SMYD3 is a SET domain-containing protein with histone methyltransferase activity on histone H3–K4. Recent studies showed that SMYD3 is frequently overexpressed in different types of cancer cells, but how SMYD3 regulates the development and progression of these malignancies remains unknown. Here, we report the previously unrecognized role of SMYD3 in estrogen receptor (ER)-mediated transcription via its histone methyltransferase activity. We demonstrate that SMYD3 functions as a coactivator of ERα and potentiates ERα activity in response to ligand. SMYD3 directly interacts with the ligand binding domain of ER and is recruited to the proximal promoter regions of ER target genes upon gene induction. Importantly, our chromatin immunoprecipitation analyses provide compelling evidence that SMYD3 is responsible for the accumulation of di- and trimethylation of H3–K4 at the induced ER target genes. Furthermore, RNA interference-directed down-regulation of SMYD3 reveals that SMYD3 is required for ER-regulated gene transcription in estrogen signaling pathway. Thus, our results identify SMYD3 as a new coactivator for ER-mediated transcription, providing a possible link between SMYD3 overexpression and breast cancer.

Estrogen receptor (ER)\(^3\)α is a member of the nuclear receptor superfamily and the primary biosensor for estrogen (1, 2). Upon activation by estrogen, ER binds to specific DNA sequences called estrogen response elements (EREs) to induce expression of a number of target genes in specific organs, including the female reproductive organs, the central nervous system, and bone (1, 3, 4). ER is comprised of several structural domains that are highly conserved in the various nuclear receptors: the N-terminal transcription activation domain, the DNA binding domain, the hinge region, and the C-terminal conserved ligand binding domain (5–7). Like other nuclear receptors, the ER collaborates with a number of transcriptional cofactors to effectively modulate transcription of its target genes (3, 8–10). These cofactors appear to regulate the chromatin configuration in a highly specific manner by controlling nucleosomal rearrangement and histone modifications at the promoter (11–13). This targeted alteration of chromatin structure allows the transcriptional machinery to access the chromatin DNA and form functional preinitiation complexes, thereby facilitating transcription initiation (14–16).

Two major types of chromatin remodeling have been widely investigated for ER transcription. The remodeling activities include the ATP-dependent chromatin remodeling factors, which alter structure and position of nucleosomes at the promoters of ER target genes. These include proteins such as brahma-related BRG1 (also known as hBRG1 or hSNF2) and BRM, both of which are subunits of the mammalian homologue of the yeast SWI/SNF complex (17, 18). The second class of remodeling factors includes a diverse group of single/multisubunit factors that effect post-translational modifications of the histone tails protruding from the surface of the nucleosome (17–19). Among the well known histone-modifying factors acting in ER-mediated transcription are histone acetyltransferases, including p300/CBP and GCN5/PCAF, and histone methyltransferases, including the arginine methyltransferases CARM1 and PRMT1, as well as SET domain lysine methyltransferases such as G9a, RIZ1, NSD1, and MLL2 (10, 14, 20–22). These remodeling factors are recruited to the promoter proximal region of the ER target genes (23–26) and facilitate either remodeling or removal of the underlying nucleosome, thereby increasing the accessibility of promoter regions to the transcription machinery.

Recent studies identified that SMYD3 possesses histone methyltransferase activity responsible for catalyzing methylation of histone H3 at K4 (27). SMYD3 contains a SET domain, which is crucial for HMT activity, and an MYND-type zinc-finger domain (zf-MYND) domain, which is common to developmental unit factors that effect post-translational modifications of the histone tails protruding from the surface of the nucleosome (3, 8–10). These cofactors appear to regulate the chromatin configuration in a highly specific manner by controlling nucleosomal rearrangement and histone modifications at the promoter (11–13). This targeted alteration of chromatin structure allows the transcriptional machinery to access the chromatin DNA and form functional preinitiation complexes, thereby facilitating transcription initiation (14–16).

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Estrogen Receptor Activation by SMYD3

levels of SMYD3 have been observed in breast cancer tissues as well as breast cancer cell lines with associated effects on cancer growth (28). ER serves as a sequence-specific transcription factor to regulate a cascade of gene targets whose products mediate the initiation, development, and metastasis of breast cancers. Thus, these results support the idea that SMYD3 might play a functional role in the transactivation of ER-mediated gene transcription in breast cancer cells.

As a starting point for the study of transcriptional processes regulated by SMYD3, we checked a possible role of SMYD3 in the ER signaling process. From molecular and cellular studies, we have obtained evidence indicating that SMYD3 is critically involved in ligand-activated, ER-mediated transcription, by methylating histone H3–K4 at the ERE in the promoter regions of target genes. The function of SMYD3 in ER-mediated transcription requires its direct interaction with ER, which in turn allows its recruitment to promoter regions of ER target genes. Down-regulation of SMYD3 expression and concomitant reduction of H3–K4 methylation substantially repressed expression of ER target genes, revealing a major role for SMYD3 as regulator of ER-mediated target gene transcription.

EXPERIMENTAL PROCEDURES

Plasmid Construction—For mammalian expression of SMYD3, SMYD3 cDNA was PCR-amplified from the pool of MCF-7 cDNA using a 5’ primer (5’-AAGGAAAAAGCGC-GCGATCCATATGCTCTGCCCCG) and a 3’ primer (5’-CGCGGATCCCTAGATGCTCTGATGTTGGC), which introduced BamHI and NotI sites at the 5’ and 3’ ends, respectively. The PCR products were digested with NotI-BamHI and inserted into plasmids containing FLAG and HA tags to generate the plasmid for mammalian expression. For bacterial expression, SMYD3 cDNA was PCR-amplified with a forward primer (5’-GGAATTCATATGCTCTGATGTTGGC) and a reverse primer (5’-CGCGGATCCCTAGATGCTCTGATGTTGGC), introducing NotI and BamHI sites at the 5’ and 3’ ends, respectively. The corresponding products were digested with NotI-BamHI and inserted into NotI and BamHI sites of pET11 containing FLAG tag. The same procedure was followed to construct the bacterial expression plasmids encoding the mutant SMYD3 (ΔNHSC, ΔEEL, and ΔNHSC and EEL) except that the original SMYD3 cDNA was first deleted at NHSC and/or EEL motifs by using the QuickChange mutagenesis kit (Stratagene). All mutations were confirmed by DNA sequencing.

Pulldown and Immunoprecipitation Assays—For in vitro interaction assays with SMYD3, FLAG epitope-tagged SMYD3 was synthesized in vitro by using TNT-Quick-coupled transcription/translation system (Promega, Madison, WI) and incubated with GST-ER coupled to glutathione-Sepharose beads (Amersham Biosciences) at 4 °C in 1 mL of binding buffer (20 mM Tris-HCl, pH 7.3, 0.2 mM KCl, 0.2 mM EDTA, 20% glycerol, and 0.01% Nonidet P-40) for overnight. After washing three times with 500 μL of binding buffer, the beads were subjected to 10% SDS-PAGE and Western blot analysis using anti-FLAG antibody. For interaction assays with AF-I and AF-II domains of ERα, the domains were synthesized by using the TNT-Quick-coupled transcription/translation system (Promega) and incubated with recombinant FLAG-tagged SMYD3 prepared from Escherichia coli along with FLAG-M2 agarose beads (Sigma). For coimmunoprecipitation assays, FLAG-tagged and untagged SMYD3 proteins were expressed in 293T and MCF-7 cells, and whole cell lysates were prepared from the cells with lysis buffer (20 mM HEPES, pH 7.8, 150 mM NaCl, 10 mM EDTA, 2 mM EGTA, and 2 mM dithiothreitol, and 0.1% Nonidet P-40). The cell lysates were reacted with anti-FLAG antibody (Sigma) and rotated at 4 °C for overnight before removal of the supernatant. The resulting samples were analyzed by Western blot analysis using anti-FLAG and anti-ER antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). To coimmunoprecipitate endogenous SMYD3 and ER, 293T cell lysates were reacted with anti-ER antibody or normal rabbit IgG (Santa Cruz Biotechnology) for overnight. After centrifugation, immunoprecipitates in the supernatants were precipitated with Protein A/G-Sepharose (Millipore) and separated on 15% SDS-PAGE. Immunoblot analyses were performed with anti-ER and anti-SMYD3 antibodies.

Histone Methyltransferase Assay—HMT assays were performed as described previously (29). 293T cells were transfected with empty expression plasmid (negative control) or plasmids expressing FLAG-HA-tagged wild-type SMYD3 (FLAG-HA-pIRES-SMYD3), mutant SMYD3 (FLAG-HA-pIRES-ΔEEL, FLAG-HA-pIRES-ΔNHSC, and FLAG-HA-pIRES-ΔEEL&ΔNHSC), and SET7/9 protein (positive control), or pFLAG-HA-pIRES (negative control), and the proteins were purified by immunoprecipitation with anti-FLAG antibody. Recombinant histone octamers (1 μg) were incubated with SMYD3 for 1 h at 30 °C in HMT reaction buffer (100 mM HEPES, pH 7.8, 300 mM KCl, 2.5 mM EDTA, 25 mM dithiothreitol, 50 mM sodium butyrate) in the presence of 2.3 μM [3H]S-adenosylmethionine (AdoMet) or 50 μM cold AdoMet. Proteins were resolved on 15% SDS-PAGE gel and visualized by fluorography. The antibodies used for detection of mono-/di-/trimethyl H3–K4 were from Abcam.

Chromatin Immunoprecipitation—ChIP experiments were performed with MCF-7 cells according to the procedure described by Kim et al. after estrogen treatment (100 nM) (14). The immunoprecipitated DNA was amplified by qPCR using the following primers: 5’-GGCCCTCTTATAGCAAATGTT-3’ (ps2 forward), 5’-CCTCTTCTGCTCCAAAAGG-3’ (ps2 reverse), 5’-GCCAACAGGCGACCTTTAGTC-3’ (CTSD forward), 5’-CATTCAACCCCTACCCTTT-3’ (CTSD reverse), 5’-TGTGGCTAGTGGACCTTTTG-3’ (GREB1 forward), 5’-CTGCCCCAACAATCTGAAGA-3’ (GREB1 reverse), 5’-CCATCATGCTGAGTCTAGTG-3’ (pS2 upstream forward), 5’-GTGAGTATTTTGAGAAGTG-3’ (pS2 upstream reverse), 5’-CCTCAACAGGTCATGCTCA-3’ (CTSD upstream forward), 5’-AGGAAAGGTTGAGATGGT-3’ (CTSD upstream reverse), 5’-ATTCTCATTTGCTTCTGCTG-3’ (GREB1 upstream forward), and 5’-AGGGGCTCCACAGGACATGA-3’ (GREB1 upstream reverse). An antibody for ER was from Santa Cruz Biotechnology, and antibody for SMYD3 was from Abcam.

SMYD3 shRNAs—For RNA interference depletion of SMYD3, MCF-7 cells were transfected with 3 μg of shSMYD3 by using Lipofectamine (Invitrogen). 48 h post-transfection,
mRNA was extracted using TRIzol (Invitrogen), and changes in gene expression were assessed by real-time PCR. ChIPs were also performed after shRNA-mediated knockdown of SMYD3. The sequences for shRNAs used in the assays were as follows:

5’-GATCCGGATCCACATCTACCAGCTGAAAGGTGTCTAGAGAGATACCTTGAGTTTGAGGTGTTCTTGGAGAATGGAAC-3’ and 5’-AGCTTTTCCAAAAAACATCTACCAGCTGAAAGGTGTTCTTGGAGAATGGAAC-3’.

RESULTS

SMYD3 Interacts with ER via Distinct Domains—As a first step toward exploring a potential role of SMYD3 in ER transcription, we analyzed the ability of SMYD3 to interact with ER N-terminal and DNA binding domain (NTD+DBD) and ER ligand binding domain (LBD) (Fig. 1A). FLAG-tagged SMYD3 was incubated with equimolar amounts of GST-fused NTD+DBD and GST-fused LBD that were immobilized on glutathione-Sepharose beads. After extensive washing of the beads, SMYD3 binding was analyzed by Western blot analysis with indicated antibodies.

Western blot analysis of anti-ER immunoprecipitates from MCF-7 cell lysates with anti-SMYD3 antibody. As shown in Fig. 1C, SMYD3 was able to bind to the ER LBD (lane 8), but not to the ER NTD+DBD (lane 7). The lack of SMYD3 interaction with GST alone (lane 6) further confirmed the specificity of the binding reactions. Reverse binding experiments using FLAG-SMYD3 immobilized on M2 affinity beads also showed the identical interaction of ER LBD, further confirming the specific interaction between SMYD3 and ER (Fig. 1C).

To determine whether SMYD3 is able to interact with ER in cellular environments, immunoprecipitation was performed after transiently expressing untagged ER and FLAG-tagged SMYD3 in 293T cells (Fig. 1D). Cell lysates were prepared and subjected to immunoprecipitation of ectopic SMYD3 with anti-FLAG M2 agarose beads, and the stable association of ER was analyzed by Western blot analysis using anti-ER antibody. Consistent with our in vitro interaction results, immunoprecipitation of ectopic SMYD3 resulted in coprecipitation of ER (lane 8). To further verify cellular interaction between SMYD3 and ER in physiological conditions, we immunoprecipitated MCF-7 cell lysates with anti-ER antibody and checked the immunoprecipitation of endogenous SMYD3. As shown in Fig. 1E, endogenous SMYD3 was readily detected by Western blot analysis of anti-ER immunoprecipitates from MCF-7 cell extracts (lane 3) but not immunoprecipitates obtained with a control IgG (lane 2). The lack of interaction of ER with linker histone H1 further confirmed the specificity of the interaction between ER and SMYD3 (lane 3). Collectively, these experiments demonstrate the direct interaction of SMYD3 with ER in vitro and in vivo, which is dependent upon ER LBD.

SMYD3 Requires Both NHSC and EEL Motifs For Its HMT Activity—It has been shown that SMYD3 can methylate histone H3 at H4 by means of its set domain that contains NHSC and EEL motifs (Fig. 2A) (27, 30). In this case, however, only histone H3 was used as a substrate to analyze the HMT activity of SMYD3, and the possible ability of SMYD3 to methylate other core histones was not analyzed. Thus, we examined SMYD3 HMT activity using recombinant histone octamers reconstituted with bacterially expressed four core histones. Recent studies showed that recombinant SMYD3 methylates H3 to a very limited extent, whereas cellular SMYD3 has a strong HMT
activity (27). These results imply that a post-translational activation might be required to potentiate SMYD3, possibly through a conformational change of the protein. For this reason, we employed the FLAG-tagged SMYD3 immunoprecipitated from transfected cells and 3H-labeled AdoMet for our HMT assays (Fig. 2B). In agreement with previous reports (27, 28), our HMT assays showed that histone H3 is specifically methylated by SMYD3 in reconstituted histone octamers (Fig. 2B, lane 4). Because the NHSC and EEL motifs are highly conserved among HMT family members (27, 30), we next checked the possible requirement of these two motifs for SMYD3 HMT activity. Deletion of the NHSC or EEL motif (SMYD3-ΔNHSC and SMYD3-ΔEEL) abolished the ability of SMYD3 to methylate histone H3 (lanes 5 and 6), exactly mirroring those reported with the similar SMYD3 mutants in 293T cells (27). Consistent with these observations, concomitant deletions of NHSC and EEL motifs (SMYD3-ΔNHSC&ΔEEL) also showed no detectable methylation of histone H3 (lane 7). To further characterize SMYD3 activity, HMT reactions were analyzed by Western blotting using antibodies recognizing mono-/di-/trimethylation of H3–K4. Our studies proved the critical requirement of NHSC and EEL motifs in SMYD3 HMT activity. A possible explanation for the above data is that changes in the HMT activity are likely to be responsible for the repressed transcription caused by the deletion of the NHSC and EEL motifs. However, another possibility is that NHSC and EEL motifs of SMYD3 are critical for the physical interaction between SMYD3 and ER; thus the lack

methylation state of histone H3 was analyzed by probing with H3–K4 mono-, di-, and trimethyl-specific antibodies. Our Western blotting of the HMT reaction detected a high signal for di- and trimethylation of H3–K4 (Fig. 2C, lane 4). In contrast, the reaction product showed a very weak signal for monomethylation (lane 4). Collectively, these data confirm the intrinsic preference of SMYD3 for di- and trimethylation of histone H3–K4 as well as the critical requirement of NHSC and EEL motifs for SMYD3 HMT activity.

**SMYD3 Functions as a Co-activator for ER-mediated Transcription**—Because recent microarray analyses indicate that SMYD3 is associated with the expression of several genes (27), we next evaluated its possible role as a coactivator in ER-mediated transcription. The human breast cancer MCF-7 cells were transfected with luciferase reporter plasmid and ER expression vector along with SMYD3 expression vector, and luciferase activity was measured 48 h after transfection. As expected, ER activated the expression of a transiently transfected reporter gene in an estrogen-dependent manner (Fig. 3A). Remarkably, when increasing amounts of wild-type SMYD3 were expressed together with ER, the ligand-dependent activation of reporter gene transcription was significantly enhanced (Fig. 3A). We also observed a modest enhancement of ligand-independent, ER-mediated activation of reporter gene expression induced by SMYD3 (Fig. 3A). Next, to check whether the NHSC and EEL motifs are important for coactivator function of SMYD3, the reporter gene assays were repeated with SMYD3 mutant constructs lacking NHSC and/or EEL motifs. The coactivator function of SMYD3 was significantly impaired by independent or simultaneous deletion of NHSC and EEL motifs, confirming the requirement of these two motifs for the coactivator function of SMYD3 (Fig. 3B). To confirm the above results, we also repeated the experiments using human epithelial 293T cells; similar results were obtained from these parallel experiments (Fig. 3, C and D).

**FIGURE 2. Requirement of NHSC and EEL motifs for SMYD3 HMT activity.** A, conserved amino acid sequences within SET domains. B, H3-targeted activity of SMYD3. HMT assays were performed with full-length SMYD3 or NHSC/EEL-deleted SMYD3 proteins expressed in 293T cells using [3H]AdoMet and recombinant histone octamers. Recombinant SET7/9 was included as a control. C, di-/trimethylation of H3–K4 by SMYD3. HMT assays were performed as in Fig. 2B, but using cold AdoMet. H3–K4 methylation was determined by Western blot with antibodies recognizing mono-/di-/trimethylation of H3–K4.
FIGURE 3. Coactivator function of SMYD3 in ER transcription. A and C, effect of SMYD3 on ER-mediated transcription in vivo. MCF-7 (A) and 293T (C) cells in 12-well plates were transiently transfected with MMTV-ERE reporter gene together with ER expression vector and various amounts of SMYD3 expression vectors in uninduced and estrogen-induced conditions as indicated. The representative data from three independent experiments are shown, and the error bars indicate as the means ± S.E. B and D, requirement of HMT activity for SMYD function. Reporter gene assays were as in Fig. 3A and 3C, but with mutant SMYD3 expression vectors. E, in vitro interaction of ER with SMYD3 lacking NHSC/EEL motifs. ER NTD + DBD and LBD fused to GST were incubated with SMYD3 lacking NHSC/EEL motifs, and SMYD3 binding was analyzed by immunoblot with anti-SMYD3 antibody.
Estrogen Receptor Activation by SMYD3

A

\[
pS2 \quad 3kb \quad \text{Upstream} \quad \text{ERE binding Site} \quad \text{TATA} \quad 150bp \quad +1
\]

\[
\begin{align*}
\alpha-ER & \quad \text{ERE} & \quad \text{IgG} & \quad \text{ERE} & \quad \alpha-ER & \quad \text{ERE} \\
\text{TIME (min)} & 0 & 30 & 60 & 90 & 120 & 0 & 30 & 60 & 90 & 120 & 0 & 30 & 60 & 90 & 120
\end{align*}
\]

\[
\begin{align*}
\alpha-SMYD3 & \quad \text{ERE} & \quad \text{IgG} & \quad \text{ERE} & \quad \alpha-SMYD3 & \quad \text{ERE} \\
\text{TIME (min)} & 0 & 30 & 60 & 90 & 120 & 0 & 30 & 60 & 90 & 120 & 0 & 30 & 60 & 90 & 120
\end{align*}
\]

B

\[
CTSD \quad 18kb \quad 753bp \quad \text{Upstream} \quad \text{ERE binding Site} \quad \text{TATA} \quad +1
\]

\[
\begin{align*}
\alpha-ER & \quad \text{ERE} & \quad \text{IgG} & \quad \text{ERE} & \quad \alpha-ER & \quad \text{ERE} \\
\text{TIME (min)} & 0 & 30 & 60 & 90 & 120 & 0 & 30 & 60 & 90 & 120 & 0 & 30 & 60 & 90 & 120
\end{align*}
\]

\[
\begin{align*}
\alpha-SMYD3 & \quad \text{ERE} & \quad \text{IgG} & \quad \text{ERE} & \quad \alpha-SMYD3 & \quad \text{ERE} \\
\text{TIME (min)} & 0 & 30 & 60 & 90 & 120 & 0 & 30 & 60 & 90 & 120 & 0 & 30 & 60 & 90 & 120
\end{align*}
\]
of these motifs might modulate downstream transcription activities. To check this possibility, we analyzed the binding of wild-type and deletion mutant SMYD3 proteins to a fixed concentration of LBD and NTD/DBD of ER. As expected from our initial binding data (Fig. 1, B and C), full-length SMYD3 was able to bind to GST-LBD fusion (Fig. 3E, lane 4), but not to GST-NTD/DBD fusion (Fig. 3E, lane 3). Furthermore, identical binding assays with the deleted forms of SMYD3 showed a comparable binding capacity of these mutant SMYD proteins to ER (lanes 7, 10, and 13). The results from these binding experiments, together with the results from our HMT assays, strongly suggest that inactivation of SMYD3 HMT activity, not changes in SMYD3-ER interaction, are the major cause of impaired transcription caused by the deletion of NHSC and EEL motifs.

SMYD3 Augments ER Target Gene Expression via Histone H3–K4 Methylation—Having shown the ability of SMYD3 to coactivate ER-mediated transcription, we sought to determine the participation of SMYD3 in transcription of three endogenous ER target genes. Thus, MCF-7 cells were treated with 100 nM estrogen for 0, 30, 60, 90, and 120 min, and the recruitment of ER and SMYD3 to the promoters of three target genes (pS2, CTSD, and GREB1) were examined by ChIP (Fig. 4). The presence of the promoter regions in the chromatin immunoprecipitates was analyzed by quantitative real-time PCR using specific pairs of primers spanning the EREs in the promoters. Immunoprecipitation with the ER-specific antibody showed an obvious increase with respect to ER occupancy of the target gene promoters within 30 min of estrogen treatment (Fig. 4, ER). ER promoter occupancy was significantly declined and returned to baseline after 60 min of estrogen treatment. The second phase of ER recruitment to the promoter was also evident after 90 min of estrogen treatment. These results are consistent with previous indications that ER acts in a transient but repeated fashion on the same target gene promoter following estrogen stimulation (8).

We next investigated the promoter recruitment of SMYD3 and the relationship of this process to the ER occupancy and H3–K4 methylation state at the three promoters. SMYD3 showed its accumulation on the promoters to a very high level at the 30-min point but showed an apparent dissociation from the promoters at the 60-min point (Fig. 4, SMYD3). The second cycle of promoter occupancy began at the 90-min point and returned to the baseline level at the 120-min point. Parallel analysis over the same time period showed that the level of di- and trimethyl K4 of histone H3 peaked at 30- and 90-min time points (Fig. 4, H3K4me2 and H3K4me3). In contrast, we did not see any apparent change in monomethylation of H3–K4 after estrogen treatment (H3K4me1). In all cases, SMYD3 recruit-
ment and H3–K4 methylation were targeted to the ERE region, because their localizations were minimal/undetectable in a distal upstream region (Fig. 4, **Upstream**). The coincidental appearance and decrease of SMYD3 and di-/trimethyl H3–K4 strongly suggest that the accumulation of di-/trimethyl H3–K4 is due to the enhanced recruitment of SMYD3 following the onset of estrogen treatment. Moreover, a very similar timing of SMYD3 occupancy and the corresponding H3–K4 methylation in all three target genes strongly supports that ER employs similar dynamics to recruit SMYD3 for H3–K4 methylation. These studies thus confirm that ER can recruit SMYD3 to its natural target genes and mediate **de novo** methylation of H3–K4 in the process of gene transcription.

To assess the requirement of SMYD3 in ER transcription activity **in vivo**, we examined the effect of manipulating SMYD3 levels on ER target gene expression in MCF-7 cells. We generated a short hairpin RNA (shRNA) construct for SMYD3 and tested its knockdown effects on SMYD3 expression. The reverse transcription-PCR confirmed that cell transfection with SMYD3 shRNA decreased the SMYD3 mRNA level by 75% in the absence of E2 and 50% in the presence of E2 (Fig. 5B, **SMYD3**). The reduced protein level was also confirmed by Western blot analysis of endogenous SMYD3 using anti-SMYD3 antibody (Fig. 5A). This shRNA-induced silencing of SMYD3 gene significantly reduced the ligand-induced expression of pS2, CTSD, and GREB1 genes compared with negative control (non-target shRNA) cells (Fig. 5B). Albeit the expression of ER target genes was much weaker without estrogen treatment, we also could detect a moderate reduction in ER transcription activity after SMYD3 shRNA transfection in this uninduced condition (Fig. 5B, **pS2**, **CTSD**, and **GREB1**).

To further investigate the effect of SMYD3 knockdown on promoter occupancy of H3–K4 methylation, we performed ChIP assays on pS2 gene with SMYD3-depleted and control MCF-7 cells following E2 treatment (Fig. 6A). As expected, SMYD3 depletion resulted in a dramatic reduction in immunoprecipitation of the ERE region using SMYD3 antibody (Fig. 6C). However, the depletion had no obvious effect on the cyclic promoter occupancy of ER after the addition of E2 (Fig. 6B). Importantly, similar ChIP analysis showed that RNA interference-mediated depletion of SMYD3 distinctly diminished promoter-targeted accumulation of di-/trimethylation of H3–K4 (Fig. 6, **E** and **F**). In contrast, the depletion had minimal effect on the level of monomethylation of H3–K4 at pS2 promoter upon E2 treatment (Fig. 6D), indicating that SMYD3 is mainly responsible for establishing H3–K4 di-/trimethylation, but not H3–K4 monomethylation. Taken together, these data indicate that endogenous SMYD3 plays an important role in the augmentation of ER-mediated transcription in the ligand-treated environment.

**DISCUSSION**

In this study, we investigated a possible role of SMYD3 histone methyltransferase in activating ER target genes. The present data demonstrate that (i) SMYD3 physically interacts with ER both **in vitro** and **in vivo**, (ii) SMYD3 acts as a transcriptional coactivator of ER that enhances ER-mediated transcription, (iii) SMYD3-imparted transactivation correlates with SMYD3 recruitment and H3–K4 methylation at ER target genes, and (iv) SMYD3 knockdown significantly reduces the ligand-induced expression of ER target genes. These results reveal an

**FIGURE 5. Requirement of SMYD3 for ER transcription.** A, validation of SMYD3 knockdown. MCF-7 cells were transfected with SMYD3 shRNA or control shRNA, and expression of SMYD3 protein was checked by Western blotting of whole cell lysates using anti-SMYD3 antibody. Actin was used as an internal control (lower panel). B, repression of ER transcription by SMYD3 knockdown. Cells were transfected with SMYD3 shRNA, and treated with 100 nM estrogen 24 h post-transfection. mRNA levels were analyzed by real-time PCR. Error bars indicates as the means ± S.E. from the experiments performed in duplicate, and the experiments were repeated three times.
The essential role of SMYD3 in modulating ER-mediated transcription and provide an example of epigenetic regulation of ER function.

The ability of ER to activate transcription requires the repeated cycling of various coregulators onto its target gene promoters in the presence of continuous stimulation by estrogen (3, 8). Currently, at least two mechanistic models have been proposed to describe the function of these coregulators. First, they transmit the signal of ligand-induced ER conformational change to the basal transcription machinery (31). Second, they are associated with targeted chromatin remodeling by ER (32). Recent biochemical and genetic studies support that methylation of histone H3 at K4 is characteristic to gene activation, and removal of this modification is involved in transcriptional repression (24, 33, 34). The data presented here demonstrate that SMYD3, through its HMT activity, plays a significant role in dictating the transcriptional activity of ER. That the effects of SMYD3 were found to be dependent upon the ability to interact with ER LBD implies that SMYD3 is a functionally important component of estrogen-stimulated ER transcription. Combined with the observation that shRNA-induced silencing of SMYD gene inhibits ER target gene expression, these results argue strongly in favor of SMYD3 as an integral component of the ER response. Although our analyses have been restricted to ER-dependent function of SMYD3, previous studies indicated that activation of other nuclear receptors also involves H3–K4 methylation (35, 36). As such, elucidation of a possible role of SMYD3 in the promoter-localized H3–K4 methylation and the consequent activation of transcription at other nuclear receptor target genes is an important issue that warrants further investigation.

Many SET domain-containing proteins with HMT activity harbor two conserved amino acid sequence called NHSC and EEL motifs (27, 30). SMYD3 also possesses these motifs within its SET domain, and they have been shown to be critical for SMYD3 enzymatic activity in HMT reaction (27). Our studies performed with SMYD3 mutants demonstrated that, although the NHSC and EEL motifs within SET domain are essential for SMYD3 HMT activity, these motifs are dispensable for SMYD3 binding to ER (Fig. 3E). Importantly, when these deletion mutants were checked in ER luciferase reporter assays, they failed to show coactivator function (Fig. 3, A–D). A simple
interpretation of these results is that HMT activity of SMYD3 is required for its action on the transactivation of reporter gene following estrogen stimulation. These characteristics ascribed to NHSC and EEL motifs fit very well with the generic properties previously assigned to the NHSC/EEL motif-containing SET domains in regulating and mediating the enzymatic activity of HMT proteins (27, 30). These results also suggest that NHSC and EEL motifs of SMYD3 could provide the molecular target for regulation of H3–K4 methylation-dependent transcriptional responses by ER. More thorough domain mapping and mutagenesis experiments will be required to provide further insights into the ER-SMYD3 interactions, which facilitate SMYD3 recruitment and H3–K4 methylation in ER-mediated transcription.

We have shown that ER promoter occupancy upon E2 treatment coincides with promoter recruitment of SMYD3 and appearance of di-/trimethyl H3–K4 (Fig. 4, A–C). This similar timing of ER occupancy and SMYD3 recruitment strongly supporting that H3–K4 methylation per se endows coactivator properties of SMYD3 in regulating ER-mediated transcription. In further support of a regulatory role of SMYD3-mediated H3–K4 methylation, SMYD3 depletion showed a significant effect on the level of di-/trimethylation, but not monomethylation, of H3–K4 at the promoter of pS2 gene upon E2 treatment (Fig. 6). However, it is also possible that SMYD3 augments the activities of other transcription components at the initial stage of gene induction. In fact, a recent study demonstrated that SMYD3 interacts with an RNA helicase to form a complex with RNA polymerase II (27). Hence, SMYD3 could act as a “bridge” protein that mediates functional interaction between ER and RNA polymerase II to coordinate the tightly integrated processes of chromatin remodeling and transcription in ER-driven transcription (31, 37). Furthermore, increasing evidence suggests that coactivator proteins such as p300, PRMT1, and CARM1 act together as potential regulators of ER-induced transcription (2, 12, 18, 38, 39). Thus, it is possible that a distinct group of coactivators play a crucial role in orchestrating ER-mediated transcription. In this respect, it will be interesting to determine the relative contribution of SMYD3, together with other coactivators that associate with ER target gene expression.

In conclusion, the analysis of SMYD3 described here establishes its role in ER-mediated transcription as a coactivator. H3–K4 methylation appears to be critical for SMYD3 function in ER transcription. Identifying cofactors that influence ER has been important in the development of effective therapies and prevention of breast cancer. Because our studies provide clues as to the requirement of SMYD3 HMT activity for its function, the development of HMT inhibitors may be of therapeutic benefit in modulating SMYD3 action in ER transcription.

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VOLUME 284 • NUMBER 30 • JULY 24, 2009
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