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Wdr82 Is a C-Terminal Domain-Binding Protein That Recruits the Setd1A Histone H3-Lys4 Methyltransferase Complex to Transcription Start Sites of Transcribed Human Genes[▽]

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Histone H3-Lys4 trimethylation is associated with the transcription start site of transcribed genes, but the molecular mechanisms that control this distribution in mammals are unclear. The human Setd1A histone H3-Lys4 methyltransferase complex was found to physically associate with the RNA polymerase II large subunit. The Wdr82 component of the Setd1A complex interacts with the RNA recognition motif of Setd1A and additionally binds to the Ser5-phosphorylated C-terminal domain of RNA polymerase II, which is involved in initiation of transcription, but does not bind to an unphosphorylated or Ser2-phosphorylated C-terminal domain. Chromatin immunoprecipitation analysis revealed that Setd1A is localized near the transcription start site of expressed genes. Small interfering RNA-mediated depletion of Wdr82 leads to decreased Setd1A expression and occupancy at transcription start sites and reduced histone H3-Lys4 trimethylation at these sites. However, neither RNA polymerase II (RNAP II) occupancy nor target gene expression levels are altered following Wdr82 depletion. Hence, Wdr82 is required for the targeting of Setd1A-mediated histone H3-Lys4 trimethylation near transcription start sites via tethering to RNA polymerase II, an event that is a consequence of transcription initiation. These results suggest a model for how the mammalian RNAP II machinery is linked with histone H3-Lys4 histone methyltransferase complexes at transcriptionally active genes.

Eukaryotic gene expression can be regulated by modification of chromatin structure through the enzymatic control of covalent histone modifications. Methylation of histone H3 at lysine 4 is associated with an active chromatin structure that is permissive to transcription (24, 26, 42). The physiologic functions of histone H3-Lys4 methylation are largely elusive, but identification of proteins recognizing H3-Lys4 methylation marks address possible functions. For example, histone H3-Lys4 trimethylation is recognized by the plant homeodomain fingers of NURF, an ISWI-related remodeling factor, and ING2, which is associated with a histone deacetylase (39). The WD40 domain-containing Wdr5 protein recognizes histone H3-Lys4 and is found in mammalian Set1-like histone H3-Lys4 methyltransferase complexes (5, 40, 52). These findings suggest that histone H3-Lys4 methylation modulates chromatin structure by recruiting chromatin remodeling and histone-modifying activities. Recently, it was proposed that histone H3-Lys4 methylation in higher eukaryotes counters the generally repressive chromatin environment imposed by histone H3-Lys9 and H3-Lys27 methylation (2).

The C-terminal domain (CTD) of the RNA polymerase II (RNAP II) large subunit is comprised of 25 to 52 tandem copies of the consensus repeat heptad $Y_1S_2P_3T_4S_5P_6S_7$ and is evolutionarily conserved from yeast to humans (34). The phosphorylation state of the CTD dramatically changes as RNAP II progresses through the transcription cycle (7). RNAP II containing CTD phosphorylated at serine 5 (Ser5-P CTD) is localized near the transcription start site and is associated with transcription initiation, whereas RNAP II containing CTD phosphorylated at Ser2 (Ser2-P CTD) is localized throughout the coding region of genes and is associated with the elongation process (29). The CTD serves as a scaffold to recruit various CTD-binding factors during the transcription cycle and plays a major role in coordinating pre-mRNA processing (34). Recent reports indicate that chromatin modification of transcribed genes is regulated by CTD phosphorylation (45). Epigenome mapping in *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and humans showed that histone H3-Lys4 trimethylation at the transcription start site of transcribed genes is strongly correlated with RNAP II containing Ser5-P CTD (1, 35, 43). Indeed, the yeast Set1/COMPASS histone methyltransferase complex associates with RNAP II containing Ser5-P CTD (32), a recruitment that is mediated by the Paf1 complex (19). Similarly, RNAP II containing Ser2-P CTD interacts with the Set2 Rpb1-interacting domain of yeast Set2 and mediates H3-Lys36 methylation within the body of genes during the elongation process (6, 12, 16). The human MLL1 complex, a Set1-like histone H3-lysine 4 methyltransferase complex, interacts with RNAP II containing Ser5-P CTD and associates with the transcription start site of transcribed genes, and MLL1 occupancy is strongly correlated with histone H3-Lys4 trimethylation (28). However, the molecular mechanism of this association is not known.

The yeast Set1/COMPASS complex is the sole histone H3-Lys4 histone methyltransferase in that organism and consists of the catalytic Set1 protein and seven other noncatalytic proteins (27, 30, 38). A *Set1* null mutant lacks histone H3-Lys4 methylation, is viable with a slow-growth phenotype, and exhibits changes of expression in ribosomal DNA genes (3). In contrast, mammalian cells contain numerous factors that exhibit

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histone H3-Lys4 histone methyltransferase activity (13). Based on homology with the yeast Set1 protein, six mammalian Set1-like factors have been identified, including Setd1A (formerly known as Set1A), Setd1B (formerly known as Set1B), MLL1, MLL2, MLL3, and MLL4 (10, 13, 21, 22). Mammalian Set1-like complexes are widely expressed and provide nonredundant functions in vivo. For example, chromosomal translocations involving the gene encoding MLL1 are frequently found in leukemia (8), and genetic disruption of the murine *MLL1* or *MLL2* gene leads to embryonic lethality (13, 55). Lastly, confocal microscopy has revealed that the human Setd1A and Setd1B proteins exhibit a largely nonoverlapping subnuclear distribution, strongly suggesting that they localize at distinct subsets of target genes (22). It is thus likely that the complexity of histone H3-Lys4 histone methyltransferases found in mammalian cells reflects the greater complexity of chromatin regulation required to control intricate developmental programs.

The human Setd1A and Setd1B histone methyltransferase complexes are identical, with the exception of the identity of the catalytic component (21, 22). Each complex contains six human homologues of the yeast Set1/COMPASS complex, including Setd1A or Setd1B, Ash2 (homologous to yeast Bre2), CXXC finger protein 1 (CFP1; homologous to yeast Spp1), Rbbp5 (homologous to yeast Swd1), Wdr5 (homologous to yeast Swd3), and Wdr82 (homologous to yeast Swd2). Human Setd1A and Setd1B exhibit extensive homology with yeast Set1, including an N-terminal RNA recognition motif (RRM), an N-SET domain, and a C-terminal catalytic SET domain. All of the mammalian Set1-like complexes contain Ash2, Rbbp5, Wdr5, and a Set1-like enzyme. These four components constitute the minimal fully active MLL1 histone methyltransferase complex in vitro (10). In contrast, the CFP1 and Wdr82 subunits of the human Setd1A and Setd1B complexes, which are homologues of yeast Set1/COMPASS components, have not been detected in the other mammalian Set1-like histone methyltransferase complexes. These observations led us to conclude that the human Setd1A and Setd1B complexes are analogous to the yeast Set1/COMPASS complex (21, 22).

Here, we show that the human Setd1A complex associates with RNAP II containing Ser5-P CTD. In contrast to the yeast Set1/COMPASS complex, which requires Paf1 for interaction with RNAP II, the human Setd1A complex is tethered to RNAP II by Wdr82, an integral component of the Setd1A complex. Wdr82 associates with the RRM domain of Setd1A and directly recognizes Ser5-P CTD. Depletion of Wdr82 decreases Setd1A expression and decreases occupancy of the Setd1A complex and histone H3-Lys4 trimethylation near the transcription start site of transcribed genes. However, depletion of Wdr82 does not alter the occupancy of RNAP II containing Ser5-P CTD at these sites or the expression levels of these target genes. These results indicate that CTD phosphorylation regulates histone H3-Lys4 methylation near the transcription start site in mammals and suggest a model for how the RNAP II machinery is linked with histone H3-Lys4 histone methyltransferase complexes at transcriptionally active genes.

MATERIALS AND METHODS

Plasmids and cell lines. Expression vectors carrying human Setd1A or Wdr82 cDNAs were prepared using the pcDNA5/TO vector (Invitrogen), which carries an amino-terminal FLAG epitope, as previously described (21). Inducible T-REx HEK293 cells carrying each expression construct were established and maintained as previously described (22).

To establish doxycycline-inducible Wdr82 knockdown cells, the multi-microRNA (multi-miRNA) hairpin method described by Sun et al. (46) was performed with slight modifications. A 118-nucleotide modified miR-30 hairpin that targets Wdr82 was amplified by PCR, using a partial-length single-strand template sequence and overlapping PCR primers. Two pairs of primers were utilized in parallel reactions to produce PCR fragments with distinct restriction enzyme sites at their ends to facilitate tandem cloning into the pcDNA5/TO vector that contains a C-terminal green fluorescent protein cDNA. The Wdr82 template sequence was 5′-TGCTGTTGACAGTGAGCGCGGACAAGATTAACTGCTTCGATAGTGAAGCCACAGATGTATCGAAGCAGTTAATCTTGTCCGTGCCTACTGCCTCGGA-3′ (the sequence that will produce shRNA is underlined). PCR primer sequences were as follows: HindFor1, 5′-GCTAAGCTTGATCCAAGAAGGTATATTGCTGTTGACAGTGAGCG-3′; EcoRI-Rev1, 5′-CTAGAATTCATCGTAGCCCTTGAAGTCCGAGGCAGTAGGCA-3′; EcoRI-For2, 5′-GCTGAATTCGATCCAAGAAGGTATATTGCTGTTGACAGTGAGCG-3′; BamHI-Rev2, 5′CTAGGATCCATCGTAGCCCTTGAAGTCCGAGGCAGTAGGCA-3′ (restriction enzyme sites are underlined). Scrambled miR-30 hairpin was similarly prepared as a negative control, using the template sequence 5′-TGCTGTTGACAGTGAGCGCCAGCTTGACGAGTAGACTATATAGTGAAGCCACAGATGTATATAGTCTACTCGTCAAGCTGGTGCCTACTGCCTCGGA-3′. T-REx HEK293 cells were transfected, selected, and maintained as described elsewhere (22).

Immunoprecipitation and Western blotting analysis. Preparation of nuclear extracts, immunoprecipitation, and Western blot analysis were performed as previously described (21). Anti-FLAG antibody was obtained from Sigma. Anti-Ash2, anti-Rbbp5, anti-Paf1, and anti-MLL1 antisera were obtained from Bethyl Laboratories. Anti-Brg1 and anti-RNAP II large subunit (RPB1) antisera were obtained from Santa Cruz Biotechnology. Anti-Ser5-P CTD (monoclonal H14), anti-Ser2-P CTD (monoclonal H5), and anti-unphosphorylated CTD (monoclonal 8WG16) antibodies were obtained from Covance Ltd. Anti-RNAP II small subunit (RPB2) antiserum was obtained from Aviva System Biology. Anti-HCF1 antiserum was a gift from Winship Herr (Cold Spring Harbor Laboratory). Production of anti-Setd1A, anti-Wdr82, anti-CFP1, anti-Wdr5, and anti-Setd1B antisera was previously described (22).

Purification of GST-RRM and FLAG-Wdr82. The cDNA encoding the RRM domain of human Setd1A was subcloned into the pGEX 4T vector (Pharmacia Ltd.), and constructs were transformed into *Escherichia coli* BL21 cells. Transformants were grown and induced by 0.2 mM isopropyl-β-D-thiogalactoside for 4 h at 25°C. Glutathione *S*-transferase (GST)–RRM fusion protein was purified using glutathione-agarose affinity beads, and purified proteins were dialyzed with phosphate-buffered saline containing 10% glycerol. A FLAG-Wdr82 cDNA fragment derived from a pcDNA3 FLAG-Wdr82 vector was subcloned into the pFASTBAC vector (Life Technologies, Inc.). Generation of recombinant baculovirus and expression of recombinant FLAG-Wdr82 in Sf9 cells were performed according to the manufacturer's instructions. For the purification of recombinant FLAG-Wdr82, Sf9 cells were harvested and resuspended in 10 ml of lysis buffer [10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid, pH 7.0, 300 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 0.5% Triton X-100]. Cells were homogenized using a Dounce homogenizer, and lysates were centrifuged. Supernatants were incubated with anti-FLAG M2 agarose beads for 4 h at 4°C, and beads were extensively washed. Bound proteins were eluted with 250 μg/ml FLAG peptide (Sigma) and dialyzed against phosphate-buffered saline containing 10% glycerol.

In vitro pull-down assays. In vitro CTD phosphorylation was performed using purified human CTD or GST-CTD (Proteinone, Bethesda, MD) and MAPK2 kinase (New England Biolabs), as described previously (25). For GST pull-down assays, purified proteins were incubated in 1 ml of pull-down buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.2% NP-40, and 0.5% deoxycholate) for 2 h, and glutathione-agarose beads were added and incubated at 4°C for 2 h. Beads were extensively washed with pull-down buffer, and bound proteins were denatured by sodium dodecyl sulfate (SDS) sample buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting or Coomassie staining. For FLAG pull-down assays, anti-FLAG M2 agarose beads were used and reactions were performed as described above. For peptide pull-down assays, biotin-labeled CTD peptides containing various patterns of phosphorylation were synthesized and purified by Anaphase Ltd. as described previously (17). Purified proteins were incubated with 0.5 μg of each purified peptide, captured using streptavidin-agarose (Invitrogen Inc.), and extensively washed. Bound proteins were analyzed as described above.

ChIP. Chromatin immunoprecipitation (ChIP) assays were performed as previously described with slight modifications (20, 29). Cells were fixed, lysed, and soni-

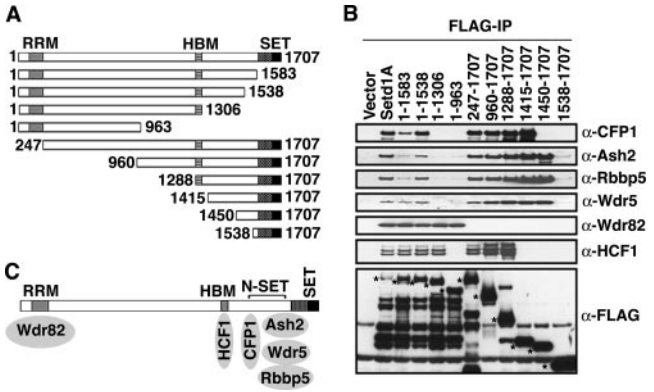

FIG. 1. Protein-protein interactions within the human Setd1A histone methyltransferase complex. A. Diagram of Setd1A expression constructs. N-SET, N-terminal region of the SET domain; SET, catalytic SET histone methyltransferase domain. Numbers indicate amino acid residues of the human Setd1A protein. All constructs contain an amino-terminal FLAG epitope. B. Inducible T-REx HEK293 cell lines that express various FLAG-tagged human Setd1A fragments were induced for 3 days with 0.5 μg/ml doxycycline. Nuclear extracts were prepared and immunoprecipitated by FLAG-IgG agarose beads, and immunoprecipitates were analyzed by Western blotting using the indicated antisera. Asterisks in the FLAG Western blot indicate the expected size of each FLAG-tagged protein. C. Summary of protein-protein interactions within the human Setd1A complex.

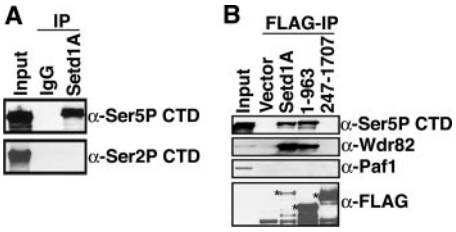

FIG. 2. Setd1A interacts with RNAP II through its RRM domain. A. Nuclear extracts from HEK293 cells were immunoprecipitated as described in Materials and Methods, and immunoprecipitates were analyzed by Western blotting using the indicated phosphorylation-specific CTD antibodies. B. Inducible cell lines that express various human FLAG-tagged Setd1A fragments were induced with doxycycline. Nuclear extracts were prepared, immunoprecipitated, and analyzed by Western blotting using the indicated antisera.

cated. Sonicated lysates equivalent to 4×10^6 cells were subjected to ChIP analysis using a ChIP assay kit (Upstate Biotechnology) following the manufacturer's instructions. ChIP products were analyzed by quantitative (real-time) PCR using Taqman probes or Cybergreen dye with an Applied Biosystems 7500 real-time PCR system. The comparative ΔC_T method was used to determine relative expression compared with input, which was then averaged over three independent experiments. PCR primer sequences were as follows: PPIA-0.5kb-For, 5′-GCGCGAGACCGGGTT-3′; PPIA-0.5kb-Rev, 5′-GACGGCAATGTCGAAGAACAC-3′; PPIA-5kb-For, 5′-GCTCGCAGTATCCTAGAATCTTTGT-3′; PPIA-5kb-Rev, 5′-GGAGGGAACAAGGAAAACATGGAA-3′; PABPC1-0.5kb-For, 5′-CAGCGGCAGTGGATCGA-3′; PABPC1-0.5kb-Rev, 5′-GGACAAAAATCAACCGGAATTG-3′; PABPC1-23kb-For, 5′-CATGG GTGGAGCTGGTCAAT-3′; PABPC1-23kb-Rev, 5′-TCCTAGCAGAGATCCATGCAGAT-3′; GAPDH-0.5kb-For, 5′-AGAAGTTCCCCAACTTTCCCGCCT-3′; GAPDH-0.5kb-Rev, 5′-ACACTAGGGAGTCAAGGACGGGGA-3′; GAPDH-4kb-For, 5′-AAGAGCACAAGAGGAAGAGAGAGA-3′; GAPDH-4kb-Rev, 5′-GGGTCTACATGGCAACTGTGA-3′; CD4-0.5kb-For, 5′-TGTGCTCTGCCCAGTTGTCT-3′; CD4-0.5kb-Rev, 5′-GCTCATGACCAGTTCCAAGAGAA-3′; Synapsin II-0.5kb-For, 5′-AGAAGTACATACTGTTGTTGTCCC-3′; Synapsin II-0.5kb-Rev, 5′-AGAAATACCTCTTCTAGGGAGGAA-3′; HOXC8-For, 5′-GGTACTCGTGAGCCAGAGG-3′; HOXC8-Rev, 5′-GACGAAGTAGGAGCTCATGCT-3′. Taqman probes were as follows: PPIA-0.5 kb-FAM probe, 5′-CTCGTGCCGTTTTGCA-3′; HOXC8-pro-FAM probe, 5′-CCGCGGGTTTTCAT-3′. Anti-H3K4me1 and anti-H3K4me3 antisera were obtained from Abcam, and anti-H3K4me2 antiserum was obtained from Upstate Biotechnology.

RT-PCR. First-strand cDNA template was synthesized as previously described (20). cDNA was analyzed using a Taqman gene expression assay kit following the manufacturer's instructions (Applied Biosystems). rRNA 18S transcripts were used as an internal control to normalize RNAP II-driven PPIA and PABPC1 transcripts. Taqman probes were as follows: 18S-FAM probe, 5′-CATTGGAGGGCAAGTCTGGTGCCAG-3′; PPIA-FAM probe, 5′-CATCTGCACTGCCAAGACTGAGTGG-3′; PABPC1-FAM probe, 5′-TGGTGCTCCGGGCTCGGAACACAC-3′.

RESULTS

Protein-protein interactions within the human Setd1A histone methyltransferase complex. Protein-protein interactions between Setd1A and the other components of the histone methyltransferase complex were investigated to gain insights into the functional significance of individual Setd1A complex components. Various deletion fragments of Setd1A containing an N-terminal FLAG epitope were expressed in inducible T-REx HEK293 cells (Fig. 1). Nuclear extracts were prepared and subjected to FLAG immunoprecipitation and Western blotting analysis as previously described (21). The common components of mammalian Set1-like histone methyltransferase complexes, such as Ash2, Rbbp5, and Wdr5, interact with the region of amino acids 1450 to 1537 of Setd1A, which is highly conserved in all Set1-like histone methyltransferase complexes. This region of Setd1A includes a portion of the N-SET domain and the catalytic SET domain. These results are consistent with previous reports for the human MLL1 complex (10, 54). CFP1 interacts with the N-terminal portion of the N-SET domain of Setd1A (amino acid residues 1415 to 1450). Unexpectedly, each of these factors interacts more weakly with the amino acids 1 to 1583 Setd1A construct compared to the amino acid 1 to 1538 construct, suggesting perhaps that the slightly longer Setd1A fragment does not fold normally. Host cell factor 1 (HCF1) interacts with the HCF1 binding motif (HBM) domain as previously reported (51). In contrast, Wdr82 interacts with all C-terminal Setd1A deletion mutants but does not interact with a fragment lacking the N-terminal 246 amino acids that includes the RRM domain of Setd1A. The CFP1-interacting region and the RRM domain are highly conserved in Setd1A and Setd1B but not in other mammalian Set1-like proteins.

Human Setd1A associates with initiating RNAP II through its RRM domain. Because the yeast Set1/COMPASS complex associates with RNAP II (32), we asked whether human Setd1A is also associated with RNAP II. Nuclear extracts from HEK293 cells were subjected to immunoprecipitation by anti-Setd1A antiserum and analyzed by Western blotting using monoclonal antibodies H14 (specific to Ser5-P CTD) and H5 (specific to Ser2-P CTD). As shown in Fig. 2A, Setd1A associates with RNAP II containing Ser5-P CTD, a form of the polymerase that is implicated in transcription initiation. However, Setd1A is not associated with RNAP II containing Ser2-P CTD, which is associated with transcription elongation. Deletion mutants of Setd1A were similarly analyzed to further investigate the association of Setd1A with RNAP II (Fig. 2B). Consistent with the results in Fig. 2A, full-length Setd1A in-

teracts with RNAP II containing Ser5-P CTD. Similar results were obtained with the fragment with amino acids 1 to 963 of Setd1A, which lacks all of the histone methyltransferase complex component interaction domains except for the RRM domain. In contrast, the amino acid 247 to 1707 fragment of Setd1A does not interact with RNAP II. This fragment of Setd1A retains all complex component interaction domains with the exception of the RRM domain (Fig. 1B). Consistent with the results presented in Fig. 1, the full-length protein and the fragment with amino acids 1 to 963 of Setd1A interact with Wdr82, but the fragment with amino acids 247 to 1707 of Setd1A fails to interact with Wdr82. These results indicate that the RRM domain is necessary for interaction between Setd1A and the initiating form of RNAP II. Because the Paf1 complex is implicated in the recruitment of the yeast Set1/COMPASS complex to RNAP II (19), we also examined the possibility that the human Paf1 complex mediates interaction between Setd1A and RNAP II. However, Setd1A fails to coimmunoprecipitate Paf1.

Wdr82-dependent interaction between the Setd1A RRM domain and Ser5-P CTD in vitro. In vitro pull-down assays using purified components were performed to determine whether Wdr82 directly interacts with the RRM domain of Setd1A. Purified FLAG-Wdr82 was pulled down by GST-RRM in vitro, but not by GST (Fig. 3A). Similar results were obtained with a reciprocal FLAG pull-down assay. These results indicate that the RRM domain of Setd1A is sufficient to directly interact with Wdr82.

Additional studies were performed to assess whether Wdr82 interacts with the CTD of RNAP II. Biotin-labeled peptides were synthesized to contain three tandem repeats of the consensus CTD motif containing either Ser2-P or Ser5-P (or unphosphorylated). Peptides were incubated with FLAG-Wdr82 and GST-RRM and analyzed in a peptide pull-down assay with streptavidin-agarose (17). The biotin-labeled CTD peptide that contains Ser5-P pulls down both FLAG-Wdr82 and GST-RRM, but the unmodified peptide and the peptide that contains Ser2-P fail to interact with FLAG-Wdr82 and GST-RRM (Fig. 3B). As expected, Ser5P-CTD directly interacts with FLAG-Wdr82 but fails to directly interact with GST-RRM. However, addition of GST-RRM significantly increases the affinity of FLAG-Wdr82 for Ser5-P CTD. This indicates that Ser5-P CTD of RNAP II, Wdr82, and the RRM of Setd1A form a ternary complex and that three consensus C-terminal repeats containing phosphorylation at the Ser5 residue are sufficient for this interaction. Furthermore, this interaction occurs in the absence of Paf1, consistent with the failure to detect Paf1 in FLAG-Setd1A coimmunoprecipitations (Fig. 2B).

MAPK2, which preferentially phosphorylates the Ser5 residue of CTD repeats (25), was used to phosphorylate purified human CTD in vitro. FLAG-Wdr82, GST-RRM, and MAPK2-treated CTD were incubated in various combinations and analyzed by GST pull-down assay to investigate the physical arrangement of Setd1A, Wdr82, and CTD within the ternary complex (Fig. 3C). GST-RRM alone does not interact with CTD but instead interacts with CTD in the presence of FLAG-Wdr82, indicating that Wdr82 mediates the interaction between Setd1A and RNAP II. To further investigate the interrelationship between component interactions, proteins were incubated in various combinations and analyzed by FLAG pull-down assay (Fig. 3D). The interaction between GST-RRM and FLAG-Wdr82 was not affected by the presence of CTD (Fig. 3D, lower panel). However, consistent with results presented in Fig. 3B, the interaction between Wdr82 and CTD is greatly increased in the presence of GST-RRM (Fig. 3D, upper panel). These results indicate that the binding of the Setd1A RRM domain to Wdr82 modulates the binding affinity of Wdr82 to the Ser5-P CTD of RNAP II.

Wdr82 and Setd1A are associated with the transcription start site of actively transcribed genes. Because RNAP II containing Ser5-P CTD is associated with the transcription start site of transcribed genes (1, 35, 43), we asked whether Setd1A and Wdr82 are also localized near the transcription start site of transcribed genes. *PPIA* (peptidylprolyl isomerase A), *PABPC1* [poly(A) binding protein, cytoplasmic 1]), and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) are housekeeping genes and are transcribed by RNAP II, while *CD4* and *SYNAPSIN II* are lineage-restricted genes that are not expressed in HEK293 cells. PCR primers for ChIP analysis were designed approximately 500 bp downstream of each transcription start site and additionally within the 3′ region of each housekeeping gene (Fig. 4). Primers were also designed for the promoter region of the *HOXC8* gene, which is a target of the MLL1 histone methyltransferase complex (52). Consistent with previous reports (29, 49), RNAP II containing Ser5-P CTD and histone H3-Lys4 trimethylation were localized near the transcription start site of the *PPIA*, *PABPC1*, and *GAPDH* genes, and RNAP II containing Ser2-P CTD was associated with the internal regions of each gene (Fig. 4). Neither the phosphorylated form of RNAP II nor histone H3-Lys4 trimethylation was apparent near the transcription start site of the nonexpressed *CD4* and *SYNAPSIN II* genes. Similar to RNAP II containing Ser5 CTD and histone H3-Lys4 trimethylation, Setd1A and Wdr82 proteins were both found near the transcription start site of the expressed genes. Interestingly, Setd1A and Wdr82 are not associated with the promoter region of the *HOXC8* gene, despite the presence of histone H3-Lys4 trimethylation, indicating that this MLL1 target gene is not bound by Setd1A, thus providing further evidence that Wdr82 is not a component of the MLL1 histone methyltransferase complex.

Depletion of Wdr82 leads to decreased histone H3-Lys4 trimethylation and decreased occupancy of the Setd1A complex near the transcription start site of target genes. Tandem miRNA hairpins specific for the Wdr82 transcript were expressed under control of the tetracycline repressor in T-REx HEK293 cells (Fig. 5A). Cells stably transfected with pcDNA5/TO 2x miRNAs were induced by 1 μg/ml doxycycline for 5 days, and proteins were analyzed by Western blotting. The level of Wdr82 protein does not change upon induction in cells stably transfected with empty vector or scrambled miRNAs, but two independent clones stably transfected with Wdr82-specific miRNA showed an approximately 80% decrease of Wdr82 protein upon induction (Fig. 5B). Depletion of Wdr82 also led to reduced levels of the Setd1A and CFP1 components of the Setd1A histone methyltransferase complex but did not affect levels of Ash2 and Rbbp5, which are found in all mammalian Set1-like histone methyltransferase complexes. Interestingly, it was previously reported that the yeast Set1/COMPASS complex is less stable in an *Swd2* (Wdr82 homologue) mutant strain of *Schizosaccharomyces pombe* and

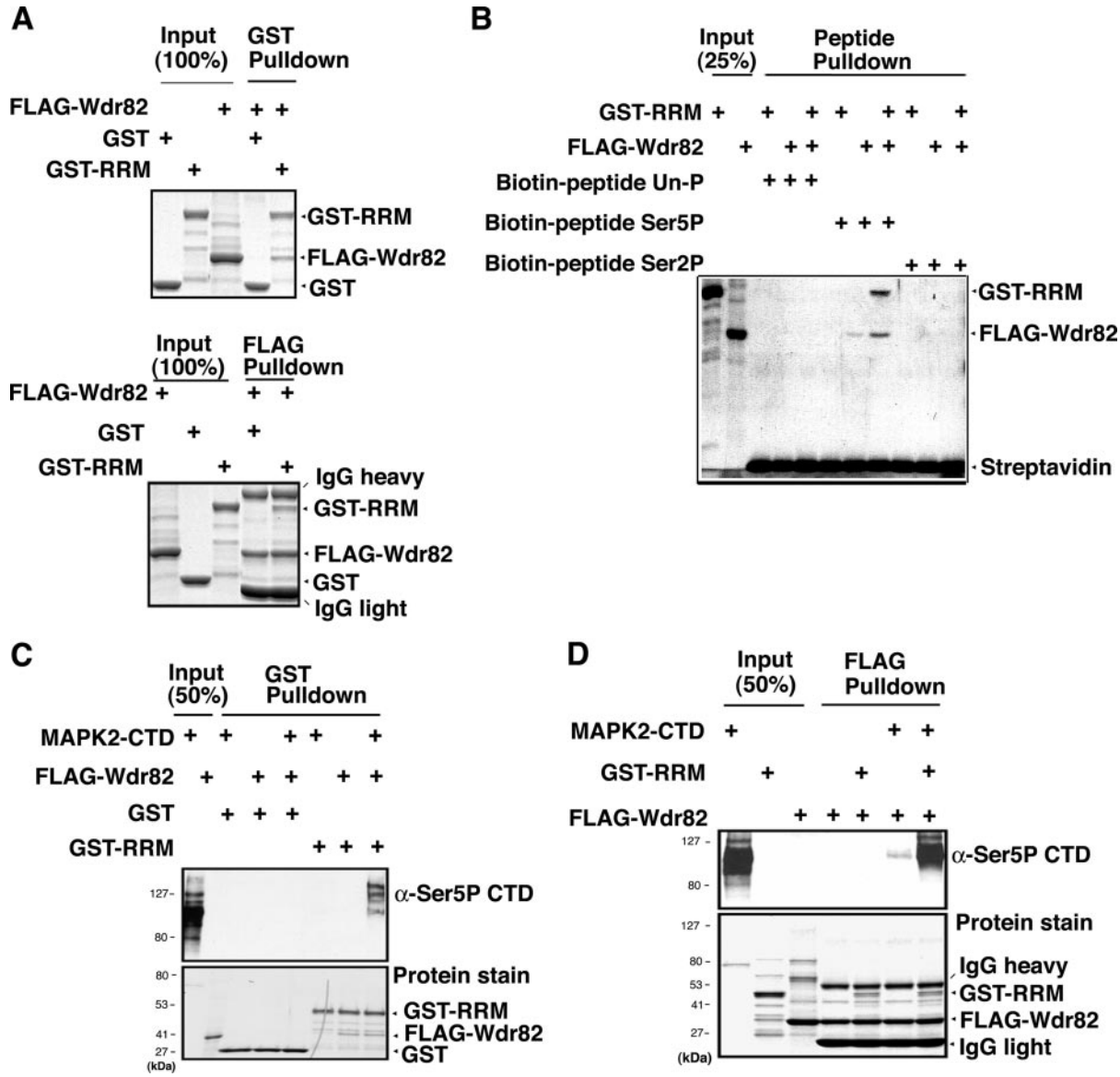

FIG. 3. The Setd1A interaction with Ser5-P CTD is mediated by Wdr82 in vitro. A. The GST-Setd1A RRM domain and FLAG-Wdr82 were purified from *E. coli* and insect cells, respectively. Purified proteins were used for in vitro GST (top panel) and FLAG (bottom panel) pull-down assays. Proteins were separated by SDS-PAGE and detected by Coomassie staining. Arrows indicate identities of protein bands. B. Biotin-labeled CTD peptides containing various phosphorylation states were synthesized and used for in vitro pull-down assays. Peptides were incubated with purified FLAG-Wdr82 and GST-RRM and captured using streptavidin-agarose. Bound proteins were denatured by SDS sample buffer, separated by SDS-PAGE, and visualized by Coomassie staining. C. Purified GST-RRM, FLAG-Wdr82, and MAPK2-treated full-length CTD were used for in vitro GST pull-down assays. Purified GST was used as a negative control. Proteins were separated by SDS-PAGE and analyzed by Western blotting (top) and Coomassie staining (bottom). D. Purified GST-RRM, FLAG-Wdr82, and MAPK2-treated CTD were used for in vitro FLAG pull-down assays. Proteins were separated by SDS-PAGE and analyzed by Western blotting (top) and Coomassie staining (bottom).

that a mutated Set1 protein lacking the RRM domain is less stable in *Saccharomyces cerevisiae* (9, 11). The results reported here indicate that the interaction of Wdr82 with the RRM domain of Setd1A regulates the steady-state level of the Setd1A histone methyltransferase complex in mammalian cells.

Because Setd1A, Wdr82, and RNAP II containing Ser5-P CTD are localized near the transcription start site of active human genes, ChIP and real-time PCR were utilized to analyze histone H3-Lys4 methylation levels in Wdr82-depleted cells. Cells stably transfected with scrambled miRNAs did not show any differences in the level of histone H3-Lys4 methylation (data not shown). Cells that were stably transfected with Wdr82-specific miRNAs showed dramatic increases of histone H3-Lys4 monomethylation and significant decreases of histone H3-Lys4 di- and trimethylation near the transcription start site of transcribed genes (Fig. 5C). In contrast, the *HOXC8* gene locus, a target of the MLL1 histone methyltransferase complex but not the Setd1A histone methyltransferase complex, does not show significant changes of histone H3-Lys4 methylation upon depletion of Wdr82 (Fig. 5C). Because a decreased state-

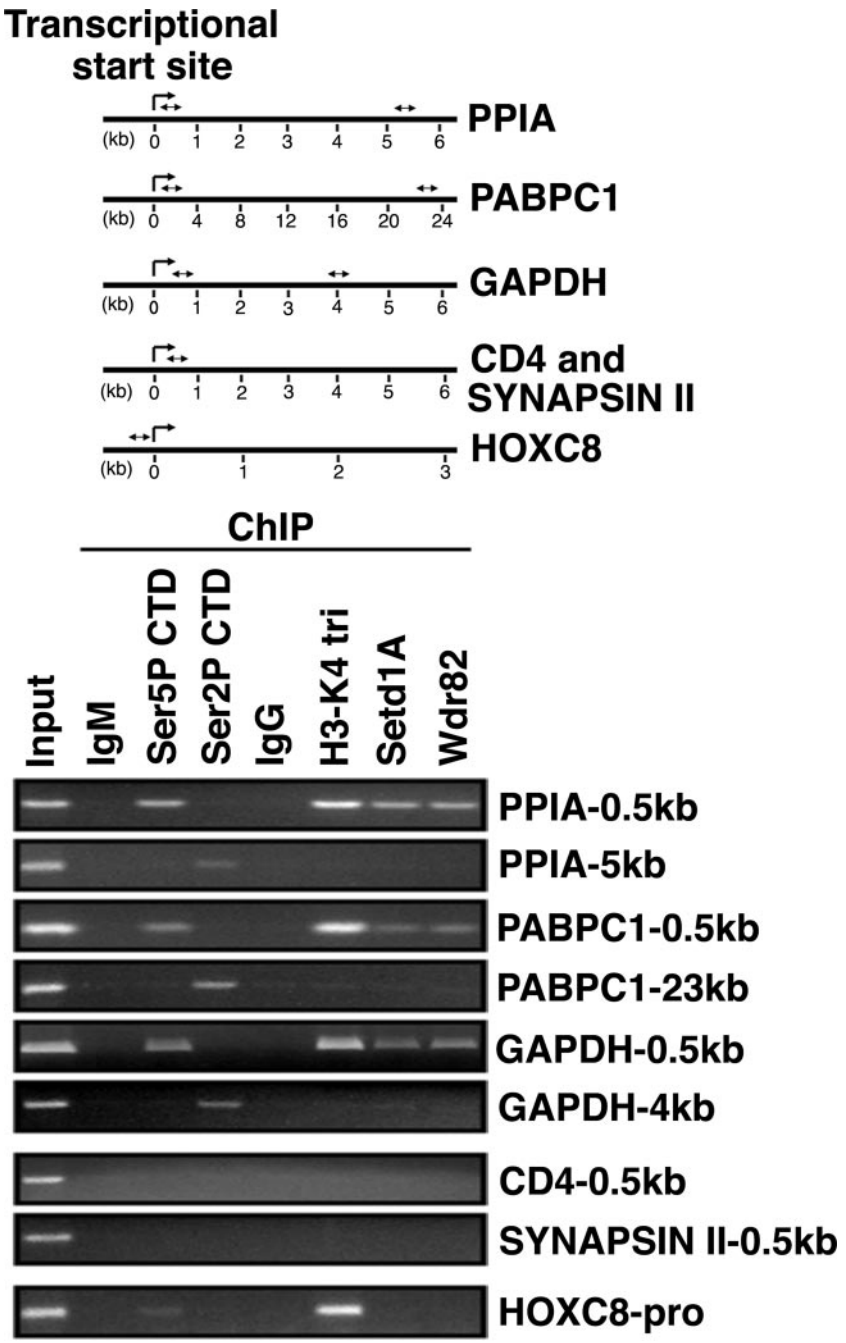

FIG. 4. The Setd1A complex is associated with the transcription start site of active genes. HEK293 cells were fixed and sonicated, and soluble chromatin was immunoprecipitated by the indicated antibodies as described in Materials and Methods. ChIP products were analyzed by PCR and agarose gel electrophoresis. The housekeeping genes *PABPC*, *PPIA*, and *GAPDH* were chosen as genes that are actively transcribed by RNAP II. In contrast, the *CD4* and *Synapsin II* genes were chosen as inactive genes that are not expressed in HEK293 cells. The *HOXC8* gene is a target of the MLL1 histone methyltransferase complex. PCR primers were designed to amplify 500-bp regions immediately downstream of each transcription start site (and within the *HOXC8* gene promoter) and additionally within the 3′ coding regions of the *PPIA*, *PABPC*, and *GAPDH* genes. Locations of primers within each gene locus are shown in the diagram as double-sided arrows.

steady level of the Setd1A complex was observed in Wdr82-depleted cells, we investigated whether loss of Wdr82 affects the occupancy of the Setd1A complex and RNAP II near the transcription start site. Occupancy levels of Wdr82, Setd1A, and Rbbp5 were decreased by 50% to 60% upon depletion of Wdr82 (Fig. 5D). Surprisingly, however, occupancy of RNAP II containing Ser5-P CTD was not changed. Reverse transcription and real-time PCR were performed to investigate the transcriptional activity of the *PPIA* and *PABPC1* genes in Wdr82-depleted cells. Consistent with the persistence of RNAP II occupancy at these loci, transcript levels for PPIA and PABPC1 were unchanged compared with untreated cells

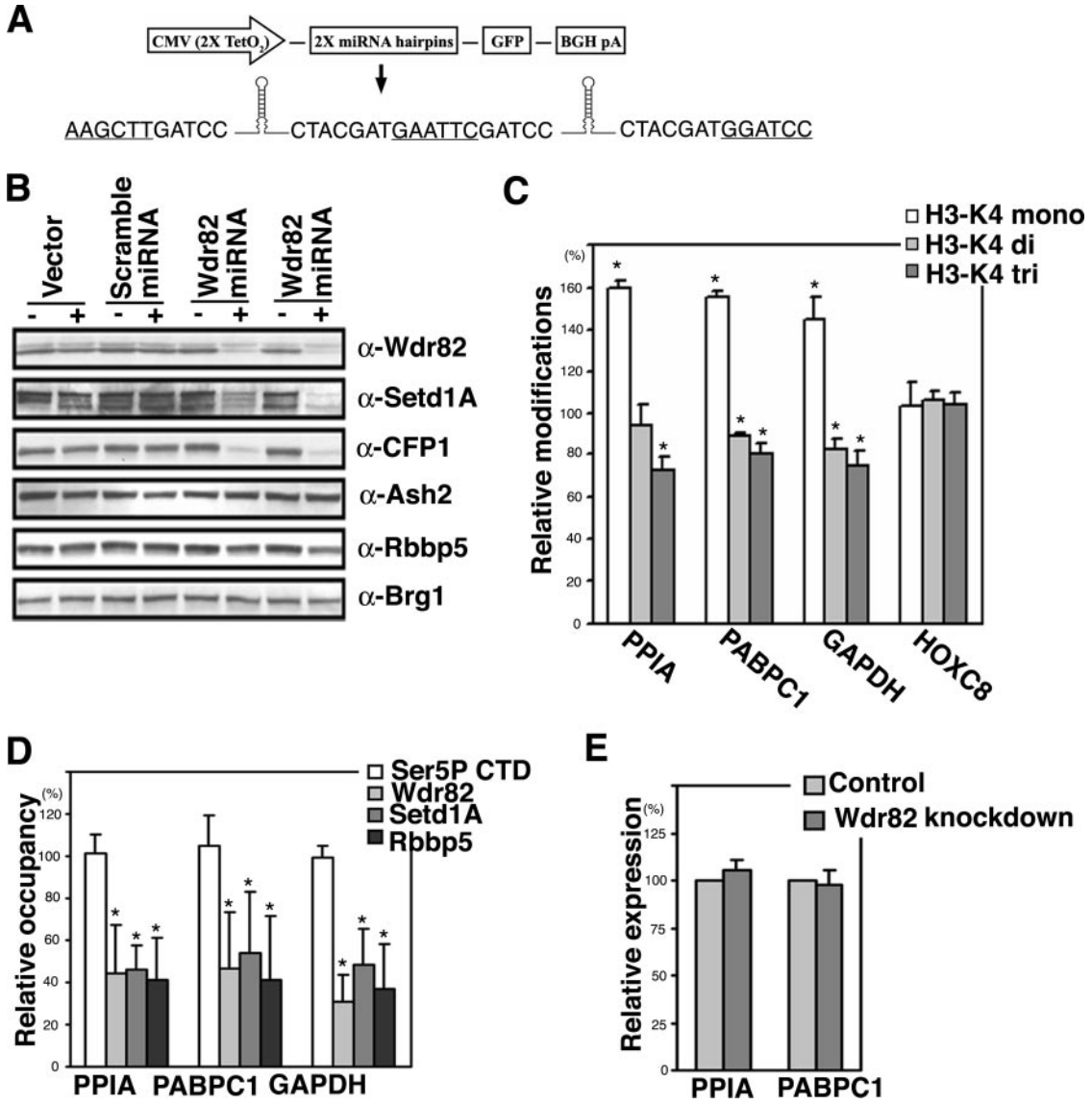

FIG. 5. Depletion of Wdr82 decreases Setd1A occupancy and histone H3-Lys4 trimethylation near the transcription start site. A. Diagram of the inducible Wdr82 miRNA construct. Two 118-nt modified miR-30 hairpins for Wdr82 were tandemly subcloned into the pcDNA5/TO vector, which contains a C-terminal green fluorescent protein cDNA and is expressed under control of a tetracycline operator. Restriction enzyme sites used for subcloning are underlined. B. Inducible T-REx HEK293 cell lines that express tandem miRNA of human Wdr82, tandem scramble miRNA, or vector only were induced for 5 days with 1 μg/ml doxycycline. Nuclear extracts were prepared and analyzed by Western blotting using the indicated antisera. The results are shown for two independent clones that express the tandem miRNA of human Wdr82. C. Cell lines that express the tandem miRNA of Wdr82 were induced for 5 days with 1 μg/ml doxycycline. Histone H3-Lys4 methylation near the transcription start site of the *PPIA*, *PABPC1*, and *GAPDH* genes and the promoter region of the *HOXC8* gene were analyzed by ChIP and real-time PCR. The level of histone H3-Lys4 methylation in uninduced cells was set at 100%. The data represent a summary of three independent experiments. Error bars indicate the mean standard deviations, and *P* values were determined by a standard *t* test. Asterisks indicate *P* values of <0.05. D. Cell lines that express the tandem miRNA for Wdr82 were induced for 5 days with 1 μg/ml doxycycline. Occupancy by RNAP II containing Ser5-P CTD, Wdr82, Setd1A, and Rbbp5 near the transcription start sites of the indicated genes was analyzed by ChIP and real-time PCR. Occupancy in uninduced cells was set at 100%. The data represent a summary of three independent experiments. Error bars indicate the mean standard deviations, and *P* values were determined by a standard *t* test. Asterisks indicate *P* values of <0.05. E. Cell lines that express tandem miRNA for Wdr82 were induced for 5 days with 1 μg/ml doxycycline. Transcripts were analyzed by reverse transcription and real-time PCR. PPIA and PABPC1 transcripts were normalized to 18S transcripts that are produced by RNA polymerase I. The level of transcripts present in uninduced cells was set at 100%. The data represent a summary of three independent experiments. Error bars indicate the mean standard deviations, and *P* values were determined by a standard *t* test.

(Fig. 5E). These results indicate that Wdr82 is required for histone H3-Lys4 trimethylation and the occupancy of the Setd1A histone methyltransferase complex near the transcription start site of transcribed genes and that depletion of Wdr82 does not affect the occupancy of RNAPII nor the transcriptional activity of these loci.

Wdr82 associates with Setd1A and Setd1B, but not MLL1. Wdr82 carries seven WD40 domains, which serve as β-propel-

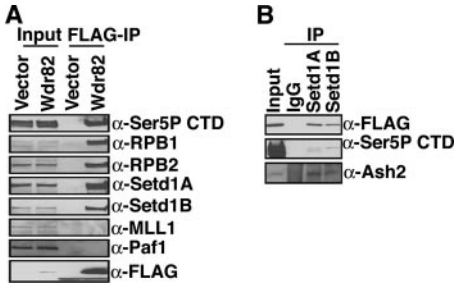

FIG. 6. Wdr82 associates with a subset of mammalian Set1-like proteins. A. Inducible cell lines that express FLAG-Wdr82 or empty vector were induced for 3 days with 0.5 μg/ml doxycycline. Nuclear extracts were prepared and immunoprecipitated with FLAG-IgG agarose beads, and immunoprecipitates were analyzed by Western blotting using the indicated antisera. B. Nuclear extracts from HEK293 cells carrying FLAG-Wdr82 were immunoprecipitated as described in Materials and Methods. The immunoprecipitates were analyzed by Western blotting using the indicated antisera.

ler-like platforms to which proteins can bind (44, 53). WD40 domains are found in a large family of proteins with diverse biological functions, including adaptor/regulatory modules in signal transduction, pre-mRNA processing, cytoskeleton assembly, and cell cycle control (23). Database analysis indicates that the Wdr82 protein is highly conserved from yeast to human (data not shown). Human Wdr82 exhibits 30% identity and 62% similarity to yeast Swd2 and 65% identity and 93% similarity to *Drosophila melanogaster* Wdr82. To investigate whether the Wdr82 protein is associated with other mammalian Set1-like proteins, FLAG-Wdr82-associated proteins were analyzed by coimmunoprecipitation (Fig. 6A). As expected, Wdr82 immunoprecipitates the RNAP II large and small subunits (RPB1 and RPB2). Consistent with previous reports (21, 22) and other data in this report, Wdr82 also associates with Setd1A and Setd1B. However, Wdr82 does not interact with MLL1, indicating that Wdr82-mediated tethering to RNAP II is not a universal mechanism for all mammalian histone H3-Lys4 histone methyltransferase complexes. Paf1, which is implicated in the recruitment of the yeast Set1/COMPASS complex to RNAP II, does not associate with Wdr82. This is consistent with the data presented in Fig. 2, which failed to detect a Paf1 interaction with Setd1A. We also tested whether the Setd1B histone methyltransferase complex is similarly associated with RNAP II containing Ser5-P CTD. As expected, both Setd1A and Setd1B interact with FLAG-Wdr82, RNAP II containing Ser5-P CTD, and the Ash2 component of the histone methyltransferase complexes (Fig. 6B). These results indicate that the mammalian Setd1A and Setd1B histone methyltransferase complexes associate with RNAP II containing Ser5-P CTD and strongly suggest that the Setd1B histone methyltransferase complex is similarly recruited to RNAP II via a Wdr82 interaction with its RRM domain.

DISCUSSION

Wdr82-mediated recruitment of the Setd1A histone methyltransferase complex to transcription start sites. Investigation of the yeast Set1/COMPASS complex and mammalian Set1-like histone H3-Lys4 methyltransferase complexes has provided insight into the mechanisms responsible for characteristic histone modifications found near the transcription start site. The yeast Set1/COMPASS complex associates with RNAP II containing Ser5-P CTD, which is implicated in the initiating and early elongation phases of transcription, thus generating the unique distribution of histone H3-Lys4 trimethylation immediately downstream of the transcription start site at actively transcribed genes (32). The yeast Paf1 complex is required for recruitment of the Set1/COMPASS histone methyltransferase complex to RNAP II. The subunits of these complexes interact both physically and genetically, and the interaction of these complexes is required for histone H3-Lys4 methylation (19).

Milne et al. (28) reported that the mammalian MLL1 complex physically interacts with RNAP II containing Ser5-P CTD and mediates histone H3-Lys4 methylation at a subset of transcriptionally active genes. Guenther et al. (14) also reported that MLL1 binds near the transcription start site of most RNAP II-occupied genes and that MLL1 occupancy is strongly correlated with histone H3-Lys4 trimethylation. The MLL2/ALR complex is also implicated in the histone H3-Lys4 trimethylation of promoters and transcription start sites of a subset of RNAP II-occupied genes, particularly those involved in the regulation of adhesion-related cytoskeletal events (18). Despite these observed associations, little is known regarding the detailed molecular mechanisms responsible for the targeting of mammalian Set1-like histone H3-Lys4 methyltransferase complexes near the transcription start site. It was recently reported, however, that the coactivator HCF1 is required for recruitment of Setd1A and MLL1 and generation of histone H3-Lys4 trimethylation at an HCF1-dependent promoter (31). In this context, it is interesting that the Setd1B protein lacks the HBM domain that serves as the HCF1 interaction domain and that the human Setd1A and Setd1B proteins exhibit a largely nonoverlapping subnuclear distribution as revealed by confocal microscopy (22), suggesting that HCF1 may participate generally in the gene-specific recruitment of some mammalian Set1-like enzymes.

This report demonstrates that RNAP II containing Ser5-P CTD physically associates with the human Setd1A histone methyltransferase complex and regulates histone H3-Lys4 trimethylation near the transcription start site of three housekeeping genes. In contrast to previous findings in yeast, the interaction between the human Setd1A histone methyltransferase complex and the Ser5-P CTD of RNAP II does not require Paf1 but instead is mediated by Wdr82, an integral component of the Setd1A complex that directly interacts with the RRM domain of Setd1A. These data do not formally exclude the possibility that the Paf1 complex may participate in the genomic targeting of the Setd1A complex in vivo. Similar analyses using Paf1-deficient cells will be required to fully address this issue. The RRM domain is conserved in yeast Set1 and mammalian Setd1A and Setd1B proteins, but not in MLL1, MLL2, MLL3, or MLL4. These results indicate that RNAP II-directed histone H3-Lys4 trimethylation of transcription start sites by Set1 histone methyltransferase complexes is conserved between yeast and humans. Given the observation of Wdr82-mediated binding of human Setd1A to RNAP II, further studies may be warranted to investigate whether Swd2 (the Wdr82 homologue) provides a similar function in yeast. Similarly, because Wdr82 does not interact with the MLL1

histone methyltransferase complex, it will be interesting to determine the mechanism by which the mammalian family of MLL histone H3-Lys4 methyltransferase complexes are recruited to the transcription start site. Importantly, Wdr82 cannot be solely responsible for gene-specific methyltransferase recruitment, as it is a component of both the Setd1A and Setd1B complexes that exhibit distinct subnuclear localizations (22).

Surprisingly, reduction of Setd1A occupancy and histone H3-Lys4 trimethylation following Wdr82 depletion had no effect on RNAP II occupancy or steady-state transcript levels for the examined housekeeping target genes. It remains possible that effects on target gene expression would be observed if Wdr82 expression were completely abrogated and Setd1A occupancy further depleted. However, these results suggest that histone H3-Lys4 methylation is a downstream consequence of transcription at these target genes. This is consistent with an earlier hypothesis that yeast Set1/COMPASS-mediated histone H3-Lys4 trimethylation serves as a molecular memory of recent transcriptional activity (32). A more extensive analysis of a diverse set of mammalian genes following Wdr82 depletion will be required to determine the generality of this observation.

Transcription-mediated histone H3-Lys4 methylation by the Setd1A histone methyltransferase complex. Several lines of evidence presented in this report support a model in which the human Wdr82 protein facilitates histone H3-Lys4 trimethylation via recruitment of the Setd1A histone H3-Lys4 methyltransferase complex to Ser5-P CTD of RNAP II: (i) the RRM domain of Setd1A directly interacts with Wdr82, (ii) Wdr82 recognizes Ser5-P CTD, (iii) both Wdr82 and Setd1A specifically associate near transcription start sites that are occupied by RNAP II containing Ser5-P CTD, and (iv) depletion of Wdr82 causes decreased occupancy of Setd1A and decreased histone H3-Lys4 trimethylation near the transcription start sites of target genes. A number of studies have investigated the functions of the Set1 RRM domain and Swd2 protein (Wdr82 homologue) in yeast. A yeast strain that expresses a mutated form of Set1 lacking the RRM domain does not rescue histone H3-Lys4 trimethylation in Set1-deficient cells (11, 33, 41), and yeast strains lacking Swd2 show a global loss of histone H3-Lys4 di- and trimethylation (4, 37, 41). However, it is unclear how loss of the Set1 RRM domain or the Swd2 protein causes defects in histone H3-Lys4 methylation. It has been suggested that the RRM domain interacts with the SET domain to regulate its catalytic activity in yeast (41).

The data presented in this report suggest a molecular mechanism for histone H3-Lys4 methylation by the Setd1A complex near transcription start sites (Fig. 7) in which the Wdr82 subunit of the Setd1A complex directly recognizes the Ser5-P CTD mark, which is generated by CTD kinase (TFIIH) (34, 36), and the Setd1A complex introduces histone H3-Lys4 trimethylation marks on the neighboring nucleosomes. As the nascent RNA elongates, the Setd1A complex dissociates from RNAP II. It is not clear what signals trigger the Setd1A complex to dissociate from RNAP II. However, a recent report highlighted the importance for regulation of the transition between transcription initiation and elongation, as a genome-wide analysis revealed that the majority of protein-encoding genes initiate transcription, even in the absence of transcription elongation (15). Tresaugues et al. (47) described a second RRM domain in yeast Set1 that exhibits in vitro RNA-binding activity. The analogous region of the Setd1A protein (amino acid residues 279 to 452) exhibits 10% identity and 48% similarity to the yeast sequence. It is possible that this second RRM domain interacts with nascent RNA and changes the binding activity of Wdr82 to either the Setd1A complex or the Ser5-P CTD of RNAP II, as proposed for the yeast Set1/COMPASS complex (12, 16). Alternatively, changes in the phosphorylation status of the CTD may change the binding affinity of Wdr82 to the CTD and result in the release of the Setd1A complex. Ser2 residues of CTD repeats are phosphorylated by CTDK-1 (34, 36), and Wdr82 may exhibit reduced affinity for doubly phosphorylated CTD. Lastly, Trinkle-Mulcahy et al. (48) reported that protein phosphatase 1 (PP1) isoforms interact with Wdr82, and PP1 has been implicated as a Ser5 CTD phosphatase (50). These results suggest the possibility that PP1 action leads to release of the Setd1A complex following removal of Ser5-P within the CTD and loss of Wdr82 binding affinity. Additional studies will be required to investigate both the mechanistic details and functional significance of Setd1A dynamics during the transcription cycle.

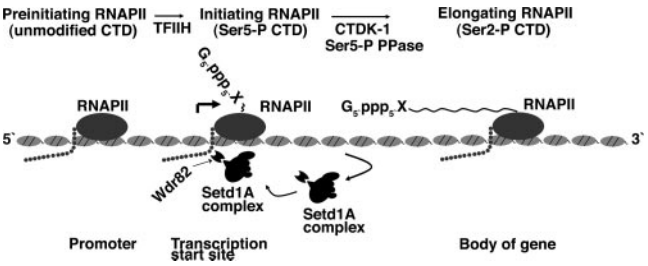

FIG. 7. Role of Wdr82 on histone H3-Lys4 methylation during the transcription cycle. After the formation of the preinitiation complex, CTD kinase (TFIIH) phosphorylates Ser5 residues of CTD repeats within the initiating RNAP II large subunit. The Ser5-P mark of CTD is directly recognized by the Wdr82 subunit of the Setd1A complex, and the Setd1A complex introduces histone H3-Lys4 trimethylation marks on neighboring nucleosomes. As the nascent RNA grows, the Setd1A complex dissociates from RNAP II by an unknown mechanism. CTDK1 phosphorylates Ser2 residues of CTD repeats during the elongation phase of transcription.

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