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Modification of *de novo* DNA methyltransferase 3a (Dnmt3a) by SUMO-1 modulates its interaction with histone deacetylases (HDACs) and its capacity to repress transcription

Yan Ling, Umesh T. Sankpal, Andrea K. Robertson, James G. McNally[1], Tatiana Karpova[1] and Keith D. Robertson*

Epigenetic Gene Regulation and Cancer Section, and [1]Fluorescence Imaging Group, LRBGE/NCI/NIH, Building 41, 41 Library Dr., Bethesda, MD 20892, USA

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ABSTRACT

The *de novo* DNA methyltransferase Dnmt3a is one of three mammalian DNA methyltransferases that has been shown to play crucial roles in embryonic development, genomic imprinting and transcriptional silencing. Despite its importance, very little is known about how the enzymatic activity and transcriptional repression functions of Dnmt3a are regulated. Here we show that Dnmt3a interacts with multiple components of the sumoylation machinery, namely the E2 sumo conjugating enzyme Ubc9 and the E3 sumo ligases PIAS1 and PIASxα, all of which are involved in conjugating the small ubiquitin-like modifier polypeptide, SUMO-1, to its target proteins. Dnmt3a is modified by SUMO-1 *in vivo* and *in vitro* and the region of Dnmt3a responsible for interaction maps to the N-terminal regulatory domain. Functionally, sumoylation of Dnmt3a disrupts its ability to interact with histone deacetylases (HDAC1/2), but not with another interaction partner, Dnmt3b. Conditions that enhance the sumoylation of Dnmt3a *in vivo* abolish its capacity to repress transcription. These studies reveal a new level of regulation governing Dnmt3a whereby a post-translational modification can dramatically regulate its interaction with specific protein partners and alter its ability to repress transcription.

INTRODUCTION

DNA methylation patterns in mammalian cells are established and maintained by a complex interplay between three DNA methyltransferases, DNMT1, DNMT3a and DNMT3b (1–3). DNMT1, which is the most abundant and catalytically active DNA methyltransferase, is thought to function primarily as a maintenance methyltransferase by copying DNA methylation patterns from the parental to the daughter strand following DNA replication (1,4). Dnmt3a and Dnmt3b are referred to as *de novo* DNA methyltransferases since they are essential for the complex series of *de novo* methylation events which occur in the wake of the genome-wide demethylation of the embryonic genome shortly after fertilization (5). The function of a fourth DNMT family member, DNMT2, remains unknown (6). DNA methylation is also critically involved in X chromosome inactivation, genomic imprinting, suppression of the detrimental effects of repetitive elements on genome stability, transcriptional repression and the maintenance of heterochromatin (1–4,7,8).

DNA methylation patterns are not randomly distributed throughout the genome, but rather are compartmentalized within specific regions. Heterochromatin, including centromeres, repetitive DNA and parasitic DNA elements, tend to be hypermethylated and transcriptionally silent (1–3,9). In contrast, gene promoters having a dense concentration of CpG dinucleotides, also known as CpG islands, tend to be hypomethylated and the associated genes active (8). Just how the three DNMTs work to establish this compartmentalization remains unknown, but given the relatively minimal DNMT recognition site, CpG, this region-specific methylation is most likely mediated by direct interactions between the DNMTs and other chromatin-associated proteins (1). The generation of mice with targeted disruptions of each of the *Dnmt* genes has revealed that proper DNA methylation patterning is essential for mammalian embryonic development (10,11). Mutations of the *DNMT3B* gene in a rare recessive human genetic disorder known as ICF (immunodeficiency, centromere instability, facial anomalies) syndrome give rise to a number of serious developmental and immunological abnormalities (10,12). Numerous studies of tumor cells have demonstrated that disruption of normal DNA methylation patterns is an early event in the tumorigenesis process that directly contributes to tumor suppressor gene silencing and genomic instability. Within the tumor cells, the normal compartmentalization is reversed, with repetitive sequences being hypomethylated and CpG island promoter regions being hypermethylated (1–3). Thus a better understanding of the processes regulating both cellular DNA methylation patterns

*To whom correspondence should be addressed. Tel: +1 301 594 8055; Fax: +1 301 496 4951; Email: robertk@mail.nih.gov

as well as the DNA methyltransferases, will give insight into how tumors arise.

Structurally, DNMT1, 3a and 3b can be divided into two domains. The C-terminal regions of all three proteins contain the highly conserved motifs involved in catalysis of the methyl transfer reaction. The N-terminal regions of each protein, often referred to as the regulatory domain, are not highly conserved (1,4). Dnmt3a and Dnmt3b do share some similarity within their N-terminal domains, characterized by two motifs, a PWWP domain and a plant homeodomain (PHD) finger-like region (13). The function of the PWWP motif remains unclear although it may have a role in DNA binding (14), while the PHD domain is thought to be involved in mediating protein–protein interactions and is a motif commonly found in chromatin-associated proteins (15). It has been shown that the N-terminal domains of both Dnmt3a and Dnmt3b can repress transcription independently of their catalytic domain (16,17). In addition, a direct interaction between the PHD domain of Dnmt3a and histone deacetylase (HDAC) 1 has been demonstrated, which is consistent with the finding that the majority of the repressive activity mediated by the Dnmt3a N-terminal domain can be relieved by treatment of cells with the HDAC inhibitor trichostatin A (TSA) (16). The role of the interaction of Dnmt3a and HDAC1 remains unclear, but may be involved in targeting Dnmt3a to transcriptionally silenced regions to carry out *de novo* methylation. Hypoacetylated histones are also a common characteristic of transcriptionally silent, hypermethylated regions. Thus the association of these two activities is likely to be critical for the establishment and maintenance of heterochromatic regions (8,9,18).

The post-translational modification of a growing number of nuclear proteins by the small ubiquitin-like modifier SUMO-1 (also known as sentrin, GMP1, PIC1 and SMT3) has emerged as an important mechanism for regulating transcription. SUMO-1 is a small polypeptide of 11 kDa which is ligated to lysine residues within its target proteins via a three-step enzymatic process involving first a heterodimeric E1 activating enzyme (SAE1/SAE2), an E2 conjugating enzyme (Ubc9) and a number of E3 ligating enzymes (PIAS proteins and RanBP2) (19–22). Sumoylation is thought to modulate several important aspects of protein function including subcellular localization, protein–protein interactions, protein stability, protein–DNA interactions and enzymatic activity (19,23). For example, sumoylation of the Sp3 transcription factor reduces its transcriptional activation capability (24), sumoylation of RanGAP1 regulates its ability to interact with RanBP2 and accumulate at the nuclear pore (25), and sumoylation of the PML (promyelocytic leukemia) protein is required for PML body formation and recruitment of other PML-associated proteins (26). Furthermore, sumoylation of IκBα occurs at the same lysine modified by ubiquitin, therefore stabilizing IκBα by making it resistant to the ubiquitin degradation pathway (27). Lastly, it has been demonstrated that sumoylation of heat shock factors HSF1 and HSF2 stimulates their DNA binding activity (28,29).

In the present study, we sought to identify and characterize the protein interaction partners of Dnmt3a to gain a better understanding of the factors that regulate this important enzyme. We show that Dnmt3a interacts with multiple components of the sumoylation machinery, namely Ubc9, PIAS1 and PIASxα (protein inhibitor of activated STAT) in a yeast two-hybrid screen, as well as *in vitro* and *in vivo* in mammalian cells. We map the interaction sites between Dnmt3a and Ubc9/PIAS1/PIASxα to the N-terminal regulatory domain of Dnmt3a. The functional consequences of Dnmt3a sumoylation were also investigated. Co-immunoprecipitation studies reveal that SUMO-1 modification of Dnmt3a disrupts its ability to interact with both HDAC1 and HDAC2. Consistent with this observation, over-expression of SUMO-1, or the PIAS proteins, abolished Dnmt3a's capacity to repress transcription of a reporter gene. Taken together, these findings reveal an important new mode of regulation of DNA methyltransferases in mammalian cells and begin to shed light on how their functions may be fine-tuned by post-translational modification.

MATERIALS AND METHODS

Yeast two-hybrid screens

The Matchmaker Two-Hybrid System 3 (Clontech) was utilized to screen a human fetal liver cDNA library (Clontech) with full-length Dnmt3a as bait according to the manufacturer's instructions. Expression of the Dnmt3a-bait fusion protein was confirmed by western blotting (not shown). The library screen was performed using yeast strain AH109 under high stringency conditions (Ade^-, His^-, Leu^-, Trp^- media) where approximately 10^7 clones were screened. Colonies growing from the primary screen were re-streaked on the same media and positives were confirmed with a beta-galactosidase assay. Plasmid DNA from positive colonies was isolated, propagated in *E.coli*, and sequenced to identify clones. Following this, bait and positive library clone plasmids were transformed into yeast strain Y187 (using Trp^-, Leu^- media) and another beta-galactosidase assay performed to verify positive interactions. The beta-galactosidase activity was calculated using the formula: $1000 \times OD_{420}/(t \times V \times OD_{600})$, where t = incubation time, V = 0.1 ml × dilution factor, and $OD_{600} = A_{600}$ of 1 ml of culture.

Plasmids

Cloning of the full-length murine Dnmt3a cDNA has been described previously (30). Full-length cDNAs for Ubc9, PIAS1, PIASxα and SUMO-1 (1–97) were prepared by PCR from HeLa cell cDNA using Herculase DNA polymerase (Stratagene) according to the manufacturer's instructions. Restriction enzyme sites were incorporated into the PCR primers (sequences available upon request). Primers were synthesized by Sigma-Genosys. Dnmt3b1, HDAC1 and HDAC2 were PCR amplified from cDNA plasmids (kindly provided by En Li and Ed Seto, respectively) using *Pfu* DNA polymerase (Stratagene) according to the manufacturer's instructions. All constructs were confirmed by sequencing. Briefly, the Dnmt3a and Dnmt3b1 PCR products were digested with EcoRI and BamHI, Ubc9 with EcoRI and XhoI, PIAS1 with EcoRI and XhoI, PIASxα with EcoRI and XhoI, HDAC1 with EcoRI and BamHI, and HDAC2 with EcoRI and BamHI, and cloned into one or more of the following plasmids (depending on the particular application or assay) utilizing the same restriction enzyme sites: pGBKT7 [fusion with the GAL4-DNA binding domain (DBD) for yeast

expression, Clontech], pGADT7 (fusion with the GAL4-activation domain for yeast expression, Clontech), pcDNA3.1 (untagged mammalian expression vector, Invitrogen), pEGFP-C1 (fusion with the green fluorescent protein, Clontech), pGEX5X-1 (fusion with the glutathione *S*-transferase protein for bacterial expression, Amersham-Pharmacia), pM (fusion with the GAL4-DBD for mammalian expression, Clontech), and pCMVTag-2 (fusion with the FLAG epitope, Stratagene). Deletion mutants of Dnmt3a were created by PCR using pcDNA3.1-Dnmt3a as a template, *Pfu* DNA polymerase and primers containing appropriate restriction enzyme sites (EcoRI and BamHI). For N-terminal deletion mutants, an ATG was also incorporated into the 5′ PCR primer sequence. PCR products were digested and cloned into pGBKT7 at the same restriction sites. The 5X-GAL4 binding-site firefly luciferase plasmid was created by cloning the HindIII–AccI fragment derived from pSV2CAT (containing the SV40 enhancer region) into the *Kpn*I site of pG5luc (Promega) to create WpKR160 (5X GAL4-BS-luciferase). Sites were blunted with T4 DNA polymerase and the orientation of the insert was determined by restriction digestion. The *Renilla* luciferase plasmid (pRL-SV40) from Promega is used as a control for transfection efficiency. The RING-finger deletion mutant of PIAS1 (PIAS1 Δ341–536) was provided by Jiann-an Tan and Frank French (31).

Cell lines and tissue culture

Cell lines utilized in this study include HCT116 (human colorectal adenocarcinoma), COS-7 (monkey kidney) and NIH3T3 (immortalized murine fibroblast). All cell lines were purchased from the American Type Culture Collection and grown in McCoy's 5-a media supplemented with 10% fetal calf serum and 2 mM L-glutamine (Invitrogen).

Whole cell extract preparation, co-immunoprecipitations and western blotting

Whole cell extracts from transfected cells for direct western detection of sumo modification, or for immunoprecipitation reactions, were prepared by lysing cells in 400 µl of RIPA buffer [1× PBS (pH 7.5), 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 10% glycerol] containing 1.0 µg/ml aprotinin, leupeptin, pepstatin A, AEBSF, E64 and 200 mM N-ethylmaleimide (NEM). Lysates were then clarified by centrifugation and stored at –80°C until use. For immunoprecipitations, 400 µl of whole cell extract was diluted to 1.0 ml in 1× PBS, 0.5% NP-40, and precleared with 20 µl of protein A/G agarose beads (Santa Cruz Biotechnology) for 1 h. Samples were centrifuged, and 15–20 µl of antibody was added to the supernatants for 2 h, followed by 20 µl of protein A/G agarose beads overnight. Samples were washed four times with 1× PBS, 0.5% NP-40, sample buffer was added to the beads, and bound proteins analyzed by western blotting following SDS–PAGE and electrophoretic transfer to PVDF membrane (32). All reactions were performed on a rotator at +4°C. Antibodies used are as follows: mouse anti-GAL4-DBD RK5C1 (Santa Cruz Biotechnology, 1:1000 for western), rabbit anti-GAL4-DBD sc-577 (Santa Cruz Biotechnology, 1:500 for western), goat anti-Dnmt3a P-16 (Santa Cruz Biotechnology, 1:500 for western), rabbit anti-HDAC1 and HDAC2 (Affinity Bioreagents, 1:2000 for western), mouse anti-FLAG M2 (Sigma, 1:1000 for western), mouse anti-GFP 11E5 (Q Biogene, 1:1000 for western), goat anti-PIAS1/3 N-18 (Santa Cruz Biotechnology, 1:500 for western), and mouse anti-SUMO-1 (Zymed Laboratories, 1:1000 for western). HeLa nuclear extracts were prepared as described in Robertson *et al.* (32).

Luciferase assays

HCT116 cells were transfected in six-well plates with Lipofectamine reagent according to the manufacturer's instructions (Invitrogen) for 24 h with the amounts of DNA indicated in the text and figure legends. Whole cell extracts were prepared in 300 µl of Passive Lysis Buffer and both firefly and *Renilla* luciferase measured from the same sample with the Dual Luciferase Assay Kit (Promega) according to the manufacturer's instructions, using an EG&G Berthold Microlumat LB96P luminometer (delay time of 2 s, integration time of 10 s). All reactions were performed in duplicate.

Protein production and GST pull-downs

All cDNAs for protein production were cloned into the GST-fusion expression vector pGEX5X-1 as described above. *E.coli* BL21 (DE3) Codon Plus-RP cells were utilized for protein production according to the manufacturer's instructions (Stratagene). Briefly a 50 ml overnight culture grown in LB media containing 100 µg/ml ampicillin (Sigma) was diluted into 500 ml of the same media, grown for 2 h, then induced by the addition of IPTG (Invitrogen) to 0.2 mM final concentration for a further 4 h. Cells were pelleted, washed, then extensively sonicated in 1× PBS containing 20% glycerol and 1% Triton X-100. Approximately 300 µl of glutathione-Sepharose beads (Amersham-Pharmacia) were added to the clarified lysates for 30 min with gentle agitation at room temperature. Beads were washed three times with 1× PBS/20% glycerol/1% Triton X-100 and the beads stored in the same buffer at –80°C. Elution of desired samples was performed in buffer containing 50 mM Tris (pH 8.0) and 10 mM reduced glutathione (Sigma). GST pull-downs were performed essentially as described in Robertson *et al.* (32) using 5–10 µg of GST fusion protein and 3 µl of ^{35}S-labeled *in vitro* transcribed/translated protein (made using the Promega TNT T7 Quick Coupled Kit). Binding buffer is 60 mM Tris (pH 7.5), 200 mM NaCl, 0.67% NP-40 and 1.25 mM EDTA. After binding, unbound proteins were removed with six washes of the same buffer used for the binding reaction. Sample buffer was added to the beads and the specifically bound proteins analyzed by SDS–PAGE followed by autoradiography.

Immunofluorescence staining

Both NIH3T3 and COS-7 cells (data not shown) were utilized with similar results. For transfection and subsequent immunofluorescence, cells (~1–2 × 10^5) were plated on glass coverslips in a six-well plate and grown overnight. Cells were transfected with 1–2 µg of DNA using either Lipofectamine (Invitrogen) or Fugene 6 transfection reagent (Roche Applied Sciences) according to the manufacturer's instructions. The cells were fixed 24 h later with 4% paraformaldehyde, washed with PBS and permeabilized with 0.5% Triton X-100. Fixed cells were incubated with primary antibody (at appropriate dilutions) for 1 h, followed by fluorescent-labeled secondary antibody (1:300 dilution) for

1 h. Nuclei were stained using Hoechst 33342 (Sigma) and the coverslips mounted onto glass slides using Fluoromount-G mounting media (Southern Biotechnology). Images were collected as a Z-series (z step was set to 0.07 μm) on an IX70 inverted microscope equipped with a 100× 1.35 NA, oil immersion objective (both from Olympus, Tokyo, Japan), and a charge-coupled device camera (Photometrics, Tucson, AZ) configured at 0.07 μm pixels. Images were deconvolved with the SoftWoRx software package (Applied Precision) using Decon3d. The following antibodies were used in these studies: anti-Flag (Sigma, 1:100 dilution), anti-Ubc9 N-15 (Santa Cruz Biotechnology, 1:25 dilution); secondary antibody, TRITC-conjugated anti-mouse antibody and Texas Red-conjugated anti-goat antibody (Southern Biotechnology).

In vitro sumoylation

One microliter of ^{35}S-labeled *in vitro* transcribed/translated Dnmt3a (made using the Promega TNT T7 Quick Coupled Kit) was utilized as the substrate for the *in vitro* sumoylation reaction. Each reaction contained 200 ng of recombinant SAE1/SAE2, 1 μg recombinant Ubc9, 10 μg recombinant SUMO-1 (1–97) (Alexis Biochemicals) or GST-SUMO-1 (1–97), 10 mM ATP, 10 mM phosphocreatine, 20 U/ml creatine phosphokinase, 0.6 U/ml inorganic pyrophosphatase (Sigma), 1 mM DTT, 50 mM Tris (pH 7.5), and 5 mM $MgCl_2$ in a 15 μl reaction volume. GST-SUMO-1 (1–97) and GST-PIASxα were cloned and purified as described above. Reactions were performed for 1 h at 37°C in duplicate, one with SUMO-1, and one without as a negative control. Sample buffer was then added and the products separated on an SDS–PAGE gel, followed by autoradiography.

RESULTS

Dnmt3a interacts with multiple components of the sumoylation machinery

To better understand the regulation of the *de novo* DNA methyltransferases, we sought to identify proteins that interact with Dnmt3a and may regulate its catalytic activity and repressive functions. We performed yeast two-hybrid screens with full-length Dnmt3a as bait (fused to the GAL4-DNA binding domain) to screen a human fetal liver cDNA library, the latter being derived from a tissue that we have previously shown to express high levels of Dnmt3a (33). Previous yeast two-hybrid studies have employed only isolated sub-domains of Dnmt3a as bait and may have missed important interactions (16). Our screen led to the identification of multiple independent clones containing sequences derived from the sumo E2 conjugating enzyme Ubc9, and the sumo E3 ligating enzymes PIAS1 and PIASxα (Fig. 1) (19,21,23). All clones scoring positive for growth on the selective media were confirmed using a liquid beta-galactosidase assay (Fig. 1). These interactions were specific as two irrelevant proteins (p53 and lamin B) did not yield appreciable beta-galactosidase activity and the GAL4-Dnmt3a bait alone gave only background levels of activity. Ubc9 has been shown to interact with a large number of proteins, many of which have been consequently demonstrated to be targets for sumoylation (20).

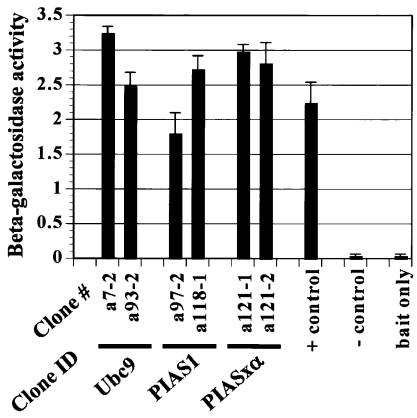

Figure 1. Dnmt3a binds to Ubc9, PIAS1 and PIASxα in a yeast two-hybrid screen. Full-length Dnmt3a was fused to the GAL4-DBD and used as bait in a yeast two-hybrid screen against a human fetal liver cDNA library in AH109 yeast cells under high stringency selection conditions. Clones scoring positive for growth on selective media were confirmed using a beta-galactosidase assay in Y187 yeast cells, then sequenced. The identity of two representative, independently isolated clones is shown below the graph of beta-galactosidase activity (as relative units). The positive control (+) is the interaction between p53 and the SV40 T antigen, the negative control (–) is the interaction between p53 and lamin B, and 'bait only' is the GAL4-Dnmt3a construct alone under the appropriate selection conditions. Expression of full-length Dnmt3a in AH109 cells was confirmed by western blotting (data not shown). Values are the mean of three independent experiments and the error bar is the standard deviation from the mean.

Dnmt3a interacts with Ubc9, PIAS1 and PIASxα *in vitro* and *in vivo* via the N-terminal regulatory domain

To confirm the three novel interactions identified in the yeast two-hybrid screen, we performed co-immunoprecipitations using cells transiently transfected with tagged versions of each of the proteins in question. Green fluorescent protein (GFP)-tagged Dnmt3a co-immunoprecipitated with Ubc9 (Fig. 2A) and with PIAS1 and PIASxα (Fig. 2B) when co-transfected into COS-7 cells. We noted that endogenous levels of Ubc9 were high and we could therefore detect an interaction between Dnmt3a and Ubc9 with and without transfection of the Ubc9 expression vector (Fig. 2A and data not shown). To further confirm the interactions, we performed *in vitro* glutathione *S* transferase (GST) pull-down assays. We were able to detect interactions, pairwise, between GST-Ubc9, GST-PIAS1 and GST-PIASxα and ^{35}S-labeled, *in vitro* transcribed and translated full-length Dnmt3a (Fig. 2C, top panel). Similarly, GST-Dnmt3a bound efficiently to both ^{35}S-labeled PIAS1 and PIASxα (Fig. 2C, bottom panel). We were unable to detect Ubc9 binding to GST-Dnmt3a, likely due to its small size and poor labeling by *in vitro* translation.

Since we had initially used full-length Dnmt3a in the yeast two-hybrid screen, we then sought to define the domain(s) of Dnmt3a responsible for these interactions. A series of Dnmt3a deletion mutations was constructed and fused to the GAL4-DNA binding domain. These were co-transfected into yeast cells with full-length versions of Ubc9, PIAS1 or PIASxα fused to the GAL4-activation domain, and their ability to interact was assessed using a beta-galactosidase assay. These experiments first confirm that full-length Dnmt3a interacts with full-length Ubc9, PIAS1 and PIASxα, and also indicate

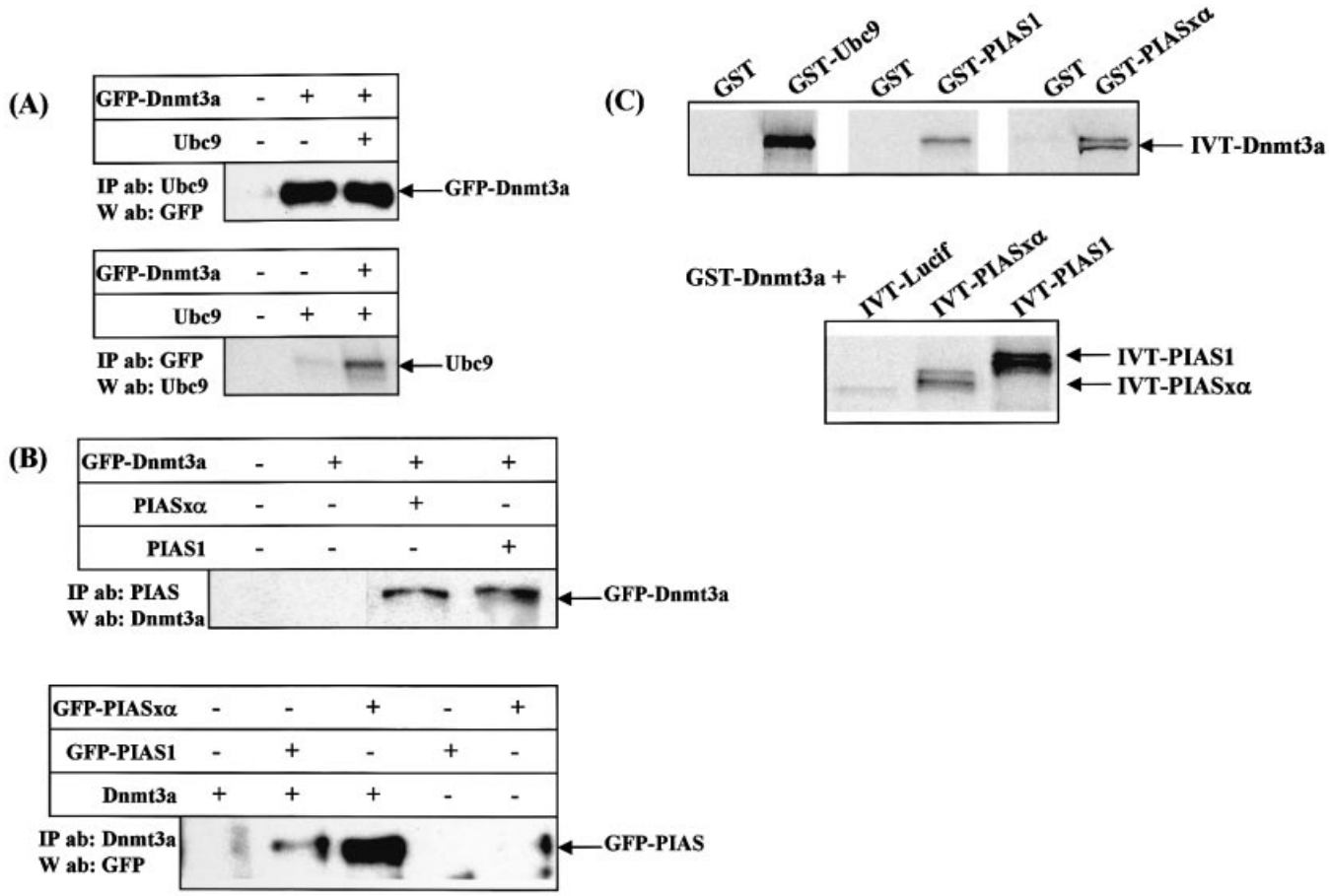

Figure 2. Dnmt3a interacts with Ubc9, PIAS1 and PIASxα *in vivo* and *in vitro*. (**A**) Dnmt3a co-immunoprecipitates with Ubc9 in whole cell extracts from transfected COS-7 cells using either an antibody directed against a tagged Dnmt3a or against Ubc9. Endogenous levels of Ubc9 were high (not shown), and we could detect the Dnmt3a–Ubc9 interaction with or without co-transfection of a Ubc9 expression vector. (**B**) Dnmt3a co-immunoprecipitates with PIAS1 and PIASxα using antibodies directed against either Dnmt3a (or a tagged Dnmt3a) or the PIAS proteins. In each case, COS-7 cells were transfected with 2.0 μg of each of the indicated expression constructs for 48 h. Whole cell extracts were then prepared and immunoprecipitated with the antibodies indicated at the left of each panel (IP ab), washed, the remaining bound proteins resolved on an SDS–PAGE gel, then subjected to western analysis with the antibody indicated at the left of each panel (W ab). (**C**) Dnmt3a binds to Ubc9, PIAS1 and PIASxα *in vitro* in a GST pull-down assay. GST alone, or the GST fusion proteins indicated across the top of each panel (bound to glutathione-Sepharose beads), were incubated with *in vitro* transcribed and translated ^{35}S-labeled proteins (IVT) and washed extensively. GST alone served as a control for non-specific binding to IVT-Dnmt3a in the top panel while IVT-luciferase served as a control for non-specific binding to GST-Dnmt3a in the lower panel. The presence of specifically bound protein is indicated with an arrow at the right of each panel. In some cases, the IVT reaction yielded two closely spaced products, most likely due to initiation at an internal methionine residue.

that the region of Dnmt3a necessary for these interactions lies between amino acids 408 and 473, a region between its PWWP and PHD domains (Fig. 3). The yeast two-hybrid interaction studies also indicate that Ubc9 can interact with a second region of Dnmt3a, encompassing amino acids 1–207 (Fig. 3, leftmost graph). This binding site may not be accessible in the context of the full-length protein. Taken together, these data indicate that Dnmt3a interacts with Ubc9, PIAS1 and PIASxα via a region within its N-terminal regulatory domain.

Dnmt3a co-localizes with components of the sumoylation machinery

To gain further insight into the nature of the interactions between Dnmt3a and Ubc9, PIAS1 and PIASxα, we examined their cellular localization and degree of co-localization using immunofluorescence staining and microscopy for each of the proteins in transfected cells. Transfected GFP-tagged Dnmt3a was found in a reticular to punctate-like pattern exclusively in the nucleus of NIH3T3 cells (Fig. 4A). Ubc9 exhibited a similar localization pattern and co-localized strongly with Dnmt3a (Fig. 4A, rightmost panel). GFP-tagged PIAS1 and PIASxα were found to reside in a more punctate pattern in the nucleus that also co-localized with Dnmt3a staining, although less than Ubc9's co-localization with Dnmt3a (Fig. 4B and C). We did not observe any dramatic change in Dnmt3a localization upon co-transfection of PIAS1, PIASxα or SUMO-1 (data not shown). These studies further support the notion that a significant fraction of Dnmt3a interacts with components of the sumoylation machinery *in vivo*.

Dnmt3a is sumoylated both *in vivo* and *in vitro*

Previous studies have shown that many of the proteins that interact with Ubc9 are also modified by SUMO-1 (19,20). To determine whether Dnmt3a is sumoylated *in vivo*, we co-transfected a plasmid expressing GAL4-DBD-tagged Dnmt3a

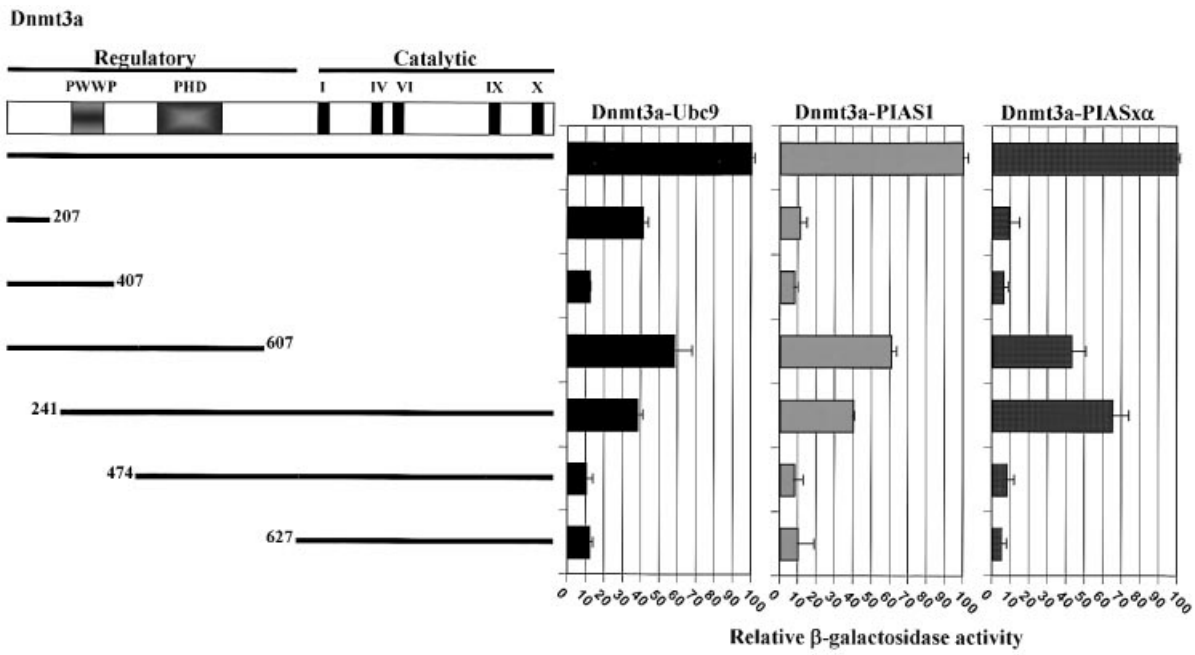

Figure 3. Ubc9, PIAS1 and PIASxα interact with Dnmt3a via a region within the N-terminal regulatory domain. A schematic diagram of the Dnmt3a protein is shown at the top with the PWWP, PHD and catalytic domains indicated. Each of the Dnmt3a deletion mutants, fused to the GAL4-DBD, was co-transformed with GAL4-AD fusions of Ubc9 (left), PIAS1 (middle), or PIASxα (right) into AH109 yeast cells. Thick lines denote the regions of Dnmt3a that were fused to the GAL4-DBD. Three colonies growing under high stringency selection for each transformation were selected, grown in liquid culture, then used for a beta-galactosidase assay (relative units). Graphs show the mean of three independent experiments (colonies) relative to the full-length Dnmt3a set at 100%. Error bars are the standard deviation from the mean.

along with an expression vector for SUMO-1, since it has been reported that the pool of endogenous free SUMO-1 is very low (20). We lysed the transfected cells in the presence of a strong inhibitor of SUMO hydrolyases (22) (N-ethyl-maleimide or NEM, a cysteine protease inhibitor), then prepared whole cell extracts for western analysis and immunoprecipitation. A SUMO-1-specific 'band shift' of tagged-Dnmt3a was readily observable in whole cell extracts from transfected cells by western blotting using an anti-GAL4-DBD antibody (Fig. 5A, left panel). As additional evidence that Dnmt3a is sumoylated, we immunoprecipitated the transfected Dnmt3a via the GAL4-DBD tag, then probed the subsequent western blot with a second GAL4-DBD antibody, to determine the molecular weight for the total pool of transfected Dnmt3a. Figure 5A (middle panel) shows that GAL4-Dnmt3a migrates at a position consistent with its predicted molecular weight (Dnmt3a plus the GAL4 tag) in the absence of co-transfected SUMO-1. A small amount of higher molecular weight GAL4-Dnmt3a can also be observed under this condition and is consistent with low-level modification by endogenous SUMO-1. When an expression vector for SUMO-1 is co-transfected with GAL4-Dnmt3a, essentially all of the GAL4-Dnmt3a is converted into the ~20 kDa larger, SUMO-1 modified form (Fig. 5A, middle panel). This is a characteristic feature of proteins modified by SUMO-1 (20). When this blot was stripped and re-probed with an anti-SUMO-1 antibody, only the low-level, higher molecular weight SUMO-1 modified GAL4-Dnmt3a band was observed in the absence of added SUMO-1 (Fig. 5A, right panel, middle lane). In the presence of co-transfected SUMO-1, only the SUMO-1 modified GAL4-Dnmt3a was detectable. Interestingly, we could also discern a small amount of an even higher molecular weight species of GAL4-Dnmt3a (~40 kDa larger) consistent with the addition of two sumo molecules to Dnmt3a (Fig. 5A, right panel, third lane). The band-shifts were specifically enhanced only when SUMO-1 was co-transfected and required the presence of NEM in the cell lysis buffers (not shown). The results clearly demonstrate that one, and possibly two molecules of sumo become covalently linked to Dnmt3a *in vivo*. Lastly, we employed recombinant E1 (SAE1/SAE2), Ubc9 and SUMO-1, along with ATP and an ATP regeneration system, to show that ^{35}S-labeled *in vitro* transcribed/translated Dnmt3a was modified by SUMO-1 *in vitro*. A band shift of ~20 kDa was observed only when recombinant SUMO-1 was added to the *in vitro* reaction, or a shift of ~40 kDa was observed when recombinant GST-tagged SUMO-1 was added to the reaction (Fig. 5B). *In vitro* sumoylation was less efficient than that observed under *in vivo* conditions, indicating that other factors are likely to be important in regulating the modification reaction.

The E3 ligases, PIAS1 and PIASxα, do not dramatically alter SUMO-1 modification of Dnmt3a

A number of recent reports have revealed that members of the PIAS protein family (PIAS1, -3, -x, and -y) function as sumo E3 ligases and enhance sumoylation of target proteins (21,22,34–36). Other work has shown that PIAS proteins can alter the transcription functions (ability to activate or repress target genes) of the proteins with which they interact without acting as an E3 ligase directly on the target protein (37,38). To determine which scenario was operating with Dnmt3a, we co-transfected plasmids expressing tagged

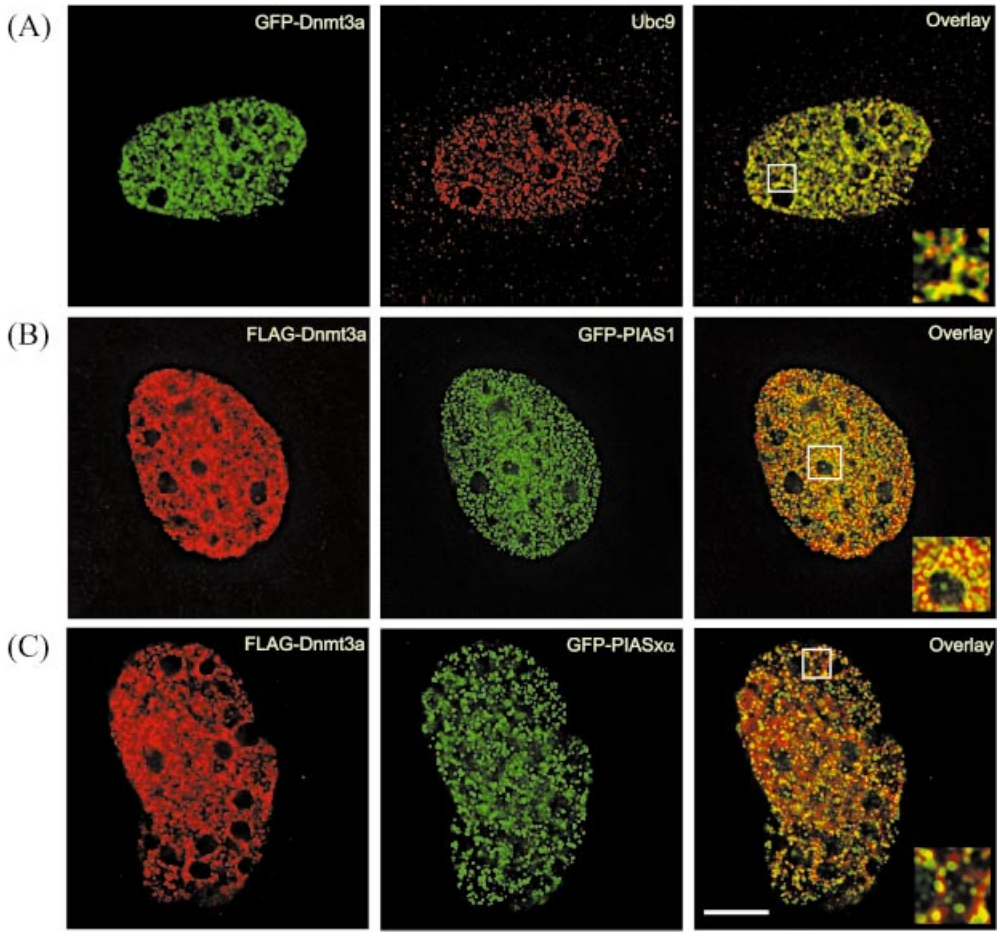

Figure 4. Dnmt3a co-localizes with Ubc9, PIAS1 and PIASxα in transfected cells. (**A**) NIH3T3 cells grown on glass cover slips were co-transfected for 24 h with GFP-Dnmt3a and Ubc9, (**B**) FLAG-Dnmt3a and GFP-PIAS1 or (**C**) FLAG-Dnmt3a and GFP-PIASxα. Immunofluorescence labeling was performed with an anti-Ubc9 antibody in (A) or the anti-FLAG-M2 antibody in (B–C) followed by incubation with an appropriate secondary antibody as described in Materials and Methods. Images were collected with an Olympus IX70 inverted microscope equipped with a 100× 1.35 NA oil immersion objective. The two images are merged in the right-most panel where yellow color represents co-localization. Boxed areas are enlarged and shown in the lower-right corner of each overlay panel. The scale bar in the lower right panel corresponds to 5 μm.

Dnmt3a, along with expression vectors for SUMO-1, PIAS1, or PIASxα into COS-7 cells. Whole cell extracts were prepared in the presence of NEM and analyzed by western blotting with the GAL4-DBD tag antibody (for Dnmt3a detection). Figure 6A shows that a low level of sumoylated Dnmt3a can be seen without added SUMO-1, but the ratio of the modified to the unmodified bands increases toward the modified form when SUMO-1 is co-expressed. When either PIAS1 or PIASxα are co-transfected with Dnmt3a and SUMO-1, there is little change in the amount of sumo modified Dnmt3a. The effects of PIASxα on sumo modification of Dnmt3a were also tested using the same *in vitro* sumo modification assay described in Figure 5B. When recombinant SUMO-1 was added to the *in vitro* sumo reaction, a band shift was clearly observed; however, similar to the *in vivo* transfection assay, little additional enhancement was observed when recombinant GST-PIASxα was added to the reactions (Fig. 6B). Thus PIAS1 and PIASxα do not appear to act as E3 ligases for Dnmt3a under the experimental conditions we have employed.

Functional consequences of SUMO-1 modification of Dnmt3a: modulation of protein–protein interactions

Previous studies have shown that Dnmt3a can interact with both HDAC1 (16) as well as with Dnmt3b1 (39). Given that one of the functions of sumo modification is to modulate protein–protein interactions (19), we examined whether the interactions of Dnmt3a with several of its known interaction partners were affected by SUMO-1 conjugation. We first tested the effects of sumo modification on the ability of Dnmt3a to interact with HDAC1. Using co-immunoprecipitation assays from HCT116 cells co-transfected with vectors expressing HDAC1 and Dnmt3a, we first confirmed that Dnmt3a interacts with HDAC1. Furthermore, we found that this interaction is dramatically reduced when an expression vector for SUMO-1 was co-transfected into HCT116 cells

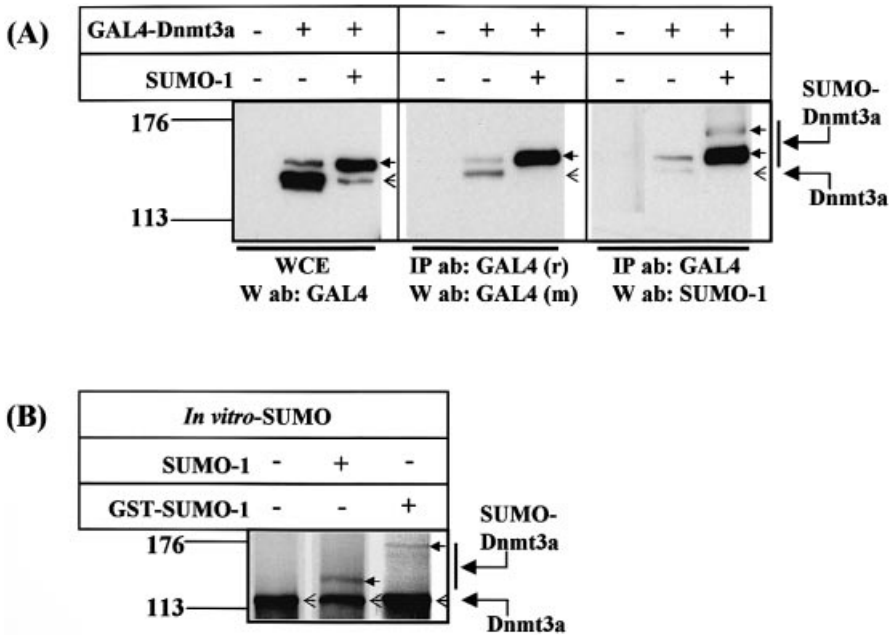

Figure 5. Dnmt3a is sumoylated *in vivo* and *in vitro*. (**A**) GAL4-DBD-tagged Dnmt3a (2 μg) was co-transfected with or without a SUMO-1 expression vector (1.5 μg) into COS-7 cells. Whole cell extracts (WCE) were prepared in the presence of NEM and the migration position of the transfected Dnmt3a determined by western blotting with an anti-GAL4-DBD antibody (left panel). Dnmt3a was also immunoprecipitated from the transfected WCEs with a rabbit polyclonal GAL4-DBD antibody ('r'), and the molecular weight of the total pool of GAL4-Dnmt3a determined by western blotting with a mouse monoclonal GAL4-DBD antibody ('m', middle panel). The presence of slower migrating forms of Dnmt3a in the '+SUMO-1' lane indicates that Dnmt3a is sumoylated. When the blot is stripped and re-probed with a SUMO-1 antibody, only the slower migrating species are detectable and these higher molecular weight forms are specifically enriched in the '+SUMO-1' reaction (right panel). (**B**) Dnmt3a is sumoylated *in vitro*. *In vitro* sumoylation reactions were carried out in the presence of ^{35}S-labeled *in vitro* transcribed/translated (IVT) Dnmt3a, 200 ng recombinant SAE1/SAE2, 1 μg Ubc9, 2 mM ATP, ATP regeneration system, and 10 μg of SUMO-1 or 5 μg GST-SUMO-1 as indicated at the top of each panel. Molecular weight markers (kDa), are indicated at the left. The open arrow denotes the position of the unmodified GAL4-Dnmt3a and the filled arrow shows the migration position of the sumo-modified Dnmt3a.

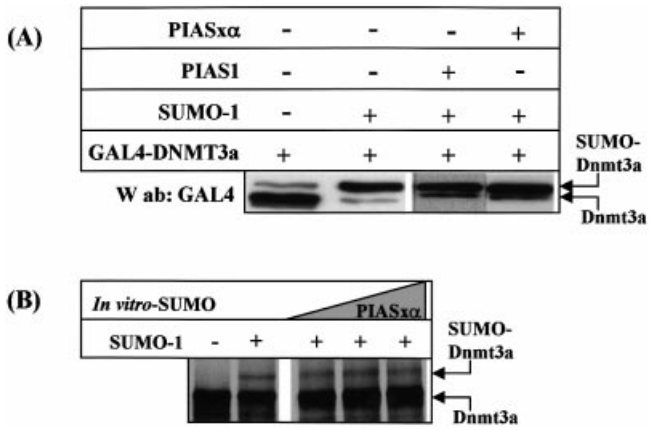

Figure 6. PIAS1 and PIASxα do not enhance sumoylation of Dnmt3a *in vivo* or *in vitro*. (**A**) Two micrograms of full-length GAL4-Dnmt3a was transfected without (lane 1) or with 1.5 μg of SUMO-1 expression vector (lane 2) into COS-7 cells and whole cell extracts were prepared for western analysis with anti-GAL4-DBD antibody. In lanes 3 and 4, four micrograms of PIAS1 or PIASxα expression vector, respectively, are co-transfected with the SUMO-1 and GAL4-Dnmt3a expression vectors. (**B**) *In vitro* sumoylation reactions containing ^{35}S-labeled-IVT Dnmt3a, performed as described in Figure 5B, were supplemented with recombinant GST-PIASxα (1, 5, 10 μg) and the reaction products analyzed on a 6% SDS–PAGE gel.

(Fig. 7A). Given the high degree of homology between HDAC1 and HDAC2, and the finding that they often co-exist in other protein complexes (40–42), we asked if Dnmt3a could also interact with HDAC2. Indeed, we were readily able to co-immunoprecipitate Dnmt3a and HDAC2 from transfected HCT116 cells. Interestingly, this interaction was also disrupted by co-transfection of the SUMO-1 expression vector (Fig. 7A). The effects were specific for the Dnmt3–HDAC interaction since SUMO-1 co-transfection did not change the endogenous levels of HDAC1, HDAC2 and GAL4-Dnmt3a (Fig. 7A, lower two panels, 'inputs'). In fact, transfection of HDAC expression vectors was found to be unnecessary since the cell line employed expressed high levels of endogenous HDAC1/2 (data not shown). To further demonstrate that the ability of SUMO-1 expression to disrupt the Dnmt3a–HDAC interaction is not due to non-specific phenomenon, we examined the effects of SUMO-1 over-expression on the ability of Dnmt3a to interact with Dnmt3b1. We first confirmed the previously published observation that Dnmt3a and Dnmt3b1 can interact (39), and also showed that SUMO-1 co-transfection does not affect this interaction (Fig. 7B). Taken together, these data indicate that modification of Dnmt3a by SUMO-1 markedly inhibits its interactions with both HDAC1 and HDAC2 but does not affect the interaction between Dnmt3a and Dnmt3b1.

Functional consequences of SUMO-1 modification of Dnmt3a: effect on its ability to repress transcription

An important function of the DNA methyltransferases, in addition to their ability to catalyze the methyl transfer reaction, is to act as transcriptional repressors independent of their catalytic activity in a largely HDAC-dependent

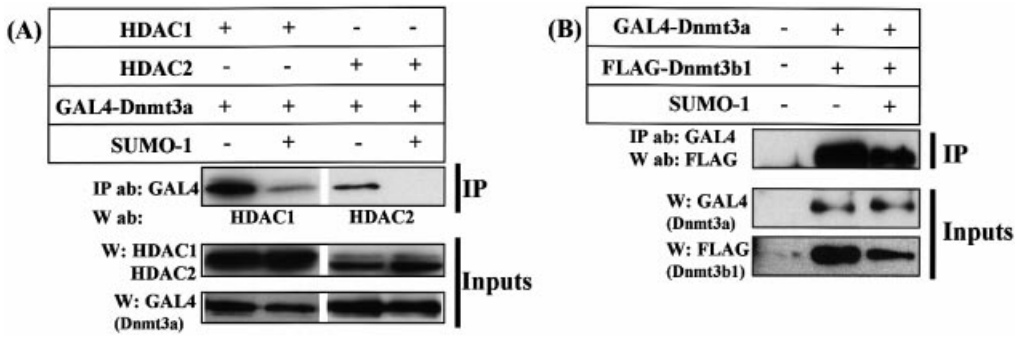

Figure 7. Sumoylation differentially affects several known protein–protein interactions involving Dnmt3a. (**A**) HCT116 cells were transfected for 24 h with 2.0 μg of each of the expression constructs listed along the top. Whole cell extracts were prepared and subjected to immunoprecipitation with a mouse anti-GAL4-DBD tag antibody to pull-down Dnmt3a. Subsequent western blotting with antibodies against HDAC1 (left panel), or HDAC2 (right panel), revealed that Dnmt3a interacts with both HDAC1 and HDAC2 and that this interaction is markedly reduced under conditions favoring sumoylation of Dnmt3a (co-transfection of 1.5 μg of SUMO-1 expression vector). Levels of HDAC1/2 and GAL4-Dnmt3a were not affected by SUMO-1 over-expression (lower two panels, 'Inputs'). (**B**) HCT116 cells were transfected with tagged Dnmt3a and Dnmt3b1 and whole cell extracts prepared as described above. Immunoprecipitations were carried out with anti-GAL4-DBD antibody and western blotting of the bound material was performed with an anti-FLAG antibody to detect the presence of Dnmt3b1. SUMO-1 expression did not alter the ability of Dnmt3a to interact with Dnmt3b1 (upper panel), nor did it affect the levels of the transfected proteins (lower two panels, 'Inputs'). Similar results were obtained using COS-7 cells (not shown).

manner. This is emphasized by findings that all three of the active DNMTs (DNMT1, 3a and 3b) can repress transcription and interact with HDACs (16,17,32,43). Since modification of Dnmt3a by SUMO-1 disrupted its ability to interact with HDAC1 and HDAC2, we examined the effects of SUMO-1 modification on the capacity of Dnmt3a to repress transcription. To accomplish this, we co-transfected HCT116 cells with a vector expressing full-length GAL4-Dnmt3a, a firefly luciferase reporter construct driven by five GAL4 DNA binding sites (BS) and the SV40 enhancer region, and a constitutively expressed *Renilla* luciferase reporter construct to normalize for transfection efficiency. The 5X GAL4-BS-luciferase reporter construct has a high level of basal promoter activity, which is repressed in a dose-dependent manner upon co-transfection of increasing amounts of GAL4-Dnmt3a (Fig. 8A). Repression was only observed when Dnmt3a was tethered to the promoter by fusion to the GAL4-DBD, since increasing amounts of untethered Dnmt3a did not affect reporter activity (Fig. 8A). To test the effect of SUMO-1 modification on the ability of GAL4-Dnmt3a to repress transcription, a fixed amount of GAL4-Dnmt3a, yielding a significant amount of transcriptional repression, was co-transfected with increasing amounts of SUMO-1 expression vector. Results showed that SUMO-1 over-expression completely relieved GAL4-Dnmt3a-mediated transcriptional repression of the reporter construct in a dose-dependent manner (Fig. 8B). Over-expression of SUMO-1 in the absence of GAL4-Dnmt3a had no effect on reporter gene activity (Fig. 8B).

Results presented in Figure 6 showed that expression of PIAS1 and PIASxα did not significantly alter the amount of sumoylated Dnmt3a protein in cells. To determine what, if any, effects PIAS proteins might have on Dnmt3a-mediated repression, we co-transfected GAL4-Dnmt3a with increasing amounts of PIAS1 or PIASxα expression vector. Repression of the luciferase reporter construct by GAL4-Dnmt3a was also relieved by PIAS protein expression in a dose-dependent fashion (Fig. 8C). Both PIAS proteins produced similar effects, although PIAS1 appeared slightly more potent at relieving repression. Although the PIAS proteins did not appear to act as E3 ligases for Dnmt3a, we sought to determine whether the domain necessary for E3 activity (a RING-finger-like domain) was required for PIAS1 to relieve Dnmt3a-mediated transcriptional repression. All PIAS proteins contain a RING-finger-like domain, a feature they share with RING-type ubiquitin ligases such as c-Cbl and MDM2 (34,44). We therefore transfected increasing amounts of a mutant PIAS1 (Δ341–536, deleted for the RING-finger domain) expression plasmid with GAL4-Dnmt3a and observed that this mutant had no effect on repression (Fig. 8C, middle set of bars). PIAS1 Δ341–536 retains the ability to interact with Dnmt3a in a GST pull-down assay (data not shown). This result indicates that the E3-ligase activity of PIAS1 is necessary for it to exert its transcriptional regulatory functions on Dnmt3a even though it does not appear to act as an E3 for Dnmt3a. As a control, over-expression of PIAS proteins in the absence of GAL4-Dnmt3a was performed and this did not significantly alter reporter gene activity (Fig. 8C, right two graphs). Therefore, one of the functional consequences of Dnmt3a sumoylation is to disrupt the Dnmt3a–HDAC1/2 interaction and this is most likely why sumoylation of Dnmt3a abrogates its ability to repress transcription. We also tested for the effects of Ubc9 expression on GAL4-Dnmt3a-mediated repression, but no significant changes were observed, most likely because the cells used already expressed high levels of endogenous Ubc9 (data not shown).

DISCUSSION

In this study, we have performed yeast two-hybrid screening to identify three proteins, Ubc9, PIAS1 and PIASxα, which interact with Dnmt3a both *in vitro* and *in vivo*. Interestingly, all three of these newly identified interaction partners are components of the sumoylation pathway. Ubc9 (the E2 conjugating enzyme) acts at the second step in the process of conjugating the 11 kDa SUMO-1 protein to its target proteins, while PIAS1 and PIASxα act as sumo ligase enzymes (E3s) that are believed to enhance sumo modification

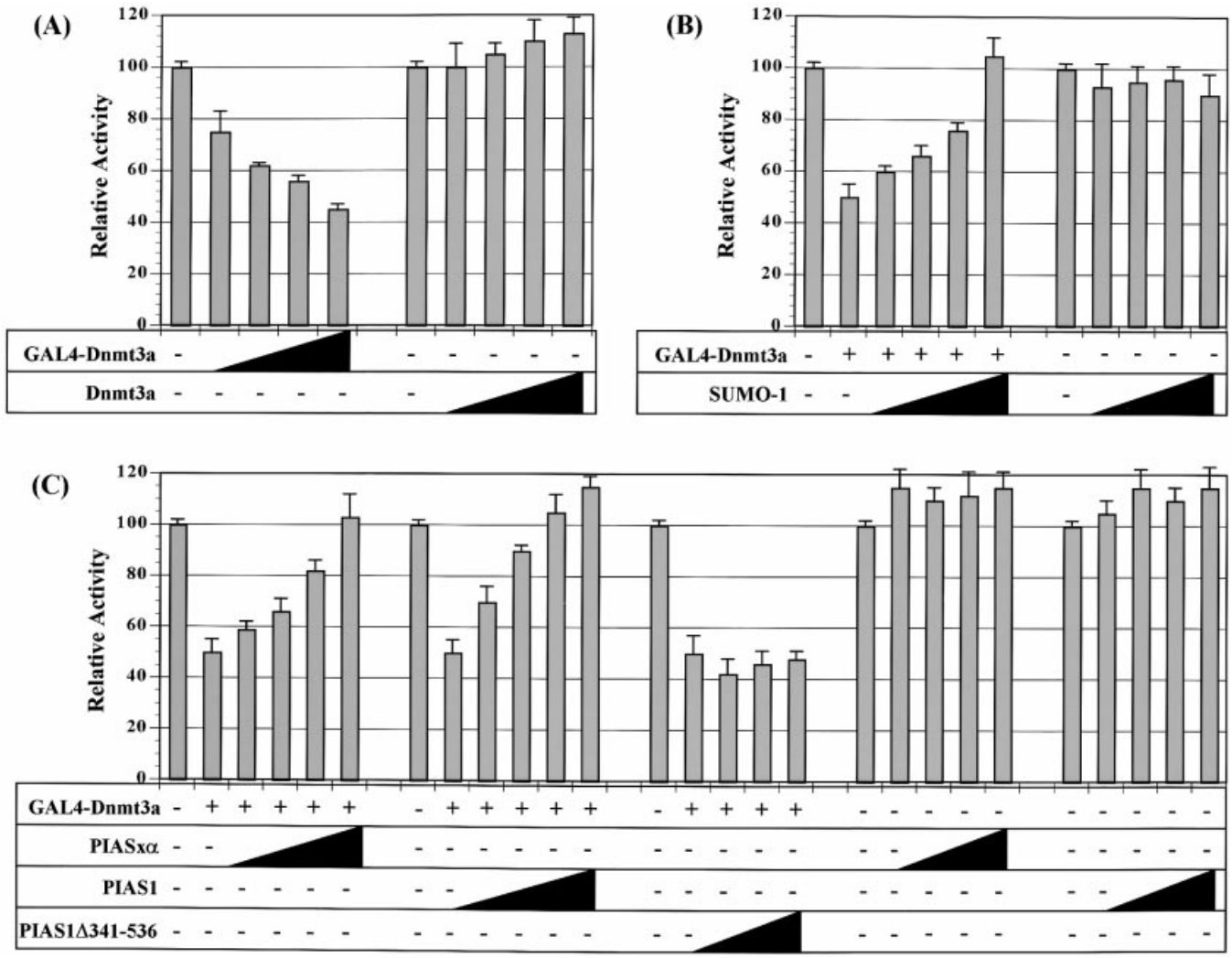

Figure 8. SUMO-1 modification of Dnmt3a eliminates its capacity to repress transcription. (**A**) Full-length Dnmt3a represses transcription when fused to the GAL4-DBD. Increasing amounts (indicated by the black wedge) of GAL4-Dnmt3a (0.5, 1.0, 2.5, 5.0 μg) or untethered Dnmt3a (0.5, 1.0, 2.5, 5.0 μg) were co-transfected into HCT116 cells along with 10 ng of an SV40-*Renilla* luciferase construct (to control for transfection efficiency), and 1.0 μg of a firefly luciferase reporter gene driven by five GAL4 DNA binding sites. Both firefly and *Renilla* luciferase activities were measured from the same whole cell extract preparation after 24 h using the Promega Dual Luciferase Assay kit. (**B**) GAL4-Dnmt3a (4.0 μg) was co-transfected with increasing amounts of SUMO-1 expression vector (1.0, 2.5, 5.0, 8.0 μg) into HCT116 cells. Transfection of equivalent amounts of SUMO-1 expression vector in the absence of GAL4-Dnmt3a did not alter promoter activity. (**C**) Effects of PIAS proteins on Dnmt3a transcriptional repression. GAL4-Dnmt3a (4.0 μg) was co-transfected with increasing amounts of PIASxα or PIAS1 expression vectors (1.0, 2.5, 5.0, 8.0 μg) or a mutant form of PIAS1 (Δ341–536, 2.5, 5.0, 8.0 μg) deleted for the RING-finger-like domain, into HCT116 cells and reporter activity determined after 24 h. Transfection of PIAS1 or PIASxα (2.5, 5.0, 8.0 μg) expression plasmids in the absence of GAL4-Dnmt3a did not affect reporter gene activity. All values were normalized for transfection efficiency (firefly luciferase/*Renilla* luciferase) and then set relative to the reporter activity of the 5× GAL4-BS-luciferase construct alone set at 100% (first bar in each series). Values are the average of two independent experiments and error bars are the range. Total DNA content was kept constant in each transfection by addition of 'empty' parental expression vector.

of at least some of the target proteins with which they interact (20–22). Consistent with these functions, we find that Dnmt3a is modified by SUMO-1 *in vitro* and *in vivo*. The region of Dnmt3a responsible for interacting with Ubc9, PIAS1 and PIASxα is located within the N-terminal regulatory domain of Dnmt3a. Our data are consistent with a previous study (45) which demonstrated that the other *de novo* DNA methyltransferase, Dnmt3b, interacts with Ubc9 and is sumo modified. Interestingly, we have found that Dnmt3b also interacts with PIAS1 and PIASxα in a yeast two-hybrid screen, whereas DNMT1 does not (K.D.Robertson, unpublished data), making sumoylation a feature which distinguishes the *de novo* from the maintenance DNA methyltransferases. The functional consequences of sumo modification of Dnmt3a were investigated using a number of assays. Co-immunoprecipitation studies revealed that the ability of Dnmt3a to interact with HDAC1 was severely impaired under conditions favoring Dnmt3a sumoylation. We present the first evidence that Dnmt3a also interacts with HDAC2 and show that this interaction is also disrupted under conditions favoring sumo modification of Dnmt3a. That SUMO-1 modification of Dnmt3a specifically disrupts its ability to interact with HDACs was demonstrated by showing that SUMO-1 modification of Dnmt3a did not affect its ability

to interact with Dnmt3b1. Lastly, transient transfection assays revealed that conditions which favor sumo modification of Dnmt3a completely eliminate the ability of Dnmt3a to repress transcription. Even though the PIAS proteins did not appear to act as E3 ligases for Dnmt3a, they too were capable of disrupting Dnmt3a-mediated repression. Thus we have identified an important new mechanism for regulating at least one major function of the Dnmt3a *de novo* DNA methyltransferase in mammalian cells.

A large and diverse group of proteins are modified by SUMO-1. Many of these proteins are in some way involved with regulating transcription; in particular, a role for sumoylation in the negative regulation of transcription appears to be emerging (19). For example, sumoylation of the transcription factors Sp3, c-Jun, several nuclear hormone receptors, c-Myb and AP-2 results in a down-regulation of their transactivation functions (24,35,46–49). In other cases, sumo modification of proteins involved in repression, such as HDAC1, HDAC4 and HDAC6, have been reported. Studies of HDAC1 and HDAC4 have shown that mutation of the sumoylation sites reduces their capacity to repress transcription (50,51). It would appear from our studies that Dnmt3a falls into the latter group of proteins, since Dnmt3a can efficiently repress transcription but this repression function is abolished by sumo modification.

The PIAS proteins also interact with a large number of other proteins, including p53 and several steroid hormone receptors (22,34,37). While the founding member of this family, PIAS1, was first identified as an inhibitor of activated Stat1 transcription (52), the PIAS protein family members have since been shown to have roles in transcriptional regulation and enhancement of sumo modification of their binding partners. It has also been found that PIAS proteins play roles in regulating transcription independent of their ability to enhance sumoylation (37). Over-expression of PIAS proteins did not result in an enhancement of Dnmt3a sumoylation; however, it remains possible that PIAS1 and PIASxα are modulating Dnmt3a transcriptional repression in additional ways that do not involve sumo modification of Dnmt3a. For example, PIAS protein binding to Dnmt3a may act as a bridge to other sumoylated proteins that could in turn alter the properties of Dnmt3a, PIAS proteins could be altering the sumo modification of other factors interacting with Dnmt3a, or binding of PIAS proteins to Dnmt3a may block interaction sites for other proteins.

The mechanism(s) by which sumo modification modulates the activities of proteins to which it is linked is not entirely clear. It has been proposed that sumoylation may alter protein–protein interactions and therefore affect the assembly of macromolecular protein complexes. This could be due to alterations in the three-dimensional structure of the modified protein, conformation changes, disruption or potentially the creation of a protein–protein interaction surface (19,23). For Dnmt3a, it is clear that sumoylation can modify its ability to interact with certain proteins, but not others. The disruption of Dnmt3a's capacity to repress transcription by SUMO-1 modification is entirely consistent with the finding that SUMO-1 modification reduces the ability of Dnmt3a to interact with HDACs. It also remains possible that sumoylation of Dnmt3a alters its capacity to repress transcription in other HDAC-independent mechanisms, since previous findings have shown that inhibition of HDAC activity with TSA does not completely relieve Dnmt3a-mediated transcriptional repression (17). HDAC1 has also been shown to be sumoylated (50). Sumo modification of HDAC2 has not been reported. Thus sumoylation of both Dnmt3a and HDAC1 may be contributing to the functional effects we have observed. Future studies will be aimed at dissecting the relative contribution of sumoylation of each protein in regulating protein–protein interactions and transcriptional repression.

Clearly sumoylation of Dnmt3a may have additional effects on Dnmt3a function, such as altering its catalytic activity, targeting it to certain regions of the nucleus, or altering its stability. Although we did not observe significant re-localization of Dnmt3a upon co-transfection of SUMO-1, it may be that there are subtle changes in its localization or function during the cell cycle, which could be highly relevant to mechanisms by which regional DNA methylation patterns are established and maintained. Reversible sumo modification of Dnmt3a could provide a highly dynamic mechanism for 'targeting' DNA methylation to specific regions of the genome. For example, the HDACs may be among the first chromatin modifying enzymes to be recruited to a gene promoter destined for silencing. Deacetylation of chromatin in the promoter region is likely sufficient for transcription silencing. Depending on whether the gene will need to be reactivated in the future, regulated recruitment of Dnmt3a by the bound HDACs, dependent on the sumo modification state of Dnmt3a, will determine whether the gene is silenced but can be reactivated relatively easily by recruitment of histone acetyltransferases. In this situation, the promoter chromatin would be deacetylated but Dnmt3a is sumoylated and not able to bind to HDAC, therefore no *de novo* DNA methylation occurs. Alternatively, if the gene is destined for 'permanent' silencing, then the promoter chromatin would be deacetylated but Dnmt3a is not sumo modified and can be recruited by bound HDACs to carry out *de novo* methylation of the region. Future studies will be aimed at testing this important idea.

In summary, we have shown, using yeast two-hybrid screening, that three proteins catalyzing the sumoylation pathway specifically interact with Dnmt3a, and we have further demonstrated that Dnmt3a is indeed post-translationally modified by SUMO-1 and that this modification specifically alters several of Dnmt3a's gene regulatory functions. In the course of dissecting the functional consequences of Dnmt3a sumoylation we found that Dnmt3a could interact with HDAC2 in addition to HDAC1. Given that sumo modification is a highly dynamic process that is tightly regulated, it will be important to define the role of sumoylation of Dnmt3a in more detail. For example, in addition to disrupting Dnmt3a's ability to repress transcription, sumoylation of Dnmt3a may also alter its ability to methylate DNA indirectly by altering its access to chromatin via its interaction with HDACs. Furthermore, sumoylation levels may vary during the cell cycle or in different tissues or at different stages of embryonic development. Given the many critical roles of DNA methylation in development, chromatin structure, and gene expression, as well as the potentially mutagenic effects a *de novo* DNA methyltransferase could exert on the genome, it is perhaps not surprising that post-translational modifications, such as sumoylation, play an important role in controlling the enzymes responsible for establishing and maintaining

genomic DNA methylation patterns. Further characterization of this and other potential modifications will no doubt provide insight into the means by which methylated, transcriptionally repressed regions of the genome are established.

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