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Nuclear receptor SHP inhibition of Dnmt1 expression via ERR γ

Yuxia Zhang and Li Wang*

Departments of Medicine and Oncological Sciences, Huntsman Cancer Institute, University of Utah School of Medicine, Salt Lake City, UT 84132

Abstract

We describe a transcriptional mechanism regulating the expression of Dnmt1 by nuclear receptors. We show that ERR γ functions as a transcriptional activator of mouse and human Dnmt1 expression by direct binding to its response elements (ERE1/ERE2) in the dnmt1/DNMT1 promoters. The induction of Dnmt1 by ERR γ is repressed by SHP through SHP inhibition of ERR γ transactivity, diminishing ERR γ recruitment to the Dnmt1 promoter, and altering the conformation of local chromatin from an active mode by ERR γ to an inactive mode. Our study provides the first evidence for nuclear receptor mediated regulation of Dnmt1 expression through ERR γ and SHP crosstalk.

Keywords

nuclear receptor; gene regulation; DNA methyltransferase

1. Introduction

DNA cytosine-5-methyltransferases (Dnmts) catalyze the methyl transfer from S-adenosyl-L-methionine to the cytosine in CpG dinucleotides [1]. Gene silencing due to DNA hypermethylation of promoter regions is mainly controlled by three Dnmts (Dnmt1, Dnmt3 α and Dnmt3b) [2]. Increased Dnmt expression has been reported in several human cancers, including human hepatocellular carcinoma (HCC) [3]. Despite evidence for their critical roles in carcinogenesis, limited information is available regarding how the expression of Dnmts are transcriptionally regulated [4–7]. Understanding the molecular mechanisms controlling Dnmt expression is essential for learning how changes in DNA methylation result in or predispose to the development of liver cancer.

Nuclear receptor small heterodimer partner (SHP, NROB2) is a transcriptional regulator of a number of genes that play critical roles in hepatic lipid metabolism [8–12]. Our recent studies show that SHP functions as a tumor suppressor in HCC. Deletion of the SHP gene promotes spontaneous hepatoma formation in mice due to hepatocyte hyperproliferation [13] and decreased apoptosis [14], and SHP expression is diminished in HCC in humans due to promoter hypermethylation [15]. These studies provide strong evidence for a critical role of SHP in hepatocarcinogenesis.

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*Correspondence: Li Wang, Tel: 801-587-4616; Fax: 801-585-0187; l.wang@hsc.utah.edu.

No conflicts of interest exist for all authors.

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In this report, we characterized a novel nuclear receptor-mediated mechanism regulating the transcription of *Dnmt1*. We show that SHP is a transcriptional repressor of *Dnmt1* expression via crosstalk with estrogen related receptor gamma (*ERRγ*). This is the first report demonstrating that the expression of *Dnmt1* is controlled by nuclear receptor signaling.

2. Materials and Methods

2.1. Cell lines and liver biospecimens

Human cervix adenocarcinoma cells (Hela; ATCC CCL-2), human hepatoma cells (Huh7; Health Science Research Resources Bank JCRB0403), and mouse liver cells (Nmuli; ATCC CRL-1638) were maintained as described previously [14,16]. Liver fragments from *ERRγ*^{+/+} and liver specific *ERRγ*^{-/-} mice were obtained from Dr. Ronald Evans's laboratory.

2.2. Antibodies and siRNAs

Antibodies used in immunoprecipitation, ChIP and immunoblot analyses include M-280 sheep anti-rabbit or mouse IgG Dynabeads (Invitrogen Dynal As), rabbit normal IgG (Sigma, R-2004) and antibodies against Flag (Sigma, F-7425), GFP (Sigma, G-1544), histone H3 acetyl antibody (H3Ac) (Millipore, #06-599), histone H4 acetyl antibody (H4Ac) (Millipore, #06-866), histone H3 dimethyl Lys4 antibody (H3K4Me2) (Millipore, #07-030), histone H3 dimethyl Lys9 antibody (H3K9Me2) (Millipore, #17-648), *ERRγ* (Sigma, AV-31655), and DNMT1 (Cell signaling, #5032). *ERRγ* RNAi (PDSIRNA SASI-Hs01-00042292, SASI-Hs01-00042292-AS) and non-specific RNAi (SIC001) were purchased from Sigma. Co-immunoprecipitation (Co-IP) and Western blots (WB) were performed as described previously [14,16].

2.3. Real-time qPCR analysis

The method are provided in our recent publications [14,16,17]. *HPRT1* was used as internal control for *Dnmt1* mRNA expression. The primer sequences are: forward 5'-CCCCTGAGCCCTACCGAAT-3' and reverse 5'-CTCGCTGGAGTGGACTTGTG-3' for DNMT1; forward 5'-CCGGTCTCTTTTCGTTTGAGG-3' and reverse 5'-CATCCTGAAGCTTCTGAACGG-3' for h*ERRγ*; forward 5'-TGACACTGGCAAACAATGCA-3' and reverse 5'-GGTCCTTTTCACCAGCAAGCT-3' for h*HPRT1*; forward 5'-CCCAGGGCTTTCCAGATAGC-3' and reverse 5'-AACTGCAGCTGATGCGCTC-3' for *dnmt1*; forward 5'-CACCTGCATCTCACAGCCACT-3' and reverse 5'-GCCAACCCAAGCAGGAAGA-3' for mSHP; and forward 5'-CGTCGTGATTAGCGATGATGA-3' and reverse 5'-CACACAGAGGGCCACAATGT-3' for m*HPRT1*.

2.4. Transient transfection and promoter activity assays

Detailed methods can be found in our recent publications [14,16,18–21]. The mouse *dnmt1* (Gene ID: 13433) promoter and its deletion mutation luciferase constructs (*mdnmt1* Luc) were engineered in our laboratory. All plasmids were described previously [16].

2.5. ChIP and ChIP-ReChIP assays

Chromatin immunoprecipitation (ChIP) assays were done as described previously [14,16]. To analyze the reciprocal association between *ERRγ* and SHP immunoprecipitates, first ChIP was performed with antibody against Flag, washed, and the bound immune-DNA complexes were released in 50 μ l of elution buffer (1X TE containing 2% SDS, 15 mM DTT and protease inhibitors) for 30 min at 37°C and resuspended in 30 times volume of IP dilution buffer (Tris pH 8.0 50 mM, EDTA 5 mM, NaCl 200 mM, NP40 0.5%) for ReChIP

with antibody to GFP. Real-time qPCR was performed using ChIP primer sets specific for the ERR γ binding sites in the dnmt1 and DNMT1 promoters: mouse p1 forward 5'-CTGGCTTTCTCTGTGTGGCTC-3' and reverse 5'-CATGCCTGCAGTCCCTTACAC-3', mouse p2 forward 5'-CAAGACCAGAGAGAGGCAGGA-3' and reverse 5'-CCACATCCAGCCACTCGAA-3'; human p1 forward 5'-TGAGCCGTAGTGACCCACCTA-3' and reverse 5'-GGGATAAAGCAGCGAGAAGCC-3', human p2 forward 5'-GTTTGGACTATGGGCACATGC-3' and reverse 5'-CGCTTGAGCCCAGGAGTTT-3'. A control region from -1489 to -1310 in dnmt1 promoter (forward 5'-CTGAGCCATCTCTCCATCC-3' and reverse 5'-CCGAGGACCAGAAAGACAGC-3') or -1364 to -1260 in DNMT1 promoter (forward 5'-TTCTGGCTGGTTACGGTGG-3' and reverse 5'-CATGTTGGCCAGGCTAGTCTG-3') serves as negative control.

2.6. Statistical analysis

Data are expressed as mean \pm SEM. Statistical analyses were carried out using Student's unpaired t test; $p < 0.01$ was considered statistically significant.

3. Results

SHP inhibits ERR γ transactivation of the dnmt1 promoter

Putative binding elements for nuclear receptors estrogen receptor alpha (ER α), glucocorticoid receptor (GR), hepatocyte nuclear factor 4 α (HNF4 α), ERR γ , liver receptor homologue 1 (LRH-1), and retinoid X receptor alpha (RXR α) were predicted in the mouse dnmt1 promoter. We cloned a 1.5 kb proximal promoter of dnmt1 into a luciferase (luc) reporter. ERR γ , but not ER α , GR, HNF4 α , LRH-1, and RXR α , markedly activated the dnmt1 promoter dose-dependently (Fig. 1A). SHP was previously shown to interact with and inhibit ERR γ transactivation [10,22]. As expected, co-expression of SHP sufficiently abrogated the activity of ERR γ (Fig. 1B). Similar but less striking results were observed in human Huh7 (Fig. 1C) and in mouse Nmuli (Fig. 1D) cells. Two putative ERE sites were located in the dnmt1 promoter (Fig. 1E, left). We generated two mutant (mut) dnmt1 luc constructs (mutLuc 798 and 628). Although the basal luciferase activity of each mutant promoter was different, single deletion of ERE2 (798) or double deletion of ERE2/ERE1 (628) markedly attenuated dnmt1 promoter activation by ERR γ (Fig. 1E, right). The data suggest that both ERE sites may be functional for ERR γ binding. We also confirmed the physical association of SHP with the ERR γ protein in Hela cells using Co-IP and WB (Fig. 1F).

SHP decreases ERR γ recruitment to the Dnmt1 promoter

Putative ERE sites were also located in the human DNMT1 promoter (Fig. 2A). ChIP analysis revealed that ERR γ had an approximately 4 fold higher affinity with mERE1 than with mERE2 in the dnmt1 promoter, which was markedly diminished by SHP co-expression (Fig. 2B, left). The association of SHP with mERE1 was stronger as compared to mERE2 (Fig. 2B, right). The physical association of ERR γ and SHP with the endogenous DNMT1 promoter was examined. Similar to what was observed with the dnmt1 promoter, ERR γ bound preferentially to hERE1 than to hERE2, which was abrogated by SHP (Fig. 2C, left). In addition, SHP exhibited favorable binding to hERE1 than to hERE2 (Fig. 2C, right) in the DNMT1 promoter.

We performed primary ChIP followed by a secondary immunoprecipitation (ReChIP) to determine whether ERR γ and SHP were enriched on similar ERE region of the dnmt1/DNMT1 promoter or whether the two receptors were recruited independently of each other. Consistent with the results in Fig. 2, ChIP analysis revealed a stronger binding of ERR γ to

mERE1/hERE1 than to mERE2/hERE2 by both PCR (Fig. 3A, 3C, 1° ChIP vs. Input) and q-PCR (Fig. 3B, 3D, left). The ReChIP experiments showed binding of SHP on ERR γ immunoprecipitates (Fig. 3A, 3C, 2° ReChIP; Fig. 3B, 3D, right).

To further validate the finding that ERR γ binding was specific to the DNMT1 promoter, ERR γ -siRNA was used to knockdown the endogenous ERR γ in HeLa cells. The enrichment of ERR γ to hERE1 and hERE2 was markedly reduced by ERR γ RNAi (Fig. 3E).

SHP reverses active histone modifications of the dnmt1 promoter by ERR γ

Gene expression could be regulated by epigenetic modification and more particularly by posttranslational modification of histones [23]. We examined histone modifications in the dnmt1 promoter by ERR γ and SHP using antibodies specific to transcriptionally active (H3Ac, H4Ac, and H3K4Me2) and inactive (H3K9Me2) histone marks [24]. We observed a marked increase in dnmt1 promoter occupancy by ERR γ , as indicated by the increased levels of H3Ac and H3K4Me2 and decreased levels of H3K9Me2 (Fig. 4). ERR γ did not alter H4Ac occupancy in the dnmt1 promoter. On the contrary, SHP co-expression reversed the effect of ERR γ by decreasing H3K4Me2 and increasing H3K9Me2. SHP also markedly decreased H3Ac and H4Ac occupancy in the dnmt1 promoter. The results suggest that ERR γ results in transcriptionally active configuration of the dnmt1 promoter which is inhibited by SHP.

SHP inhibits ERR γ induction of Dnmt1 expression

The endogenous DNMT1 mRNA was induced by ERR γ which was down-regulated by SHP (Fig. 5A, left). Interestingly, SHP also decreased the endogenous ERR γ mRNA (Fig. 5A, middle) to undetectable levels, suggesting that SHP may counteract the effect of ERR γ by repressing ERR γ gene expression, in addition to interacting with ERR γ to repress the transactivation of its target gene [22]. ERR γ protein was not completely repressed by SHP (Fig. 5A, right) compared to its mRNA, suggesting that a post-translational mechanism may be involved in regulating ERR γ protein stability. The levels of exogenously expressed ERR γ protein were not inhibited by SHP co-expression (Fig. 5B), confirming that the SHP inhibition of ERR γ activity in Fig. 1 represents the effect of a direct protein-protein interaction between ERR γ and SHP. The mRNA of dnmt1 was moderately but not significantly decreased in liver of ERR $\gamma^{-/-}$ mice (Fig. 5C, left). SHP can be constitutively activated by ERR γ [22] and SHP mRNA was markedly decreased in ERR $\gamma^{-/-}$ mice (Fig. 5C, right). The reduced SHP expression may result in a derepression of Dnmt1 therefore mask the lost activation of ERR γ on dnmt1. ERR γ knockdown using siRNA reduced the levels of DNMT1 mRNA (Fig. 5D) and protein (Fig. 5E), whereas ERR γ overexpression increased DNMT1 protein (Fig. 5F). These results further suggest a crosstalk between ERR γ and SHP in regulating the expression of dnmt1 and DNMT1.

4. Discussion

Previous work has shown that the dnmt1 promoter of mouse is independently activated by Sp1/Sp3 [4] and E2F [5,25] transcription factors, and that human DNMT1 is activated by STAT3 [7]. A recent study demonstrated that p53 is a negative regulator of Dnmt1 promoter activity [26]. However, no information is available whether Dnmt1 gene transcription is regulated by nuclear receptors.

In this study, we identified nuclear receptor SHP as a transcriptional repressor of both mouse dnmt1 and human DNMT1 expression by inhibiting the activity of ERR γ . Such regulation was observed in several cancer cell lines including HeLa and Huh7 cells, but was stronger in HeLa cells, suggesting that this regulatory mechanism may play an important role in

controlling DNMT1 function in cervical cancer. However, it should be noted that the expression of Dnmt1 was only partially decreased by ERR γ knockdown, indicating that in addition to the ERR γ /SHP pathway, other mechanisms may exist to control the expression of Dnmt1.

Another observation was that the ERR γ protein was moderately repressed by SHP, which is distinct from the complete repression of its mRNA by SHP. We also observed that SHP overexpression itself did not inhibit DNMT1 mRNA and protein (not shown) in HeLa cells. This could be in part attributed to the moderate inhibitory effect of SHP on the ERR γ protein. Nevertheless, our results provide strong evidence for a novel role of SHP in repressing gene transcription of Dnmt1 via ERR γ .

Importantly, the co-recruitment of SHP with ERR γ to the dnmt1/DNMT1 promoters significantly decreased the enrichment of ERR γ . In addition, binding of ERR γ to the dnmt1 promoter induces transcriptionally active configuration of the promoter, which is reversed by co-expression of SHP. This suggests that the major mechanism for SHP inhibition of dnmt1 promoter activity is through alteration of local chromatin structure by ERR γ . SHP-mediated repression is not limited to the action of ERR γ , because SHP also decreases H4Ac occupancy which is not significantly affected by ERR γ . The results suggest that SHP may have a general function to keep the local chromatin configuration of its target genes in a repressive mode, which is consistent with its role as a universal transcriptional repressor [27].

Over sixty chromatin modifying enzymes have been identified [28,29], and many of them are associated with post-translational modification of H3Ac, H4Ac, H3K4Me2, and H3K9Me2. It remains to be determined in future studies whether SHP and ERR γ are co-recruited to a multiprotein complex including proteins governing the post-translational modification of histones, and how this would regulate the gene transcription of Dnmt1.

Overall, our findings are important because they link SHP function to DNA methylation at the molecular levels. Considering the importance of Dnmt1 in DNA methylation associated silencing of tumor suppressors [2], and the critical role of SHP in liver cancer progression [13–15], the identification of the regulatory role of SHP in controlling Dnmt1 expression improves our understanding of the epigenetic mechanisms governing the development of liver cancer.

Acknowledgments

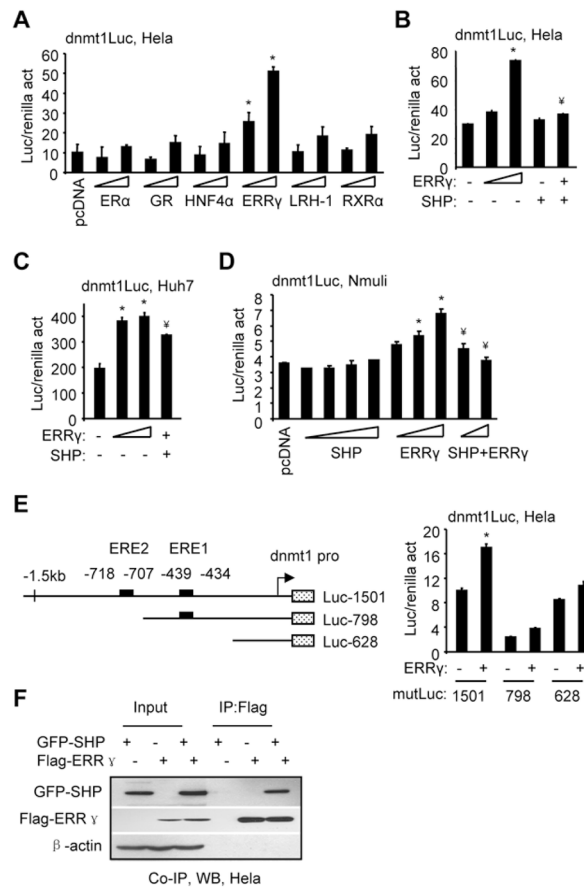
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**Fig. 1.**

SHP inhibition of dnmt1 promoter activity by ERR γ . (A) Transient transfection assays to determine dnmt1 promoter luciferase (luc) activity (act) by expression of ER α (50, 200 ng), GR (50, 200 ng), HNF4 α (50, 200 ng), ERR γ (50, 200 ng), LRH-1 (50, 200 ng), or RXR α (50, 200 ng) in HeLa cells. (B–D) Transient transfection assays to determine dnmt1 promoter luciferase activity by co-expression of ERR γ and SHP in HeLa (B) (ERR γ : 50, 200 ng; SHP, 200 ng; ERR γ +SHP, 200 ng+200 ng), Huh7 (C) (ERR γ : 100, 400 ng; ERR γ +SHP: 400 ng +100 ng), and Nmuli cells (D) (SHP: 100, 200, 400, 800 ng; ERR γ : 200, 400, 800 ng; ERR γ +SHP: 800 ng+200 ng, 800 ng+400 ng). (E) Left: Diagram showing the location of EREs in the dnmt1 promoter and its deletion constructs. Right: Transient transfection assays to determine dnmt1 promoter luciferase activity by ERR γ (100 ng) in HeLa cells. A–E: The luciferase activities were normalized by renilla activities. Data is represented as mean \pm SEM (* p <0.01 vs. control; $\ddagger p$ <0.01 vs. ERR γ alone). The experiments were repeated three times (triplicate wells/time) with similar results. One representative result is shown. (F) Immunoprecipitation (IP) and Western blots (WB) to determine the direct association of SHP with the ERR γ protein. HeLa cells were transfected with Flag-ERR γ (1 μ g, 3 cm plate) and/or GFP-SHP (1 μ g, 3 cm plate) expression vectors. Anti-Flag antibodies were used to immunoprecipitate ERR γ , and the protein levels of SHP and ERR γ were detected by WB using anti-GFP or anti-Flag antibodies, respectively.

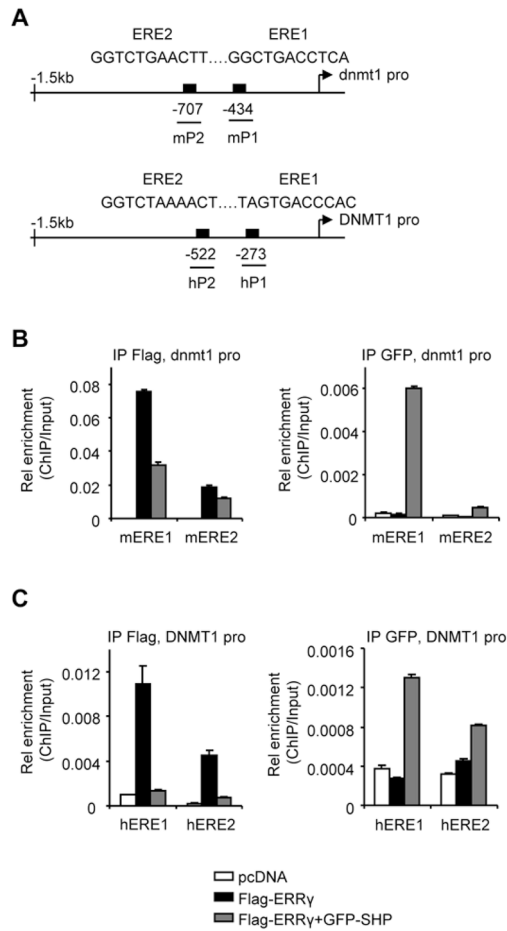
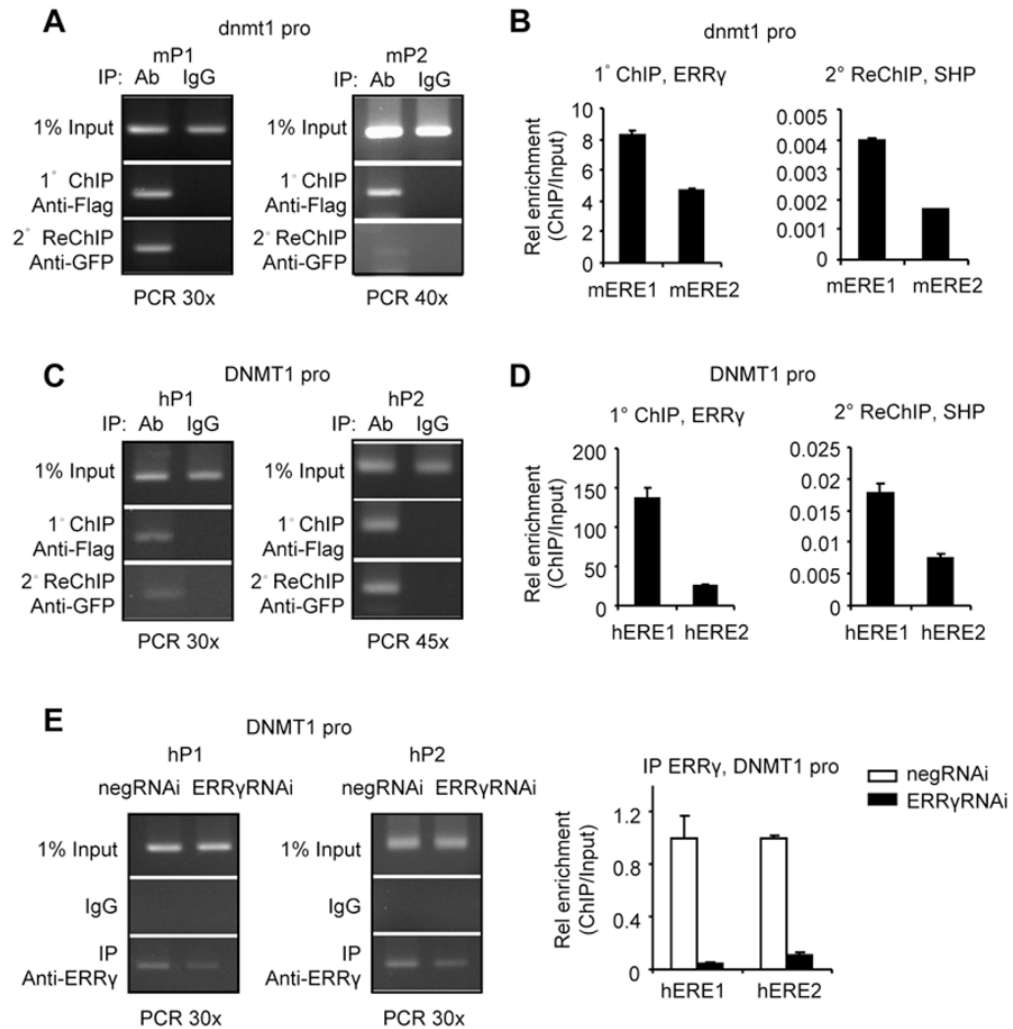


Fig. 2. SHP inhibition of ERR γ recruitment to the Dnmt1 promoter. (A) Schematic showing the putative ERR γ binding sites (EREs) in the dnmt1 and DNMT1 promoters. (B–C) ChIP assays to determine ERR γ and SHP co-immunoprecipitation (Co-IP) to the ERE region of the dnmt1 (B) or DNMT1 (C) promoter. ERR γ and SHP were exogenously expressed in HeLa cells with Flag-ERR γ (5 μ g, 10 cm plate) and/or GFP-SHP (5 μ g, 10 cm plate) plasmids, along with the dnmt1 promoter. The chromatin was co-immunoprecipitated using anti-Flag or anti-GFP antibodies. Real-time qPCR was used to determine the association of ERR γ and SHP to the exogenous dnmt1 promoter using mP1 and mP2 primers (B), and their association to the endogenous DNMT1 promoter was determined by hP1 and hP2 primers (C). PCR products were amplified from input-positive controls, IgG-negative controls, and antibody immunoprecipitates. Histograms show antibody/input ratios of PCR products quantified using qPCR, expressed as Relative (Rel) enrichment. Error bars represent SEM from three independent measurements.

**Fig. 3.**

Co-recruitment of ERR γ and SHP to the Dnmt1 promoter. (A–D) ChIP-ReChIP analysis by PCR (A, C) or qPCR (B, D) to illustrate the enrichment of ERR γ and SHP to the dnmt1 (A–B) or DNMT1 (C–D) promoter. Soluble chromatin was prepared and immunoprecipitated (1° ChIP) with anti-Flag antibodies. Immunocomplexes were collected and eluted, and soluble chromatin fraction was reimmunoprecipitated (2° ReChIP) with anti-GFP antibodies. Other procedures were done as described in Fig. 2. (E) ChIP assays by PCR (left and middle) and q-PCR (right) to determine ERR γ recruitment to the endogenous DNMT1 promoter in HeLa cells with ERR γ knockdown using siRNA (ERR γ RNAi, 100 pmol). ChIP analysis was done as described in Fig. 2.

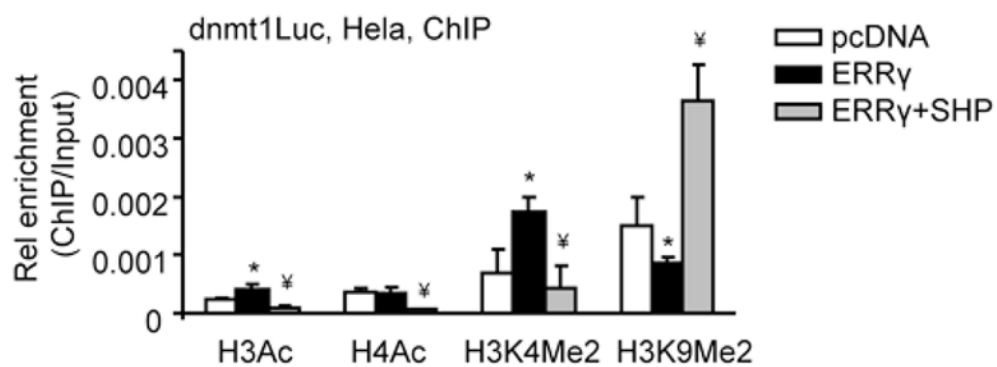


Fig. 4. Alteration of histone modifications of the dnmt1 promoter by ERR γ and SHp. The chromatin was co-immunoprecipitated using anti-H3Ac, anti-H4Ac, anti-H3K4Me2 or anti-H3K9Me2 antibodies. ChIP analysis was done as described in Fig. 2. * $p < 0.01$ vs. pcDNA control; ‡ $p < 0.01$ vs. ERR γ group.

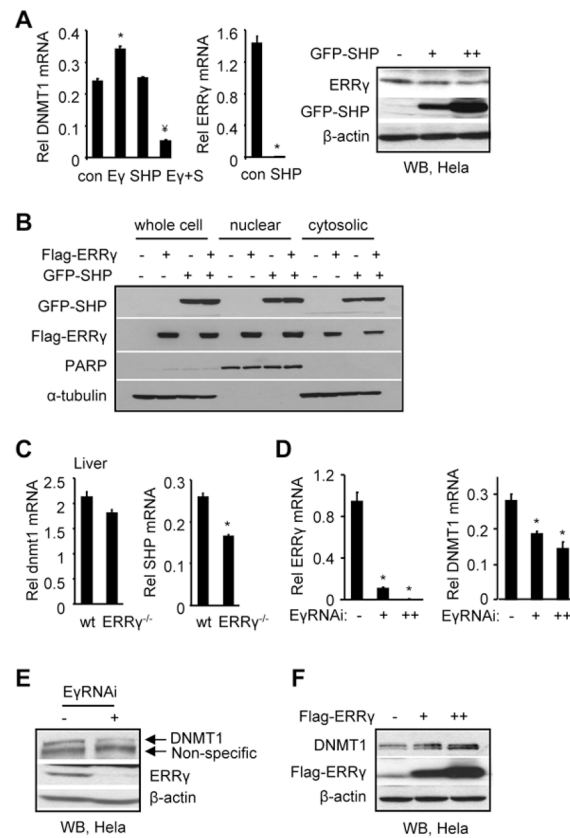


Fig. 5. SHP inhibition of Dnmt1 expression through $ERR\gamma$. (A) qPCR analysis of DNMT1 (left) and $ERR\gamma$ (middle) mRNA in HeLa cells that were overexpressed with $ERR\gamma$ (3 μ g, 6 cm plate) and/or SHP (3 μ g, 6 cm plate) expression vectors. Right: WB analysis of $ERR\gamma$ and GFP-SHP protein in HeLa cells that were overexpressed with GFP-SHP plasmids (0, 0.5, 1 μ g, 12 well plate). Anti- $ERR\gamma$ and anti-GFP antibodies were used, respectively. (B) WB analysis of $ERR\gamma$ and SHP protein in HeLa cells that were overexpressed with Flag- $ERR\gamma$ and GFP-SHP plasmids. The protein levels of $ERR\gamma$ and SHP in the whole cell lysate, nuclear fraction and cytosolic fraction were detected using anti-Flag and anti-GFP antibodies, respectively. PARP was used as a marker for nuclear proteins and α -tubulin was used as a marker for whole cell and cytosolic proteins. (C) qPCR analysis of hepatic *dnmt1* and SHP mRNA in liver specific $ERR\gamma$ -knockout ($ERR\gamma^{-/-}$) mice. (D) qPCR analysis of $ERR\gamma$ and DNMT1 mRNA in HeLa cells with $ERR\gamma$ knockdown using siRNA (-, non-specific siRNA control; +, 100 pmol $ERR\gamma$ RNAi; ++, 200 pmol $ERR\gamma$ RNAi). A, C, D: Data are represented as mean \pm SEM of three independent assays (n=three). * p <0.01 vs. control; $\ddagger p$ <0.01 vs. $ERR\gamma$ alone. Rel, relative. (E) WB analysis of DNMT1 and $ERR\gamma$ protein in HeLa cells with $ERR\gamma$ knockdown using siRNA (-, non-specific siRNA control; +, 100 pmol $ERR\gamma$ RNAi). Anti-DNMT1 and anti- $ERR\gamma$ antibodies were used, respectively. (F) WB analysis of DNMT1 and Flag- $ERR\gamma$ protein in HeLa cells with Flag- $ERR\gamma$ overexpression (0, 1, 4 μ g, 6 cm plate). Anti-DNMT1 and anti-Flag antibodies were used, respectively. A weak non-specific band was seen in the first lane.