ABSTRACT
The Mixed Lineage Leukemia protein 1 (MLL1) plays an essential role in the maintenance of the histone H3 lysine 4 (H3K4) methylation status for gene expression during differentiation and development. The methyltransferase activity of MLL1 is regulated by three conserved core subunits, WDR5, RBBP5 and ASH2L. Here, we determined the structure of human RBBP5 and demonstrated its role in the assembly and regulation of the MLL1 complex. We identified an internal interaction between the WD40 propeller and the C-terminal distal region in RBBP5, which assisted the maintenance of the compact conformation of the MLL1 complex. We also discovered a vertebrate-specific motif in the C-terminal distal region of RBBP5 that contributed to nucleosome recognition and methylation of nucleosomes by the MLL1 complex. Our results provide new insights into functional conservation and evolutionary plasticity of the scaffold protein RBBP5 in the regulation of KMT2-family methyltransferase complexes.

INTRODUCTION
The methylation of Histone H3 Lysine 4 (H3K4) is crucial to the epigenetic regulation of gene transcription (1,2). The dimethylation and trimethylation of H3K4 mainly occur at the promoters and coding regions of actively transcribed genes, while H3K4 monomethylation is primarily enriched at the enhancers (3,4). The methylation of H3K4 is predominantly mediated by the KMT2-family histone methyltransferases (5,6). Set1 is the only KMT2-family protein discovered in Saccharomyces cerevisiae (7,8). At least six Set1 homologs have been identified in mammalian cells, including SET1A, SET1B, and four Mixed Lineage Leukemia proteins (MLL1, MLL2, MLL3, and MLL4) (5,6). Of these, MLL1, the founding member of KMT2-family methyltransferase, has drawn the most attention because chromosome translocations of MLL1 lead to acute lymphoid and myeloid leukemia (9).

Although MLL1 and other KMT2-family proteins contain a conserved C-terminal catalytic SET [SU(VAR)3-9, E(Z) and TRX] domain, their intrinsic histone lysine methyltransferase (HKMT) activities remain low (10). Three regulatory proteins, WDR5, ASH2L and RBBP5, can bind to MLL1 and stimulate the HKMT activity of MLL1 (10,11). WDR5, ASH2L and RBBP5 are conserved from yeast to human (5,12). Their yeast homologs Swd3, Bre2, and Swd1 are also irreplaceable for the assembly and activity regulation of the yeast Set1-containing complex, COMPASS (COMplex of Proteins Associated with Set1) (6).

Recent studies on the structure of KMT2-family complexes have shed light on how these regulatory proteins bind and activate KMT2-family proteins (13–15). The splA and ryanodine receptor (SPRY) domain of ASH2L (ASH2L-SPRY, residues 286–505) forms a heterodimer with a short fragment of RBBP5 (RBBP5AS-ABM, residues 330–360) to stimulate the HKMT activity of MLL-family methyltransferases (15). The crystal structure of the minimized MLL3SET-RBBP5AS-ABM-ASH2L-SPRY complex revealed that RBBP5AS-ABM-ASH2L-SPRY constrained the flexibility of the SET-I motif of MLLSET, thereby facilitating substrate binding and catalytic activation (15). Furthermore, the structures of yeast COMPASS complexes revealed the assembly and regulation mechanisms of an active holoenzyme (13,14). The yeast COMPASS complex adopts a Y-shaped structure in which Swd1 (an RBBP5 ortholog) and Swd3 (a WDR5 ortholog) form the two arms of the Y-shape. Set1 sits at the central fork and Bre2 (an
ASH2L ortholog) is located at the bottom of the Y-shape (13,14). Low-resolution cryo electron microscopy analysis suggested that the ML1 core complex may adopt a similar architecture as the yeast COMPASS complex (16). However, the detailed structural models of different human KMT2-family complexes remain to be determined.

Among KMT2-associated regulators, RBBP5 and its yeast ortholog Swd1 play a pivotal role in the assembly and activity regulation of KMT2-family complexes (13–15). Both RBBP5 and Swd1 contain a WD40 propeller domain followed by a long C-terminal tail (17). The C-terminal tail can be further divided into two regions, a WD40 repeat proximal (WDRP) region and a C-terminal distal (CTD) region (Figure 1A) (14). The conserved WDRP region of RBBP5/Swd1 contains three adjacent short motifs that mediate interactions with KMT2SET, ASH2L/Bre2, and WDR5/Swd3, respectively (14,15). Although RBBP5 orthologs are highly similar in sequence, they exhibit variations that may relate to distinct roles of RBBP5 in different species. First, the WD40 propeller of human RBBP5 features a ring structure along the axis arising from arginine side-chains (hereinafter referred to as arginine-ring), which is involved in DNA/RNA-binding. This unique feature is absent in yeast Swd1 (17). Second, the C-terminal distal region of RBBP5 is highly variable in length in different species, from 25 aa (residues 403–427) in Saccharomyces cerevisiae to 158 aa (residues 381–538) in Homo sapiens. Kluyveromyces lactis COMPASS structure shows that the short CTD of Swd1 (residues 422–439) extending from the Swd3-binding motif is sandwiched between Swd3WD40 and Swd1WD40 to further secure Swd3 binding (14). Removal of the CTD 422–439 from Swd1 completely abolished the binding of Swd3 (14). However, in human RBBP5, the CTD region (residues 381–538) is dispensable for the binding of RBBP5 to WDR5 (18). Therefore, the conformation and function of the extended CTD region in human RBBP5 still require further characterization.

Although RBBP5 and its yeast ortholog Swd1 have been extensively studied, there are still controversies regarding its structural feature and functional roles in the regulation of KMT2-family complex. In our work, we elucidated an internal interaction between the WD40 propeller and the CTD region in human RBBP5 by structural analysis and biochemical assays. This internal interaction promotes a compact conformation of the ML1 complex and fine-tunes the activity of the ML1 complex. We also uncovered a novel DNA-binding motif in the CTD region that is involved in nucleosome binding and methylation of nucleosomes mediated by the ML1 complex.

**MATERIALS AND METHODS**

**Protein expression and purification**

ML1\_WIN-SET (ML1\_1754–1969) containing both WIN motif and SET domain, full-length RBBP5, full-length ASH2L, and WDR5\_A22 (WDR5\_333–334) were purified as previously described (15). RBBP5 WD40-containing constructs and CTD-containing fragments were expressed as His-Sumo fusion proteins in Rosetta cells. The proteins were purified by Ni-NTA affinity column, and on-beads digestion using Ulp1 protease. The eluted proteins were further purified by size-exclusion chromatography (SEC) on a HIlaid Superdex 75 or 200 column. Purified proteins were concentrated, snap-frozen in liquid nitrogen, and stored at −80°C.

**Crystalization, data collection and structural determination**

After extensive screening, we obtained the complex crystal from the following constructs: WD40 propeller with residues 10–325 (RBBP5\_10–325) and C-terminal distal region from 390 to 480 with the 422–443 loop deletion (RBBP5\_390–480d). The RBBP5\_10–325–RBBP5\_390–480d complex was obtained by mixing two separately purified proteins at 1:2 molar ratio, followed by size-exclusion chromatography on a HIlaid Superdex 200 column in the buffer containing 150 mM NaCl, 25 mM Tris–HCl, pH 8.0 and 5 mM DTT. Native or Se-Met RBBP5 complex was crystallized in 0.1 M HEPES-Na, pH 7.5, 2% (w/v) PEG 400, 2.0 M ammonium sulfate at 16°C by vapor diffusion method. A 1.8 Å selenomethionine-SAD (single-wavelength anomalous dispersion) dataset was collected at the Se peak wavelength at beamline BL19U1 of National Facility for Protein Science Shanghai (NFPS) in Shanghai Synchrotron Radiation Facility (SSRF). The dataset was processed by HKL3000 (19). The crystal belongs to the P1 space group, and there are two complexes in one asymmetric unit. AutoSHARP was used for selenium site search, solvent flattening, and automatic model building (20). Eight selenium atoms were located and refined, and an initial model was automatically built with good quality using ARP/WARP (21). Crystallography refinement was then carried out in PHENIX (22) together with manual model building in COOT (23).

**GST-pull down assays**

GST-ASH2L\_FL, WDR5\_333–334, ML1\_1754–1969, and RBBP5 proteins were incubated with 10 μl glutathione Sepharose 4B beads (GE Healthcare, USA) in 100 μl binding buffer containing 150 mM NaCl, 25 mM Tris–HCl, pH 8.0, 1 mM PMSF, 10 μM ZnSO4 and 2 mM DTT. After 2 h incubation at 4°C, the resin was washed three times with a total volume of 1.2 ml binding buffer. The bound proteins were then eluted by 25 μl elution buffer (150 mM NaCl, 25 mM Tris–HCl, pH 8.0, 4 mM DTT, 20 mM reduced GSH), and analyzed by SDS-PAGE on 8–12% Tris-glycine gel.

**Isothermal titration calorimetry**

The equilibrium dissociation constants between RBBP5\_2–333 and different C-terminal constructs of RBBP5 were determined by the MicroCal iTC200 calorimeter (Malvern Panalytical Ltd, UK). Different C-terminal constructs of RBBP5 (1 mM) were titrated into 0.1 mM RBBP5\_2–333 in the assay buffer of 300 mM NaCl, 25 mM Tris–HCl, pH 8.0, unless stated. To dissect the effect of ion-strength on the interaction between RBBP5\_2–333
and RBBP5\textsubscript{381-538}, three different salt concentrations were tested (150 mM NaCl, 300 mM NaCl and 800 mM NaCl) in the 25 mM Tris–HCl, pH 8.0 buffer. To evaluate the effects of mutations in the CTD region of RBBP5, RBBP5\textsubscript{390-480}d or its mutants (1 mM) was injected into a sample cell containing 0.1 mM RBBP5\textsubscript{2-333} in the assay buffer (300 mM NaCl, 25 mM Tris–HCl, pH 8.0). The dissociation constants between different RBBP5 constructs and WDR5 (WDR5\textsubscript{23-334}) were determined in the assay buffer of 150 mM NaCl, 25 mM Tris–HCl, pH 8.0. A total of 20 injections were carried out at 20°C with a reference power of 5 μcal/s. Binding data were plotted and analyzed in Origin 7 (OriginLab, USA). The ITC measurements for each interaction pair were repeated twice. The dissociation constants ($K_d$) and the fitting errors are derived from one representative ITC curve by data fitting using one-site binding model.

**Fluorescence polarization assay**

RBBP5 proteins were serially diluted into the buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% BSA) from 400 to 0.2 μM in 12 reactions tubes with a final volume of 30 μl. An equal volume of FAM-labeled 167-bp dsDNA was added to each reaction tube to reach a final concentration of 0.1 μM DNA. The reaction mixture was incubated at 25°C for 30 min in the dark. The fluorescence anisotropy of each tube was measured using Synergy Neo Multi-Mode Reader (BioTek, USA). The $K_d$ values and the fitting errors are calculated with Prism 6.0 software (GraphPad, USA) by using the sigmoidal dose-response model.

**Biological Small-Angle X-ray Scattering**

Small-angle X-ray scattering (SAXS) experiments were performed at beamline BL19U2 of National Facility for Protein Science Shanghai (NFPS) in Shanghai Synchrotron Radiation Facility (SSRF) (24). The wavelength of X-ray is 0.918 Å. Scattered X-ray intensities were collected using a Pilatus 1M detector (DECTRIS Ltd, Switzerland). The sample-to-detector distance was set such that the detecting distance of momentum transfer $q = 4\pi \sin \theta /\lambda$, where $\theta$ is the scattering angle of SAXS experiments was 0.008–0.47 Å$^{-1}$. A flow cell made of a cylindrical quartz capillary with a diameter of 1.5 mm and a wall of 10 μm was used. The MLL1 complexes were obtained by mixing MLL1\textsubscript{3754-3969}, WDR5\textsubscript{23-334}, ASH2L\textsubscript{FL}, and RBBP5 at the molar ratio of 2:1:1:1, followed by the purification on the Superdex 200 column (10/300 GL) column. Then they were diluted to three different concentrations (0.5, 1 and 2 mg/ml) in the buffer of 300 mM NaCl, 25 mM Tris, pH 8.0, 4% glycerol and 1 mM TCEP. SAXS data were collected as 20 × 1-s exposures and scattering profiles for the 20 passes were compared at 10°C using 60 μl sample. The 2D scattering images were converted to 1D SAXS curves through azimuthally averaging after solid angle correction and then normalizing with the intensity of the transmitted X-ray beam, using the software package BioXXTAS RAW (25). Background scattering was subtracted using PRIMUS in ATSAS software package (26). Linear Guinier plots in the Guinier region ($q^2 R_g < 1.3$) were confirmed in all experimental groups. The pair distribution functions of the particles $P(r)$ and the maximum sizes $D_{max}$ were computed using GNOM (27). The ab initio shapes were determined using DAMMIF (28).
with 15 DAMMIF runs for each experimental group and DAMAVER (29) was used to analyze the normalized spatial discrepancy (NSD) between the 15 models. The models with an NSD greater than the average NSD ± 2 standard deviation were excluded, and the remaining aligned models were averaged to get the representative molecular envelope.

Cross-linking mass spectrometry

The MLL1 complex was obtained by mixing MLL13754–3969, WDR523–334, ASH2L-FL, and RBBP5-FL at the molar ratio of 2:1:1:1, followed by the purification on the Superdex 200 increase (10/300 GL) column. The purified complex was diluted to 5 mM in the buffer of 150 mM NaCl, 25 mM HEPES, pH 7.0 with a final volume of 20 μl. EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) was first mixed with MLL1 complex at a final concentration of 1 mM. After incubation at room temperature for 1 h, the reaction was quenched by addition of 20 mM 2-mercaptoethanol. Then Sulfo-NHS (N-hydroxsulfoosuccinimide) was added to the reaction mixture at a final concentration of 2 mM, followed by incubation at room temperature for 2 h. Finally, the reaction was quenched by adding 20 mM Tris (pH 8.0). The reaction products were separated by SDS-PAGE on 8–10% Tris-glycine gels and visualized by staining with Coomassie blue.

The gel band of cross-linked MLL1 complex was excised and cut into small pieces. The gel sample was then destained with methanol/H2O, followed by TCEP reduction (10 mM), iodoacetamide alkylation (55 mM) and trypsin digestion. Trypsin digestion was performed with sequencing-grade modified trypsin (Promega, USA) at 37°C overnight. The tryptic digested peptides were extracted and desalted with monoSpin C18 columns (GL Science, Japan), followed by separation in a Proxen EASY-nLC liquid chromatography system (ThermoFisher Scientific, USA) by applying a step-wise gradient of 0–80% acetonitrile in 0.1% formic acid. Peptides eluted from the LC column were directly electrosprayed into the mass spectrometer with a distal 2 kV spray voltage. Tandem mass spectrometry (MS/MS) analysis was performed on a Q-Exactive instrument (ThermoFisher Scientific, USA) in a 120-minute gradient. Cross-linked peptides were identified and evaluated using pLink2 software (30).

MALDI-TOF-based methyltransferase assay

The MLL1 complex was prepared by mixing MLL13754–3969, WDR523–334, ASH2L-FL, and RBBP5 at the molar ratio of 1:1:1:1 without further purification. For a 50 μl reaction, 250 μM SAM, 10 μM histone peptide (ARTKQTARY) and 1 μM MLL1 complex were mixed in the buffer containing 20 mM HEPES (pH7.8), 10 mM NaCl, and 5 mM DTT and incubated at 25°C. At different time points, 4 μl reaction mixture was taken out and stopped by adding 20 mM Tris (pH 8.0). The reaction was initiated by adding 20 μM SAM and incubated at 30°C. At different time points (1, 3, 8, 15 min), 8 μl reaction mixture was taken out and stopped by adding 2 μl 0.5% tri-fluoroacetic acid. Then we followed the protocol described in the kit manual to measure the amount of 5-adenosyl-L-homocysteine (SAH) produced. The assays were performed in triplicate, and the averaged data points were used for linear fitting of the amount of SAH generated to give an estimation of the initial reaction rate (nM SAH/min).

Preparation of nucleosome

*Xenopus laevis* histones were expressed in *Escherichia coli* BL21 (DE3) and purified from inclusion bodies as described before (31). The nucleosome was reconstituted from histone octamers and the 167-bp Widom 601 DNA (31). The reconstituted NCP was further purified through Superose 6 increase 10/300 gel filtration.

Electrophoretic mobility shift assay (EMSA)

The MLL1 complexes were obtained by mixing MLL13754–3969, WDR523–334, ASH2L-FL and RBBP5 at the molar ratio of 1:1:1:1 and incubate on ice for 30 minutes without further purification. The reaction assays were performed with 20 μM SAM, 1 μM nucleosome and 1 μM MLL1 complex in the buffer containing 20 mM HEPES, pH 7.8, 10 mM NaCl and 5 mM DTT. The reaction was incubated at 25°C for 1 h. Each reaction mixture was divided into three parts that were separated on a 15% SDS-PAGE. The methylation products of H3K4 were detected by western blot using corresponding antibodies (H3K4me1 antibody, #39297, Active motif; H3K4me2 antibody, # 07-030 Millipore; H3K4me3 antibody, #9751, CST).

MTase-Glo™ methyltransferase assay

To monitor the progression of the nucleosome-methylation reaction, we used the MTase-Glo™ Methyltransferases Assay Kit (Promega, USA). MLL1 complex (50 nM containing MLL13754–3969, WDR523–334, ASH2L-FL and RBBP5 at the molar ratio of 1:1:1:1) and nucleosome (1 μM) were mixed in the buffer of 25 mM HEPES-Na, pH 7.4, 100 mM NaCl, 0.1 mg/ml BSA and 1 mM DTT. The reaction was initiated by adding 20 μM SAM and incubated at 30°C. At different time points (1, 3, 8, 15 min), 8 μl reaction mixture was taken out and stopped by adding 2 μl 0.5% tri-fluoroacetic acid. Then we followed the protocol described in the kit manual to measure the amount of 5-adenosyl-L-homocysteine (SAH) produced. The assays were performed in triplicate, and the averaged data points were used for linear fitting of the amount of SAH generated to give an estimation of the initial reaction rate (nM SAH/min).
the molar ratio of 2:1:1:1, followed by the purification on the Superdex 200 column. MLL1 complexes were diluted into a series of concentrations (4–0.125 μM) in six reaction tubes with a final volume of 5 μl in the buffer containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 10% glycerol. Then 5 μl of 0.2 μM nuclease was added to each tube. After incubation on ice for 1 h, the reaction mixture was separated on 6% native polyacrylamide gels and visualized by staining with ethidium bromide (EB).

RESULTS

Both WD40 and CTD of RBBP5 contribute to activity regulation of MLL1 complex

RBBP5WDRP (residues 330–381) is the primary region required for activity stimulation of KMT2-family complexes (15). However, our previous data showed that an RBBP5WDRP-ASH2L286–505 complex stimulated MLL3 HKMT activity to a level of ~70% of full-length RBBP5ASH2L2L (15). Results from Wilson lab also declared that the HKMT activity of MLL1 complex containing the WD40 region of RBBP5 may also participate in the activation of KMT2-family complexes. Thus, we first quantitatively characterized the HKMT activities of the MLL1 core complexes in the presence of different RBBP5 domains by a mass-spectrometry-based methyltransferase assay. We used an H3 peptide as the substrate. As expected, core complexes in the presence of different RBBP5 did not lose activity, and the activity boost was only observed in the presence of RBBP52–538 containing both WD40 and CTD (Figure 1B). After extensive screening, we found that deletion of the WD40 region of RBBP5 (residues 286–505) is in close contacts with RBBP5 ASH2L-binding motif (ABM, residues 344–360). SET-N and SET-I modules of MLL1SET were crosslinked with RBBP5AS+ABM fragments. Unexpectedly, we observed extensive crosslinking between RBBP5381–538 (named as RBBP5CTTD) and WDR5 WD40 propeller. In addition, RBBP5CTTD was also found to have a higher affinity to WDR5 WD40 (residues 23–334) in our assay condition (Figure 2B), suggesting that they may be in spatial proximity. In contrast, RBBP5CTTD interacted with GST-WD40 propeller (residues 2–333) with a dissociation constant ($K_d$) of 6.8 ± 0.9 μM at 150 mM NaCl concentration (Figure 2C). The binding of RBBP5CTTD to its WD40 propeller was primarily driven by hydrophobic interactions because increasing salt concentrations only mildly reduced the affinity between these two domains (Figure 2C).

**RBBP5CTDM wraps around RBBP5WD40**

To further characterize the internal interaction between WD40 propeller and C-terminal regions of RBBP5, we generated a series of RBBP5 C-terminal constructs to assess their interactions with RBBP52–333 by ITC assays (Supplementary Figure S3A and B). A minimal RBBP5 C-terminal construct (390–480) retained the ability to bind with RBBP52–333 (Supplementary Figure S3A and B). After extensive screening, we found that deletion of a disordered loop (residues 422–443) from RBBP5390–480 (RBBP5390–480d) promoted the crystallization of the complex composed of RBBP5390–480d and RBBP5501–525 (Supplementary Figure S3A and B). Hereafter, we refer to RBBP5390–480d as RBBP5CTDM (C-Terminal Distal Minimized) and RBBP5501–525 as RBBP5WD40. Selenomethionine-substituted crystals diffracted up to 1.8 A resolution, and we were able to solve the complex structure by single-wavelength anomalous dispersion.

**The internal interaction between RBBP5WD40 and RBBP5CTTD**

Then we employed crosslinking mass spectrometry (CX-MS) to map potential RBBP5-mediated interaction sites in the MLL1 complex. We purified the MLL11754–396WDR523–334–RBBP5FL–ASH2FL complex and subjected it to chemical crosslinking using a zero-length crosslinker, EDC-Sulfo-NHS. The crosslinked products were digested by trypsin and then analyzed by liquid chromatography-mass spectrometry. From the MS data, we identified 37 intermolecular cross-links and 38 intramolecular cross-links (Figure 2A and Supplementary Figure S2). Most of the crosslinked patterns confirmed the previously-known interaction interfaces (Figure 2A). For example, ASH2LSPRY (residues 286–505) is in close contacts with RBBP5 ASH2L-binding motif (ABM, residues 344–360). SET-N and SET-I modules of MLL1SET were crosslinked with RBBP5AS+ABM fragments. Unexpectedly, we observed extensive crosslinking between RBBP5381–538 (named as RBBP5CTTD) and WDR5 WD40 propeller. In addition, RBBP5CTTD was also found to have a higher affinity to WDR5 WD40 (residues 23–334) in our assay condition (Figure 2B), suggesting that they may be in spatial proximity. In contrast, RBBP5CTTD interacted with GST-WD40 propeller (residues 2–333) with a dissociation constant ($K_d$) of 6.8 ± 0.9 μM at 150 mM NaCl concentration (Figure 2C). The binding of RBBP5CTTD to its WD40 propeller was primarily driven by hydrophobic interactions because increasing salt concentrations only mildly reduced the affinity between these two domains (Figure 2C).
Figure 2. The internal interactions in RBBP5. (A) Schematic representation of inter-protein crosslinks detected by CX-MS analysis of the MLL1 complex. RBBP5 intra-protein crosslinks are also labeled. The width of the linking line is correlated to the peptide amount of one specific crosslink appeared in the MS. (B) WDR523–334 has no interaction with RBBP5CTD (residues 381–538) as shown by a representative ITC binding curve. The assay buffer is 150 mM NaCl, 25 mM Tris–HCl, pH 8.0. (C) RBBP5WD40 (residues 2–333) has a direct interaction with RBBP5CTD (residues 381–538) as shown by representative ITC binding curves at three different salt concentrations (150, 300 and 800 mM NaCl in the presence of 25 mM Tris–HCl, pH 8.0 buffer). The binding affinity slightly decreased with the increase of salt concentration. The purple curve is the buffer titrated with RBBP5CTD. The dissociation constants ($K_d$) and the reported fitting errors were determined from the representative ITC curves by data fitting using one-site binding model.

Table 1. Data collection, phasing and refinement statistics for the crystal structure

| Data collection | ScMet RBBP510–325-RBBP5390–480d |
|-----------------|---------------------------------|
| Space group     | P1                               |
| Cell dimensions | a, b, c (Å)                      | 57.6, 68.8, 72.8 |
| a, β, γ (°)     | 99.2, 99.3, 113.8                |
| Wavelength (Å)  | 0.9785(Se peak)                 |
| Resolution (Å)  | 50–1.8                           |
| $R_{merge}$     | 0.08(0.42) *                     |
| $I/I_{ave}$     | 20.0 (2.5)                       |
| Completeness (%)| 96.3 (89.2)                      |
| Redundancy      | 6.7/5.5                          |
| Refinement      |                                  |
| Resolution (Å)  | 34.8–1.8                         |
| No. reflections | 86 883                           |
| $R_{work}/R_{free}$ (%) | 16.5/19.4 |
| No. atoms       |                                  |
| Protein         | 538                              |
| Solvent & ligands| 576                             |
| $B$-factors (Å$^2$)|                       |
| Protein         | 30.8                             |
| Solvent & ligands| 45.4                            |
| R.m.s. deviations| Bond lengths (Å) 0.008 |
|                 | Bond angles (°) 0.962            |

* Highest resolution shell is shown in parenthesis.

Table 1. Data collection, phasing and refinement statistics for the crystal structure

The electron density map unambiguously allowed us to build the atomic model of the majority of RBBP5WD40 (residues 12–324) and two segments of RBBP5CTDM (residues 396–404 and 452–474) (Figure 3 and Supplementary Figure S4A-C).

RBBP5WD40 forms a canonical β-propeller fold with seven WD40 repeats (Figure 3A). The seventh repeat is comprised of three strands from the C-terminal region (RBBP5296–324) and one strand from the N-terminal region (RBBP512–22), thereby sealing the β-propeller. The RBBP5WD40 conformation is almost identical to that of apo-RBBP5WD40 with a root-mean-square deviation value of 0.39 Å for 319 Cα pairs, indicating that CTDM binding induces no dramatic structural rearrangement of RBBP5WD40 (Supplementary Figure S4D). Some loop regions of WD40 propeller contacting CTDM slightly change their configurations (Supplementary Figure S4D).

RBBP5CTDM exhibits an extended conformation and wraps the WD40 propeller through two segments, burying 1472 Å$^2$ of the surface area at the interface. The first segment of RBBP5CTDM (CTD1) contains residues 396–404 and sits on the top surface of RBBP5WD40. The second segment (CTD2) meanders its way around the side face of RBBP5WD40 and also pairs with the repeat 2 of the WD40 propeller to form a five-stranded β-sheet. After a sharp turn, CTD2 further extends along the bottom face and ends in the central cavity of the β-propeller fold (Figure 3). CTD1 and CTD2 are connected by a disordered loop which is invisible in the crystal structure.

The interfaces between RBBP5CTD1 and RBBP5WD40

The first segment of CTDM (CTD1) is rich in non-polar residues (L396SKALLYLPI404) and runs across a hydrophobic groove formed by repeats 1 and 2 of RBBP5WD40 (Figures 3 and 4A). Three leucine residues (L399, L400 and L402) in CTD1 fit snugly into the hydrophobic groove formed by residues from RBBP5WD40, including W35, L38, I50, W74, V95, L96, and the aliphatic side chain of K60 (Figure 4B). Consistent with the structural model, alanine substitution of hydrophobic residues on RBBP5CTDM (L399A, L400A) resulted in ~13-fold de-
creased binding affinities between between RBBP5_{2-333} and RBBP5_{CTDM} (Figure 4C). The activity assays showed that RBBP5_{2-333} mutation mildly decreased the HKMT activities of MLL1 core complexes and the activity with RBBP5_{2-333}L399A mutation was not significantly different from wild type RBBP5_{2-333} (Figure 4D). It suggests that the decreased CTDM-WD40 interactions of RBBP5 L399A and L400A mutations (K_d \approx 70 \mu M) are still strong enough to maintain the activities of MLL1 core complexes.

In addition to the hydrophobic contacts observed in the structure, electrostatic interactions also participate in stabilizing RBBP5_{WD40}–RBBP5_{CTD1} interaction. Although the acidic N-terminus of RBBP5_{CTD1} (390DEELED395) is invisible in the crystal structure, this N-terminal extension is presumably located near the central arginine ring of the WD40 propeller and may interact with RBBP5_{WD40} through electrostatic attractions (Figure 4A). This model is partly supported by the observation that the RBBP5_{393-480} construct with fewer acidic residues in CTD1 possessed a reduced affinity with RBBP5_{2-333} (Supplementary Figure S3A). In summary, the hydrophobic contacts and accompanying electrostatic interactions determine the specific recognition of RBBP5_{CTD1} by RBBP5_{WD40}.

It is worth noting that a similar CTD1 motif in yeast Swd1 also mediates an internal interaction with the Swd1 WD40 propeller (14). Superimposition of the WD40 domains from hRBBP5 and KlSwd1 reveals that a portion of Swd1_{CTD1} adopts a similar configuration as RBBP5_{CTD1} and binds to a hydrophobic cleft of Swd1_{WD40} (Supplementary Figure S3A-D). Notably, RBBP5_{CTD1} from higher organisms contains a hydrophobic core flanked by two acidic segments, but yeast orthologs have much fewer acidic residues (Supplementary Figure S5B). Correspondingly, the RBBP5 orthologs in yeast species also do not contain the arginine-ring residues that are involved in CTD1-binding (Supplementary Figure S5A), so the acidic feature of Swd1_{CTD1} may not be necessary for its association with the WD40 propeller. Another major difference between human RBBP5_{CTD1} and yeast Swd1_{CTD1} is that yeast CTD1 ends with a short \alpha helix binding to the WDR5 ortholog, Swd3 (Supplementary Figure S5C). Swd1_{CTD1} is absolutely essential for binding Swd3 and removal of this short CTD1 completely abolished Swd3-Swd1 interaction (14). On the contrary, hRBBP5_{CTD1} only plays a negligible role in stabilizing WDR5–RBBP5 interaction, because full-length RBBP5 (RBBP5_{52-538}) and RBBP5_{WDRP} (RBBP5_{330-381}) have comparable binding affinities with WDR5 (Supplementary Figure S5E). Taken together, the CTD1 motif of RBBP5 is a conserved module and has gained structural and functional plasticity in the process of evolution.

The interface between RBBP5_{CTD2} and RBBP5_{WD40}

The second segment of RBBP5_{CTDM} wraps the side and the bottom faces of RBBP5_{WD40}. The absence of CTD2 in yeast species (Supplementary Figure S5B) indicates a more specific role of CTD2 in higher organisms. The CTD2 motif is docked to a relatively hydrophobic pocket formed by repeats 2, 3 and 4 (Figures 3 and 4E). The interface between RBBP5_{CTD2} and RBBP5_{WD40} consists of a set of hydrophobic residues, including I457, L459, V462, P469, L470 and L471 from RBBP5_{CTD2} and L28, C70, F106, I110, L111, P127, V133, V144 and L299 from RBBP5_{WD40} (Figure 4F). Mutations of the hydrophobic residues (L470A, I457A and L459A) on CTD2 diminished the interaction between RBBP5_{CTDM} and RBBP5_{WD40} (Figure 4C). Combined mutation of CTD1 and CTD2 (L399A/L400A/I457A/L459A, named as RBBP5_{CTDM-4A mutant}) further disrupted the RBBP5_{CTDM–RBBP5_{WD40}} interaction (Figure 4C) and reduced the HKMT activity of the MLL1 complex to the activity level comparable with that of the complete-CTD-deletion construct (RBBP5_{381}) (Figure 4D). These results reinforced the notion that both CTD1 and CTD2 are required for stable association with RBBP5_{WD40}, and this internal interaction fine-tunes the methyltransferase activity of the MLL1 complex.

The internal interaction of RBBP5 compacts the MLL1 complex

To further investigate how the internal interaction of RBBP5 contributes to the assembly of the MLL1 core
Figure 4. The interaction interface between RBBP5WD40 and RBBP5CTDM. (A) The RBBP5CTD1-binding pocket on RBBP5WD40. RBBP5WD40 surface is colored according to its electrostatic potential (positive potential, blue; negative potential, red). RBBP5CTD1 is shown in cyan. One possible path of the absent N-terminal acidic extension of RBBP5CTD1 is indicated. (B) Details of hydrophobic interactions between RBBP5WD40 and RBBP5CTD1. The critical residues are presented as ball-and-stick models. RBBP5WD40 residues are colored in red and RBBP5CTD1 residues in cyan. Hydrogen bonding interactions are shown as dashed magenta lines. (C) Effects of mutations in the RBBP5CTDM on the interaction between RBBP5–333 and RBBP5CTDM (RBBP5390–480d) analyzed by ITC assays. ITC buffer is 25 mM Tris–HCl, 300 mM NaCl, pH 8.0. The RBBP5–4A mutation is L399A/L400A/I457A/L459A mutation. The dissociation constants ($K_d$) and the reported fitting errors for each mutant were determined from one representative ITC curve by data fitting using one-site binding model. (D) HKMT activities of the MLL1 complexes (MLL1 3754–3969–WDR523–334–ASH2L–RBBP5) containing different RBBP5 mutants determined by the MALDI-TOF-based methyltransferase assays. The HKMT activities are normalized to the activity of RBBP5–480-containing MLL1 complex. Mean ± s.d. (n = 3) are shown. The ANOVA test was used to determine the statistical difference of HKMT activities between groups. The label ‘ns’ stands for ‘not significant’ (P > 0.05). (E) The RBBP5CTD2-binding surface on RBBP5WD40. RBBP5WD40 surface is colored according to its electrostatic potential (positive potential, blue; negative potential, red). RBBP5CTD2 is shown in cyan. (F) Details of hydrophobic interactions between RBBP5WD40 and RBBP5CTD2. The critical hydrophobic residues are presented as ball-and-stick models. Salt bridge and hydrogen bonding interactions are shown as dashed magenta lines.
complex, we utilized small-angle X-ray scattering (SAXS) to characterize the conformation of the MLL1 complex. To test this idea, we carried out the electron microscopic mobility shift assay (EMSA) to characterize the interactions between MLL1 complexes and nucleosomes. We first tested the HKMT activities of the MLL1 complexes with recombinant mononucleosomes by a western-blot-based methyltransferase assay. The MLL1 complex assembled with RBBP5 WD40 (RBBP52–480) has a weak methyltransferase activity on nucleosomes (Figure 6A, lane 1). The inclusion of either the WD40 propeller or CTD only slightly increased the activity of the MLL1 complex (Figure 6A, lanes 2 and 3). A considerable increase in the monomethylation and dimethylation levels of nucleosomal H3 was observed in the MLL1 complex assembled with RBBP52–480, which retains the interaction between RBBP5 WD40 and RBBP5 CTD (Figure 6A, lane 4). The MLL1 complex assembled with the RBBP5A mutant that disrupted RBBP5 WD40–RBBP5 CTD interaction had much weaker methyltransferase activity than the complex containing RBBP52–480 (Figure 6A, lane 7). These results thus confirmed the critical role of the RBBP5 WD40–RBBP5 CTD interaction in stimulating the methyltransferase activity of MLL1. Interestingly, the inclusion of a C-terminal tail (RBBP5480–538) moderately increased the methylation of nucleosomal H3 (Figure 6A, lane 5), indicating an important role of the uncharacterized tail of RBBP5 in activity regulation of the MLL1 complex.

In the western-blot-based methyltransferase assay, equal concentrations of enzymes and the nucleosome substrates were used. The result may not quantitatively describe the activities of the MLL1 complexes containing different RBBP5 fragments since western blot signals can be easily saturated. To accurately compare the activities of the MLL1 complexes, we performed steady-state kinetic analysis of the methyltransfer reaction using 50 nM enzymes and 1 μM substrates. We found that the MLL1 complex assembled with RBBP52–381 had negligible activity towards nucleosomes at such a condition, and the initial reaction rate could not be accurately measured (Figure 6B). Time-course monitoring of methylation progression manifested that RBBP52–480 substantially increased the activity of MLL1 complex on nucleosomes and RBBP52–538 generated a ~3-fold higher activity than RBBP52–480 (Figure 6B). It is noteworthy that RBBP52–538 and RBBP52–480 had the same methylation activities on H3 peptides (Figure 4D). These data suggest that RBBP5480–538 played an additional supporting role in mediating efficient methylation of nucleosomes. Here we name the RBBP5480–538 segment RBBP5 CTD.

RBBP5 CTD may stimulate the HKMT activity of the MLL1 complex by facilitating the nucleosome binding to the MLL1 complex. To test this idea, we carried out the electrophoretic mobility shift assay (EMSA) to characterize the interactions between MLL1 complexes and nucleosomes. The EMSA data showed that the MLL1 complex assembled with RBBP52–538 had a much higher binding affinity with nucleosomes than the MLL1 complex assembled with RBBP52–480 (Figure 6C). This result thus implies that RBBP5 CTD contains extra binding interfaces with nucleosomes and increases the MLL1 complex association with nucleosomes.

A novel CTD3 motif is important for methylation on nucleosomes

The above studies of MLL1 activity regulation by RBBP5 are limited to the methylation of H3 peptides. Then we are eager to extend the landscape to know if the RBBP5 WD40–RBBP5 CTD interaction is also crucial for histone methylation on nucleosomes. We first tested the HKMT activities of the MLL1 complexes with recombinant mononucleosomes by a western-blot-based methyltransferase assay. The MLL1 complex assembled with RBBP5 WD40 (RBBP52–538) has a weak methyltransferase activity on nucleosomes (Figure 6A, lane 1). The inclusion of either the WD40 propeller or CTD only slightly increased the activity of the MLL1 complex (Figure 6A, lanes 2 and 3). A considerable increase in the monomethylation and dimethylation levels of nucleosomal H3 was observed in the MLL1 complex assembled with RBBP52–480, which retains the interaction between RBBP5 WD40 and RBBP5 CTD (Figure 6A, lane 4). The MLL1 complex assembled with the RBBP5A mutant that disrupted RBBP5 WD40–RBBP5 CTD interaction had much weaker methyltransferase activity than the complex containing RBBP52–480 (Figure 6A, lane 7). These results thus confirmed the critical role of the RBBP5 WD40–RBBP5 CTD interaction in stimulating the methyltransferase activity of MLL1. Interestingly, the inclusion of a C-terminal tail (RBBP5480–538) moderately increased the methylation of nucleosomal H3 (Figure 6A, lane 5), indicating an important role of the uncharacterized tail of RBBP5 in activity regulation of the MLL1 complex.

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RBBP5 CTD may stimulate the HKMT activity of the MLL1 complex by facilitating the nucleosome binding to the MLL1 complex. To test this idea, we carried out the electrophoretic mobility shift assay (EMSA) to characterize the interactions between MLL1 complexes and nucleosomes. The EMSA data showed that the MLL1 complex assembled with RBBP52–538 had a much higher binding affinity with nucleosomes than the MLL1 complex assembled with RBBP52–480 (Figure 6C). This result thus implies that RBBP5 CTD contains extra binding interfaces with nucleosomes and increases the MLL1 complex association with nucleosomes.

**Figure 5.** SAXS analyses of MLL1 complex. (A) Overlay of P(r) distributions from the MLL1 complexes assembled from different RBBP5 constructs. RBBP52–480–4A is RBBP52–480 with the L399A/L400A/L457A/L459A mutation. (B) Superimposition of the molecular envelopes of the RBBP52–480-containing MLL1 complex (yellow) and the RBBP52–381-containing MLL1 complex (red). The sizes of the longest dimension of different complexes are indicated.
Figure 6. RBBP5<sub>CTD3</sub> contributes to methylation on nucleosomes. (A) Methyltransferase activity assays were carried out using 167-bp nucleosomes as substrates. MLL1 complex (1 μM), nucleosome (1 μM), and SAM (20 μM) were incubated at 25 °C for 1 h. At this condition, no H3K4me3 signal was detected. The band densities were quantified by setting the band density from the sample of MLL1<sub>3754–3969–WDR523–334–ASH2LFL–RBBP5<sub>330–381</sub> as one. RBBP5<sub>2–538KMA</sub> is a mutation with 10 lysine residues in CTD3 mutated into alanine. RBBP5<sub>2–480L399A/L400A/I457A/L459A</sub> contains the RBBP5<sub>2–480</sub> L399A/L400A/I457A/L459A mutation. (B) Time-course monitoring of methyltransferase activities of different MLL1 complexes by MTase-Glo™ Methyltransferases Assay Kit. The assay system contains 50 nM MLL1 complex, 1 μM nucleosome, and 20 μM SAM. Each data point was presented as mean ± s.d. from triplicate measurements. The reaction rate and reported fitting errors were derived by linear fitting of the averaged dataset. (C) EMSA showed that MLL1 complexes bound to 167-bp nucleosomes. 0.1 μM nucleosome was incubated with increasing amounts of MLL1 complexes with different RBBP5 constructs as indicated. (D) The dissociation constants of the RBBP5-DNA interactions were determined by fluorescence polarization assays. 0.1 μM FAM-labelled 167-bp dsDNA was used. Each data point was shown as mean ± s.d. from triplicate measurements. Some error bars were not shown because the error bar was shorter than the size of the symbol. The dissociation constants (K<sub>d</sub>) and the reported fitting errors were determined from the averaged data points by fitting with the sigmoidal dose-response model.

The CTD3 motif has a DNA-binding activity

Examination of the RBBP5<sub>CTD3</sub> sequence provides us some clues on how RBBP5<sub>CTD3</sub> contributes to nucleosome binding. We noticed that RBBP5<sub>CTD3</sub> is rich of basic residues. There are 14 K/R out of 59 residues in RBBP5<sub>CTD3</sub> (residues 480–538). We hypothesized that these K/R residues might be involved in DNA binding, thereby increasing the binding of MLL1 complex to nucleosomes. To test this hypothesis, we checked the binding ability of RBBP5 to a fluorescently labeled 167-bp DNA by fluorescence polarization (FP) assay. As expected, RBBP5<sub>CTD3</sub> bound strongly to DNA (K<sub>d</sub> = 13.3 ± 2.4 μM), comparable to the full-length RBBP5 (K<sub>d</sub> = 11.5 ± 0.9 μM) (Figure 6D). Removal of CTD3 from RBBP5 dramatically disrupted its binding to DNA (the RBBP5<sub>2–480</sub> curve, Figure 6D). Additionally, we mutated ten lysine residues in CTD3 (K480/L482/L488/K491/L495/L500/L502/L505) to alanine and found that this full-length RBBP5 mutant (RBBP5<sub>KMA</sub>) substantially attenuated the interaction with DNA (Figure 6D). This DNA-binding-deficient RBBP5 mutant also severely impaired the association of the MLL1 complex with nucleosomes (Figure 6C). As a result, the methylation activity of the MLL1 complex assembled with RBBP5<sub>KMA</sub> was decreased (Figure 6A and B). Collectively, these data reveal that RBBP5<sub>CTD3</sub> is a novel DNA-binding motif in RBBP5. This new element can stabilize the MLL1
complex associated with nucleosomes and stimulates the HKMT activity of the MLL1 complex on nucleosome H3.

DISCUSSION

RBBP5 is a conserved scaffold protein to orchestrate the assembly of KMT2-family methyltransferase complexes (13–15). In the present work, we identified an internal interaction in RBBP5 and revealed its roles in MLL1 complex assembly and activity regulation. The dissociation constant for the interaction between RBBP5WD40 and RBBP5CTD is around sub micro-molar range as determined by ITC assays (Figure 2C). Given that the binding affinity is measured from two purified proteins separated in solution, the actual interaction between these two regions on a single RBBP5 polypeptide is expected to be much stronger than that reported here. Thus, this internal interaction in RBBP5 might be comparable with other RBBP5-mediated intermolecular interactions in the MLL1 complex (e.g. WDR5-RBBP5, $K_d = 1.8 \pm 0.1 \mu M$; ASH2L-RBBP5, $K_d = 0.46 \pm 0.04 \mu M$) (18,32). Such a robust internal interaction may have a profound effect on the assembly of the MLL1 complex. We indeed observed that the interaction between RBBP5WD40 and RBBP5CTD helps the MLL1 complex to form a compact conformation, as shown by the SAXS analyses (Figure 5). The compact conformation of the MLL1 complex driven by the RBBP5WD40–RBBP5CTD interaction may facilitate the proper coordination of structural elements required for efficient methylation reactions (Figure 7).

Another intriguing finding in our study is the novel DNA-binding CTD3 motif found at the end of RBBP5. It is worth noting that Mittal et al. recently reported that RBBP5WD40 domain could bind dsDNA ($K_d = 29.7 \pm 4.2 \mu M$) and ssRNA ($K_d = 22.5 \pm 3.2 \mu M$), presumably mediated by the arginine-rich surface of WD40 propeller (17). We also found that RBBP52–325 binds to dsDNA with a similar affinity ($K_d = 35.1 \pm 8.7 \mu M$) (Figure 6D). However, RBBP52–480 that includes WD40, WDRP, CTD1 and CTD2 had a negligible DNA-binding activity (Figure 6D). This result can be explained by our structural model, in which the arginine-rich surface of RBBP5WD40 is involved in the binding of the acidic segment of RBBP5CTD1 (Figure 4A), thereby masking the potential DNA-binding surface on the WD40 propeller. Instead, the CTD3 motif serves as the primary DNA-binding element in RBBP5, as the mutation in CTD3 substantially decreased the DNA-binding activity of full-length RBBP5 (Figure 6D). This novel DNA-binding element is important for nucleosome recognition, as deletion or mutation of the CTD3 motif decreased the nucleosome-binding affinity of the MLL1 complex (Figure 6C). We propose that when the MLL1 complex binds to nucleosomes, RBBP5 is positioned close to the nucleosome and RBBP5CTD3 directly contacts with nucleosomal DNA (Figure 7). RBBP5 CTD3 acts as an additional buckle to secure nucleosome recognition and also helps orient the H3 tail to the active site of MLL1SET, thereby increasing the MLL1 methylation activity on nucleosomal substrates.

Finally, the structural comparison of human RBBP5 and yeast Swd1 allowed us to understand the evolutionary plasticity of RBBP5. The primary sequences of the N-terminal WD40 propeller and the central WDRP region of RBBP5 are well conserved across species (Supplementary Figure S5A). In contrast, the C-terminal distal regions in different species are highly variable (Supplementary Figure S5B). Due to the absence of the CTD2 and CTD3 motifs in RBBP5 yeast orthologs (Supplementary Figure S5B), the
internal interaction between WD40 and CTD is not necessarily conserved in yeast species. The CTD2 motif that wraps around RBBP5-WD40 is specific for animal and plant species. The CTD3 motif involved in nucleosome recognition is exclusively found in animal species. It is worth noting that the differences in CTD3 motifs are closely correlated with the complexity of the animal species. For example, C. elegans RBBP5 has a very short CTD3, while vertebrate RBBP5 has a much longer CTD3 with more positively charged residues. Differences in CTD regions strongly support the idea that RBBP5 becomes more complicated during evolution to accommodate its role in precise regulation of gene expression in higher organisms. How RBBP5 mediates functional specification of the KMT2-family methyltransferase complexes in different species awaits further investigation.

DATA AVAILABILITY

Coordinate and structure factor have been deposited in the Protein Data Bank under accession code 6KM7 (RBBP510–325-RBBP5390–480d). The SAXS data have been deposited in the Small Angle Scattering Biological Data Bank with the accession codes: SASDGD4, MLL1 complex with RBBP52–381; SASDGE4, MLL1 complex with RBBP510–325-RBBP5390–480d. The SAXS data have been deposited in the Small Angle Scattering Biological Data Bank with the accession code: 6KM7.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank staffs from the BL19U1 and BL19U2 beamlines of National Facility for Protein Science Shanghai (NFPS) at Shanghai Synchrotron Radiation Facility (SSRF) for X-ray crystal and SAXS data collection. We are grateful to the Mass Spectrometry staffs of NFPS, Zhangjiang Lab, China for their instrumental support and technical assistance in CX-MS analysis. We thank the staff members of the Large-scale Protein Preparation System at NFPS for providing technical support. We also thank Shu Quan for the conceptual advice and Jun C. Mencius for the critical reading of the manuscript.

FUNDING

Strategic Priority Research Program of the Chinese Academy of Sciences [XDB08010201 to Y.C.]; National Natural Science Foundation of China [31470737, 31670748 to Y.C.]. Funding for open access charge: National Natural Science Foundation of China.

Conflict of interest statement. None declared.

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