SUMOylation enhances DNA methyltransferase 1 activity

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DNMT1 was the first well characterized eukaryotic DNMT and is involved in S-phase-associated maintenance methylation. It localizes to replication foci through several independent domains [8] and methylates newly synthesized daughter strands. It comprises a large N-terminal domain and a highly conserved C-terminal catalytic domain. The N-terminal region interacts with many chromatin-associated proteins. The far N-terminus of DNMT1 interacts with DMAP1 (DNMT1-associated protein 1) for transcriptional repression [9]. It contains a nuclear localization signal, a PCNA (proliferating cell nuclear antigen)-interacting domain [10], a replication targeting region [8] and a cysteine-rich Zn2+-binding domain. DNMT1 also contains a domain showing homology to the polybromo-1 protein from birds [11]. This domain contains two BAH (bromo-adjacent homology) domains thought to mediate protein–protein interactions. The N-terminal part of DNMT1 interacts with the Rb (retinoblastoma) protein to repress the E2F1-responsive genes [12]. Also, DNMT1 directly interacts with a histone methyltransferase SUV39H1 [13], HDAC1 (histone deacetylase 1) and HDAC2 [9,12], MBDs (methyl CpG-binding proteins) [14,15] and HP1 (heterochromatin-binding protein 1) [13]. All these interactions are involved in transcriptional repression. Finally, DNMT1 also interacts with the de novo methylases, DNMT3a and DNMT3b [16]. Clearly, DNMT1 forms multiple, complex networks with other proteins involved in gene regulation and epigenetic signalling.

DNMT1 is also posttranslationally modified. Glickman et al. [17] reported that murine DNMT1 is phosphorylated at Ser114 with unknown biological consequences. In addition, it is also ubiquitylated. Two other reports showed that DNMT1 is degraded through a proteasomal pathway [18,19]. DNMT1 interacts with E3 ubiquitin ligase Cdh1, which is a component of the anaphase-promoting complex and is involved in late mitotic or early G1 degradation of cell cycle regulatory proteins [18]. DNMT1 degradation is dependent on an N-terminal 120 amino acid domain.

INTRODUCTION

A major form of epigenetic information in mammals is DNA methylation, in which a methyl group is added covalently to the 5-position of cytosine, mostly within the CpG dinucleotide repeats. DNA methylation has a significant and potentially long term impact on gene expression and genome stability [1]. The enzymatic machinery that establishes and maintains DNA methylation involves a number of cytosine-5 DNMTs (DNA methyltransferases), including DNMT1, DNMT2, DNMT3a and DNMT3b. DNMT1 was the first DNMT discovered. It has a strong preference for hemimethylated DNA and is generally the most abundant DNMT isoform in somatic cells [2]. One established role of DNMT1 is to maintain the methylation patterning during DNA replication by methylating newly synthesized daughter strands, although we recently reported that DNMT1 may act as a de novo methylase at sites of homologous recombination repair [3]. DNMT3a and 3b are thought to be de novo methylases with equal preference for hemimethylated and unmethylated DNA. They are required for de novo methylation of the genome (for example, following embryo implantation) as well as methylation of newly integrated retroviral sequences [4]. The third member of methyltransferase is DNMT2. It has a catalytic domain with homology to prokaryotic DNMT and recent reports suggest that it may also act as a methyltransferase activity for tRNA [5].

Although the majority of CpGs are methylated, the overall DNA methylation patterns appear to be non-randomly distributed throughout the genome. Heterochromatin regions are generally hypermethylated and transcriptionally inactive [6]. In contrast, gene promoters having dense concentrations of CpG dinucleotides tend to be hypomethylated [7]; however, in cancer, methylation patterns are often altered. Specifically, repetitive and parasitic DNA elements are hypomethylated (causing genomic instability) and CpG island promoter regions are hypermethylated (causing inappropriate gene silencing) [6].

SUMOylated DNMT1 is catalytically active on genomic DNA in vivo and we find that SUMOylation significantly enhances the methylase activity of DNMT1 both in vitro and in chromatin. These data suggest that SUMOylation modulates the endogenous activity of a prominent epigenetic maintenance pathway in somatic cells.

Key words: DNA methylation, DNA methyltransferase 1 (DNMT1), small ubiquitin-related modifier 1 (SUMO1), SUMOylation.
and is markedly reduced upon treatment with a proteasome inhibitor, MG132 [19]. Ubiquitylation can be regulated by the phosphorylation of target proteins, since phosphorylation may create binding sites for E3 ligases [20] or in other cases stabilize proteins by inhibiting E3 ligase interactions [21]. The relationship between phosphorylation and ubiquitylation of DNMT1 is not known, although DNMT1 phosphorylation levels are not significantly altered under conditions of aza-dC (5-aza-2'-deoxycytidine)-induced degradation [18].

A SUMO (small ubiquitin-related modifier) modification, SUMOylation, is a newly identified post-translational modification. It was discovered in studies on nuclear import [22]. SUMO is 18% identical to ubiquitin and has a similar protein structure [23] involving a lysine linkage to the target protein. This reaction is ATP-dependent and requires the E1 activating enzyme Aos1/Uba2, as well as the E2 conjugating enzyme Ubc9. Compared with the ubiquitin-conjugating system (where E3 ligases recognize targets), in the SUMOylation pathway an E2 enzyme (Ubc9 in this case) mediates partial recognition of target proteins. Ubc9 recognizes a SUMOylation consensus sequence, ψKXE/D (ψ is a hydrophobic residue) [24]. Although SUMOylation is quite similar to ubiquitylation, the biological role and consequences of the modification can be very different. It modulates several important functions of target proteins, such as protein–protein and protein–DNA interactions and subcellular localization, in addition to protein stability [25]. One distinct function of SUMOylation is its ability to antagonize ubiquitin-mediated degradation. Among mammalian DNMTs, mouse Dmnt3a and 3b have been reported to be SUMOylated, thereby affecting protein–protein interactions leading to transcriptional repression [26,27].

During the investigation of post-translational modification of DNMT1, we found that human DNMT1, a large (>1600 residue) protein, has more than 10 putative SUMOylation sites distributed throughout its primary amino acid sequence [27a]. In the present study, we show that DNMT1 is modified by SUMO1. In addition, the SUMOylated endogenous DNMT1 displays elevated DNA methylase activity both in vitro and in vivo. The data suggest that DNMT1 SUMOylation regulates the catalytic action of DNMT1 in chromatin.

**MATERIALS AND METHODS**

**Plasmids**

DNMT1–V5, HA (haemagglutinin)–SUMO1, HA–SUMO1-AA and Myc–Ubc9 were PCR amplified from cDNA plasmids (provided by K.D. Robertson, Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, FL, U.S.A.) using Accuprime Pfx DNA polymerase (Invitrogen) according to the manufacturer’s instructions. Restriction enzyme sites were incorporated into the PCR primers. Briefly, DNMT1 PCR products were digested with EcoRI and NotI (or XhoI for DNMT3a) and cloned into pcDNA3.1/V5-HisA plasmid (Invitrogen) using the same restriction enzyme sites. SUMO1 was digested with BglII and XhoI and SUMO1 conjugates, nuclear extracts (1 mg protein) were diluted into the above deletion mutants using the NotI site. The orientation of the cloned catalytic domain was determined by restriction enzyme digestions. Mutation of the active site cysteine (C1226A) of DNMT1 and replacement of two glycine residues of SUMO1 with alanine residues were generated by PCR using a site-directed mutagenesis kit (Stratagene). HA–PIASy [protein inhibitor of activated STAT (signal transducer and activator of transcription)] plasmid was provided by H. Yu (Department of Pharmacology, University of Texas Southwestern, Dallas, TX, U.S.A.).

**Cell culture and transfection assays**

Wild type HCT116, dnmt1−/−, dnmt3b1−/− cells (from B. Vogelstein, Program in Cellular and Molecular Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A.) and HEK (human embryonic kidney)-293FT cells purchased from Invitrogen were grown in Dulbecco's modified Eagle’s medium (Gibco) with 10% (v/v) fetal bovine serum. Cells were transfected in six-well plates with Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer's instructions.

**Co-immunoprecipitation and Western blotting**

Whole cell extracts were prepared by lysing cells in RIPA buffer (1 × PBS, pH 7.5, 1% Nonidet P40, 0.5% deoxycholate, 0.1% SDS and 10% glycerol) containing 1× protease inhibitor cocktail (Roche), 1 mM EDTA (dithiothreitol), 1 mM MgCl₂, 2 mM PMSF and 100 mM NEM (N-ethylmaleimide). For co-immunoprecipitation, the whole cell extracts were diluted 4 times with 0.5× PBS containing 1× protease inhibitor cocktail, 1 mM EDTA, 1 mM EDTA and 2 mM PMSF. Antibody (1 µg) was added to samples for 3 h, followed by 30 µl of Protein A-Sepharose 4B beads (Zymed) at 4°C for 18 h. Beads were washed three times with 1× PBS, 0.5% Nonidet P40 or RIPA buffer (for SUMO modification detection), and bound proteins were eluted with sample buffer and analysed by Western blotting. The antibodies and their commercial sources were as follows: anti-V5 (Invitrogen), anti-DNMT1 (NEB), anti-HA11 (Covance), anti-SUMO1 (Zymed) and anti-Myc (Upstate). To examine SUMOylation of endogenous DNMT1, nuclear extracts were prepared by resuspending cells in buffer A (10 mM Heps/KOH, pH 8.0, 1.5 mM MgCl₂, 10 mM KCl and 1 mM DTT). The cells were incubated on ice for 15 min and disrupted in a type S Dounce homogenizer. Nuclei were collected by centrifugation at 10000 g for 1 min and washed twice with buffer A. The nuclei were resuspended in buffer B (20 mM Tris/HCl, pH 7.5, 1.5 mM MgCl₂, 10% glycerol, 500 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF and 1× protease inhibitor cocktail (Roche)) and kept on ice for 30 min. Nuclear extracts were prepared by centrifugation at 20000 g for 20 min. The supernatants were used for further immunoprecipitation experiments. To immunoprecipitate the endogenous DNMT1 or SUMO1 conjugates, nuclear extracts (1 mg protein) were diluted twice with buffer B without NaCl. DNMT1 antibody (5 µg, Santa Cruz, N-16) or SUMO1 antibody (10 µg, Santa Cruz, D-11) was added to samples for 3 h, followed by 50 µl of Protein A-Sepharose 4B beads (Zymed) at 4°C for 18 h. Beads were washed three times with buffer C (20 mM Tris/HCl, pH 7.5, 300 mM.
NaCl, 0.5 mM EDTA, 1 mM PMSF and 0.25% Nonidet P40. The bound proteins were eluted and analysed by Western blotting.

The ICM assay

We performed the ICM assay as described in Liu et al. [28]. Briefly, cells treated with aza-dC were lysed with 1% sarkosyl in TE (10 mM Tris/HCl, pH 7.5, and 1 mM EDTA). The lysates were layered onto a step CsCl gradient followed by centrifugation (34,296 rev./min, SW50.1 rotor for 20 h at 22 °C). The gradient was fractionated into 0.4 ml aliquots and DNA fractions were pooled and their concentrations determined by UV spectroscopy. Typically, two or three concentrations of DNA were placed on a slot blot device and the membrane was probed with the antibody specified in the experiment. Immune complexes were detected with Amersham’s enhanced chemiluminescence kit. Signals were quantified using a GeneTools (SynGene, Cambridge, UK) program.

Recovery of DNMT1 from ICM DNA pools

DNA was purified by CsCl centrifugation as described in the ICM protocol. The DNA fractions were pooled, ethanol precipitated and resuspended in digestion buffer (20 mM Tris/HCl, pH 8.0, 5 mM NaCl, 2.5 mM MgCl₂, 2.5 mM CaCl₂, and 5% glycerol), S7 nuclease (200 units, Roche) and 100 units of DNase I (Roche) were added and incubated for 1 h at 37 °C. For immunoprecipitation, the reaction mixture was diluted 10-fold with 0.5 x PBS containing 1 mM DTT and 1 mM PMSF. Anti-V5 antibody (1 μg) was used for immunoprecipitation.

In vitro SUMOylation

Recombinant DNMT1 purified from insect cell lines (Baculovirus; Methylation, Ltd.) was used as the substrate for the in vitro SUMOylation reaction. Reactions contained 500 ng of DNMT1, 900 ng of SAE1 (SUMO-activating enzyme 1)/SAE2, 620 ng of Ubc9, and 1.25 μg of SUMO1 or SUMO1-AAA. All components of the SUMOylation were purified from Escherichia coli BL21 (DE3) Codon Plus cells (Stratagene). The reaction was carried out at 30 °C for 3 h in the presence of 5 mM ATP, 50 mM Heps/KOH (pH 7.5), 50 mM NaCl and 10 mM MgCl₂ in a 60 μl reaction volume. The reaction was stopped by adding 100% trichloroacetic acid and precipitated. Pellets were washed with cold acetone and re-dissolved in SDS/PAGE buffer (0.125 M Tris/HCl, pH 6.8, 4% SDS, 20% glycerol, 0.01% bromophenol blue and 10% 2-mercaptoethanol). The samples were separated on a SDS/PAGE gel, followed by Western blotting.

In vitro DNA methylation assays

DNA methylation activity of the in vitro SUMOylated DNMT1 was measured by the incorporation of tritiated methyl group from radiolabelled SAM ([5-adenosyl-L-[(methyl-³H] methionine (PerkinElmer)] into an oligonucleotide substrate containing a single CpG site in hemimethylated form (D1subHMF: 5'-GAACTCGGACCTCCGGCCAGGAGGTGCACA-3', D1sub-UMF: 5'-TTGCACTCTTCTGGCGAGGAGGCTCTTC-3', where M denotes 5-methylcytosine). Oligonucleotides were purchased from Integrated DNA Technologies. Double-stranded oligonucleotides were annealed by mixing equal amounts of complimentary oligonucleotides, heating to 95 °C for 5 min, 65 °C for 10 min, and cooling down to 20 °C. The methylation reaction was carried out at a concentration of 0.5 μM DNA, 0.5 μCi of SAM and 100 ng of in vitro SUMOylated DNMT1 in methylation buffer (20 mM Tris/HCl, pH 7.5, 5 mM EDTA, 5 mM DTT, 1 mM PMSF and 10% glycerol) at 37 °C. At defined times, the reactions were stopped by adding phenol/chloroform and DNA was precipitated by adding the same volume of isopropyl alcohol. The DNA pellet was dissolved in TE buffer and transferred to Whatman filter paper. Radioactivity was determined using a LS6500 scintillation counter (Beckman Coulter).

RESULTS

DNMT1 is SUMOylated both in vivo and in vitro

To examine DNMT1 SUMOylation in vivo, we co-transfected a plasmid expressing DNMT1–V5 along with expression vectors for HA–SUMO1 and Myc–Ubc9. Whole cell extracts were prepared in the presence of an inhibitor of the SUMO hydrolase, NEM. Since the size of V5 tagged-DNMT1 is relatively large (187 kDa), the SUMO1-specific band shift of DNMT1–V5 is difficult to resolve in whole cell extracts or immunoprecipitates (Figure 1A, lower panel); however, when the immunoprecipitates were probed with anti-HA11 antibody, the SUMOylated form of DNMT1–V5 was clearly seen (Figure 1A, lanes 1 and 5). The bands seen in the upper panel (Figure 1A, lane 1) were not seen in the negative controls (omitting DNMT1–V5 or HA–SUMO1), showing dependence on all three expression components. Co-expression of Myc–Ubc9 with DNMT1–V5 and HA–SUMO1 increased the level of DNMT1 SUMOylation (compare lanes 1 and 5 in Figure 1A). Examination of the SUMOylated DNMT1 revealed two or more HA–SUMO1-specific bands of DNMT1–V5.

To confirm the SUMOylation data, we employed a recombinant heterodimer of E1 activating enzyme (SAE1/SAE2), plus Ubc9 and SUMO1 to show that purified recombinant DNMT1 was modified by SUMO1 in vitro. As shown in lane 1 in Figure 1(B), two bands were observed in Western blots probed with anti-SUMO1 antibody. The signal seen in lane 1 was dependent on co-incubation of DNMT1 and SUMO1 (Figure 1B, compare lanes 1, 2 and 3).

To verify that SUMOylation occurs on endogenous DNMT1, we performed immunoprecipitation experiments. First, we immunoprecipitated endogenous DNMT1 using a highly specific DNMT1 antibody and probed the Western blot membrane with anti-SUMO1 antibody. As a negative control, we used nuclear extracts from HCT116 dnmt1−/− cells. Figure 1(C) demonstrates that endogenous DNMT1 was SUMOylated. The bands shown in lane 2 of Figure 1(C) were not observed in HCT116 dnmt1−/− nuclear extracts (Figure 1C, lane 3) or no antibody control (lane 1). The SUMO1 antibody-specific bands that appear with anti-DNMT1 antibody immunoprecipitation show that endogenous DNMT1 is modified by SUMO1. To confirm this result, we immunoprecipitated total SUMO1 conjugates from nuclear extracts (using anti-SUMO1 antibody) and checked for the presence of DNMT1 (Figure 1D). The band shown in lane 2 of Figure 1(D) was slightly larger than the unmodified form of DNMT1, as expected (Figure 1D, lane 4) and was not detected in control lanes (Figure 1D, lanes 1 and 3). Detecting DNMT1 in the SUMO1 immunoprecipitates is consistent with Figure 1(C); therefore the collective data strongly support the idea that endogenous DNMT1 is subject to SUMO1 modification.

DNMT1 interacts with Ubc9

The E2 conjugating enzyme, Ubc9, has been shown to interact with a large number of proteins, many of which are targets for SUMOylation [25]. To support the notion that DNMT1 is

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SUMOylated, we performed co-immunoprecipitation using cells transiently transfected with tagged versions of each protein. Myc-tagged Ubc9 co-immunoprecipitated with DNMT1–V5 when co-transfected into HEK-293FT cells, and vice versa (Figure 2A). The co-immunoprecipitation of Myc–Ubc9 was dependent on DNMT1–V5 (compare lanes 1 and 2 in Figure 2A).

To define the domains responsible for the interaction, we constructed a series of V5-tagged DNMT1 deletion mutants for immunoprecipitation (Figure 2B). These were co-transfected into HEK-293FT cells with Myc–Ubc9, and their ability to interact was assessed using co-immunoprecipitation experiments as shown in Figure 2(C). The lysates of co-transfected cells were immunoprecipitated with anti-V5 antibody and the immunoprecipitates were analysed by Western blotting. Only the 1–419 deletion failed to interact with Ubc9. We normalized co-immunoprecipitated Ubc9 band intensity based on the total recovery level for each deletion mutant (lower blot in Figure 2C) to reveal absolute levels of Ubc9 (Figure 2C, right panel). As shown in Figure 2(C, right panel), the 412–1113 mutant showed the strongest interaction with Ubc9, and the domain could be narrowed down to amino acids between 645 and 1113, containing the two contiguous BAH domains. Also, the 1114–1616 catalytic domain mutant showed weak interaction with Ubc9. The data indicate that, except for the 1–419 deletion, all other deletion mutants may potentially be SUMOylated.

**SUMOylation of DNMT1 deletion mutants**

As shown above (Figure 1A), multiple high molecular mass bands are detected in the DNMT1 SUMOylation analysis, suggesting that the DNMT1 is modified at multiple sites. We additionally

Figure 1 DNMT1 is SUMOylated in vivo and in vitro

(A) The in vivo analysis of DNMT1 SUMOylation is shown. V5-tagged DNMT1 was co-transfected with HA–SUMO1 and Myc–Ubc9 expression vectors into HEK-293FT cells. DNMT1–V5 was immunoprecipitated from the transfected whole cell extracts using anti-V5 antibody, as described in the Materials and methods section. Bound proteins were resolved by SDS/PAGE then subjected to Western blot analysis with an anti-HA antibody to detect HA–SUMO1. (B) The in vitro SUMOylation of DNMT1 is shown. In vitro SUMOylation reactions were carried out in the presence of 500 ng of purified DNMT1, 900 ng of SAE1/SAE2, 620 ng of Ubc9, 5 mM ATP and 1.25 μg of SUMO1. The reactions were analysed by SDS/PAGE, transferred to membrane and stained with a Ponceau S dye (right panel) or probed with anti-SUMO1 antibody (left panel). (C and D) The SUMOylation of endogenous DNMT1 is shown. DNMT1 (C) or all SUMO1 conjugates (D) were immunoprecipitated from the nuclear extracts of HCT116 WT or dnmt1−/− cells. Bound proteins were analysed by Western blot using anti-SUMO1 (C) or anti-DNMT1 (D) antibody.
found several potential sites (similar to the consensus for SUMO modification) in DNMT1 [25]. We mutated many of these sites (replacing lysines with arginines at residues 148, 194, 675, 739, 957, 1135 and 1609) and checked for SUMOylation; however, we failed to detect any impairment in the overall SUMOylation levels (results not shown), suggesting the existence of multiple SUMO1 sites and/or the presence of non-consensus sites. To examine the possibility of multiple sites, we co-expressed deletion mutants with HA–SUMO1 and Myc–Ubc9 in HEK-293FT cells and analysed their SUMOylation status (Figure 3). The mutant proteins were immunoprecipitated and beads were extensively washed with the modified 0.5× or 1× RIPA buffer to help distinguish between specific and non-specific bands. Three different deletion mutants showed SUMOylation products (Figure 3A); however, the 1–419 deletion, which did not interact with Ubc9, was not SUMOylated. The 412–1113 mutant protein, which strongly binds to UBC9 (Figure 2), appears to have formed multiple SUMOylated forms, whereas the 645–1113 protein displayed only a single SUMO1 form. The 1114–1616 mutant, consisting of only the catalytic domain, showed two distinguishable SUMOylated products. Thus, there are multiple SUMOylation sites in DNMT1.

**SUMOylated DNMT1 methylates genomic DNA in vivo**

Previously, we reported an in vivo methylase assay, ICM, that quantifies DNMTs trapped on the genome in cells labelled with aza-dC [28]. This antibody based-assay is ideal for monitoring the methylation activity of DNMT1 in a chromosomal setting; thus, we examined whether DNMT1 detected by the ICM assay is modified by SUMO1. The results (Figure 4A) clearly show that a SUMO1 signal was detected in the DNA peak. Since
Figure 3  SUMOylation of DNMT1 deletion mutants in vivo

Each V5-tagged DNMT1 deletion mutant was co-transfected with HA–SUMO1 and Myc–Ubc9 into HEK-293FT cells. DNMT1 deletion mutants were immunoprecipitated with anti-V5 antibody. Equivalent numbers of beads were divided into two tubes and washed extensively with 0.5× or 1× RIPA buffer. (Washing with 1× RIPA buffer is more stringent and allowed us to distinguish non-specific bands.) The immunoprecipitates were resolved by SDS/PAGE and subjected to Western blot analysis with an anti-HA11 antibody to detect HA–SUMO1. Arrowheads indicate the SUMOylated form of each mutant and asterisks indicate non-specific bands, which are shown in all samples regardless of the size of each deletion mutant.

Figure 4  ICM analysis of endogenous DNMT1 SUMOylation in colon cancer cells

(A) Results of the ICM assay with HCT116 WT and dnmt1−/− cell lines. Approx. 4 × 10⁶ cells were treated or untreated with 10 μM aza-dC for 1 h. The DNA was banded in CsCl and the gradient was fractionated as described in the Materials and methods section. Individual fractions were slot blotted onto membranes and probed with the antibody indicated on the left side of each blot. (B) Results of the ICM assay with HCT116 WT and dnmt3b1−/− cell lines. The DNA peak fractions were collected and pooled, and concentrations were determined by absorbance at 260 nm. Either 0.5 or 1.0 μg of DNA was blotted on to the membrane, and the membrane was probed with either DNMT1 antibody or SUMO1 antibody, as indicated below each blot.
the signal is only seen in WT (wild-type) HCT116 cells but not dnmt1−/− cells, and is dependent upon aza-dC labelling, we conclude that the SUMO1 signal in DNA peak is due to DNMT1. To validate this result further, we tested another mutant cell line lacking DNMT3b1. To facilitate comparison between cell lines, we normalized the Western blot signals on a per genome basis, by spotting the same amount of genomic DNA (0.5 and 1 μg) from each cell line (Figure 4B). The amount of DNMT1 bound on genomic DNA in the dnmt3b1−/− cells was elevated over the WT line (Figure 4B, left blot) and this increase was reflected in the SUMO1-probed blot (Figure 4B, right blot).

Next, transient transfections were carried out in HCT116 dnmt1−/− cells with plasmids harbouring DNMT1–V5, HA–SUMO1 and Myc–Ubc9. After 24 h post-transfection, cells were treated with 10 μM aza-dC for 1 h, lysed, and overlaid onto CsCl gradients. DNA fractions of each gradient were pooled and concentrations were determined to ensure that a fixed amount of DNA (0.3, 0.6 or 0.9 μg) was spotted on the membrane. The membranes were then probed with anti-V5 antibody or anti-HA11 antibody (indicated on the bottom of each blot). (A) HCT116 dnmt1−/− cells were transiently transfected with V5-tagged DNMT1 along with HA–SUMO1 and Myc–Ubc9. After 24 h post-transfection, cells were treated with 10 μM aza-dC for 1 h, lysed, and overlaid onto CsCl gradients. DNA fractions of each gradient were pooled and concentrations were determined to ensure that a fixed amount of DNA (0.3, 0.6 or 0.9 μg) was spotted on the membrane. The membranes were then probed with anti-V5 antibody or anti-HA11 antibody (indicated on the bottom of each blot). (B) Recovery of DNMT1–V5 from ICM DNA pools. DNA pools were digested with S7 nuclease and DNase I to release bound DNMT1–V5. Free DNMT1–V5 was immunoprecipitated with anti-V5 antibody and subjected to Western blotting. The authors performed an analysis of SUMOylation of DNMT1 mutants trapped on genomic DNA.

**Analysis of SUMOylation of DNMT1 mutants trapped on genomic DNA**

Recently, Spada et al. [29] reported that HCT116 dnmt1−/− cells express a truncated form of DNMT1, which is responsible for maintaining the genomic methylation level in the dnmt1−/− line. The truncated form of DNMT1, DNMT1ΔE3−6, lacks residues from 40 and 200, including the part of the DMAP- and PCNA-binding domains [9,10]. The ICM experiment shown in Figure 4(A) did not detect the truncated mutant because our antibody probe maps within the deletion (results not shown); therefore, its presence cannot be evaluated with this particular antibody probe. In theory, the endogenous SUMO1-modified DNMT1ΔE3−6 should be present in the DNA peak in this experiment; however, we did not detect any signal in HCT116 dnmt1−/− cells as shown in Figure 4(A) (bottom row). This could be due to low levels of expression of the truncated mutant (as reported by Spada et al. [29]), combined with the fact that the endogenous SUMO1 signals are rather weak (for example, compare SUMO1 and DNMT1 signals).
Figure 6  ICM assay of V5-tagged DNMT1 deletion mutants

(A) A schematic representation of V5-tagged DNMT1 deletion mutants. (B) HCT116 dnmt1−/− cells were transiently transfected with the V5-tagged the deletion mutants indicated on the right side of the blot, HA–SUMO1 and Myc–Ubc9. At 24 h post-transfection, cells were treated with 5 μM aza-dC for an additional 24 h prior to harvest. The DNA fractions from each CsCl gradient were pooled and fixed quantities of genomic DNA slot blotted onto membranes and probed with anti-V5 antibody (left) or anti-HA11 antibody (right). (C) The expression level of each of the V5-tagged DNMT1 deletion mutants.

signals, Figure 4A). Alternatively, the truncated mutant may lack key domains for SUMO1 modification (e.g. PCNA, DMAP). To examine these possibilities, a series of DNMT1 deletions (Figure 6A) were tested for methylase activity in vivo using the ICM assay and transient expression. Expression levels for the various mutants were confirmed by Western blotting (Figure 6C). The data show that the truncated mutant (DNMT1ΔΔE3−6) retained catalytic activity in a chromatin setting (Figure 6B, left blot), consistent with prior data showing that this truncation mutant is active in the DNMT1 knockout cell line [29]. The amount of bound protein was similar for both WT and DNMT1ΔΔE3−6 mutants, suggesting that level of endogenous activity is similar for both with regard to genome-wide methylation. SUMOylation also appears to be similar for these two proteins (Figure 6B). The same analysis was carried out with the other deletion mutants listed in Figure 6(A). The mutant containing only the catalytic domain (1114−1616) was incapable of methylating the genome and its SUMOylation status could not be evaluated in this experiment. The N-terminal mutants (412−1616 and 645−1616) were much less active than WT DNMT1 in terms of catalytic activity (Figure 6B, left panel). SUMOylation levels were also reduced as more of the N-terminus was deleted (Figure 6B, right panel).

SUMO1 modification of DNMT1 increases the methyltransferase activity of DNMT1 in vitro

SUMOylation affects various aspects of target protein function. To investigate the role of DNMT1 SUMOylation, we examined whether the enzymatic activity of DNMT1 is affected by SUMOylation. Purified DNMT1 was SUMOylated in vitro and its catalytic activity was measured using S-[methyl-3H]adenosyl methionine as a methyl donor with a hemimethylated oligonucleotide substrate. As a control, DNMT1 was also mock SUMOylated with an inactive mutant, SUMO1-AA, in which C-terminal diglycine residues of SUMO1 are mutated to two alanine residues; therefore, these two reactions contained identical
sets of purified proteins except that one reaction was SUMO1-negative (compare lanes SUMO1 and SUMO1- AA in Figure 7A). As shown in Figure 7(B), the SUMOylation of DNMT1 increased the catalytic activity of DNMT1 by as much as 10-fold (range of 4–10-fold). Thus, SUMOylation strongly stimulated DNMT1 activity on hemimethylated substrates.

### DISCUSSION

In the present study, we show that human DNMT1 is modified by SUMO1 in vivo and in vitro. Consistent with this modification, DNMT1 interacts with an E2 conjugating enzyme, Ubc9, and SUMOylation of endogenous DNMT1 stimulates its methylation activity on genomic DNA. The following pieces of evidence support the notion that DNMT1 is subject to SUMOylation control.

Firstly, DNMT1 is trapped on aza-dC-labelled DNA inside HCT116 cells and is SUMOylated as a bound protein. A dnmt1−/− knockout HCT116 line did not display any SUMO1 modified methylase in the DNA fraction (Figure 4); thus we conclude that the SUMO1 signal detected in WT cells is attributable to DNMT1 and not to other methylase isoforms (de novo enzymes for example). Additional support comes from an analysis of DNMT1 on genomic DNA in HCT116 dnmt3b1−/− mutants (Figure 4). Since these data are normalized based on DNA content, we can directly compare signals in different cell lines. Compared with WT cells, the DNMT3b1 deletion showed higher levels of DNMT1 methylase activity, along with elevated SUMO1-modified DNMT1. In the absence of DNMT3b1, it is possible that DNMT1 is complementing for this defect, thereby generating higher amounts of genomic DNA-directed methylation. The fact that SUMO1 was elevated in a commensurate fashion suggests that SUMO1 modifications correlate with elevated activity in vivo. Finally, it is important to emphasize that the experiments shown in Figures 1(C), 1(D) and 4 involved endogenous levels of reactants and not overexpressed constructs; therefore the findings hold strong physiological relevance.

Secondly, we can rule out the presence of SUMO1-modified DNMT3a or 3b complexes in the DNA fraction of the ICM experiments with WT HCT116 cells (Figure 4). As noted below,
Figure 8  SUMOylation increases the formation of DNMT1/DNA covalent complexes in vivo

(A) ICM assays with transiently transfected HCT116 cells. Cells were transfected with various amounts of HA–PIASy. At 24 h post-transfection, cells were treated with 10 μM aza-dC for 1 h. Genomic DNA purified on CsCl gradients was slot blotted onto the membrane (0.3 μg), which was then probed with anti-DNMT1 or anti-HA11 antibody. Band intensities were quantified using GeneTools (SynGene, Cambridge, UK) and the relative level of each band from slot blot was plotted. The statistical data are based on two independent experiments (*P < 0.05 compared with WT).

(B) The interaction of DNMT1–V5 with PIASy. HEK-293FT cells were transfected with DNMT1–V5, HA–SUMO1, Myc–Ubc9 and HA–PIASy. DNMT1–V5 was immunoprecipitated from the transfected whole cell extracts using anti-V5 antibody. Bound proteins were resolved on a SDS/PAGE gel, then subjected to Western blot analysis with an anti-HA11 antibody to detect co-immunoprecipitated HA–PIASy. Arrowheads indicate the co-immunoprecipitated HA–PIASy. SUMO1-modified DNMT1–V5 is shown by a bracket.

the SUMO1 signal derived from these cells is immunoprecipitated by DNMT1 antibody, and is the expected size; therefore DNMT1 must be the SUMO1 target in the DNA fraction. Also, DNMT1 is by far the most prominent endogenous methylase present in these cells (based on ICM and Western blot band-shift experiments; results not shown). In our previous analysis of de novo methylase activity by the ICM assay, DNMT3a and 3b were not detected in HCT116 cells, but were easily seen in a P19 mouse cell line that expressed sufficiently high levels of these activities [28]. In WT HCT116 cells, DNMT3a and 3b are not robust endogenous methylase activities, compared with DNMT1.

Third, there is evidence in the literature that the dnmt1−/− cells used here actually contain a mutant form of the gene with an internal deletion that would be undetected using our anti-DNMT1 antibody reagent (the epitope maps to the internal deletion) [27]. When we repeated the analysis with overexpressed DNMT1ΔE3–6, we detected strong methylation and SUMOylation in vivo. Thus, when overexpressed, the deletion mutant is highly active in chromatin and is also subject to SUMOylation control. We did not evaluate the endogenous activity of this mutant in the dnmt1−/− cells since we lack a suitable antibody; however, we did not detect any SUMOylation signatures associated with DNA from dnmt1−/− cells (Figure 4A), suggesting that the endogenous DNMT1ΔE3–6 mutant is not subjected to robust SUMO1 modification. Further experimentation will be required to explain the fact that data derived from the overexpression experiments are at odds with
the endogenous target results. The endogenous data are of course physiologically relevant and may provide important clues to our understanding of a complex problem.

Fourth, we resorted to transient expression assays to examine specific mutations and methylation in vivo. These experiments are possible since V5-tagged DNMT1 is active in vivo (Figure 5). When we co-transfected HA-SUMO1 and DNMT1-V5 genes, we showed that the latter was also SUMOylated as a DNA-bound methylase. Co-transfections with a mutant, SUMOylation negative, HA-SUMO1 showed that DNMT1-V5 bound to genomic DNA lost its SUMO1 signature. Interestingly, we consistently detected lower levels of DNA-bound DNMT1-V5 in co-transfections with mutant SUMO1-AA, compared with WT SUMO1. This result supports the notion that SUMO1 modifications enhance DNMT1 methylase activity (Figure 5A, left blot).

Fifth, we co-transfected a mutant DNMT1 that lacks catalytic activity (C1226A) with the WT HA–SUMO1 gene, and assayed in vivo methylase activity. In the aza-dC treated cells, we did not detect any bound DNMT1 (anti-V5 probe) nor any SUMO1-modified protein (anti-HA11 probe; Figure 5). This important control demonstrates that a catalytically inactive DNMT1 cannot be detected by the ICM assay, and there is a concurrent loss of SUMO1 signals in the genomic DNA peak. This argues strongly against the prospect that SUMO1 detected in the DNA peak is due to unrelated activities (SUMO1 bound to a non-methylase target protein or another methylase isoform, etc.) or some sort of artifact (SUMO1 carry over into the DNA fraction but unbound to genomic DNA).

Sixth, we showed that a physical association exists between SUMO1-DNMT1 and DNA in cells treated with aza-dC (Figure 5B). Note that aza-dC substituted DNA will trap endogenous DNMT1 on the genome in a covalent complex. When these complexes are extensively digested with nuclease, it is possible to recover the bound proteins and evaluate their size on Western blots. Due to a massive amount of carryover of degraded DNA, backgrounds were high in these blots; however, we detected both unmodified DNMT1 and SUMO1-modified polyepitides in the blots. This confirms the identity of signals detected on slot blots.

Seventh, we compared in vitro methylase activity of DNMT1 that was unmodified or SUMO1 modified. Reactions were carried out by incubating DNMT1 with SUMOylating proteins (SAE1, SAE2, Ubc9) plus SUMO1 or the SUMO1-AA mutant as a negative control. Thus the reactions contained identical reactants and the only difference was the mutant form of SUMO1. SUMOylation of DNMT1 was attended by a 5–10-fold increase in methylase activity (Figure 7).

Eighth, we tested the effects of overexpressing PIASy (an E3 ligase active on DNMT1) on endogenous DNMT1 methylation in cells. These conditions strongly favour SUMOylation of target proteins. Using the ICM as a readout for endogenous methylation, we showed that increasing PIASy gene dosages stimulates the binding of DNMT1 on aza-dC-labelled genomic DNA (Figure 8). This increase was commensurate with the increase in SUMO1 modification of DNMT1. In this experiment (Figure 8A), we examined endogenous levels of DNMT1 (not transfected constructs). This suggests that our results reflect the in vivo situation with physiological levels of methylase.

The modification of proteins by the reversible covalent attachment of SUMO protein is a flexible, yet powerful, method of regulating protein activity. Among the major DNMTs in mammals, the SUMOylation of murine Dnmt3a and Dnmt3b has been reported [26,27,31]. The SUMOylation of Dnmt3a disrupts its ability to interact with HDACs and impairs its capacity to repress transcription [27]. The role of SUMOylation of Dnmt3b is not known [26]. DNMT1 has many putative SUMOylation sites fitted in a consensus ψKXE/D motif, which prompted us to investigate the SUMOylation of DNMT1. Since the level of SUMOylation of target proteins is extremely low in general, we over-expressed SUMO1, Ubc9 and DNMT1 to analyse and dissect the process. Over-expression of each protein allowed us to detect the SUMOylated form of DNMT1 in HEK-293FT cells and its SUMOylation was further confirmed through an in vitro SUMOylation assay, as noted above. The in vitro SUMOylation efficiency is low compared with the in vivo situation, implying that additional endogenous factors are required in regulating this modification. Such factors include E3 ligases, which determine substrate specificity and increase SUMOylation efficiency. Currently, three classes of E3 ligases have been reported: PIASy, RanBP2 (RAN binding protein 2) and Pc2 (polycomb protein 2). Dnmt3a and 3b interact with the PIAS proteins, PIAS1 and PIASxα; however, DNMT1 does not [27]. Neither PIAS1 nor PIASxα stimulate the Dnmt3a SUMOylation, whereas Pc2 is an E3 ligase acting on Dnmt3a [31]. The E3 ligase PIASy interacts with DNMT1; however, we have not examined other E3 ligases for DNMT1 specificity.

Ubc9 is the sole conjugating enzyme for SUMOylation. We showed that DNMT1 interacts with Ubc9 and mapped the interaction region of DNMT1 to residues 645–1113. This region contains two BAH domains [11]. Proteins containing BAH domains are typically involved in replication, methylation and transcriptional regulation. For example, a yeast Orc1p (origin recognition complex subunit 1) is a member of the origin replication complex which directs DNA replication and transcriptional silencing at the mating-type locus with another BAH domain-containing protein, Sir3p (silent information regulator 3) [32]. The BAH domain of Drosophila Orc1 binds to HP1, suggesting a role in chromatin packaging that results in transcriptionally inactive chromatin [33]. Other methyltransferases, such as CMT1 (chromomethylase 1) in Arabidopsis thaliana [34] and the Ascoplasm immerses DNMT, masc2 [35], have BAH domains. SUMO modifications of these proteins have not been reported. To the best of our knowledge, this is the first report of a BAH domain–Ubc9 interaction. In addition, the BAH domain of DNMT1 is also involved in targeting DNMT1 to replication sites [8]. In vivo methylation analyses with endogenous DNMT1 showed that a deletion mutant lacking 644 N-terminal residues (mutant 645–1616) was partially active in vivo, whereas a mutant containing only the catalytic domain (mutant 1114–1616) was not. The 645–1616 mutant is missing its PCNA binding domain and primary nuclear localization domain, therefore it was surprising that we detected any activity in cells at all. In all of the mutants that showed DNMT1 methylation activity in chromatin (defined by their ability to covalently bind aza-dC substituted genomic DNA), we detected at least some level of SUMO1 modification (Figure 6).

Considering the role of BAH domain in other proteins and our data together, it is possible that the SUMOylation of DNMT1 might be associated with late-replicating events for several reasons. First, replication of heterochromatin regions occurs mainly in late S-phase [36]. Second, the heterochromatin protein HP1 interacts with DNMT1 and increases DNA methylation on DNA [37]. Third, the BAH domain is necessary and sufficient to target the 645–1616 mutant to DNA and interacts with Ubc9. Thus, the SUMOylation of DNMT1 is more likely involved when DNMT1 methylates DNA at heterochromatic regions. Supporting this idea, a previous study showed a high abundance of SUMOs in the heterochromatin region [38]. Future work will focus on dissecting the contribution of the BAH domain on SUMOylation of DNMT1.

SUMOylation regulates numerous biological functions, such as protein–protein interactions, enzyme activity and subcellular localization [39]. DNMT1 has a large N-terminal regulatory
that purified DNMT1 is activated
activity of DNMT1; however, SUMO1 stimulation appears to
in vivo
endogenous DNMT1 bound to genomic DNA (based on the
along with SUMO1 and Ubc9 also increased the level of DNMT1
SUMOylation of DNMT1. Co-expression of DNMT1 and PIASy
sites reside in non-consensus motifs or that deleting one site
to detect changes in the SUMO banding patterns relative to
plasmid; and Dr B. Vogelstein for HCT116 and dnmt1
individuals for supplying critical reagents: Dr K. Robertson for DNMT1, DNMT3a, Ubc9
The capable technical assistance of J. Kim is acknowledged. We thank the following
bacteriophage T7. We also thank Dr. B. Vogelstein for the gift of the HCT116 cell line.
the catalytic domain mutant has no endogenous
methylation activity, in agreement with published data [42]. At
this point, a biological role for SUMOylation in the catalytic
domain of the full length DNMT1 protein is not clear, but will be
the subject of future work.

Although SUMO E3 ligases are not required for SUMOylation in vitro, these ligases may still be important in regulating
substrate specificity in vivo, particularly for substrates that
lack consensus SUMO motifs. Indeed, we tested several point
mutants of DNMT1, in which lysine residues in the SUMO
consensus motif were mutated to arginine (including Lys148, Lys152, Lys159, Lys167, Lys171, Lys197, and Lys198), but we failed to
detect changes in the SUMO banding patterns relative to WT. It is possible, therefore, that some DNMT1 SUMOylation sites
reside in non-consensus motifs or that deleting one site has no effect due to multiple or cryptic SUMO sites elsewhere.
We showed that the E3 ligase (PIASy) specifically enhances
SUMOylation of DNMT1. Co-expression of DNMT1 and PIASy
along with SUMO1 and Ubc9 also increased the level of DNMT1
SUMOylation. As noted above, PIASy increased the level of endogenous DNMT1 bound to genomic DNA (based on the
ICAM assay) showing that SUMOylation of DNMT1 enhances
methylation activity in chromatin (in vivo). Currently, it is
not clear how SUMOylation stimulates the methyltransferase
activity of DNMT1; however, SUMO1 stimulation appears to
be independent of other interacting partners, since we observed
that purified DNMT1 is activated in vitro. Presumably, SUMO1
modification results in a conformational change that stimulates
methylation or enhances DNMT1–DNA interaction, for example by
making the methylase more processive.

DNMT1 is the major DNMT and plays a central role in
maintaining, and possibly establishing, the epigenetic status in mammalian cells. DNMT1 expression and activity levels are regulated at both the transcriptional/translational levels and during interaction with numerous other cellular factors. The fact that
DNMT1 interacts with many proteins limits our understanding of
how DNMT1 is fine tuned in a micro-environment. In this regard,
the SUMOylation of DNMT1 will give us a new insight into
understanding its regulation. Our data reveal that endogenous DNMT1 is SUMOylated and that SUMOylation has direct or
indirect consequences on DNMT1-mediated methylation activity in a chromosomal setting. Future studies will be directed at the
interplay between DNMT1 and other factors whose interaction is
modulated by SUMOylation.

AUTHOR CONTRIBUTIONS
Bongyong Lee designed and performed the experiments and did statistical analyses along with data interpretation. Mark Muller directed the project and carried out data interpretation and analysis. Bongyong Lee and Mark Muller contributed equally to writing the manuscript.

ACKNOWLEDGEMENTS
The capable technical assistance of J. Kim is acknowledged. We thank the following individuals for supplying critical reagents: Dr K. Robertson for DNMT1, DNMT3a, Ubc9 and SUMO1 constructs; Dr M. Szyl for DNMT1 CAT plasmid; Dr H. Yu for HA–PIASy plasmid; and Dr B. Vogelstein for HCT116 and dnmt1+/−, dnmt3b1+/− mutant cell lines.

We thank Methylation, Ltd. for donating critical reagents for this work (DNMT1 baculovirus stock).

FUNDING
This work was supported by the National Institutes for Health (grant numbers CA172416 and CA098214).

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Received 17 February 2009/11 May 2009; accepted 18 May 2009
Published as BJ Immediate Publication 18 May 2009, doi:10.1042/BJ20090142

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