Chromatin Targeting of de Novo DNA Methyltransferases by the PWWP Domain*

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Ying-Zi Ge‡, Min-Tie Pu‡, Humaira Gowher§, Hai-Ping Wu‡, Jian-Ping Ding§, Albert Jeltsch§, and Guo-Liang Xu***

From the ‡State Key Laboratory of Molecular Biology and the **Key Laboratory of Proteomics, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, 320 Yueyang Road, Shanghai 200031, China, the §Institute für Biochemie, Fachbereich 8, Justus-Liebig Universität, Heinrich-Buff-Ring 58, 35392 Giessen, Germany, and the ¶School of Engineering and Science, International University Bremen, Campus-Ring 1, 28759 Bremen, Germany

DNA methylation patterns of mammalian genomes are generated in gametogenesis and early embryonic development. Two de novo DNA methyltransferases, Dnmt3a and Dnmt3b, are responsible for the process. Both enzymes contain a long N-terminal regulatory region linked to a conserved C-terminal domain responsible for the catalytic activity. Although a PWWP domain in the N-terminal region has been shown to bind DNA in vitro, it is unclear how the DNA methyltransferases access their substrate in chromatin in vivo. We show here that the two proteins are associated with chromatin including mitotic chromosomes in mammalian cells, and the PWWP domain is essential for the chromatin targeting of the enzymes. The functional significance of PWWP-mediated chromatin targeting is suggested by the fact that a missense mutation in this domain of human DNMT3B causes immunodeficiency, centromeric heterochromatin instability, facial anomalies (ICF) syndrome, which is characterized by loss of methylation in satellite DNA, pericentromeric instability, and immunodeficiency. We demonstrate that the mutant protein completely loses its chromatin targeting capacity. Our data establish the PWWP domain as a novel chromatin/chromosome-targeting module and suggest that the PWWP-mediated chromatin association is essential for the function of the de novo methyltransferases during development.

DNA cytosine methylation provides an epigenetic means that is indispensable for multiple cellular processes including genomic imprinting, inactivation of the X chromosome, repression of transposons, and regulation of tissue-specific gene expression (1, 2). Aberrant methylation is also known to contribute to tumorigenesis and other diseases (3, 4).

Methylcytosines account for 5% of the total cytosines in mammalian genomes. This modified base is present predominately in CpG dinucleotides (over 60% methylated). De novo methyltransferase processes in gametogenesis and early embryonic development create new methylation patterns. Once created, the methylation patterns are inherited through mitotic divisions by maintenance methylation that takes place in concert with DNA duplication. The well-characterized methyltransferase Dnmt1 has substrate preference for hemimethylated DNA generated in the S phase and performs the function of maintenance methylation (5). Consistent with its function, Dnmt1 appears at DNA replication foci in S phase (6, 7).

The generation of somatic methylation patterns in gametogenesis and embryogenesis depends on the de novo methyltransferases Dnmt3a and Dnmt3b (8, 9). Disruption of both or either of the genes in mice led to failure in methylation initiation in early embryos and caused lethal phenotypes (10). Together with Dnmt3L, Dnmt3a is also required in the establishment of methylation marks of maternally imprinted genes in oogenesis (11, 12). A defective DNMT3B gene is associated with the human ICF1 syndrome characterized by immunodeficiency, centromeric heterochromatin instability, and facial anomalies (13). As a potential underlying molecular cause of the syndrome, methylation of the classic satellite sequences is lost in patient DNA (14).

Dnmt3a and Dnmt3b have a long N-terminal region fused to the catalytic domain that resembles bacterial cytosine methyltransferases (15) and is active in the absence of the N-terminal parts (16). Their N-terminal region contains a PWWP domain and a cysteine-rich PHD zinc finger domain that interacts with the transcriptional repressor RP58, silencer heterochromatin HP1β, histone deacetylases, and histone methyltransferase SUV39H1 (17–19). The biological significance of PHD-mediated transcriptional silencing, independent of DNA methylation activity, is still an open question.

PWWP domains have been found in more than 60 eukaryotic proteins implicated in transcriptional regulation and chromatin organization (20). It was first identified in WHSC1, whose gene maps to the Wolf-Hirschhorn syndrome critical region (21). The structure of the PWWP domain of murine Dnmt3b has been solved recently (22). Although the domain binds DNA in vitro, its in vivo cellular function has yet to be resolved.

We show here that both Dnmt3a and Dnmt3b are associated with chromatin in vivo throughout the entire cell cycle; they are concentrated in heterochromatic regions during interphase and at specific loci on chromosome arms at metaphase. The PWWP domain is essential for chromatin targeting. An ICF missense mutation located in this domain (23) causes the loss

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** To whom correspondence should be addressed. Tel.: 86-21-5492-1332; Fax: 86-21-5492-1333; E-mail: glxu@sibs.ac.cn.

† The abbreviations used are: ICF, immunodeficiency, centromeric heterochromatin instability, facial anomalies; PHD, plant homeodomain; HP1, heterochromatin protein 1; RT, room temperature; HA, hemagglutinin; DAPI, 4′,6-diamidino-2-phenylindole; Pipes, 1,4-piperazineethanesulfonic acid.
of chromatin targeting ability, which is suggested to contribute to the deficiency in DNA methylation in ICF patients. Collectively our findings underscore the importance of PWPF-mediated chromatin targeting for the function of DNA methyltransferases and provide a framework for future study of regulation of DNA methylation in development and pathogenesis.

**EXPERIMENTAL PROCEDURES**

** Constructs— Full-length wild-type Dnmt3a and Dnmt3b and various fragments were cloned by PCR from original cDNA clones kindly provided from En Li (15) into a mammalian expression vector, pcDNA3-HA (a generous gift from Dr. Gang Pei). Mutations in Dnmt3a and Dnmt3b were introduced by PCR. All PCR-cloned regions were verified by sequencing.

**Antibody Preparation— Anti-Dnmt3a and anti-Dnmt3b antibodies were prepared for Western analysis and immunocytochemistry. The full-length coding sequence for both proteins was inserted into a histidine fusion vector, pET28a (Novagen). The expression and purification of the both fusion proteins were carried out according to Gowher and Jeltsch (24). Antisera were raised by immunizing two rabbits with each fusion protein.

Anti-Dnmt3a and anti-Dnmt3b were affinity-purified on a nickel-nitriilotriacetic acid column attached with the corresponding antigen protein as described previously (25). To remove cross-reactivity, the affinity-purified anti-Dnmt3a (or anti-Dnmt3b) was allowed to flow through a column of nickel-nitriilotriacetic acid attached with Dnmt3b (or Dnmt3a) protein three times. 

**Cell Culture, Transfection, and Western Blotting—** P19 (a mouse teratocarcinoma cell line, ATCC no. CRL-1825), human embryonic kidney 293T, and NIH 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Plasmid constructs were transfected into human embryonic kidney 293T cells or NIH 3T3 cells using the calcium phosphate co-precipitation method or Lithofectamine (Invitrogen). Cells were harvested into standard lysis buffer (50 mM Tris- HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, 10% glycerol). Western analysis was carried out according to a standard protocol.

**Cell Immunofluorescence—** Post-transfection (24-48 h) cells cultured on glass coverslips were fixed with 3.7% formaldehyde at RT for 10 min, permeabilized with 0.2% Triton X-100 at RT for 20 min, and then blocked at RT with blocking solution (1% goat serum, 3% bovine serum albumin, 0.1% Triton X-100, 0.1% sodium azide in phosphate-buffered saline) for 30 min. Anti-HA polyclonal antibody (Santa Cruz Biotechnology, sc-7392) diluted 1:200, rabbit polyclonal anti-Dnmt3a (this work) diluted 1:500, and rabbit polyclonal anti-Dnmt3b dilution 1:500 were used as primary antibodies. Alexa Fluor® 488 goat anti-rabbit IgG (Molecular Probes, A-11010) and Alexa Fluor® 585 goat anti-mouse IgG (Molecular Probes, A-11029) were used as secondary antibodies. Cells on coverslips were incubated with a primary antibody in blocking solution at RT for 3 h and then washed three times for 5 min with the same solution. Coverslips were next incubated with a secondary antibody in blocking solution at RT for 1 h and washed twice for 5 min with phosphate-buffered saline. Cells were further counterstained with Hoechst 33258 or DAPI (Sigma) at 0.5 μg/ml in phosphate-buffered saline at RT for 10 min. After a wash with water for 5 min, coverslips were mounted onto slides using Gel/ Mount (Biomeda, M01) and observed with a Nikon fluorescence microscope (E6000). The objective lenses were 40× and 100×.

**Metaphase Chromosome Immunofluorescence—** Metaphase chromosome spreads were prepared as described previously (26). Cells were treated with 0.4 μg/ml nucodazole for 14 h and harvested by trypsinization, washed twice with phosphate-buffered saline, and centrifuged (200 × g for 3 min). They were then resuspended and swollen in 75 mM KCl (about 200 μl-80-mm dish) at 37 °C for 20 min. The cells were then centrifuged (200 × g for 5 min) and resuspended with ice-cold fixative (freshly prepared methanol and glacial acetic acid at a 3:1 ratio) on ice for 5 min. Further fixation was 25 min on ice. Chromosomes were spread on ice-cold slides coated with polylysine. When completely dried, slides were blocked in blocking solution at RT for 30 min. Anti-HA polyclonal antibody diluted at 1:1000, anti-HA monoclonal antibody at 1:200, and anti-Dnmt3a or -Dnmt3b (this work) at 1:100 were used as primary antibodies for incubation in blocking solution at 4 °C overnight. Detection with secondary antibodies was the same as described above.

Chromosome immunofluorescence was also performed using a method by Keohane et al. (27) to confirm the chromosome binding properties of both endogenous and recombinant proteins. Cells resuspended and swollen in 75 mM KCl were added to a 24-well plate and centrifuged down onto polylsine-coated coverslips (652 × g for 5 min at 4 °C). Cells on the coverslip were fixed with 3% paraformaldehyde before or after immunofluorescence staining as described above.

**Cell Fractionation—** Cell fractionation was performed as described previously (28). Briefly about 1 × 10⁷ cells were extracted in 0.2 ml of cold cytoskeleton (CSK) buffer (10 mM Pipes, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1 mM EGTA, 0.5% Triton X-100, 1 mM dithiothreitol, 1.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) on ice for 3 min to remove the soluble proteins. Cytoskeletal frameworks and chromatin were sepa-
rated from soluble proteins by centrifugation at 600 × g for 3 min. Cells were further treated with 0.25 units/ml DNase I in CSK buffer with NaCl concentration adjusted to 50 mM for 30 min at room temperature. Ammonium sulfate was added from a 1 M stock solution to a final concentration of 0.25 M to extract chromatin. After incubation at room temperature for 10 min, the sample was centrifuged as before to separate chromatin from cytoskeletal frameworks. The pellet was washed once with CSK buffer and further extracted with CSK buffer containing 2 M NaCl. Cytoskeletal frameworks including nuclear matrix were recovered by centrifugation at 1300 × g for 6 min. Except for the DNase I digestion and ammonium sulfate extraction, all steps were performed at 4°C.

RESULTS

Dnmt3a and Dnmt3b Are Associated with Chromatin—To analyze the two de novo methyltransferases, we first raised and affinity-purified antibodies specific for Dnmt3a and Dnmt3b. Specificity is important because of the high level similarity between the two proteins (57% identity). To test our affinity-purified antibodies, Dnmt3a and Dnmt3b overexpressed in 293T cells were used in immunoblotting. A single band of both proteins was detected by their respective antibodies (Fig. 1A, left panel), and no cross-reaction was observed even after prolonged exposure (right panel). This indicates that cross-reactivity was truly eliminated during affinity purification. The antibodies do not recognize any other cellular proteins; no signals were observed in control extracts from cells transfected with the empty vector.

The expression of Dnmt3a and Dnmt3b proteins is developmentally regulated with a particularly high level in embryo and embryonic carcinoma cells (29). To facilitate our studies, we identified the multipotent teratocarcinoma cell line P19 that expresses both enzymes (Fig. 1B). For Dnmt3a, only a long isoform was detected that co-migrated with the recombinant full-length protein expressed from either transfected 293T or bacterial cells, suggesting that the alternative upstream promoter is used (29). For Dnmt3b, we detected three isoforms, which can be generated by alternative splicing as well as post-translational sumoylation (30, 31). The most intense band seems to represent the full-length form as it co-migrated with recombinant protein (data not shown). The larger and smaller isoforms are thus presumably generated by sumoylation and exon skipping, respectively.

Previous biochemical and genetic studies suggested a mutual interaction between DNA methylation and chromatin modification (32). We next examined by biochemical fractionation whether the DNA methyltransferases Dnmt3a and Dnmt3b are physically associated with chromatin in P19 cells. Cells were detergent-extracted to separate soluble cellular components from the pellet fraction by centrifugation (28). The pellet fraction is a cytoskeleton framework with chromatin attached on the nuclear matrix. Next the chromatin was separated from nuclear matrix by DNase I digestion followed by ammonium

### Fig. 2. Chromosome association of endogenous Dnmt3a and Dnmt3b in P19 cells.

A, the validation of antibody specificity by fluorescence immunostaining of 293T cells transfected with HA-tagged Dnmt3a or Dnmt3b (indicated on the left). Examples of mitotic cells displaying metaphase chromosomes coated by the enzymes (stained in red) are shown. B, metaphase chromosome staining of endogenous Dnmt3a and Dnmt3b in P19 cells. C, co-localization of Myc-Dnmt3a and HA-Dnmt3b on metaphase chromosomes of transfected 293T cells. Scale bars, 5 μm.
sulfate extraction. We found that both Dnmt3 enzymes fractionated with the chromatin (Fig. 1C). The fractionation was validated by the separation of three different markers: proliferating cell nuclear antigen for soluble cellular components, H3 histone for chromatin, and lamin B for nuclear matrix.

Both Enzymes Are Associated with Metaphase Chromosomes—Exogenous Dnmt3a and Dnmt3b in transfected 293T cells were observed to coat metaphase chromosome arms (33), but this could be caused by the overexpression as in the case of centromere protein-C (34). To address whether the endogenous enzymes are associated with chromosomes, we performed immunofluorescence staining in P19 cells. First the antibody quality for immunostaining was examined by using 293T cells overexpressing transfected Dnmt3a or Dnmt3b. Both antibodies stained exclusively their corresponding proteins (Fig. 2A) because signals detected for their counterpart paralogous protein were as low as the background defined both by untransfected surrounding cells and a separate staining with preimmune sera (data not shown).

In P19 cells in different phases of the cell cycle, both enzymes stained invariably in the nucleus (data not shown); co-staining with DAPI revealed association with chromosomes in mitotic cells. To investigate whether the enzymes coat chromosomes uniformly or site-specifically, we prepared metaphase chromosome spreads from transfected 293T cells. Some chromosomes seen at the bottom of the first row (for 3aF) and at the upper right corner of the second row (for 3aN1) might derive from untransfected cells and thus served as internal negative controls for the immunostaining.

Chromosome Targeting in Metaphase Cells Is Mediated by the PWWP Domain—Dnmt3a and Dnmt3b enzymes share three domains of conserved sequences: the catalytic domain at the C terminus and the PWWP and PHD domains at the N terminus for Western and immunofluorescence detection. The numbers above the bars are the amino acid coordinate of the polypeptide regions relative to the full-length protein. B, confirmation of expression by Western blotting. Extracts of transfected 293T cells were resolved on polyacrylamide gels prior to immunoblotting with an anti-HA antibody. Size markers are indicated (in kDa). An additional band of 47 kDa was detected for 3aP, possibly as a consequence of sumoylation in this region (31). The expression level of the mutant protein S333P was compared with the wild-type protein using co-transfected green fluorescent protein (GFP) as control (right panel). C, mapping of the chromosome-targeting domain by immunofluorescence staining of metaphase chromosome spreads prepared from transfected 293T cells. Some chromosomes seen at the bottom of the first row (for 3aF) and at the upper right corner of the second row (for 3aN1) might derive from untransfected cells and thus served as internal negative controls for the immunostaining.
terminus (9). To identify the domain(s) involved in chromosome targeting, we analyzed the chromosome binding activity of various deletion constructs of Dnmt3a (Fig. 3A). Expression of the protein constructs in 293T cells was confirmed by Western blotting using anti-HA antibody (Fig. 3B). All constructs showed the expected molecular weight. The two proteins 3aN1 and 3aN2, which contain the PWWP and PHD domains (3aN1) or the PWWP domain alone (3aN2), were associated with chromosomes like full-length wild-type 3aF (Fig. 3C). The C-terminal catalytic construct (3aC), which lacks both the PWWP and PHD domains, was excluded from chromosomes. This indicates that the PHD and the C-terminal catalytic domain are not required for chromosome targeting. The N-terminal region of 269 amino acids is also dispensable because the PWWP domain composed of 161 amino acids starting from amino acid 270 retained the targeting activity. The targeting activity was diminished by a single amino acid substitution (S333P) in the PWWP domain of the full-length Dnmt3a that is the equivalent of a DNMT3B point mutation identified in ICF patients (23) (Fig. 3C). Our data thus indicate that the PWWP domain is both necessary and sufficient for chromosome targeting. This also holds true for the paralogous Dnmt3b protein (data not shown).

The PWWP Domain Is Necessary but Not Sufficient for Heterochromatin Association of the Methyltransferases in Interphase—Exogenously expressed methyltransferase enzymes are concentrated in heterochromatin domains in the interphase nucleus of 3T3 embryonic fibroblasts and embryonic stem cells (17, 29). Immunostaining of endogenous proteins in embryonic stem cells with our purified antibodies also revealed a heterochromatin-associated pattern for both enzymes (data not shown), thus eliminating the possibility that incorporation into heterochromatin might be caused by the overexpression of the transfected proteins as found with HP1γ (35). We next sought to determine the region of the protein required for heterochromatin targeting by analyzing the subcellular localization of the deletion constructs of Dnmt3a in transfected 3T3 cells. The shortest form (3aN2) concentrated in heterochromatic domains (Fig. 4). The catalytic and PHD domains are not required but can contribute to strengthen the binding affinity to heterochromatin because deletion constructs 3aN1 and 3aN2 showed reduced association with heterochromatin when compared with 3aF. Three fragments further shortened at either end of 3aN2 (3aN3 (amino acids 94–482), 3aN4 (amino acids 1–219), and 3aN5 (amino acids 1–430); data not shown) and the catalytic domain (3aC) showed a diffuse nuclear staining. It is surprising that the PHD domain that interacts with HP1β (19) is not required for heterochromatin localization. In contrast, the PWWP domain is indispensable because substitution (S333P) of a single amino acid equivalent to the missense mutation in human DNMT3B (S282P) found in ICF syndrome (23) completely abolished the heterochromatin targeting ability of the full-length protein (Fig. 4, 3aF/S333P, bottom row). Notably the mutant 3aF/S333P tended to be excluded from the heterochromatin domains in the interphase nucleus, but this negative correlation of distribution patterns was not seen for the truncated 3aC and 3aP. The same dependence on the PWWP domain for heterochromatin association was also observed for Dnmt3b (see below).

Loss of Chromatin Binding Capacity of DNMT3B in ICF Syndrome—The human ICF genetic disease is caused by deficiency in the DNMT3B enzyme. Most mutations occur within the C-terminal catalytic domain and directly perturb the methyl transfer function (13, 16). The four mutations found in the N-terminal region are either nonsense or frameshift muta-

**FIG. 4.** The N terminus containing the PWWP domain is required for heterochromatin localization of Dnmt3a. Fluorescence immunostaining of 3T3 cells transfected with various constructs (indicated at the left, also see Fig. 3A) was carried out using anti-HA antibody. The heterochromatin domains defined by bright DAPI staining were verified by immunofluorescence staining using an anti-HP1α antibody (not shown). The percentage of cells showing heterochromatin localization of the Dnmt3a fragments was determined by analyzing >500 transfected cells and is indicated. Data for the constructs shorter than 3aN2 are not shown. Scale bar, 5 μm. Note that the pattern of the dark spots in the HA-3aF/S333P staining is similar to that of the heterochromatin domains stained by DAPI, suggesting the absence of the mutant protein in the heterochromatin domains.

To this end, we next explored the role of serine 282 (the equivalent of serine 277 in mouse Dnmt3b) and the effect of its replacement by proline on the PWWP structure, which has been solved recently (22). The domain (residues 223–357) is of great interest because it falls into the PWWP domain (23). We suspected that this point mutation might lead to deficiency in genomic methylation in patients due to the loss of chromatin targeting capacity of the affected DNMT3B enzyme. In agreement with this model, exchange of the corresponding amino acid residue in Dnmt3a did not reduce the catalytic activity of the enzyme (data not shown).

Ser-277 is located in the β4 strand and oriented toward the outer surface of the protein. Its side chain Oγ atom forms a hydrogen bond with the side chain Nε-1 atom of Trp-270, which serves to stabilize the side chain orientation of Trp-270 (β3). Trp-270, as the center of the hydrophobic core of the β-barrel
subdomain, is surrounded by Ile-240 (β1), Ala-248 (β2), Val-268 (β3), and Ile-279 (β4). The side chain of Trp-270 has extensive hydrophobic interactions with these residues. Mutation of Ser-277 to proline might affect the orientation or position of the side chain of Trp-270 due to the inability of hydrogen bond formation. As a consequence, the structure of the hydrophobic core may be destabilized. The destabilizing effect could be aggravated by the occurrence of the proline that is not well tolerated in the middle of a β-strand.

We then asked whether the apparent structural impact of the point mutation would lead to loss of chromatin targeting capacity of Dnmt3b. To this end, the S227P point mutation was incorporated into the mouse Dnmt3b, and the subcellular distribution of the mutant protein expressed in 3T3 cells was determined by immunofluorescence. Strikingly the mutant protein was dispersed in the nucleus and excluded from the chromosomes in the M phase cells (Fig. 5B). Mislocalization of the mutant protein in transfected 293T cells was also confirmed by immunostaining of metaphase chromosome spreads (data not shown). C, loss of heterochromatin association of mutant Dnmt3b. The percentage of cells showing heterochromatin localization of the Dnmt3b protein is indicated. The scale bars represent 5 μm.

FIG. 5. Chromatin targeting is disrupted by a point mutation of the ICF syndrome. A, confirmation of the expression of the Dnmt3b mutant protein (3bF/S277P) in comparison with the wild-type (3bF). Co-transfected green fluorescent protein (GFP) served as an internal normalization control. B, disruption of chromosome targeting ability by the ICF mutation (S277P) in Dnmt3b. Transfected 293T cells grown on coverslips were immunostained using an anti-HA antibody. Representative metaphase cells expressing the wild-type and mutant Dnmt3b proteins (in red) are shown. Mislocalization of the mutant protein in transfected 293T cells was also confirmed by immunostaining of metaphase chromosome spreads (data not shown). C, loss of heterochromatin association of mutant Dnmt3b. The percentage of cells showing heterochromatin localization of the Dnmt3b protein is indicated. The scale bars represent 5 μm.

It is unclear from the immunostaining whether the mutant protein dispersed in the nucleus is still associated with chromatin other than the pericentromeric heterochromatin regions defined by bright DAPI spots. To resolve this point, we conducted fractionation analysis of 293T cells transfected with the mutant protein construct. The majority of the mutant protein was located in the nuclear matrix (Fig. 6). In contrast, one-third of the wild-type protein was found in the chromatin fraction. The appearance of wild-type Dnmt3b also in soluble and matrix fractions might be caused by overexpression of the transfected protein because endogenous enzymes in P19 cells are distributed predominantly in chromatin (Fig. 1C). The reason for the presence of the mutant protein in matrix but its absence from the soluble fraction has not yet been understood, but one possibility could be due to the increased degradation of the mutant protein not associated with chromatin or matrix. Taken together, chromatin association of the methyltransferases is disrupted by the ICF mutation in the PWWP domain.
DISCUSSION

The Role of PWWP Domain in Chromatin Targeting—Our primary finding is that the mammalian de novo DNA methyltransferases are associated with chromatin in vivo through the region containing the PWWP domain. While this domain with 161 amino acids is both necessary and sufficient to bind metaphase chromosomes, extended sequences on both sides are also required for heterochromatin association. We also found that chromatin association of the methyltransferases is disrupted by a point mutation causing ICF syndrome. This highlights the functional importance of PWWP-mediated chromatin targeting in the function of DNA methyltransferases. Notably the PWWP domain is present in all isoforms of Dnmt3 enzymes generated by alternative splicing and use of different promoters (29, 30).

The structure of the PWWP domain of Dnmt3b reveals a prominent positive electrostatic surface, which is suggested to mediate the interaction with negatively charged molecules such as DNA. Not surprisingly, the PWWP domain alone can bind DNA in vitro (22). DNA binding activity was detected only for PWWP by gel shift assay (data not shown), although other domains have also been suggested to interact with DNA (22). The intrinsic DNA binding ability may partially contribute to chromatin targeting in vivo. As DNA in general is compacted in chromatin in vivo, it is conceivable that chromatin targeting involves contacts with both nucleosomal DNA and proteins.

PWWP domains are present in over 60 eukaryotic proteins implicated in various chromatin-associated processes such as DNA repair (MSH6, a mammalian ortholog of mismatch repair protein MutSβ (36); Arabidopsis ATM ortholog (37)); histone modification (Arabidopsis trithorax ATX1 and ATX2 (38); mammalian histone methyltransferases NSD1 and NSD2/WHSC1 (39)), and transcriptional activation (lens epithelial derived growth factor (40)). Interestingly loss or disruption of PWWP domains occurs frequently in myeloid leukemia due to chromosome translocations involving the two histone methyltransferases NSD1 (41) and NSD2/WHSC1 (21) and the transcriptional coactivator lens epithelial derived growth factor (42, 43). Moreover deletions in the NSD2/WHSC1 genomic region are associated with the Wolf-Hirschhorn syndrome with multiple malformations including prominent forehead with widely spaced eyes (21), whereas mutations in NSD1 cause the Sotos syndrome, a neurological disorder characterized by overgrowth from the prenatal stage through childhood (44). The functional significance of the PWWP domain encoded by these two histone methyltransferase genes still needs to be resolved as no point mutations affecting the region have been found to date.

The PWWP-containing proteins often possess other chromatin-interacting domains such as chromo, bromo, PHD, high mobility group, and SET domains (45). The linked occurrence reinforces the implication of PWWP in interaction with chromatin and that cooperation between PWWP and other domains might be required for modification of chromatin or for the read-out of chromatin signals. We also confirmed that the PWWP domains from the mismatch repair protein MSH6 and histone methyltransferase NSD2/WHSC1 are able to mediate chromosome association in metaphase cells (data not shown). This indicates that the role of the PWWP as a chromatin target module may hold true for other proteins.

Functional Importance of Chromatin Targeting by the PWWP Domain for DNA Methylation—Defects in the DNMT3B methyltransferase gene are associated with a human recessive autosomal disorder characterized by immunodeficiency, centromeric heterochromatin instability, and facial anomalies (10, 13, 46). The cytogenetic manifestation in patient lymphocytes is linked with loss of cytosine methylation in the pericentromeric satellite repeats (14). Most identified mutations are clustered in the C-terminal catalytic domain, and they can affect methylation function by directly impairing the enzymatic process. Indeed point mutations in this region greatly reduce catalytic activity of the mouse Dnmt3b, whereas deletion of the N-terminal region containing the PWWP domain has no apparent effect in vitro (13, 16, 22).

A recently identified missense mutation (S282P) in two patients falls within the PWWP domain (23). The homozygous patients exhibited all typical features including loss of methylation in the classical satellite 2 DNA but had no apparent mental retardation. The mutated position is conserved among four mammalian and two zebrafish DNMT3B homologs. Structural inspection of the murine PWWP domain reveals an overwhelming detrimental effect of the substitution of proline for serine; as a consequence, the mouse mutant counterpart (S277P) is unable to bind to the chromatin in vivo as revealed by our immunostaining and cell fractionation assays. This finding, taken together with the methylation deficiency caused by the Ser-282 mutation in humans, suggests that chromatin targeting of the de novo methyltransferases is required for their methylation function. The PWWP domain may allow the enzymes to access the DNA substrate, which is presented in chromatin.

This view is supported by our result from an in vitro methylation activity assay of truncated Dnmt3a proteins on nucleosomal DNA.2 Lacking the N-terminal region, the catalytic domain of Dnmt3a is able to catalyze methyl group transfer to naked DNA substrate at a rate similar to that of the full-length protein. However, when the DNA substrate is complexed in nucleosomes, the enzymatic activity of the catalytic domain is much more inhibited than that of the full-length Dnmt3a. We suggest that the N-terminal region containing the PWWP domain facilitates methylation on nucleosomal DNA, for example, by increasing binding affinity of the enzyme to chromatin. Loss of genomic methylation in ICF patients with S282P missense mutation of DNMT3B might thus be caused by the inability of the mutated enzyme to interact properly with chromatin rather than to mediate the correct pattern of methylation.
than a failure in the methyltransfer process.

**Chromatin Mark(s) Recognized by Dnmt3a and Dnmt3b via the PWWP Domain May Exist**—Formation of specific gene expression profiles in development is the result of genome patterning by epigenetic means (i.e. DNA methylation and histone modifications). It has been proposed that targeting of cytosine methyltransferases by alterations in the chromatin structure may generate the genomic methylation patterns in mammals (47). The targeting mechanism is not well understood. Dnmt3a and Dnmt3b themselves display little sequence preference because of DNA binding was observed for the PWWP domain (22).

DNA methylation occurs depending on the methylation status of histone H3 lysine 9 in *Neurospora* and *Arabidopsis* (48–50). Most recently, histone H3 lysine 9 trimethylation was shown as a signal to direct the localization of Dnmt3b at the pericentromeric heterochromatin foci in mammalian interphase cells and DNA methylation of pericentromeric repeats (51). The conserved PHD domain of Dnmt3b may mediate the pericentromeric localization by interacting with the heterochromatin protein HP1 (19), which in turn recognizes histone H3 lysine 9 trimethylation. Consistent with this notion, the PWWP domain failed to recognize histone H3 lysine 9 trimethylation in a peptide pull-down assay (data now shown). Our finding of the binding of both Dnmt3a and Dnmt3b to specific sites on metaphase chromosome arms via the PWWP domain lends support for the presence of additional chromatin mark(s) recognized by the enzymes. Identification of unknown chromatin mark(s) recognized by Dnmt3a and Dnmt3b would help to elucidate both the role and mechanism of these enzymes in patterning chromatin along the genomic DNA during development.

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