

Dysregulation of AKT Pathway by SMYD2-Mediated Lysine Methylation on PTEN^{1,2}

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Abstract

Phosphatase and tensin homologue (PTEN), one of the well-characterized tumor suppressor proteins, counteracts the phosphatidylinositol 3-kinase-AKT pathway through its unique lipid phosphatase activity. The functions of PTEN are regulated by a variety of posttranslational modifications such as acetylation, oxidation, ubiquitylation, phosphorylation, and SUMOylation. However, methylation of PTEN has not been reported so far. In this study, we demonstrated that the oncogenic protein lysine methyltransferase SET and MYND domain containing 2 (SMYD2) methylates PTEN at lysine 313 *in vitro* and *in vivo*. Knockdown of SMYD2 suppressed the cell growth of breast cancer cells and attenuated phosphorylation levels of AKT, indicating that SMYD2-mediated methylation negatively regulates PTEN tumor suppressor activity and results in activation of the phosphatidylinositol 3-kinase-AKT pathway. Furthermore, PTEN protein with lysine 313 substitution diminished phosphorylation of PTEN at serine 380, which is known to inactivate tumor suppressor functions of PTEN. Taken together, our findings unveil a novel mechanism of PTEN dysregulation regulated by lysine methylation in human cancer.

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Introduction

Phosphatase and tensin homologue (PTEN) was originally identified to be frequently disrupted in multiple sporadic tumor types and targeted by germline mutations in patients with cancer predisposition syndromes like Cowden disease [1,2]. Subsequently, the generation of *Pten* knockout mice demonstrated the important tumor suppressor functions of PTEN in various types of cancer [3–5]. PTEN has lipid phosphatase activity, which converts phosphatidylinositol-3,4,5-triphosphate to phosphatidylinositol-4,5-bisphosphate and antagonize the AKT signaling pathway [6]. The AKT signaling pathway is involved in multiple physiological processes including cell survival and proliferation and cellular metabolism. Dysregulation of AKT activity often causes a variety of diseases, including cancer [7]. Hence, this phosphatidylinositol-3,4,5-triphosphate lipid phosphatase activity of PTEN is widely recognized as an important tumor-suppressor function. PTEN consists of a phosphatidylinositol 4,5-bisphosphate binding domain, a phosphatase domain, a C2 domain, a carboxy-terminal tail, and a PDZ-binding domain [8]. While the N-terminal portion containing the phosphatidylinositol 4,5-bisphosphate binding domain and phosphatase domain is a catalytic core for phosphatase activity, the C2 domain is critical to recruit PTEN to the membrane, where PTEN works as a lipid

phosphatase [8]. It is well known that the PTEN functions are regulated by a variety of posttranslational modifications such as acetylation, oxidation, ubiquitylation, phosphorylation, and SUMOylation. For instance, acetylation of lysines 125 and 128 within the catalytic pocket that are mediated by p300/CBP-associated factor in response to the growth factor stimulation negatively regulates the tumor suppressor activity of PTEN [9]. Reactive oxygen species-mediated linkage of

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catalytic cysteines 124 and 71 by disulfide bond formation also inactivates PTEN [10]. In addition, the E3 ligase neural precursor cell expressed developmentally down-regulated protein 4 monoubiquitylates and polyubiquitylates PTEN; polyubiquitylation promotes rapid degradation, whereas monoubiquitylation is important for PTEN nuclear translocation [11,12]. Furthermore, multiple kinases have been reported to phosphorylate PTEN and regulate protein stability and function of PTEN [13–16]. However, methylation of PTEN has not been reported. In the present study, we describe that SET and MYND domain containing 2 (SMYD2), a protein lysine methyltransferase that is known to be involved in human tumorigenesis, methylates PTEN at lysine 313 *in vitro* and also *in vivo*. Our results imply that SMYD2-mediated methylation attenuates the tumor suppressor activity of PTEN as a regulator of the phosphatidylinositol 3-kinase (PI3K)-AKT signaling pathway, which results in the promotion of cancer cell growth.

Methods

Antibodies

The following primary antibodies were used: anti-FLAG (mouse, M2; Sigma-Aldrich, St Louis, MO; dilution used in immunocytochemistry: 1:2000), anti-human influenza hemagglutinin (rabbit, Y-11; Santa Cruz Biotechnology, Dallas, TX; dilution used in Western blotting: 1:3000, ICC: 1:1000), anti-SMYD2 (rabbit, D14H7; Cell Signaling Technology, Danvers, MA; dilution used in WB: 1:1000), anti-phosphorylated AKT (Thr 308) (rabbit, D25E6; Cell Signaling Technology; dilution used in WB: 1:1000), anti-AKT (rabbit, C67E7; Cell Signaling Technology; dilution used in WB: 1:1000), anti- α -tubulin (mouse, DM1A; Calbiochem, Billerica, MA; dilution used in WB: 1:1000), and anti-phospho-PTEN (Ser 380) (rabbit, Cell Signaling Technology, dilution used in WB: 1:1000). An anti-K313 dimethylated PTEN antibody (Sigma-Aldrich; dilution used in WB: 1:1000) was produced in rabbit immunized with a synthetic peptide.

Cell Culture

HeLa, HEK293T, and MDA-MB-231 cell lines were obtained from American Type Culture Collection (ATCC) (Manassas, VA). All cell lines were grown in monolayers in appropriate media supplemented with 10% FBS and 1% antibiotic/antimycotic solution (Sigma-Aldrich): Dulbecco's modified Eagle's medium for HEK293T cells; Eagle's minimal essential medium for HeLa cells; Leibovitz's L-15 for MDA-MB-231 cells. All cells were maintained at 37°C in humid air with 5% CO₂ condition (HEK293T and HeLa) or without CO₂ (MDA-MB-231). Cells were transfected with FuGENE6 or Fugene HD (Promega, Fitchburg, WI) according to the manufacturer's protocols [17,18].

In Vitro Methyltransferase Assay

In vitro methyltransferase assays were described previously [17,19–22]. Briefly, recombinant PTEN protein was incubated with recombinant SMYD2 and 2 μ Ci *S*-adenosyl-L-[methyl-³H]-methionine (PerkinElmer, Waltham, MA) in a mixture of methylase activity buffer (50 mM Tris-HCl at pH 8.8, 10 mM DTT, and 10 mM MgCl₂) for 1 hour at 30°C. After denaturing, samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), blotted to polyvinylidene difluoride membrane, and visualized by MemCode Reversible Stain (Thermo Fisher Scientific, Waltham, MA) and fluorography.

Mass Spectrometry

The reaction mixture of *in vitro* methyltransferase assay was analyzed by nano liquid chromatography-tandem mass spectrometry

(LC-MS/MS) using Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific GmbH, Bremen, Germany). The peptides were separated using nano ESI spray column (NTCC analytical column, C18, 75 μ m[ID] \times 100 mm[L], 3 μ m, Nikkoyo Technos Co., Ltd.) at a flow rate of 300 nl/min. The mass spectrometer was operated in the positive-ion mode, and the spectra were acquired in a data-dependent top 10 MS/MS mode. The MS/MS spectra were searched against the in-house database using local MASCOT server (version 2.2.1; Matrix Sciences, London, United Kingdom). The reaction mixture was desalted and applied to matrix-assisted laser desorption/ionization - time of flight - mass spectrometry using an ultrafleXtreme (Bruker Daltonik GmbH, Bremen, Germany).

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer at 4°C for 1 hour, permeabilized in 0.1% Triton X-100 (Sigma-Aldrich) for 3 minutes at room temperature, and blocked with 3% BSA for 1 hour at room temperature. Fixed cells were incubated with each primary antibody overnight at 4°C followed by incubation with Alexa Fluor-conjugated secondary antibody (Life Technologies, Carlsbad, CA) [23,24] and observed using Leica confocal microscopy (SP5 Tandem Scanner Spectral 2-Photon Confocal).

Western Blot Analysis

Samples were prepared from the cells lysed with CellLytic M lysis reagent (Sigma-Aldrich) supplemented with complete protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany). Whole-cell lysates or immunoprecipitated samples were separated by SDS-PAGE and blotted to nitrocellulose membrane. Protein bands were detected by incubating with HRP-conjugated antibodies (GE Healthcare, Little Chalfont, United Kingdom) and visualizing with enhanced chemiluminescence (GE Healthcare).

Small Interfering RNA Transfection

Small interfering RNA (siRNA) oligonucleotide duplexes were purchased from Sigma-Aldrich for targeting SMYD2 transcripts. siEnhanced green fluorescent protein and siNegative control (siNC), which is a mixture of three different oligonucleotide duplexes, were used as control siRNAs [25–28]. The siRNA sequences are described in Table W1. siRNA duplexes were transfected with Lipofectamine RNAi max (Life Technologies). Cell viability was examined by Cell Counting Kit-8 (Dojindo, Kumamoto, Japan).

Cell Cycle Analysis by Flow Cytometry

A 5'-bromo-2'-deoxyuridine (BrdU) flow kit (BD Biosciences, Franklin Lakes, NJ) was used to determine the cell cycle kinetics and to measure the incorporation of BrdU into DNA of proliferating cells [25]. The assay was performed according to the manufacturer's protocol. Briefly, cells (5×10^5 per dish) were seeded overnight in 10-cm diameter tissue culture dishes and treated with an optimized concentration of siRNAs for 96 hours, followed by addition of 10 μ M BrdU, and incubations continued for an additional 35 minutes. Floating and adherent cells were pooled from triplicate wells per treatment point, fixed in a solution containing paraformaldehyde and saponin, and incubated for 1 hour with DNAase at 37°C (30 μ g per sample). Fluorescein isothiocyanate-conjugated anti-BrdU antibody (1:50 dilution in wash buffer; BD Pharmingen, San Diego, CA) was added and incubated for 20 minutes at room temperature. Cells were washed in wash buffer and total DNA was stained with 7-amino-actinomycin D (7-AAD; 20 μ l per sample),

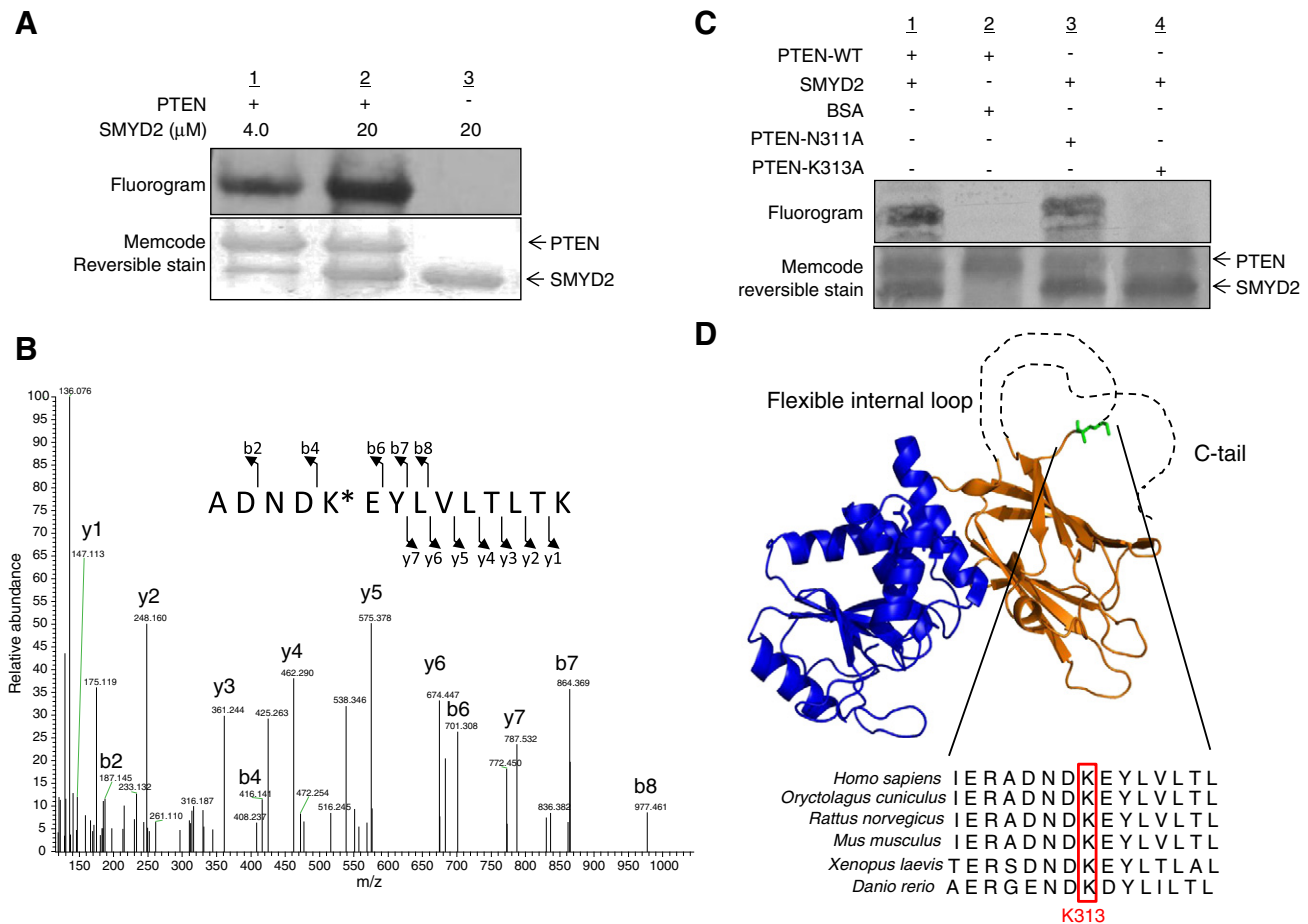


Figure 1. SMYD2 methylates PTEN at lysine 313 *in vitro*. (A) Recombinant PTEN protein was methylated by SMYD2 in a dose-dependent manner. Methylated PTEN was detected by fluorography, and lodging proteins were visualized by MemCode Reversible Protein stain. (B) LC-MS/MS spectrum of the dimethylated PTEN 309-322 peptide. PTEN recombinant protein was reacted with SMYD2 followed by SDS-PAGE. LC-MS/MS analysis was conducted after digestion of samples by trypsin. (C) Wild-type PTEN (PTEN-WT) and mutant-type PTEN (N311A and K313A) were prepared and used for an *in vitro* methyltransferase assay. Methylation signal of PTEN was diminished when lysine 313 was substituted. (D) Methylation site is displayed in the crystal structure of PTEN (PDB ID: 1D5R). Lysine 313 is located in the large flexible internal loop, and the lysine residue is evolutionally conserved.

followed by flow cytometric analysis using BD LSR II (BD Biosciences). Total DNA content (7-AAD) was determined by FlowJo software.

Results

In Vitro Methylation of PTEN by SMYD2

To clarify whether PTEN can be methylated, we conducted an *in vitro* methyltransferase assay using multiple recombinant histone lysine or arginine methyltransferases (Figure W1) and found that SMYD2 methylated PTEN in a dose-dependent manner (Figures 1A and W2). Subsequently, we conducted LC-MS/MS analysis and identified that lysine 313 of PTEN is dimethylated by SMYD2 (Figures 1B and W3). To further verify the SMYD2-mediated PTEN methylation at this lysine residue, we prepared wild-type and lysine 313-substituted recombinant PTEN proteins and performed an *in vitro* methyltransferase assay and confirmed that the PTEN methylation signal was significantly attenuated when we used the lysine 313-substituted PTEN as a substrate (Figure 1C). These results indicate that SMYD2 methylates PTEN at lysine 313 *in vitro*. This lysine residue is located in a highly flexible internal loop in the C2 domain of PTEN [8] and highly conserved among species (Figure 1D).

In Vivo PTEN Methylation

We subsequently performed immunocytochemical analysis and confirmed co-localization of SMYD2 and PTEN in the cytoplasm (Figure 2A). To verify the *in vivo* methylation, we generated a polyclonal antibody that specifically recognizes PTEN with dimethylation at lysine 313 (PTENK313me₂). The specificity of the antibody was confirmed by ELISA, which shows high affinity only to the methylated PTEN peptide (Figure W4). Then, we transfected FLAG-Mock and FLAG-SMYD2 into HEK293T cells and performed immunoprecipitation to analyze the methylation status of endogenous PTEN protein using the methylation-specific antibody. As shown in Figure 2B, the PTEN methylation signal was significantly higher in FLAG-SMYD2-transfected cells than FLAG-Mock-transfected cells. In addition, we also examined the methylation level of PTEN after knockdown of SMYD2 in the breast cancer MDA-MB-231 cells and confirmed that the methylation signal was decreased by knockdown of SMYD2 (Figure 2, C and D), indicating that SMYD2 methylates PTEN in cancer cells. Furthermore, we co-transfected FLAG-SMYD2 and HA-tagged wild-type PTEN (HA-PTEN-WT) or K313A mutant-type PTEN (HA-PTEN-K313A) into HEK293T cells and immunoprecipitated HA-PTEN proteins

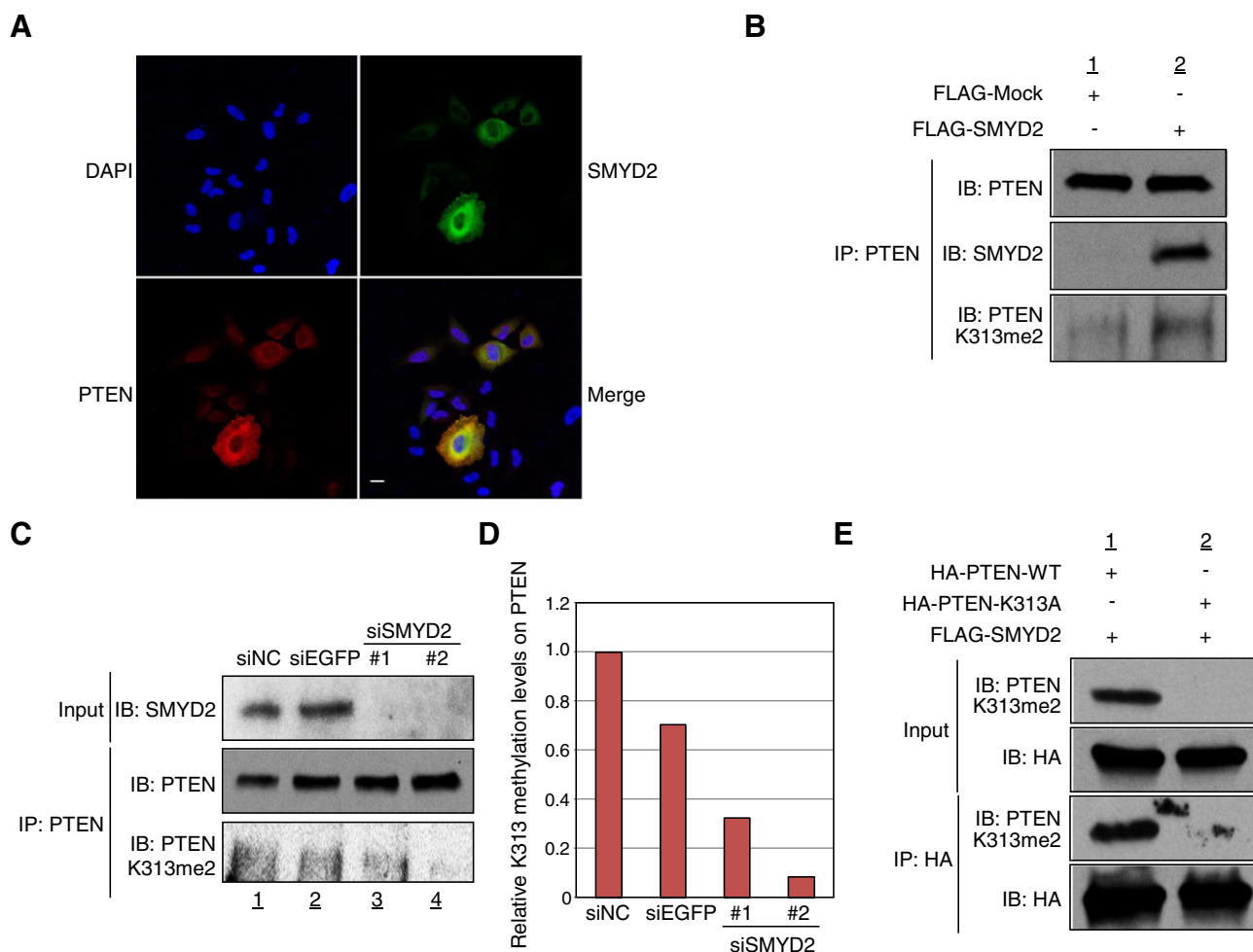


Figure 2. PTEN is methylated *in vivo*. (A) PTEN and SMYD2 were co-localized in the cytoplasm. HeLa cells were stained with anti-FLAG (for FLAG-tagged PTEN; Alexa Fluor 594, red) and anti-HA (for HA-tagged SMYD2; Alexa Fluor 488, green) antibodies and 4',6-diamidino-2-phenylindole (blue). Bar, 20 μ m. (B) The endogenous PTEN methylation level was examined after SMYD2 overexpression. FLAG-Mock and FLAG-SMYD2 were transfected into HEK293T cells, and immunoprecipitation using anti-PTEN antibody was conducted 48 hours after transfection. SMYD2 overexpression clearly increased PTEN methylation. (C) The methylation level of endogenous PTEN was examined after SMYD2 knockdown. MDA-MB-231 cells were treated with control siRNA or siSMYD2, and immunoprecipitation using an anti-PTEN antibody was conducted 96 hours after transfection. PTEN methylation was attenuated by SMYD2 knockdown. (D) Relative methylation levels of PTEN after SMYD2 knockdown. Signal intensity of the bands corresponding to K313 methylation of PTEN was quantified using a GS-800 calibrated densitometer (Bio-Rad, Hercules, CA). (E) Wild-type or lysine 313-substituted PTEN was overexpressed in HEK293T cells, and whole-cell lysate (input) and immunoprecipitated fractions were subjected to Western blot analysis using the anti-dimethylated K313 PTEN antibody (PTENK313me2). An anti-HA antibody was used as an internal control. Methylation signal was abolished in PTEN-K313A proteins.

using whole-cell lysates followed by Western blot analysis with the anti-PTEN K313me2 antibody. Consequently, we observed attenuation of the methylation-specific signal in the lysine 313-substituted PTEN protein (Figure 2E). Taken together, these results strongly reveal that PTEN is methylated at lysine 313 both *in vitro* and *in vivo* by SMYD2.

Negative Regulation of the Tumor Suppressor Activity of PTEN through SMYD2-Mediated Methylation

To address the biologic significance of the PTEN methylation in cancer cells, we examined the cell growth of breast cancer cells that overexpress SMYD2 by knockdown of SMYD2 (Figure 3A) [29]. The growth of two breast cell lines, MDA-MB-231 and MCF7 that

express wild-type PTEN [30,31], was significantly suppressed by treatment with two different SMYD2 siRNAs (Figure 3A). To further assess the mechanism of growth suppression caused by SMYD2 knockdown, we performed BrdU and 7-AAD staining and examined cell cycle status (Figure W5). The proportion of MDA-MB-231 cells at the G₁ phase was significantly increased after treatment with siSMYD2, while that at the S phase was remarkably decreased (Figure W5). This result has implied that SMYD2 is likely to play an important role in the G₁/S transition of cancer cells. Since it is well known that PTEN negatively regulates the G₁/S transition [32], SMYD2-mediated methylation might affect this PTEN function. Given that PTEN is known to counteract the activation of the PI3K-AKT pathway [33], we examined the phosphorylation status of AKT

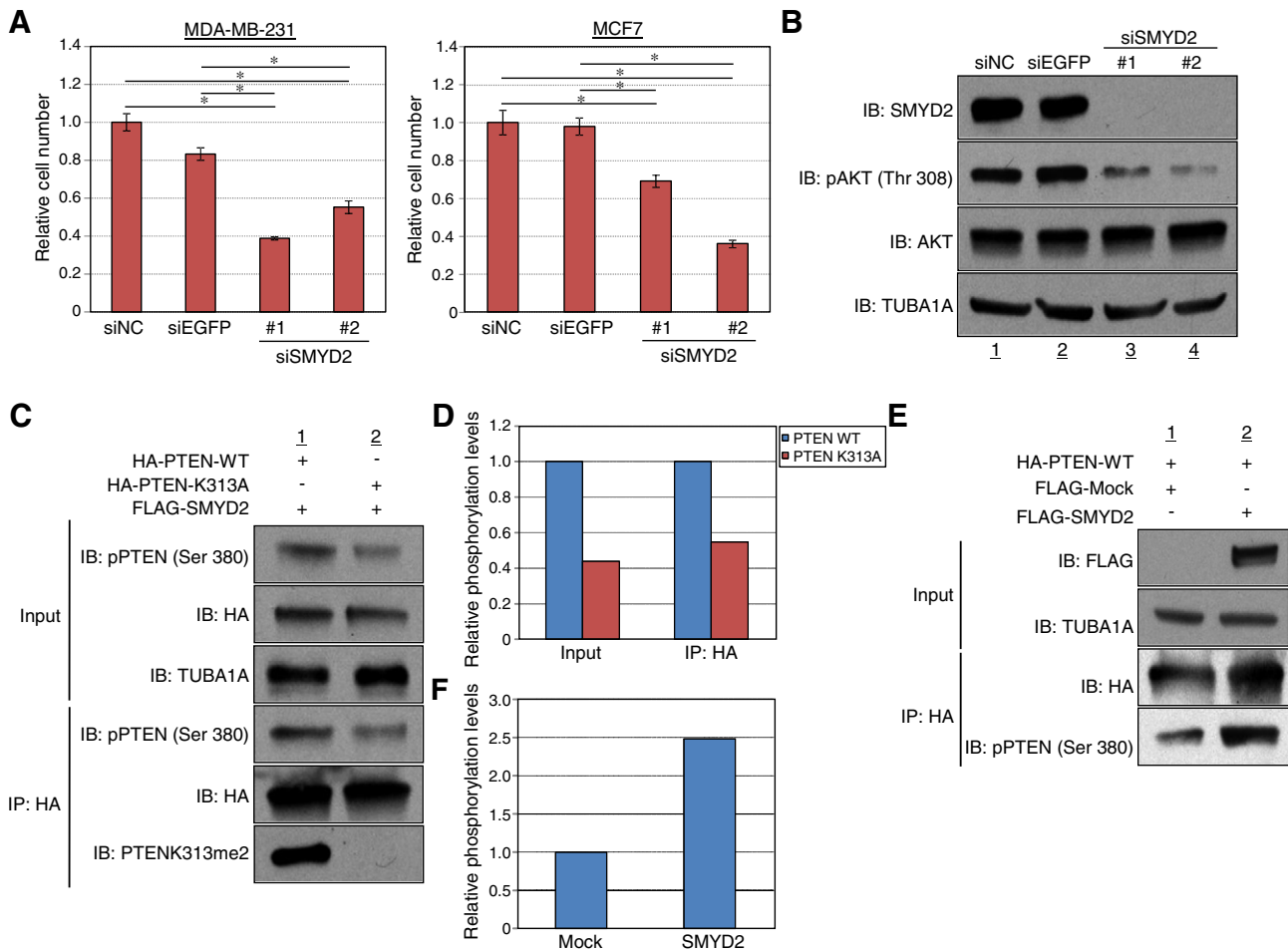


Figure 3. SMYD2-mediated methylation regulates the tumor suppressor activity of PTEN. (A) Cell growth assays of MDA-MB-231 and MCF7 cells after treatment with siNC (control), siEnhanced green fluorescent protein (control), siSMYD2#1, or siSMYD2#2. Knockdown of SMYD2 suppressed the cancer cell growth. Relative cell numbers are normalized to the number of siNC-treated cells (siNC = 1). Results are the mean of six independent experiments, and P values were calculated with Student's t test ($*P < .001$). (B) Phosphorylation levels of AKT in MDA-MB-231 cells were examined by Western blot analysis 96 hours after treatment with siRNAs. SMYD2 knockdown resulted in the attenuation of phosphorylation levels of AKT. (C) Wild-type PTEN and K313A mutant-type PTEN were overexpressed in HEK293T cells and phosphorylation levels of the proteins were examined 48 hours after transfection. The phosphorylation level of serine 380 on PTEN was diminished in K313-substituted PTEN. (D) Signal intensity of phospho-PTEN (Ser 380) was quantified and normalized by PTEN amount using a GS-800 calibrated densitometer (Bio-Rad). (E) Wild-type PTEN was co-transfected with FLAG-Mock or FLAG-SMYD2 into HEK293T cells. Cells were harvested 48 hours after transfection followed by immunoprecipitation using anti-HA agarose beads. Samples were immunoblotted with anti-FLAG, anti-tubulin, alpha 1a, anti-HA, and anti-phospho-PTEN (Ser 380) antibodies. (F) Signal intensity phospho-PTEN (Ser 380) was quantified and normalized by PTEN amount.

after SMYD2 knockdown. As shown in [Figure 3B](#), phosphorylation levels of AKT were significantly decreased after SMYD2 knockdown. Then, we hypothesized that this attenuation of AKT after SMYD2 knockdown may be caused by the increase of PTEN activity. It was reported that phosphorylation of PTEN represses its tumor suppressor functions [1]. We and others reported that lysine methylation can affect the phosphorylation status of another amino acid in the same protein [34]. Therefore, we examined the relationship between lysine 313 methylation and the phosphorylation status of PTEN. As shown in [Figure 3, C and D](#), methylation-inactive PTEN (PTEN-K313A) shows significantly lower phosphorylation levels (Ser 380) than wild-type PTEN. Furthermore, we examined phosphorylation levels of PTEN in the presence or absence of SMYD2 overexpression. The results clearly confirmed that the phosphorylation level of PTEN was significantly increased by co-overexpression of SMYD2 ([Figure 3, E and F](#)). This phosphorylation was reported to be involved in inactivation of

PTEN tumor suppressor functions by promoting “closed conformation” of PTEN and resulting in dissociation of the C2 domain from the membrane [15,35]. In summary, these results suggest a model that SMYD2-mediated methylation of PTEN at lysine 313 diminishes PTEN tumor suppressor activity through enhancement of phosphorylation at serine 380 ([Figure 4](#)).

Discussion

Here, we have demonstrated that SMYD2 methylates PTEN at lysine 313 both *in vitro* and *in vivo*. Our findings suggest that the SMYD2-mediated PTEN methylation negatively regulates the tumor suppressor activity of PTEN on the PI3K-AKT signaling pathway and promotes cancer cell proliferation. Given a critical role of PTEN as a tumor suppressor and that the PI3K-AKT signaling pathway is one of the key signaling pathways in human cancer, the attenuation of PTEN tumor suppressor functions by SMYD2-mediated

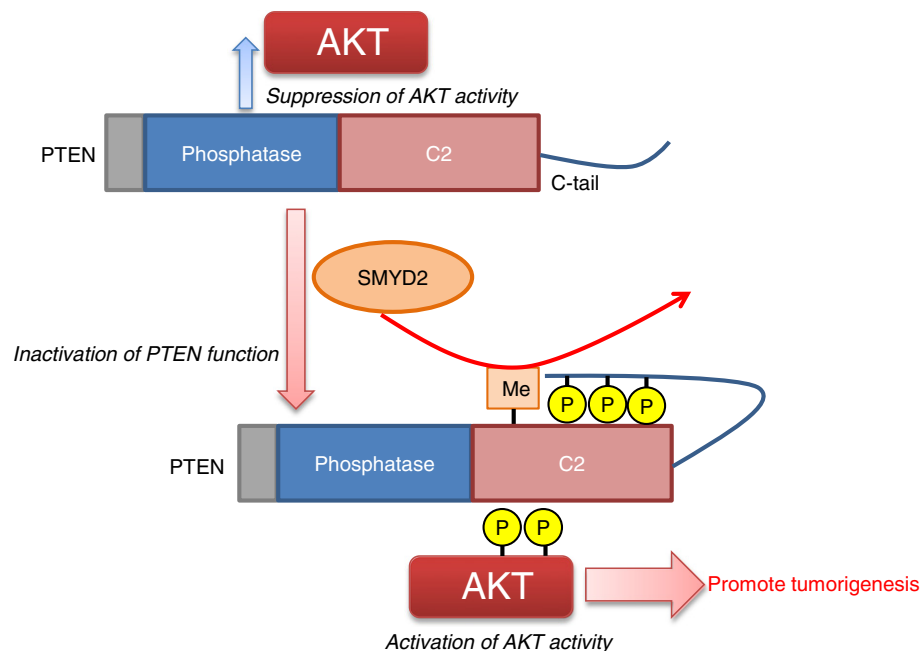


Figure 4. Model of the SMYD2-mediated PTEN methylation function. Methylation promotes phosphorylation of the C-tail of PTEN, which may induce inactive conformation.

methylation may provide novel insight into human tumorigenesis. Genetic alteration of PTEN, including somatic mutations and deletion, and/or epigenetic silencing are frequently observed in various types of cancer [36]. In addition to these known mechanisms of PTEN dysregulation, we unveil a novel mechanism of PTEN suppression mediated by lysine methylation through SMYD2. Importantly, the data set from cBioPortal for Cancer Genomics indicates that the amplification of *SMYD2* and alteration of the *PTEN* gene would be mutually exclusive in breast cancer (Figure W6) [37,38]. It may also support our hypothesis that the lysine methylation would be one of the mechanisms of PTEN functional loss.

Although SMYD2 was originally identified as a histone methyltransferase that specifically methylates H3K36 [39], recent studies identified various non-histone protein substrates of SMYD2 such as p53, retinoblastoma 1, heat shock protein 90kDa, estrogen receptor alpha, and poly (ADP-ribose) polymerase 1 [21,40–43]. Our present and previous studies demonstrated that SMYD2 is mainly localized in the cytoplasm (Figure 2A) [21,29,42], implying that the functionally important substrates of SMYD2 in human tumorigenesis may not be histone proteins but non-histone cytoplasmic proteins. To date, how SMYD2 specifically recognizes its substrates remains largely unknown. Recent structural and biochemical analyses suggested some consensus sequences for other methyltransferases, SET7/9 and SUV39H2 [44,45], but any consensus sequence has not been indicated for SMYD2. Additional *in vitro* studies may explore the substrate specificity of SMYD2.

Our study indicates that SMYD2-mediated PTEN methylation at lysine 313 promotes phosphorylation at serine 380 of PTEN. In this case, the methylation site is not adjacent to the phosphorylation site. However, since these lysine and serine residues are located in a highly flexible internal loop or loosely folded C-terminal region [8], they may stay close to each other in the tertiary structure. Although the detailed molecular mechanism by which methylation modulates phosphorylation still remains to be elucidated, it may affect the

interaction between PTEN and CK2, which phosphorylates serine 380 [15]. Cross-talk of various types of posttranslational modifications on histone proteins as well as non-histone proteins, including methylation, is widely recognized [29,40,41,46–48].

In conclusion, we identified PTEN as a novel substrate of SMYD2. The SMYD2-mediated methylation negatively regulates the PTEN tumor-suppressive function on the PI3K-AKT pathway through inducing PTEN phosphorylation, which enhances the cancer cell growth. Given that it is frequently overexpressed in various types of cancer [29], SMYD2 is likely to be a promising target for development of novel anti-cancer therapy.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.neo.2015.03.002>.

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