Structural Insight into Coordinated Recognition of Trimethylated Histone H3 Lysine 9 (H3K9me3) by the Plant Homeodomain (PHD) and Tandem Tudor Domain (TTD) of UHRF1 (Ubiquitin-like, Containing PHD and RING Finger Domains, 1) Protein

UHRF1 is an important epigenetic regulator connecting DNA methylation and histone methylations. UHRF1 is required for maintenance of DNA methylation through recruiting DNMT1 to DNA replication forks. Recent studies have shown that the plant homeodomain (PHD) of UHRF1 recognizes the N terminus of unmodified histone H3, and the interaction is inhibited by methylation of H3R2, whereas the tandem tudor domain (TTD) of UHRF1 recognizes trimethylated histone H3 lysine 9 (H3K9me3). However, how the two domains of UHRF1 coordinate to recognize histone methylations remains elusive. In this report, we identified that PHD largely enhances the interaction between TTD and H3K9me3. We present the crystal structure of UHRF1 containing both TTD and PHD (TTD-PHD) in complex with H3K9m3 peptide at 3.0 Å resolution. The structure shows that TTD-PHD binds to the H3K9me3 peptide with 1:1 stoichiometry with the two domains connected by the H3K9me3 peptide and a linker region. The TTD interacts with residues Arg-8 and trimethylated Lys-9, and the PHD interacts with residues Ala-1, Arg-2, and Lys-4 of the H3K9me3 peptide. The biochemical experiments indicate that PHD-mediated recognition of unmodified H3 is independent of the TTD, whereas TTD-mediated recognition of H3K9me3 PHD. Thus, both TTD and PHD are essential for specific recognition of H3K9me3 by UHRF1. Interestingly, the H3K9me3 peptide induces conformational changes of TTD-PHD, which do not affect the autoubiquitination activity or hemimethylated DNA binding affinity of UHRF1 in vitro. Taken together, our studies provide structural insight into the coordinated recognition of H3K9me3 by the TTD and PHD of UHRF1.

DNA (5-cytosine) methylation and histone modifications are important epigenetic regulations, which control various chromatin-based processes, including chromatin structure, transcription, and DNA repair (1, 2). Histone methylations are established by histone methyltransferases and removed by histone demethylases. Proteins that recognize these histone methylations are involved in regulation of transcriptional activation and repression, heterochromatin formation, and DNA repair (1). In mammals, the DNA methylation is established by de novo DNA methyltransferases Dnmt3a and Dnmt3b, and the maintenance of DNA methylation is mediated by DNA methyltransferase 1 (DNMT1) in DNA replication (3, 4). Although these modifications have been studied extensively, the connection between DNA methylation and histone modifications is poorly understood. Recent studies have indicated that UHRF1 (ubiquitin-like, containing PHD and RING finger domains 1) (also known as NP95 in mice and ICBP90 in humans) is an important epigenetic regulator connecting DNA methylation and histone methylations. UHRF1 recognizes histone methylations and is required for DNMT1-mediated DNA methylation through recruiting DNMT1 to hemimethylated replication forks (5–12). Deletion of UHRF1 leads to genomic hypomethy-
lation and cell cycle arrest (9, 11). Besides DNMT1, UHRF1 also interacts with PCNA, USP7 (also known as HAUSP), histone deacetylase 1 (HDAC1), H3K9 methyltransferases (Suv39H1, G9a), and histone acetyltransferase (Tip60) (9, 11, 13–20). It has been proposed that UHRF1 works together with these proteins to form a multicomponent complex, termed the epigenetic code replication machinery, to regulate DNA replication (21). UHRF1 was also characterized as an E3 ligase with autoubiquitination activity, and its substrates include histone H3 and DNMT1 (14, 22–25).

UHRF1 is mainly expressed in proliferating cells and is essential for S phase entry (26). As a cell cycle-regulated protein, UHRF1 is expressed in S phase and is absent in G0 and G1 phases (26–29). UHRF1 is preferentially localized to pericentric heterochromatin (PCH) and is involved in heterochromatin formation (22, 27, 28). UHRF1 is also localized to euchromatin and regulates gene expression, particularly the chromatin formation (22, 27, 28). UHRF1 was also characterized as an E3 ligase with autoubiquitination activity, and its substrates include histone H3 and DNMT1 (14, 22–25).

UHRF1 is expressed in S phase and is absent in G0 and G1 phases (26–29). UHRF1 is preferentially localized to pericentric heterochromatin (PCH) and is involved in heterochromatin formation (22, 27, 28). UHRF1 is also localized to euchromatin and regulates gene expression, particularly the silencing of tumor suppressor genes, including p16INK4A, p14ARF, p53, and pRb, possibly through affecting DNA methylation and histone modifications (16, 30–33). Up-regulations of UHRF1 were observed in various cancer cells, including breast, prostate, and lung cancer (24, 34, 35). In cancer cells, UHRF1 plays a key role in promoting proliferation, and its abundance remains at high levels in all phases of the cell cycle (24, 31, 36, 37). Thus, UHRF1 is a potential drug target for cancer therapy and a biomarker for diagnosis (38).

UHRF1 is composed of at least five recognizable structural modules: an N-terminal ubiquitin-like domain, followed by a tandem tudor domain (TTD), a plant homeodomain (PHD), a tandem tudor domain (TTD), a plant homeodomain (PHD), a SET and RING-associated (SRA) domain, and a C-terminal RING (really interesting new gene) domain (39). The SRA domain preferentially binds to hemimethylated CpG, and the PHD binds to trimethylated histone H3 lysine 9 (H3K9me3) (6, 9, 11). The PHD is involved in large scale reorganization of pericentric heterochromatin (40). Recently, we and other groups demonstrated that the PHD of UHRF1 specifically recognizes unmodified histone H3, and the interaction is inhibited by methylation on H3R2, which links UHRF1 to the regulation of euchromatic gene expression (5, 7). Both DNA methylation and H3K9me3 are hallmarks of PCH, where UHRF1 is preferentially localized (41–43). Thus, as a multidomain-containing protein, UHRF1 plays an important role in connecting DNA methylation and histone modifications.

Structures for all five domains of UHRF1 or its homolog UHRF2 are currently available in the Protein Data Bank. However, how these domains work together to connect the DNA methylation and histone methylations remains unclear. For example, the TTD and PHD of UHRF1 interact with histone H3 or H3 methylation, and these interactions are important for PCH localization of UHRF1 or large scale reorganization of PCH (6, 22, 40, 44). Our previous studies demonstrated that the PHD of UHRF1 recognizes unmodified H3 on residues Ala-1, Arg-2, and Lys-4, and the specific recognition is inhibited by the methylations of H3R2 but not H3K9 (5, 7). Recently, Nady et al. showed that the TTD of UHRF1 recognizes residues Lys-4, Thr-6, and trimethylated Lys-9 of the H3K9me3 peptide (6). Thus, how the TTD and PHD of UHRF1 coordinately recognize histone H3 methylation remains elusive.

In this report, through biochemical and structural studies, we found that TTD-PHD binds to the H3K9me3 peptide with 1:1 stoichiometry. The TTD interacts with residues Arg-8 and trimethylated Lys-9, whereas the PHD interacts with residues Ala-1, Arg-2, and Lys-4 of the H3K9me3 peptide. Both the TTD and PHD are essential for specific recognition of H3K9me3 by UHRF1. Interestingly, the H3K9me3 peptide induces a conformational change of TTD-PHD, which does not affect the autoubiquitination activity or hemimethylated DNA binding affinity of UHRF1 in vitro. Thus, our studies reveal the mechanism for coordinate recognition of H3K9me3 by the TTD and PHD of UHRF1.

**EXPERIMENTAL PROCEDURES**

**Protein Purification**—The human UHRF1 proteins TTD-PHD (residues 134–366), TTD linker (residues 126–300), and TTD (residues 126–285) were expressed in *Escherichia coli* strain BL21(DE3). The proteins were purified using affinity purification, followed by anion exchange and gel filtration chromatography. The proteins were concentrated to 20 mg/ml for crystallization, isothermal titration calorimetry (ITC), and fluorescence resonance energy transfer (FRET) assays.

**Crystallization and Data Collection**—Crystals of TTD-PHD in complex with the H3K9me3 peptide were grown at 4 °C using the hanging drop vapor diffusion method by mixing an equal volume of protein complex and crystallization buffer containing 20% PEG 3350, 200 mM ammonium tartrate, 100 mM BisTris (pH 6.0–7.0). The peptide used for crystallization is H3K9me3 (residues 1–17). Protein and H3K9me3 peptide were mixed at a molar ratio of 1:2 before crystallization. Crystals were slowly equilibrated with a cryoprotectant buffer containing reservoir buffer plus 5% glycerol (v/v) and were flash frozen in a cold nitrogen stream at −173 °C. All data sets were collected on beamline BL17U at the Shanghai Synchrotron Radiation Facility. The data were processed using the program HKL2000 (45).

**Structure Determination**—The structure of TTD-PHD-H3K9me3 was determined by molecular replacement using structures of the TTD (Protein Data Bank entry 3DB3)(6) and PHD (Protein Data Bank entry 3SHB)(7) as searching models and manual building with COOT (46). The crystal contains four molecules (TTD-PHD-H3K9me3 complex) in one asymmetric unit. Rotation and translation function searches were performed with the program PHASER (47). All refinements were performed using the refinement module phenix.refine of the PHENIX package (48). The model quality was checked with the PROCHECK program, which showed good stereochemistry according to the Ramachandran plot for the structure (49). All structure figures were generated by PyMOL (50).

**ITC**—To obtain the binding affinity between UHRF1 and different H3 peptides, 0.05 mM wild-type TTD-PHD, its mutants, and TTD linker were titrated with 0.68 mM H3K9me3 peptide (residues 1–17) and 0.4 mM H3 peptide (residues 1–17) using an iTC200 microcalorimeter (GE Healthcare) at 18 °C. Both proteins and peptides were prepared in a buffer containing 10 mM HEPES, pH 8.0, and 100 mM NaCl. The data were fitted by Origin version 7.0 software.
In Vitro FRET Measurements—CFP-TTD-PHD-YFP proteins were generated with CFP fusion at the N terminus of the TTD and YFP at the C terminus of the PHD. The wild type and mutants of CFP-TTD-PHD-YFP proteins were expressed and purified as described for TTD-PHD proteins. The protein concentration used for FRET measurements is 0.25 μM, and the H3K9me3 peptide concentration is from 2.5 nM to 100 μM, as indicated. Assays were performed in “non-binding surface flat bottom low flange” black 384-well plates (Corning Inc.) in buffer containing 10 mM HEPES, pH 8.0, 150 mM NaCl in 50 μl. Emission intensities were scanned using a Synergy 4 microplate reader (BioTek) from 450 to 600 nm, with 435 nm as the excitation wavelength. The FRET ratio was defined as the ratio of 528-nm to 485-nm emissions. All assays were performed in triplicate, and the data were processed and normalized using Origin version 7.0 (OriginLab).

In Vitro Ubiquitination Assay—WT UHRF1 full-length protein was used for autoubiquitination assays. Briefly, 250 ng of UBE1, 250 ng of Ubch5c, 5 μg of HA-ubiquitin, 0.5 μg of UHRF1, and increasing amounts of the H3K9me3 peptide were mixed in a reaction buffer consisting of 50 mM Tris, pH 7.5, 1 mM DTT, 5 mM MgCl2, 5 mM ATP, and 0.05% Triton X-100. The reactions were incubated at 37 °C for 60 min and stopped by SDS-PAGE loading buffer. Samples were subjected to Western blotting and detected by anti-UHRF1 (from the laboratory of Prof. Yang Shi) and anti-HA antibodies.

DNA Binding Assay—A 6-carboxyfluorescein-labeled double strand DNA (6-carboxyfluorescein-5′-CCATGGCTGAC-3′/5′-GTCAGXGATGG-3′, where X represents 5mC) (10 nM) was mixed with increasing amounts of UHRF1 or premixed UHRF1-H3K9me3 peptide complex. The mixtures were incubated in binding buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl) for 10 min at 25 °C. Fluorescence polarization measurements were performed at 25 °C on a Synergy 4 microplate reader (BioTek). The data were fitted using GraphPad Prism version 5.

RESULTS

Specificity of Histone Recognition by TTD-PHD of UHRF1—To investigate the specificity of histone recognition by UHRF1, we first performed an ITC experiment to measure the interactions between UHRF1 and histone peptide with various methylations. As shown in Fig. 1A, TTD-PHD of UHRF1 robustly (Kd = 0.15 μM) bound to the H3K9me3 peptide in a 1:1 stoichiometry but had undetectable binding affinity to trimethylated histone H3 lysine 27 or 36 peptides. Compared with the H3K9me3 peptide, unmodified H3 or trimethylated histone H3 lysine 4 peptide also bound to TTD-PHD with binding affinity decreased by ~11- or 33-fold, respectively. The results are consistent with our previous findings that the PHD alone specifically recognizes unmodified histone H3 and trimethylation on H3 Lys-4 slightly decreases the binding affinity, whereas the TTD of UHRF1 does not bind to unmodified H3 (7). The ITC results also indicate that the TTD binds to the H3K9me3 peptide with a binding affinity (Kd = 0.97 μM) 6-fold weaker than that for TTD-PHD, suggesting that the interaction between the TTD and the H3K9me3 peptide is significantly enhanced by the PHD (Fig. 1B). Compared with H3K9me3 peptide, the TTD had very weak binding affinity to H3K9me3 peptide and undetectable binding affinity to H3K9me3 or trimethylated H3 Lys-27 peptide, suggesting that the TTD provides the specificity for H3K9me3 recognition. Parameters for ITC measurements are summarized in supplemental Table S1, and sequences of peptides used are listed in supplemental Table S2. Taken together, TTD-PHD binds to the H3K9me3 peptide with highest binding affinity among the histone H3 trimethylations tested. The existence of the PHD increases the binding affinity between TTD and H3K9me3 peptide ~6-fold. Because UHRF1 is mainly localized to PCH, where H3K9me3 and DNA methylation are enriched, the results suggest that the TTD and PHD work together to recognize H3K9me3 and that both domains are required for the specific recognition.

FIGURE 1. Recognition of H3K9me3 by TTD-PHD of UHRF1. A and B, superimposed ITC enthalpy plots for the binding of TTD-PHD (A) or TTD alone (B) to various histone H3 peptides (syringe) with the estimated binding affinity (Kd) listed. Parameters for all ITC measurements are summarized in supplemental Table S1. H3K9me3, H3K4me3, H3K27me3, and H3K36me3, trimethylated histone H3 Lys-9, -4, -27, and -36, respectively. N/D, not determined.
VOLUME 288 • NUMBER 2 • JANUARY 11, 2013

1332 JOURNAL OF BIOLOGICAL CHEMISTRY

Structure of UHRF1 TTD-PHD

Overall Structure of TTD-PHD-H3K9me3 Complex—To investigate how the TTD and PHD recognize H3K9me3 coordinate, we determined the crystal structure of TTD-PHD of human UHRF1 in complex with the H3K9me3 peptide (residues 1–17) (Fig. 2A and supplemental Fig. S1). The crystal structure was solved by molecular replacement with structures of the TTD and PHD as searching models, and the final model was refined to 3.0 Å resolution (6, 7). The statistics for the structure determination are summarized in supplemental Table S3. Four TTD-PHD-H3K9me3 molecules were found in one asymmetric unit of the crystals (supplemental Fig. S2), which may result from crystal packing because TTD-PHD-H3K9me3 forms a monomer in solution (supplemental Fig. S3). The TTD, PHD, and H3K9me3 peptide (residues 1–10) were clearly traced in two of the four molecules, and only the TTD was found and built in the other two molecules due to a lack of electron density for the PHD and peptide. The residues of H3 peptide and critical residues for H3R2 or H3K9me3 recognition are well covered by the electron density (2Fo –Fc map), indicating that the model was correctly built (supplemental Fig. S4). The two TTD-PHD-H3K9me3 complexes are well aligned with a root mean square deviation of 0.387 Å for 203 aligned Ca atoms, and thus one complex is used in the following structural analyses.

In the TTD-PHD-H3K9me3 Complex Structure, TTD-PHD Interacts with the H3K9me3 Peptide with 1:1 Stoichiometry. As shown in Fig. 2B, the TTD and the PHD have a direct interaction, and the two domains are connected by a linker region (residues 285–301) and the H3K9me3 peptide. The PHD and TTD interact with the N terminus (residues 1–4) and C terminus (residues 8–10) of the H3K9me3 peptide, respectively. Residues 5–7 of the H3K9me3 peptide adopt a one-turn α-helical conformation and have no direct interaction with the TTD or PHD (Figs. 2B and 3A).

Structure of the PHD and Recognition of H3R2—The PHD of UHRF1 is coordinated by three zinc atoms and forms a globular structure, consisting of a pre-PHD subdomain and a canonical PHD subdomain (supplemental Fig. S5). The pre-PHD subdomain (residues 302–317) is coordinated by the first zinc atom and does not interact with other parts of TTD-PHD. The canonical PHD subdomain (residues 318–366) is coordinated by two zinc atoms and is composed of a double-stranded antiparallel β-sheet connected by loop regions in an interleaved manner, which is the characteristic structural feature of the PHD in other proteins, such as BPTF, ING2, TAF3, and BHC80 (Fig. 3C and supplemental Fig. S5).

The N terminus (residues Ala-1 to Lys-4) of the H3K9me3 peptide adopts a coil conformation and packs against the surface of the canonical PHD subdomain, and residues 5–10 of the H3K9me3 peptide do not interact with the PHD (Fig. 3, B and C). Specifically, two inter-main chain hydrogen bonds are formed between residues Arg-2 and Lys-4 of the H3K9me3 peptide and residues Met-332 and Leu-331 of the PHD. The methyl group of residue H3 Ala-1 inserts into a hydrophobic pocket formed by the side chains of residues Leu-331, Pro-353, and Trp-358 of the PHD. The N-terminal amino group of residue H3 Ala-1 forms hydrogen bonds with carbonyl oxygen atoms of residues Glu-355 and Asp-356 of the PHD. The side chain of H3R2 forms hydrogen bonds with the side chains of residues Asp-334 and Asp-337 of the PHD. The side chain of H3 Lys-4 forms hydrogen bonds with the carbonyl group of residue Cys-316 of the pre-PHD subdomain (Fig. 3C).

Structural comparison of the PHD of TTD-PHD and the isolated PHD of UHRF1 (Protein Data Bank entry 3SHB) shows that the PHDs in both structures share a similar fold with a root mean square deviation of 0.526 Å for 62 aligned Ca atoms, and thus will not be discussed here (7). Collectively, the PHDs spe-
specifically recognize the N terminus of unmodified histone H3 on residues Ala-1, Arg-2, and Lys-4, and the recognition is independent of the TTD.

**Structure of the TTD and Recognition of Trimethylated H3K9**—The TTD of UHRF1 consists of two tudor domains (referred to as TTD$_{\alpha}$ and TTD$_{\beta}$), which tightly pack against each other through extensive hydrophobic interactions and hydrogen bonds (data not shown). Each Tudor domain adopts a “royal” fold containing a characteristic five-stranded $\beta$-barrel (Fig. 2B). Trimethylated H3K9 inserts into a hydrophobic pocket formed by three highly conserved residues, Phe-152, Tyr-188, and Tyr-191, of TTD$_{\alpha}$ (Fig. 3, D and E). The side chain of histone H3 Arg-8 forms a hydrogen bond with the carbonyl oxygen atom of residue Asp-190 of TTD$_{\alpha}$. No interaction was observed between TTD$_{\beta}$ and the H3K9me3 peptide. Interestingly, the linker region (residues 285–301) adopts an extended conformation and packs against a groove formed between TTD$_{\alpha}$ and TTD$_{\beta}$ (Fig. 4A). In particular, the side chains of residues Arg-296 and Ser-298 of the linker region form hydrogen bonds with side chains of residues Asp-142, Glu-153, and Trp-238 of the TTD. Moreover, the carbonyl oxygen atoms of residues Ser-287, Met-289, Met-294, and Lys-297 of the linker region form hydrogen bonds with residues Arg-204, Tyr-140, Arg-207, and Arg-235 of the TTD. Thus, residues 287–298 of the linker region stably associate with the TTD, and residues Gly-299, Pro-300, and Ser-301 have no direct interaction with the TTD or PHD (Fig. 4A). The flexibility of these three residues (Gly-299/Pro-300/Ser-301) may contribute to the dynamic property of TTD-PHD, which will be discussed in detail below.

The NMR structure of the TTD of UHRF1 in complex with the H3K9me3 peptide (Protein Data Bank entry 2L3R) was used for the following structural comparison (6). The TTD in TTD-PHD-H3K9me3 and TTD-H3K9me3 structures share a similar tandem Tudor fold with a root mean square deviation of 1.056 Å for 125 aligned Ca atoms (Fig. 4B). In addition, residue K9me3 of histone H3 and critical residues Phe-152, Tyr-188, and Tyr-191 of the TTD for K9me3 recognition also adopt similar conformation in the two structures. However, significant difference does exist between the two structures compared. In the TTD-PHD-H3K9me3 structure, only two residues, Arg-8 and K9me3, interact with the TTD (Figs. 2E and 4D). However, in the TTD-H3K9me3 structure, residues 1–9 of the H3K9me3 peptide pack along with a shallow groove on the surface of the TTD, with residues K9me3, Thr-6, and Lys-4 involved in the interactions (Fig. 4C). The side chain of histone H3 Lys-4 interacts with residues Asp-142 and Glu-153, and residue H3 Thr-6 is stabilized by residues Asp-190 and Arg-235 of the TTD. Mutations of D142A/E153A and D190A of the TTD decreased the H3K9me3 peptide binding affinity ~50- and 2.5-fold, respectively (6). Interestingly, these interactions were not observed in the TTD-PHD-H3K9me3 structure, in which residue histone H3 Lys-4 forms a hydrogen bond with residue Cys-316 of the PHD, and H3 Thr-6 does not interact with UHRF1. The superimposed structures show that the surface on the TTD for the H3 peptide (residues Lys-4, Gln-5, Thr-6, and Ala-7) association in the TTD-H3K9me3 structure is occupied by the linker region (residues Arg-296, Lys-297, Ser-298, and Gly-299) in the TTD-PHD-H3K9me3 structure (Fig. 4D). Thus, in the context of the linker region, the TTD does not interact with the N terminus of the H3K9me3 peptide (residues 1–7), and the structure determined in this work represents a natural conformation of UHRF1 in complex with the H3K9me3 peptide.

**Coordinate Recognition of H3K9me3 by TTD-PHD**—We next performed ITC experiments to investigate amino acids of TTD-PHD that are involved in the specific recognition of H3K9me3. As shown in Fig. 5A, changing the H3R2-interacting residue Asp-334 to Ala in TTD-PHD significantly reduced the binding affinity to unmodified H3 (undetectable), indicating a critical role of residue Asp-344 of the PHD for H3 recognition. The result is consistent with a previous study indicating that mutation of D334A of the PHD significantly decreased the interaction between the PHD and H3, whereas the TTD has undetectable binding affinity to unmodified H3 (7). Mutation of D334A also significantly decreased the interaction between TTD-PHD and the H3K9me3 peptide (~30-fold reduction), indicating that the interaction between the PHD and the N terminus of H3 is important for H3K9me3 recognition by TTD-PHD (Fig. 5B). The interaction between the H3K9me3 peptide and mutation of D334A on TTD-PHD is even weaker than that for the TTD alone. The difference can be explained by the fact that the TTD has an artificial surface, which stabilizes the N terminus of H3,
whereas the surface is blocked by the linker region in TTD-PHD (Fig. 4B). Consistent with the above observations, the ITC experiment shows undetectable interaction between the H3K9me3 peptide and TTD linker (residues 126–300), indicating that the existence of the linker region indeed blocks the artificial interaction between the TTD and N terminus of H3 (supplemental Fig. S6). These results further provide supporting evidence that the TTD is not sufficient and that the PHD is required for H3K9me3 recognition by UHRF1. Previous studies showed that the mutation Y188A in the TTD significantly decreased the binding affinity to the H3K9me3 peptide (≥1000-fold reduction), which was detected by fluorescence polarization (6). Interestingly, changing trimethylated H3K9-contacting residue Tyr-188 to Ala had little effect on unmodified H3 interaction and slightly decreased the binding affinity to the H3K9me3 peptide (~2-fold reduction), whereas the mutation Y188A/D334A significantly decreased the interaction with the H3K9me3 peptide (~200-fold reduction) (Fig. 5B). The results suggest that the mutation F152A or Y188A may not completely abolish the interaction between the TTD and the H3K9me3 peptide and that the existence of the PHD facilitates the H3K9me3 recognition by TTD-PHD mutation F152A or Y188A. Taken together, these results demonstrate that the TTD of UHRF1 interacts with residues H3 Arg-8 and trimethylated H3K9 of the H3K9me3 peptide, and the specific recognition of H3K9me3 mediated by the TTD is dependent on the
PHD. In other words, both TTD and PHD are required for specific recognition of H3K9me3 by UHRF1.

**Conformational Changes of TTD-PHD upon H3K9me3 Peptide Interaction**—The crystal structure of TTD-PHD-H3K9me3 shows that the TTD and PHD do not directly interact with each other, and the two domains are connected by the H3K9me3 peptide and three residues (Gly-299/Pro-300/Ser-301) of the linker region (Fig. 4A). The three residues do not interact with other residues and thus are not restricted to a fixed conformation in the overall structure. In addition, the residues glycine and serine are both in favor of flexible conformation, and the residue proline is also found to mediate conformational change through cis/trans state transition. Thus, it is tempting to speculate that TTD and PHD are relatively flexible, and the H3K9me3 peptide may lead to a conformational change of TTD-PHD. To test the hypothesis, FRET measurements were performed using a CFP-TTD-PHD-YFP protein with CFP fusion on the N terminus of the TTD and YFP fusion on the C terminus of the PHD. The wild type CFP-TTD-PHD-YFP protein generated a clear FRET signal (decreased donor signal and increased acceptor signal), suggesting that the TTD and PHD are close enough for a FRET signal generation (Fig. 6A and supplemental Fig. S7). In contrast, the FRET signal significantly decreased with the addition of the H3K9me3 peptide, indicating an increased distance between the N terminus of the TTD and C terminus of the PHD. In the TTD-PHD-H3K9me3 structure, the N terminus of the TTD and C terminus of the PHD are in the opposite orientation with a distance of 55 Å (Fig. 2B). The results indicate that the TTD and PHD are close to each other in free state, and the H3K9me3 peptide induces the formation of the overall fold of TTD-PHD, as indicated in Fig. 2B.

We next tested whether H3K9me3 peptide-induced conformational change of TTD-PHD is dependent on its association with both TTD and PHD. Similar FRET experiments were performed using wild type or various mutations of CFP-TTD-PHD-YFP in complex with H3 or H3K9me3 peptides. As shown in Fig. 6A, although H3 interacts with TTD-PHD, the peptide did not decrease FRET signal for wild type CFP-TTD-PHD-YFP protein, suggesting that association of the TTD and trimethylated H3K9 is important for the conformational change. Moreover, the mutation Y188A significantly decreased and mutation D344A or Y188/D334A abolished the conformational change induced by the H3K9me3 peptide. These results are also consistent with the ITC findings that the mutations Y188A, D344A, and Y188A/D334A decreased binding affinity to H3K9me3 to different extents. Note that although the mutation Y188A of TTD-PHD still strongly bound to H3K9me3, the conformational change in this mutant significantly decreased, further suggesting that K9me3 recognition by TTD and Arg-2 recognition by PHD are both essential for H3K9me3 recognition by UHRF1 as well as the H3K9me3-induced conformational change of TTD-PHD. Taken together, association with both TTD and PHD is critical for the conformational change of TTD-PHD induced by the H3K9me3 peptide.

**H3K9me3-induced Conformational Change of TTD-PHD Does Not Affect Autoubiquitination Activity or Hemimethylated DNA Binding Affinity of Full-length UHRF1**—We next tested whether the H3K9me3-induced conformational change of TTD-PHD affects the function of UHRF1. UHRF1 has been reported to be an E3 ligase with autoubiquitination activity, and its substrates include histone H3 and DNMT1 (14, 22–25). Thus, we first performed an in vitro ubiquitination assay to test whether the H3K9me3 peptide affects the autoubiquitination of UHRF1. We next tested whether the H3K9me3-induced conformational change of TTD-PHD affects the function of UHRF1. UHRF1 has been reported to be an E3 ligase with autoubiquitination activity, and its substrates include histone H3 and DNMT1 (14, 22–25). Thus, we first performed an in vitro ubiquitination assay to test whether the H3K9me3 peptide affects the autoubiquitination of UHRF1. The results show that autoubiquitination activity of wild type UHRF1 protein was not enhanced or inhibited by the H3K9me3 peptide (Fig. 6B). UHRF1 recruits DNMT1 to the replication fork through recognition of hemimethylated DNA by the SRA domain (9, 11). We next performed fluorescence polarization measurement to test whether the H3K9me3 peptide affects the binding affinity between UHRF1 and hemimethylated DNA. The results indicate that with or without the H3K9me3 peptide association, wild type UHRF1 interacted with hemimethylated DNA with comparable binding affinity.
Collectively, although the H3K9me3 peptide induces conformational change of TTD-PHD, it will not alter the autoubiquitination activity or hemimethylated DNA binding affinity of full-length UHRF1 in vitro. The potential function of the conformational change occurring in TTD-PHD of UHRF1 requires further investigation.

DISCUSSION

When this manuscript was in preparation, Arita et al. (51) reported the crystal structure of TTD-PHD in complex with H3K9me3. The structures from both studies are similar, with a root mean square deviation of 0.373 Å for 632 aligned Ca atoms for all four molecules in one asymmetric unit (supplemental Fig. S2). The structural similarity is not surprising because similar TTD-PHD-H3K9me3 protein complexes were used in crystallization. Although the two studies present similar crystal structures of TTD-PHD-H3K9me3, here we reported novel findings as discussed below: 1) coordinated recognition of H3K9me3 by TTD and PHD of UHRF1 and 2) conformational changes of TTD-PHD induced by the H3K9me3 peptide and the functional studies of the conformational changes.

Specific Recognition of H3K9me by UHRF1—UHRF1 is a multidomain protein consisting of at least five domains. The SRA domain recognizes hemimethylated DNA and is critical for recruitment of DNMT1 to maintain DNA methylation in DNA replication. The TTD and PHD are both involved in histone H3 recognition. Thus, UHRF1 is an important epigenetic regulator to connect histone methylation and DNA methylation. However, how these domains of UHRF1 work together to recognize histone methylation and regulate DNA methylation remains elusive. In this report, through biochemical and structural analyses, we demonstrated that the TTD and PHD of UHRF1 coordinately recognize H3K9me3, with the TTD interacting with trimethylated H3K9 and the PHD interacting with the N terminus of H3. Previous biochemical and structural studies indicate that the TTD is sufficient for H3K9me3 interaction, and PHD is not required (6, 44). Through structural analysis, we found that the interaction between the H3K9me3 peptide and the TTD (residues 121–286) revealed in the TTD-H3K9me3 structure does not represent the natural condition. The linker region (residues 287–298) blocks this artificial interaction when the TTD is in the context of the PHD. Further ITC experiments demonstrated that H3R2-defective mutant (D334A) significantly decreased (~30-fold) the interaction between TTD-PHD and the H3K9me3 peptide. Thus, the TTD is necessary but not sufficient for H3K9me3 recognition, and the PHD is required for this specific recognition by UHRF1.

Our previous work identified that the PHD of UHRF1 specifically recognizes unmodified histone H3, and the interaction is inhibited by methylation of H3R2 (7). The repression of euchromatic target gene expression by UHRF1 is also dependent on PHD binding to unmodified H3R2 (5). The TTD is not ligase. The H3K9me3 peptide was used in three different concentrations, and the reaction products were detected by immunoblotting with the indicated antibodies. C, superimposed fluorescence polarization plots for the binding of hemimethylated DNA to wild type UHRF1 with or without the H3K9me3 peptide.
involved in this regulation because H3K9me3 was not found in the promoter regions of these genes. Thus, the PHD of UHRF1 has two functions. First, the PHD works together with the TTD to specifically recognize H3K9me3 and determine the localization of UHRF1 to PCH, which is important for the maintenance of DNA methylation by DNMT1 in DNA replication. Second, the PHD works independent of the TTD to recognize H3R2 for the regulation of the euchromatic gene expression by UHRF1, which may also involve DNA methylation. In conclusion, the PHD is necessary and sufficient for H3R2 recognition, whereas both the PHD and TTD are required for H3K9me3 recognition by UHRF1.

Conformational Changes of TTD-PHD Induced by H3K9me3 Peptide—The FRET experiments indicate that the H3K9me3 peptide not only binds to TTD-PHD but also leads to a conformational change on TTD-PHD of UHRF1. Our in vitro experiments show that such conformational change did not affect the autoubiquitination activity or hemimethylated DNA binding affinity of full-length UHRF1. However, when put into the context to the nucleosomal condition, the conformational change may regulate particular function of UHRF1, such as the E3 activity for its substrates, hemimethylated DNA interaction, or the binding affinity to other interacting proteins (DNMT1, HDAC1, USP7, or G9a). The potential function of the conformational change of TTD-PHD induced by the H3K9me3 peptide requires further investigation.

Through small angle x-ray scattering (SAXS) measurements, Arita et al. (51) found that domain orientation of TTD-PHD is not static in solution, and H3K9me3 peptide binding does not alter the overall architecture of TTD-PHD and its intrinsic dynamic motion. In general, the finding is consistent with our observation from structural analyses. However, using FRET measurements, we observed conformational change of TTD-PHD induced by the H3K9me3 peptide, which was not found from the SAXS measurements (51). The SAXS measurements is a good method to detect the overall shape but is not sensitive to the relative movement when the overall shape does not change significantly. Thus, H3K9me3 peptide association may not lead to detectable changes of the overall shape of TTD-PHD using SAXS measurements but indeed changes the distance between the N terminus of the TTD and C terminus of the PHD detected by FRET measurements.

Potential Function of Ser-298 Phosphorylation—It has been reported that UHRF1 is phosphorylated by kinases, including CDK1, CDK2, CK2, and PKA, and the phosphorylations regulate the function of UHRF1 (13, 52–54). For example, our recent study demonstrated that USP7 interacts with and stabilizes UHRF1 in S phase, and the interaction/stabilization is inhibited by Ser-639 (Ser-652 in the longer UHRF1 isoform with a total of 806 amino acids) phosphorylation of UHRF1 by CDK1-cyclin B in M phase (13). Phosphorylation of Ser-661 by CDK2 regulates the cellular localization of UHRF1 and is essential for embryogenesis of zebrafish (52). Within TTD-PHD of UHRF1, residue Ser-298 was found to be phosphorylated by PKA, and the phosphorylation increases the binding of UHRF1 to the TopoIIα gene promoter and, as a consequence, increases the expression of TopoIIα (53). Interestingly, Arita et al. (51) showed that phosphorylation on Ser-298 alters the histone H3K9me3 peptide binding affinity (~30-fold reduction). However, we did not find significant changes of binding affinity between the mutation S298E of TTD-PHD and the H3K9me3 peptide (Fig. 5B). This difference could result from the use of Glu to mimic the phosphorylation, which may not be good enough to represent the Ser-298 phosphorylation. Because Ser-298 phosphorylation of UHRF1 significantly decreases the interaction between H3K9me3 peptide and TTD-PHD of UHRF1, the phosphorylation may not only regulate TopoIIα gene expression but also have a general impact on UHRF1 function. It will be interesting to study whether Ser-298 phosphorylation regulates the functions of UHRF1, such as the PCH localization of UHRF1 and DNMT1-mediated DNA methylation in DNA replication.

In conclusion, our biochemical and structural studies demonstrate that PHD-mediated recognition of unmodified H3 is independent of the TTD, whereas TTD-mediated recognition of H3K9me3 is required for the PHD. The TTD and PHD work coordinately for specific recognition of H3K9me3 by UHRF1, and both TTD and PHD are essential for this process. The H3K9me3 peptide induces change of relative conformation between the TTD and PHD in TTD-PHD. The conformational change does not affect in vitro autoubiquitination activity or hemimethylated DNA binding affinity of full-length UHRF1. The biological function of the conformational change requires further investigation.

Acknowledgments—We thank staff members of beamline BL17U at the Shanghai Synchrotron Radiation Facility for help with data collection and structure determination and the staff members of Bio-medical Core Facility, Fudan University for their help on ITC and FRET experiments.

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