SETD8 Promotes Carcinogenesis by Deregulating PCNA Expression

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