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The Methyl Transferase PRMT1 Functions as Co-Activator of FXR/RXR and Regulates Transcription of FXR Responsive Genes

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FXR and PRMT-1

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ABBREVIATIONS

BA, Bile Acid; BSEP, bile-salt export pump; CARM1, coactivator-associated arginine methyltransferase 1; CDCA, chenodeoxycholic acid; CYP7A1, cholesterol 7α-hydroxylase cytochrome P-450; CYP8B1, sterol 12α-hydroxylase; IR, inverted repeat; ER, everted repeat; FXR, farnesoid X receptor; FXRE, FXR response element; HAT, histone acetyl transferase; HMT, histone methyltransferase; MTA, 5'-deoxy-5'-methyl-thioadenosine; NR, Nuclear Receptor; NTCP, Na+ taurocholate cotransport peptide; 9-cis RA, 9-cis retinoic acid; PRMT, protein arginine methyltransferase; RXR, 9-cis retinoid X receptor; SAM, S-adenosyl-L-methionine; 6-ECDCA, 6-ethyl CDCA; SRC-1, steroid receptor associated coactivator-1.

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ABSTRACT

The farnesoid X receptor (FXR) is a nuclear receptor (NR) that functions as an endogenous sensor for Bile Acids (BAs). FXR is bound to, and activated by bile acid and chenodeoxycholic acid (CDCA) is the natural most active ligand. Upon activation FXR heterodimerizes with 9-cis retinoic X receptor (RXR) and regulates genes involved in cholesterol and BAs homeostasis. 6-Ethyl CDCA (6-ECDCA) is a synthetic BA that binds FXR and induces gene transcription by recruits coactivators such as steroid receptor coactivator-1 (SRC-1), with histone acetyltransferase activity (HAT). In addition to acetylation, histone methylation is critically involved in regulating eukaryotic gene expression. In the present study, we demonstrated that 6-ECDCA activates FXR to interacts with Protein Arginine Methyl-Transferase type I (PRMT1), which induces upregulation of bile salt export pump (BSEP) and the Small Heterodimer Partner (SHP) mRNA expression, while causes a downregulation of P-450 cholesterol 7α-hydroxylase and Na+ taurocholate cotransport peptide genes. Chromatin immunoprecipitation assay (ChIP) suggests that 6-ECDCA induces both the recruitment of PRMT1 and the H4 methylation to the promoter of BSEP and SHP genes. We also provide evidence that a methyltransferase inhibitor blocks the activation of FXR responsive genes. Our results indicate that histone methylation, similar to acetylation, regulates transcriptional activation of genes involved in cholesterol and BAs homeostasis.

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Bile Acids (BAs) regulate their own biosynthesis and transport by binding to and activating the farnesoid X receptor (FXR), a nuclear receptor (NR) expressed in liver, intestine, gallbladder and kidney (Forman, 1995; Makishima, 1999; Parks, 1999; Wang, 1999). Chenodeoxycholic acid (CDCA), a primary BAs, is the natural most active ligand of FXR with an EC_{50} of 10-50 μM (Makishima, 1999; Parks, 1999; Wang, 1999). We have previously shown that 6α-ethyl-chenodeoxycholic acid (6-ECDCA) a semi-synthetic derivative of CDCA is a potent and selective steroidal FXR agonist with an EC_{50} of 99 nM (Pellicciari, 2002a) and protects against cholestasis and liver fibrosis when administered in vivo (Fiorucci, 2004; Fiorucci, 2005).

In liver cells, activation of FXR leads to the regulation of genes whose function is to decrease the concentrations of BAs within hepatocyte. Thus, upon ligand-induced activation FXR causes a SHP (small heterodimer partner)-dependent inhibition of the expression of P-450 cholesterol 7α-hydroxylase (CYP7A1) and oxysterol 12α- hydroxylase (CYP8B1), both of which are central to the synthesis of BAs from cholesterol (del Castillo-Olivares, 2001; Goodwin, 2000; Lu, 2000; Sinal, 2000; Zhang). In addition, FXR ligands promote the expression of canalicular transporters such as the bile-salt export pump (BSEP), the multidrug resistance associated protein (MRP)-2 (Ananthanarayanan, 2001; Sinal, 2000) and the multidrug resistance protein (MDR)-2 (Kast, 2002) involved in BAs transport across the canalicular membrane of hepatocytes providing a pathway for cholesterol and BAs excretion.

In liver diseases accumulation of toxic BAs plays mechanistic role in hepatocyte injury leading to cell necrosis, fibrosis and cirrhosis. Works from our and other laboratories have provided evidence that FXR activation with potent ligands protects

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against cholestasis (Liu, 2003) and liver fibrosis in rodents (Fiorucci, 2004; Fiorucci 2005).

FXR is an obbligate partner of the 9-cis-retinoic acid (9-*cis* RA) receptor (RXR) (Forman, 1995; Wang, 1999; Zavacki, 1997). The FXR/RXR heterodimer binds DNA sequences composed of two inverted repeats separated by one nucleotide (IR-1) and can be activated by the ligands of both receptors (BAs and/or 9-*cis* RA) (Forman, 1995; Wang, 1999; Zavacki, 1997). There is evidence that 6-ECDCA, binds FXR and induces gene transcription by recruiting coactivators with histone acetyltransferase activity (HAT) such as the steroid receptor coactivator-1 (SRC-1) (Berger, 2002; Pellicciari, 2002b).

Histones methylation occurs on lysine (K) or arginine (R) residues and is catalyzed by a family of histone methyltransferases (HMTs) that use S-AdenosylMethionine (SAM) as a methyl-group donor. Methylation of lysine residues is known to occur on histone H3 (K4, K9 and K27) and H4 (K20) (Kouzarides, 2002; Zhang, 2001b). Methylation at arginine residues occurs within the tails of histone H3 (R2, R17, R26) and H4 (R3) (Kouzarides, 2002; Strahl, 2001; Zhang, 2001b). Unlike lysine methylation, which exerts a repressive or active function dependent on the promotorial context (Kouzarides, 2002; Santos-Rosa, 2002; Zhang, 2001b), methylation of arginine residues, similar to acetylation, correlates with the active state of transcription (Bauer, 2002; Ma, 2001). There are five known protein arginine methyltransferases (PRMTs) that have a highly conserved catalytic domain (Kouzarides, 2002). PRMT1, PRMT3 and PRMT4/CARM1 catalyze the formation of asymmetric dimethylated arginine, whereas PRMT5/JBP1 catalyses symmetric dimethylation. The enzymatic activity of the PRMT2 protein has not been established yet (Kouzarides, 2002; McBride, 2001; Zhang, 2001b). PRMT1 is the predominant, if not exclusive, H4 Arginine-Methyltransferase in mammalian cells and functions as a

coactivator of several NRs, including the thyroid, the estrogen and the androgen receptor (Koh, 2001; Strahl, 2000; Strahl, 2001; Tang, 2000). Whether PRMT1 mediates chromatin remodeling in response to FXR ligands is unknown.

In the present study we demonstrate that FXR immunoprecipitates contains an HMT activity and that FXR binds to PRMT1. Moreover, by chromatin immunoprecipitation assay (ChIP) we provide evidence that natural and synthetic FXR ligands enhance the interaction between FXR/RXR and PRMT1 leading to the formation of a ternary complex on the promoter of BSEP and SHP, two FXR responsive genes. Together, these results indicate that histone H4 methylation induced by FXR activation plays a functional role in regulating cholesterol and BAs homeostasis.

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MATERIALS AND METHODS

Molecular cloning of FXRα, RXRα and PRMT1, and Plasmids construction. Human cDNAs encoding FXRα, RXRα and PRMT1 were cloned by RT-PCR from HepG2 cells. Briefly, RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA) reagent according to the manufacturer's instructions and 1 μg of RNA was random reverse-transcribed with Mo-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) in 20 μl volume reaction for 1 h at 42 °C. The cDNA was amplified using 250 ng of reverse transcribed template utilizing Phusion DNA polymerase (FINNzymes, Espoo, Finland) in a 50 μl volume reaction containing 1x Phusion reaction buffer, 200 nM dNTPs and 1 μM each primer. The sequences of the primers used were:

FXRα 5'-tggatgggatcaaaaatgaatctc-3' and 5'-catcactgcacgtcccagatttc-3';

RXRα 5'-catgagttagtcgcagacatggac-3' and 5'-gcctaagtcatttggtgcggcg-3';

PRMT1 5'-cgaactgcatcatggaggtgtcctg-3' and 5'-ctcagcgcatccggtagtcggtg-3'.

The RXR and PRMT1 cDNAs were cloned into EcoRI cloning site into pSG5 mammalian expression vector and PRMT1 cloned into pGEX-4T1 (Pharmacia biotech, Piscataway, New Jersey) in order to express the GST fusion protein. The FXR cDNA was cloned into the BglII site in pSG5 (Stratagene, La Jolla, CA). For luciferase assays, the reporter plasmid pGL3$(IR1)_3$-Luc was constructed by cloning three FXREs (IR1) upstream to luciferase into the BglII site of pGL3-Luc vector. The FXRE sequence from BSEP promoter (-70/-43 nt) was 5'-gcccttagggacattgatccttaggcaa-3' (Ananthanarayanan, 2001). The FXRE (IR1) is underlined. Plasmid pGL3-FXREmut-Luc was generated by mutating the FXRE site using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. The mutated FXRE sequence

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was 5'-gcccttagaaacattgatttttaggcaa-3'. The mutated FXRE (IR1) is underlined and the mutations are shown in italic letters.

Cell Culture, Transfection and Luciferase assays. HepG2 and HuH7 (two hepatoma cell lines) and HEK 293T cells (human embryonic kidney cells) were cultured in E-MEM and DMEM (high glucose) respectively, supplemented with 1% penicillin/streptomycin, 1% L-glutamine and 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Cells were grown at 37°C in 5% CO_2. All transfections were performed using the calcium phosphate coprecipitation method in the presence of 25 μM chloroquine. Twenty-four hours before transfection HEK 293, HepG2 and HuH7 cells were seeded onto 6-well plates at a density of 250,000-400,000 cells/well. Transient transfections were performed using 500 ng of reporter vector pGL3-$(IR1)_3$-Luc, 200 ng pCMV-βgal as internal control for transfection efficiency, and 50 ng of each receptor expression plasmid (pSG5-FXR, pSG5-RXR or pSG5-PRMT1). The pGEM vector (Promega, Madison, WI) was added to normalize the amounts of DNA transfected in each assay (2.5 μg). At 36-48 h post-transfection cells were stimulated with GW-4064 or 6-ECDCA at 1 μM, or 20 μM, respectively for 18 h, all diluted in DMSO. Control cultures received vehicle (0.1% DMSO) alone. Cells were lysed in 100 μl diluted reporter lysis buffer (Promega, Madison, WI), and 0.2 μl (HEK 293) or 10 μl (HepG2 and HuH7) and cellular lysates assayed for luciferase activity using the Luciferase Assay System (Promega, Madison, WI). Luminescence was measured using an automated luminometer. Luciferase activities were normalized for transfection efficiencies by dividing the relative light units by β-galactosidase activity expressed from cotransfected pCMV-βgal. Each data point was the average of triplicate assays and repeated three times.

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I*mmunoprecipitations*. Transfected and untransfected cells were induced with FXR ligands or DMSO (vehicle) for 18 h. Cells were first washed three times with ice cold PBS and lysed by sonication in either RIPA lysis buffer (1x PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) for immunoblotting and IPH buffer (50 mM Tis pH 8, 150 mM NaCl, 0.5% NP-40, 5 mM EDTA) for in vitro methylation. Both media were supplemented with 0.1 mg/ml phenylmethylsulfonyl fluoride and 1X complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Lysates were clarified by centrifugation at 13,000g for 10 min and the protein concentration was adjusted to 1 mg/ml. From one to four mg total proteins or 10^7 cells were pre-cleared on a rotating wheel for 1 h at 4°C using protein A Sepharose beads (Amersham, Madison, WI). Immunoprecipitaion was performed overnight at 4°C with 1 μg/ml anti-FXRα (Santa Cruz Biotechnology, SantaCruz, CA) or anti-CD3 as a control antibody in the presence of 10 μl protein A Sepharose (Amersham, Madison, WI). The resultant immunoprecipitates were washed 5 times with 1 ml of lysis buffer and then used for in vitro methylation or immunoblotting.

Histone purification, in vitro methylation and fluorography. Histones were purified from the HEK 293 basic cell line using the acid extraction method. Briefly, the cells were lysed on ice in 10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1.5 mM PMSF, and HCl 0.2 M for 30 min. Acidic proteins were separated from cellular debris by centrifugation and subjected to dialysis in 0.1 M acetic acid and then in H_2O. Proteins were quantified by the Bradford assay (Biorad, Hercules, CA). Immunoprecipitates were incubated for 30 min at 30°C in 40 μl of IPH buffer supplemented with 0.8 mM S-adenosyl-L-(methyl-3H) methionine, (^{3}H)AdoMet (79 Ci/mmol from a 12.6 μM stock solution in dilute HCl/ethanol 9:1, pH 2.0-2.5; Amersham, Madison, WI) and purified histones (1 μg). The positive control for methylation wasobtained using a GST-PRMT1 fusion protein 0.5 μg and purified

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histones (1 μg). Labeled histones were analyzed by SDS-PAGE followed by fluorography. Briefly, after electrophoresis the gel was stained in Coomassie blue for 15 min and then fixed in destaining solution (40% methanol and 10% acetic acid) for 1 h at room temp. The gel was then soaked in a volume of EN3HANCE (NEN Life Sciences, Boston, MA) equivalent to five times the gel volume for one hour under gentle agitation. The gel was then incubated in water for 30-60 min with gently shacking after which it was dried and exposed to a Kodak Biomax film at the -80°C with an intensifying screen.

Western Blot. Immunoprecipitates or cellular extract were resuspended in 2X SDS-sample Laemmli buffer, boiled 3 min, and separated by SDS-PAGE. The gel was then analyzed by Western blotting with anti-FXRα or anti-RXRα (both from Santa Cruz Biotechnology, SantaCruz, CA) or anti-PRMT1 antibodies (Abcam, Cambridge, UK). All blots were developed with HRP-conjugated secondary antibodies using the ECL system (Amersham, Madison, WI).

Chromatin Immunoprecipitation (ChIP). A ChIP assay was performed according to the manufacturer's protocols (Upstate Biotechnology, Lake Placid, NY) with minor modifications. Briefly, HepG2 cells were cross-linked with 1% formaldehyde at room temperature and then the reaction was terminated by the addition of glycine to a final concentration of 0.125 M. Cells were washed in PBS ice-cold and lysed with SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Trip-HCl pH 8). Cellular lysates were diluited with ChIP dilution buffer, sonicated and immunoprecipitated with specific antibody: anti-FXR (H-130), anti-SHP (Q-14) and anti-Cytochrome C (C-20) from Santacruz Biotech (Santa Cruz, CA); anti-PRMT1 (ab3768) from Abcam (Abcam Ltd, Cambridge, UK) and anti-methyl-H4 (Arg3) (07-213) from Upstate Biotech (Upstate Biotechnology, Lake Placid, NY). Immunoprecipitates were collected with protein A/G agarose beads (Upstate Biotechnology, Lake Placid, NY) and washed sequentially

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first with a low salt wash buffer and then a high salt wash buffer (Upstate Biotechnology, Lake Placid, NY) using manufacturer's recommended procedures. DNA was eluted by addition of 1% SDS and 0.1M NaHCO3, and the cross-linking reactions were reversed by heating the mixture to 65°C overnight. The DNA was recovered from immunoprecipitated material by proteinase K treatment at 65°C for 1 hour followed by phenol/chloroform (1:1) extraction, ethanol precipitation and dissolved into 50 µl water. Two µl were used for quantitative real-time PCR (qRT-PCR). Five microliters of PCR reactions were extracted after 40 complete cycles for visualization on agarose gels and stained with ethidium bromide.

qRT-PCR. Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) from human HepG2 and HuH7 cells stimulated 6-ECDCA (1 µM), GW-4064 (1 µM), or CDCA (20 µM) for 18 h. One µg RNA was incubated with DNaseI (Invitrogen, Carlsbad, CA) for 15 min at room temperature followed by 95°C for 5 min in the presence of 2.5 mM EDTA. RNA was reverse-transcribed with Superscript III (Invitrogen, Carlsbad, CA) with random-primers in volume of 20 µl. For real-time PCR, 100 ng template was used in a 25 µl reaction containing 0.3 µM each primer and 12.5 µl of 2X SYBR Green PCR Master Mix (Biorad, Hercules, CA). All reactions were performed in triplicate using the following cycling conditions: 2 min at 95°C, followed by 50 cycles of 95°C for 10 sec and 60°C for 30 sec using an iCycler iQ instrument (Biorad, Hercules, CA). The mean value of the replicates for each sample was calculated and expressed as cycle threshold (C_T: cycle number at which each PCR reaction reaches a predetermined fluorescence threshold, set within the linear range of all reactions). The amount of gene expression was then calculated as the difference (ΔC_T) between the C_T value of the sample for the target gene and the mean C_T value of that sample for the endogenous control (GAPDH). Relative expression was calculated as the difference ($\Delta\Delta C_T$) between the ΔC_T values of the

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test and control (WT) samples for each target gene. The relative level of expression was expressed as $2^{-\Delta\Delta C}{}_{T.}$ All PCR primers were designed using the PRIMER3-OUTPUT software and published sequence data obtained from the NCBI database. Primers were as follows:

hBSEP: 5'-gggccattgtacgagatcctaa-3'; 5'-tgcaccgtcttttcactttctg-3';

hCYP7A1: 5'-caccttgaggacggttccta-3'; 5'-cgatccaaagggcatgtagt-3';

hGAPDH: 5'-gacaacagcctcaagatcatcagc-3'; 5'-gtagaggcagggatgatgttctgg-3';

hSHP: 5'-ccaatgatagggcgaaagaa-3'; 5'-gctgtctggagtccttctgg-3';

hNTCP: 5'-ctgagcgtcatcctggtgttcatg-3'; 5'-ggtcatcacaatgctgaggtt-3'

hBSEP promoter: 5'-ctcgtatgtcactgaactgtgctt-3'; 5'-gcactgaacagaattcaaacttt-3'

hSHP promoter: 5'- gctggcttcctggcttagc-3'; 5'-cttatcagatgactcaagtg-3'

Statistical analysis. Data were analyzed with a two-tailed Student's t test using Prism 3 (Graphpad Software, Inc, San Diego, CA). Values are shown as the mean ± SE . A p value < 0.05 was considered statistically significant.

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RESULTS

F*XR ligands induce H4 specific methylation activity* — To investigate whether FXR activation by 6-ECDCA leads to the recruitment of HMT activity, HepG2 cells were exposed to 1 μM 6-ECDCA, and anti-FXR immunoprecipitates were incubated with purified core histones in the presence of S-adenosyl-L-[methyl-^{3}H]methionine ([^{3}H] SAM), as a methyl group donor for histone methyltransferases. The reactions were then subjected to SDS-PAGE followed by fluorography. As shown in Fig. 1A, HMT activity was detected in anti-FXR immunoprecipitates and was enhanced by incubating the cells with 6-ECDCA (Fig. 1A, lane 6 versus lane 4). In contrast, immunoprecipitates obtained with the unrelated antibody (anti-CD3) showed only a weak HMT activity (Fig. 1A, lanes 3 and 5). In contrast, no HMT activity was detected in immunoprecipitates with protein A Sepharose alone (Fig. 1A, lanes 1 and 2). The HMT activity found in the FXR immunocomplexes efficiently methylates H4, but not H2A, H2B, or H3 (Fig. 1A and 1B). Specificity of methylation of H4 was confirmed by incubating core histone proteins with a bacterially expressed GST-PRMT1 fusion protein which only methylates histone H4 (Fig. 1A, lane 7). Induction of HMT activity by the 6-ECDCA was not due to changes in neither the amount of core histone proteins (Fig. 1B) nor to an increased expression of FXR, RXR and PRMT1 proteins, as shown by the Western blot assay (Fig. 1C). Similar results were obtained with the natural FXR ligand CDCA (20 μM) and with GW-4064 (1 μM) a non-steroidal FXR ligand (data not shown).

6-ECDCA induces FXR to recruit PRMT1 — Since PRMT1 is the predominant arginine methyltransferase in mammalian cells (Tang, 2000) and selectively methylates Arg 3 of histone H4 (Strahl, 2001; Wang, 2001) we investigated whether the H4-specific HMT enzymatic activity revealed by the FXR immunoprecipitates was

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due to the recruitment of PRMT1 by FXR. To test this hypothesis, lysates obtained from HepG2 cells were immunoprecipitated with anti-FXR and immunoblotted with anti-PRMT1 antibody. FXR activation with natural (CDCA, not shown) and synthetic (6-ECDCA and GW4064) ligands caused PRMT1 recruitment (Fig. 2A and 2B, lane 5 versus 6 and lane 6 versus 7). No interactions were observed in immunoprecipitates obtained with an unrelated antibody (anti-CD3). The exposure of cells to FXR ligands did not change the relative protein expression in total lysates as shown in Fig. 1C and Fig. 2A and 2B, lanes 1 and 2.

PRMT1 is a coactivator of the FXR/RXR heterodimer — To investigate whether PRMT1 functions as FXR/RXR coactivator, we transiently transfected HEK 293 cells with vector alone, PRMT1, FXR and RXR, or FXR, RXR and PRMT1 expression vectors. This cell line was chosen because it express very low levels of endogenous FXR. The cells were cotransfected with a plasmid containing the luciferase reporter gene under the control of three FXR response elements [FXRE $(IR1)_3$] and the β-gal expression vector (pCMV-βgal) to normalize the transfection efficiency and then exposed to 6-ECDCA or DMSO (vehicle). While transfecting HEK 293 cells with the PRMT1 expression vector alone had no effect on the gene reporter activity and was insensitive to the addiction of 6-ECDCA (Fig. 3A), the transient expression of the FXR and RXR plasmids resulted in a little enhancement of the gene reporter activity. However, this response was significantly increased by adding 6-ECDCA (1 μM), and cotransfecting PRMT1 caused a further 3.5 fold enhancement of gene reporter activity in the presence of 6-ECDCA (Fig. 3A). The coactivator effect of PRMT1 in cells induced by 6-ECDCA was abrogated when a reporter plasmid containing the mutated FXRE-Luc was employed for transfection (Fig. 3A), indicating that an intact FXRE is required for the PRMT1 promoter induction by FXR ligands. As illustrated in Fig. 3B, the transactivation of FXRE by PRMT1 was modulated in a concentration-

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dependnet manner by the 6-ECDCA with an EC_{50} of ≈ 1 μM (n= 4; P<0.05 versus FXR/RXR). In addition, transfection of PRMT1 enhanced the FXR-dependent transactivation of CDCA or GW-4064 (Fig. 3C). The exposure of HEK293 cells to FXR ligands did not change the relative protein expression in total lysates as shown by the Western blot assay (Fig. 3D).

FXR-dependent transactivation enhanced by PRMT1 is induced by RXR ligand, 9-cis RA — Since FXR functions as a permissive heterodimer with RXR and previous data have shown that RXR ligands might transactivate FXR (Forman, 1995; Wang, 1999; Zavacki, 1997), we have then examined whether PRMT1 is involved in the FXR transactivation caused by the RXR ligand 9-*cis* RA. HEK 293 cells were cotransfected with FXR, RXR, PRMT1 and the FXRE-Luc reporter gene plasmid and then induced with 1 μM 9-*cis* RA. As shown in Fig. 4, 9-*cis* RA induced a weak transactivation of FXR/RXR heterodimer (Fig. 4, lane 2 versus lane 1). Cotransfection of PRMT1 caused a further enhancement of gene reporter activity in the presence of 9-cis RA (Fig. 4, lane 6 versus lane 2), suggesting that the RXR ligand induces the transcriptional activation of FXRE by recruiting a methyltransferase coactivator to the FXR/RXR heterodimer. However, the 9-*cis* RA antagonizes the FXR/RXR transactivation caused by 6-ECDCA (Fig. 4, lane 4 versus lane 3 and lane 8 versus lane 7; n=5, P<0.05 versus 6-ECDCA alone). Similar results were obtained with CDCA and GW-4064 (data not shown).

PRMT1 enhances transcriptional activation of FXR responsive genes induced by 6-ECDCA — To investigate whether PRMT1 regulates the transcriptional activation of FXR responsive genes in response to FXR ligands, the HepG2 cell line was transiently transfected with vector alone or PRMT1 plasmid and then stimulated with 6-ECDCA and GW-4064 (data not shown). Fig. 5 shows that the relative amounts of BSEP (Fig. 5A) and SHP (Fig. 5B) mRNAs, which are directly regulated

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by FXR, normalized by GAPDH gene expression, were significantly upregulated by PRMT1 in the presence of 6-ECDCA (n=4; P<0.05 versus vector alone). The relative expression of NTCP (Fig. 5C) and CYP7A1 (Fig. 5D) was downregulated by 6-ECDCA (n=4; P<0.05 versus untreated). However, cotransfection of PRMT1 induces a further downregulation of these two genes induced by the FXR ligand (Fig. 5C and D) (n=4; P<0.05 versus vector alone). These results correlate with the regulation of SHP, that functions as corepressor for NTCP and CYP7A1 (Goodwin, 2000; Lu, 2000). Similar results were obtained using HuH7, a human hepatoma cell line (Supplementary Fig. 1 on line).

6-ECDCA induces recruitment of PRMT1 to BSEP and SHP promoters and histone H4 methylation — There is evidence that the FXR/RXR heterodimer binds directly to the promoter of FXR responsive genes (Ananthanarayanan, 2001; Goodwin, 2000; Lu, 2000). In the presence of FXR ligands, FXR/RXR heterodimer recruits coactivators such as SRC-1, which acetylates histones (Berger, 2002; Pellicciari, 2002b), and CARM1 wich methylates histone H3 (Anantanarayanan, 2004). Therefore, we hypothesized that FXR activation might recruit PRMT1 to the promoters of BSEP and SHP. To answer this question we used the ChiP analysis. A time-course analysis of ChIP using the anti–PRMT1 antibody shown in Fig. 6A demonstrates that, in response to 6-ECDCA, occupation of BSEP (and SHP) promoter by PRMT1 peaks at 20 minutes and used this time-frame for all the following experiments (n=4; P<0.05 versus baseline). To investigate whether H4 histone methylation occurred in the BSEP and SHP promoters, the chromatin was prepared from HepG2 cells treated with 6-ECDCA for 20 minutes and immunoprecipitated with anti-FXR, anti-PRMT1, anti-methyl H4 (Arg 3) or anti-CytC antibodies (control). The qRT-PCR analysis shown in Fig. 6B demonstrates that the 6-ECDCA induces recruitment of PRMT1 to BSEP and SHP promoters and increases H4 arginine methylation. Together, our results provide firm

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evidence that 6-ECDCA activates FXR to recruits PRMT1 and H4 histones methylation, as a mechanism of transcriptional activation.

Inhibition of arginine methyltransferase activity decreases FXR-mediated transcription — Since these data supported a role for arginine methylation in FXR function, we have then investigated the potential effects of the 5'-deoxy-5'-methylthioadenosine (MTA), a methylation inhibitor, on the ability of FXR to induce gene transcription. HepG2 cells were pretreated with 0.3 mM MTA prior to incubation with 6-ECDCA or GW-4064. The expression of BSEP gene normalized by GAPDH gene expression, was upregulated 84- and 89-fold by 6-ECDCA and GW-4064, respectively (Fig. 7A, columns 3 and 4). Exposing the cells to MTA reduced the expression of BSEP induced by either 6-ECDCA or GW-4064 by ~ 80 % (Fig. 7A, columns 5 and 6). The same results, with different values, were obtained with SHP gene expression (Fig. 7B).

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DISCUSSION

Chromatin remodeling induced by FXR/RXR heterodimer is mediated by histone acetylation. Previous studies have shown that upon FXR activation SRC-1, a member of p160 family of acetyltransferases, is recruited to the DNA(Berger, 2002; Mi, 2003; Pellicciari, 2002b). Histone acetylation, in concert with H3 and H4 histone methylation, recruits different proteins or protein complexes and regulates diverse chromatin functions (Kouzarides, 2002; Strahl, 2000; Zhang, 2001b). In the present study, using immunoprecipitation and *in vitro* methylation assays, we demonstrated that in addition to histone acethylation, activation of FXR induces H4 histone methylation. The PRMT1 is the predominant histone methyltransferase in mammalian cells (Strahl, 2000; Strahl, 2001; Tang, 2000) and specifically methylates Arginine 3 on H4 histone both *in vitro* and *in viv*o (Strahl, 2001; Wang, 2001). Our immunoprecipitation and Western blot analysis indicate that exposure to natural and synthetic FXR ligands induces the interaction of FXR with PRMT1. This methyltransferase acts as a coactivator for several NRs including the estrogen, the thyroid hormone and the androgen receptors in transient transfection assays suggesting that NRs causes chromatin remodeling by inducing histone methylation in addition to histone acethylation (Koh, 2001; Strahl, 2001; Wang, 2001). By luciferase assays, we also demonstrated that PRMT1 increases the transactivation of the luciferase reporter gene induced by FXR and that this function was dependent of an intact FXRE. Finally, we provide evidence that the increase of the FXR-induced transcriptional activation by PRMT1 was dependent on the concentration of FXR ligands.

The FXR/RXR complex is a permissive heterodimer that might be activated by BAs as well as by the RXR ligand 9-*cis* RA (Forman, 1995; Wang, 1999; Zavacki, 1997). An important finding of this study was the demonstration that exposure to the 9-*cis* RA activates the FXR/RXR complex and enhances the transactivation induced

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by PRMT1. These results suggest that in addition to histone acetylation, transcriptional activation mediated by 9-*cis* RA involves histones methyltransferase and that this pathway uses PRMT1 as a coactivator. While the 9-*cis* RA activates FXR/RXR complex, the functional consequence of this interaction is not completely understood. Thus, Kassam et al., (2003) have recently shown that exposure of FXR/RXR heterodimer to 9-*cis* RA decreases, rather than activates, the recruitment of coactivators as well as the DNA binding activity in the presence of FXR ligands. Consistent with this finding, we demonstrated that 9-*cis* RA antagonizes the transcriptional activation induced by 6-ECDCA. While the mechanism(s) involved in this antagonism was not investigated at least two explanations warrant consideration. First, it might be possible that 9-*cis* RA induces the formation of RXR homodimers which bind to the RXR response element (Zhang, 1992). Alternatively, 9-*cis* RA might induce the heterodimerization of RXR with other partners such as peroxisome proliferator-activated receptors, RAR and thyroid receptor, thus reducing the availability of RXR for dimerization with FXR (Thompson, 1998) (Zhang, 1992).

Here, we have shown that PRMT1 functions in the transcriptional regulation of FXR responsive genes. By real-time PCR we demonstrated that BSEP and SHP, two FXR regulated genes, were induced by transient transfection of PRMT1 in hepatic cell lines in the presence of FXR ligands. Furthermore, NTCP and CYP7A1, which are direct targets of SHP, were also downregulated by PRMT1 cotransfection. These effects correlate with the ability of FXR ligands to increase SHP expression in target cells, which directly represses CYP7A1 and NTCP (Goodwin, 2000; Lu, 2000).

BSEP is the major BAs exporting pump and plays an integral role in lipid homeostasis by regulating the canalicular excretion of BAs. Indeed, inactivating mutations of this gene result in progressive familial intrahepatic cholestasis (type 2) and liver cirrhosis (Strautnieks, 1998). In the presence of FXR ligands, the FXR/RXR

heterodimer binds to an FXRE located in the BSEP promoters and induces the transcriptional activation of BSEP (Ananthanarayanan, 2001). The results obtained by ChIP assay suggest that 6-ECDCA induces both the recruitment of PRMT1 and H4 histone methylation to the BSEP promoter. Similar results were obtained with another FXR regulated gene, i.e. SHP, which is a master regulator of several nuclear receptors. The histone methylation was required in order to activate gene transcription upon FXR activation. Indeed, we found that exposure to MTA, a methyl-transferase inhibitor (Williams-Ashman, 1982), antagonizes BSEP and SHP mRNA induction by 6-ECDCA or GW-4064 in HepG2 cells. These results support the notion that activation of FXR regulated genes induced by FXR ligands requires protein methylation.

In conclusion, here we have provided evidence that chromatin remodeling induced by FXR ligands requires H4 histone methylation and that PRMT1 functions as FXR coactivator.

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FIGURE LEGENDS

Figure 1. FXR is associated with H4 Histone Methyltransferase Activity (HMT). A, *In vitro* methylation. HepG2 cells were induced with or without 1 μM 6-ECDCA, then lysed and immunoprecipitated with a specific anti-FXR antibody or an unrelated antibody (anti-CD3) or protein A sepharose alone (pre-clearing). Immunoprecipitates were analyzed for HMT activity in the presence of [^{3}H]SAM and core histones purified as described in the Materials and Methods. Exposure to 6-ECDCA enhances the H4 HMT activity (lanes 4 and 6) in immunoprecipitates obtained with the anti FXR antibody. In contrast, only a weak HMT activity was detected in anti-CD3 immunoprecipitates (lanes 3 and 5), while no HMT activity was detected in immunoprecipitates with protein A Sepharose (PAS) alone (lanes 1 and 2). Lane 7 is a positive control for in vitro methylation obtained with a GST-PRMT1 fusion protein that selectively methylates histone H4. B, Coomassie blue staining of purified core histones from HepG2 basic cell line indicate that histone protein levels were unmodified by treatment with 6-ECDCA. C, Immunoblot of total lysates with anti-FXR, anti-RXR and anti-PRMT1 antibodies shown that those protein levels were unmodified by 6-ECDCA treatment. Each blot is representative at least two others showing the same pattern.

Figure 2. FXR ligands activate FXR to recruits PRMT1. The cellular extracts from HepG2 cells stimulated with or without to FXR ligands were immunoprecipitated with the anti-FXR or anti-CD3 antibody and then analized by Western blotting analysis with anti-PRMT1 antibody. A, 6-ECDCA induces recruitment of PRMT1 to FXR complex. B, A non steroidal FXR ligand, GW-4064, induces recruitment of PRMT1. No interaction was detected in anti-CD3 immunoprecipitates (A, lane 1; and B, lanes

3, 4). MKs, protein markers. Each blot is representative at least two others showing the same pattern. The level of the protein PRMT1 was unmodified by treatment of the cells with 6-ECDCA (lanes 1 and 2).

Figure 3. PRMT1 functions as a coactivator of the FXR/RXR heterodimer. HEK 293 cells were cotransfected with PRMT1, FXR and RXR expression plasmid, pFXRE-luc reporter plasmid and pCMV-βGal plasmid, as control of transfection efficacy. After 48 hours post-transfection the cells were induced with DMSO, 1 μM 6-ECDCA, 20 μM CDCA or 1 μM GW4064 for further 18 hours. Relative luciferase expression units were measured and normalized by β-gal expression. A, Cotransfection of the cells with PRMT1, FXR and RXR enhances transactivation induced by FXR/RXR in the presence 1 μM 6-ECDCA. The transactivation induced by PRMT1 was almost abrogate by using a pFXRE-luc reporter plasmid with the FXRE mutated (FXRE-Mut). Transfection of PRMT1 alone does not induce transactivation of FXRE, in the presence or absence of 6-ECDCA, indicating that PRMT1 has no intrinsic coactivation function. Data are the mean ± SE of 4 separate experiments. *P<0.05 versus DMSO alone. **P<0.01 versus FXR/RXR in the presence of 6-ECDCA. B, Transactivation induced by PRMT1 is regulated by 6-ECDCA in a concentration dependent manner. Data are the mean ± SE of 4 separate experiments. *P<0.05 versus FXR/RXR alone. C, PRMT1 enhances FXR/RXR induced transactivation caused by 20 μM CDCA and 1 μM GW-4064. Data are the mean ± SE of 4 separate experiments. *P<0.05 versus FXR/RXR alone. D, The level of the proteins FXR, RXR and PRMT1 was unmodified by 6-ECDCA.

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Figure 4. The RXR ligand 9-cis RA enhances FXR/RXR transactivation induced by PRMT1. HEK 293 cells were cotransfected with FXR, RXR, PRMT1 and pGL3(IR1)$_3$-Luc expression plasmids. After 36 hours post transfection the cells were induced for 18 h with DMSO (untreated), 1 μM 6-ECDCA or 1 μM 9-cis RA alone or in combination. Luciferase activites were normalized to the expression of β-gal. The 9-cis RA induces enhancement of gene reporter activity in the cells cotransfeted with PRMT1, suggesting that the RXR ligand induces a transcriptional activation of FXRE by recruiting a methyltransferase coactivator to the FXR/RXR heterodimer. However, 9-cis RA antagonizes the FXR/RXR transactivation caused by 6-ECDCA. Data are the mean ± SE of 4 separate experiments. *P<0.05 versus untreated cells. **p<0.05 FXR/RXR alone. ***P<0.05 versus 6-ECDCA alone.

Figure 5. PRMT1 induces transcriptional activation of FXR responsive genes. HepG2 cells were transiently transfected with vector alone or PRMT1 expression plasmid and induced with DMSO (untreated) or 1 μM 6-ECDCA. Total RNA was isolated and the relative expression of the FXR responsive genes, normalized by the expression of GAPDH, was evaluated by qRT-PCR. BSEP and SHP mRNA expression was up-regulated by cotransfection of PRMT1 in the presence of 6-ECDCA. NTCP and Cyp7A1 mRNA expression was down-regulated by cotransfection of PRMT1 in the presence of 6-ECDCA. The down-regulation of the NTCP and Cyp7A1 correlates with up-regulation of SHP. Data are the mean ± SE of 4 experiments. *P<0.05 versus Untreated. **P<0.05 versus vector alone.

Figure 6. ChIP analysis. 6-ECDCA induces recruitment of PRMT1 and increases H4 arginine methylation to promoters of FXR responsive genes. Chromatin was prepared as *Material and Methods* and immunoprecipitated with anti-FXR, anti-

PRMT1, anti-methyl H4 (Arg3) antibodies or anti-Cyt C, as unrelated antibody. A, Quantitative PCR analyses of the BSEP promoter in the presence of 6-ECDCA 1 μM at different time of induction. The chromatin was prepared from HepG2 cell line and ChIP analysis was performed with anti-PRMT1 antibody and anti-Cyt C antibody as negative control (data not shown). B, Quantitative PCR shows that the 6-ECDCA induces recruitment of PRMT1 to BSEP and SHP promoters and increases histone H4 arginine methylation. The histograms represent a real-time PCR values of promoter amplification (total DNA detected with anti-FXR, anti-PRMT1 or anti-methyl H4 (Arg3) specific antibody minus the DNA quantity detected with the anti-Cyt C antibody). Data are the mean ± SE of 4 experiments. *P<0.05 versus Untreated. **P<0.05 versus vector alone.

Figure 7. The inhibitor of the proteins methylation, MTA, inhibits transcriptional activation of the FXR responsive genes BSEP and SHP. HepG2 cells were exposed to DMSO (untreated), 1 μM 6-ECDCA, GW-4064, with or without 0.3 mM MTA and after 18 hours total RNA was extracted to evaluate the BSEP and SHP gene expression by qRT-PCR. MTA significantly downregulates the expression of the BSEP (A) and SHP (B) gene induced by 6-ECDCA and GW-4064. *P<0.001 versus untreated cells. **P<0.01 versus FXR ligands.

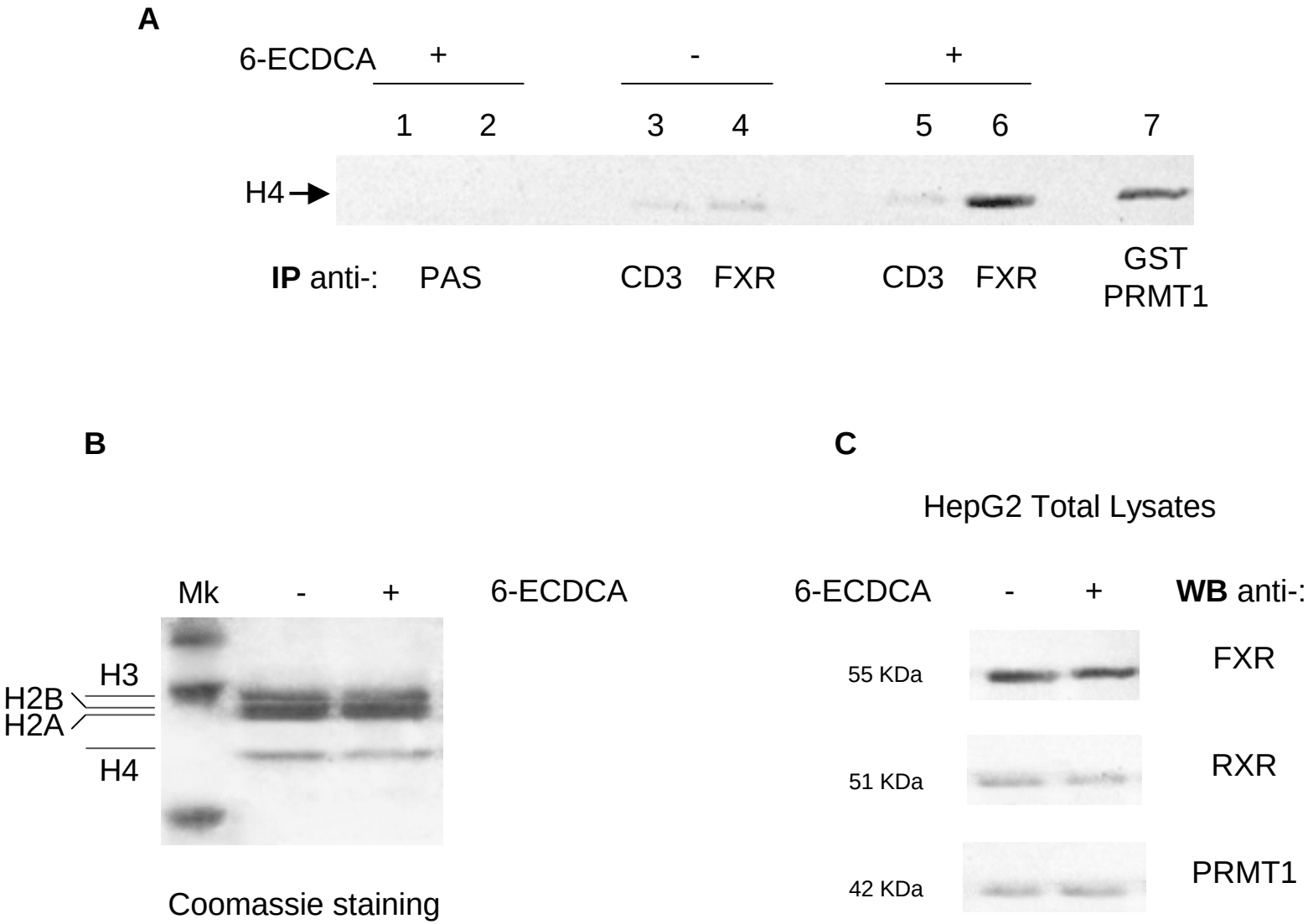

Figure 1

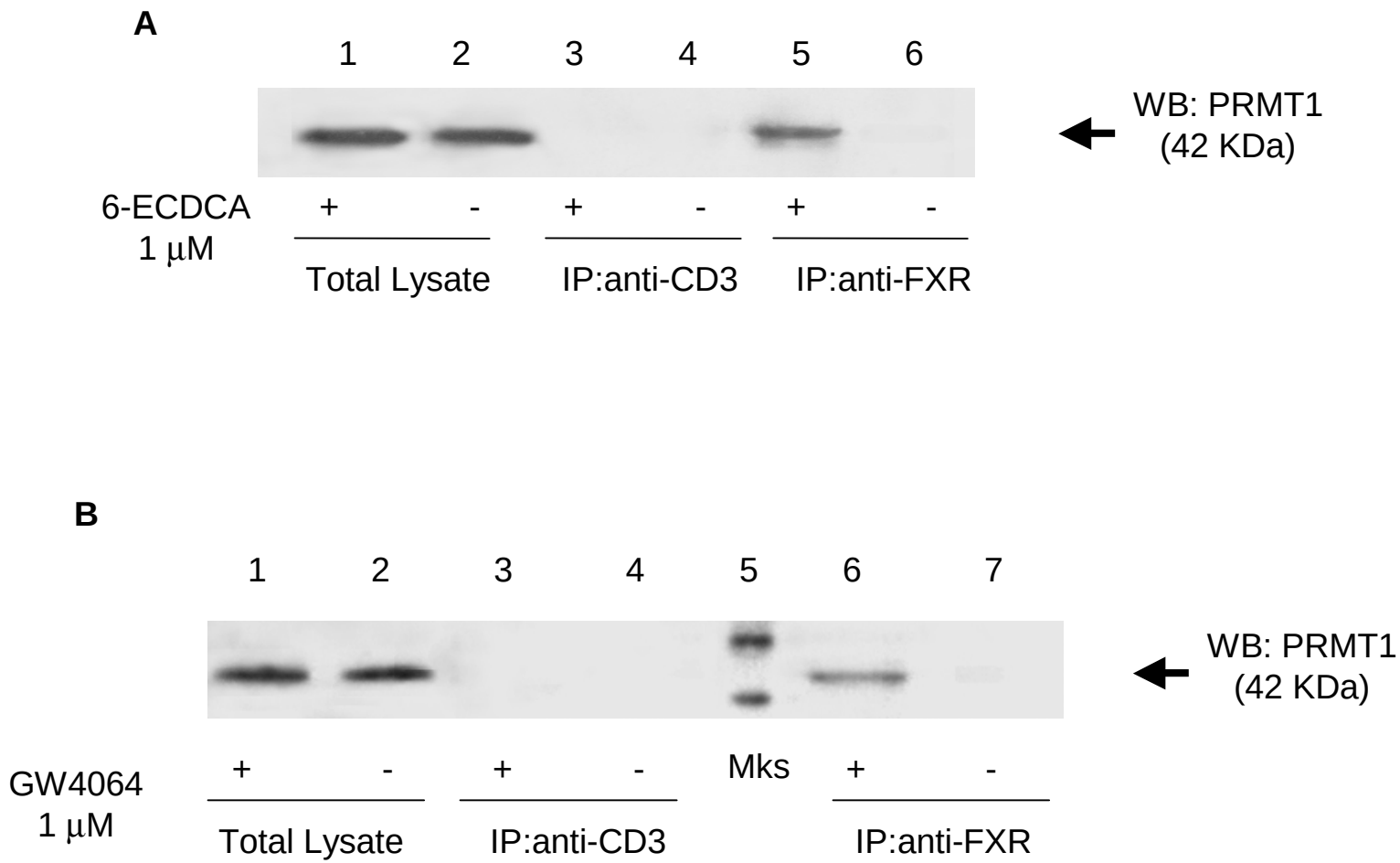

Figure 2

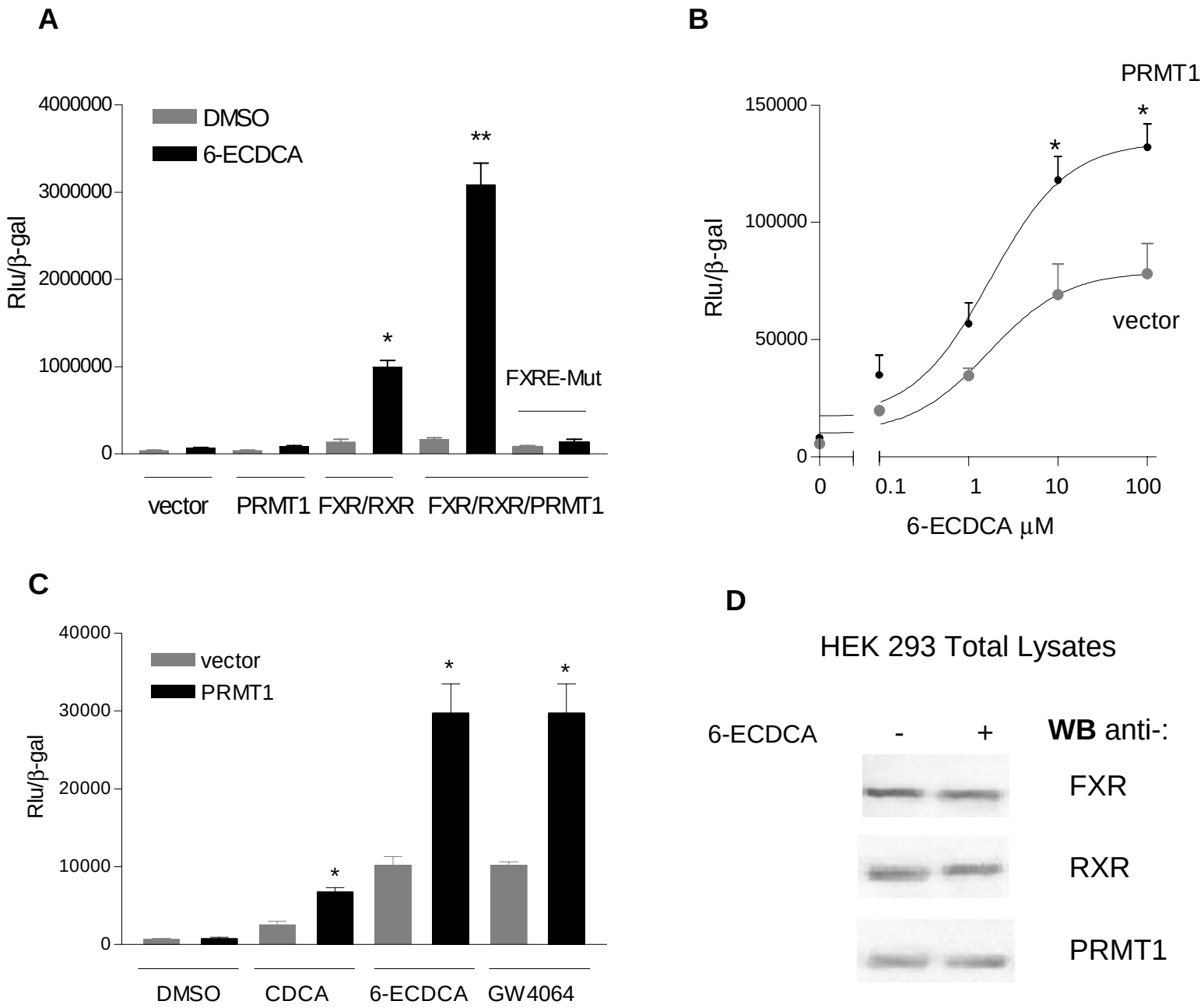

Figure 3

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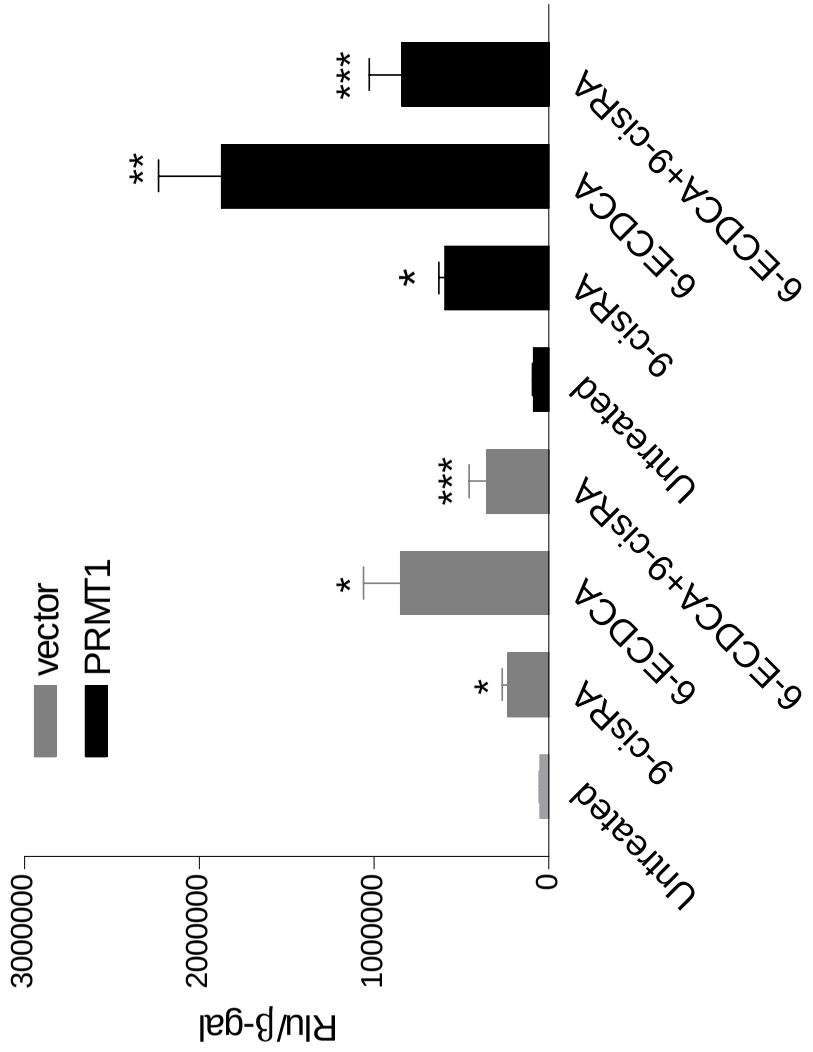

Figure 4

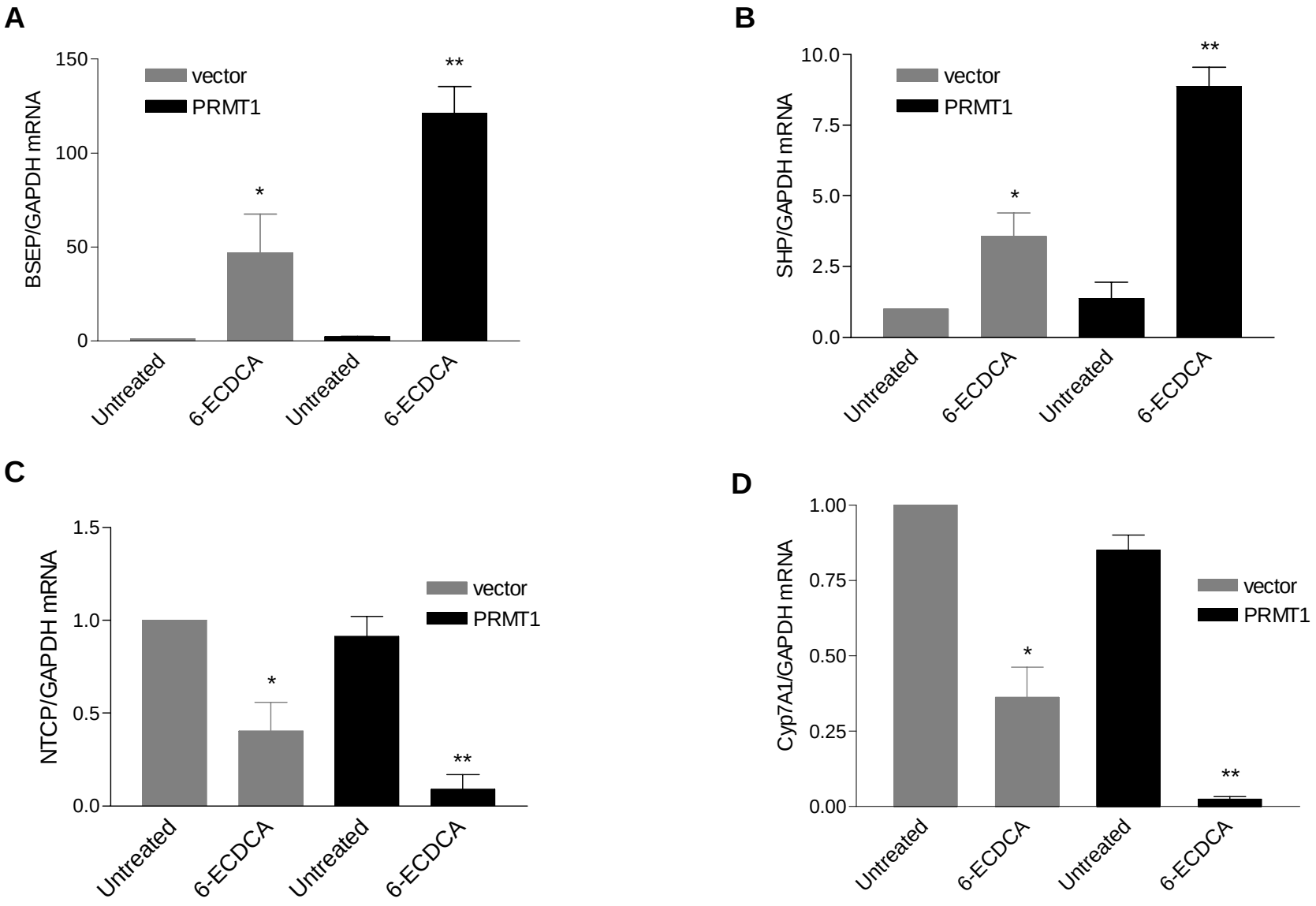

Figure 5

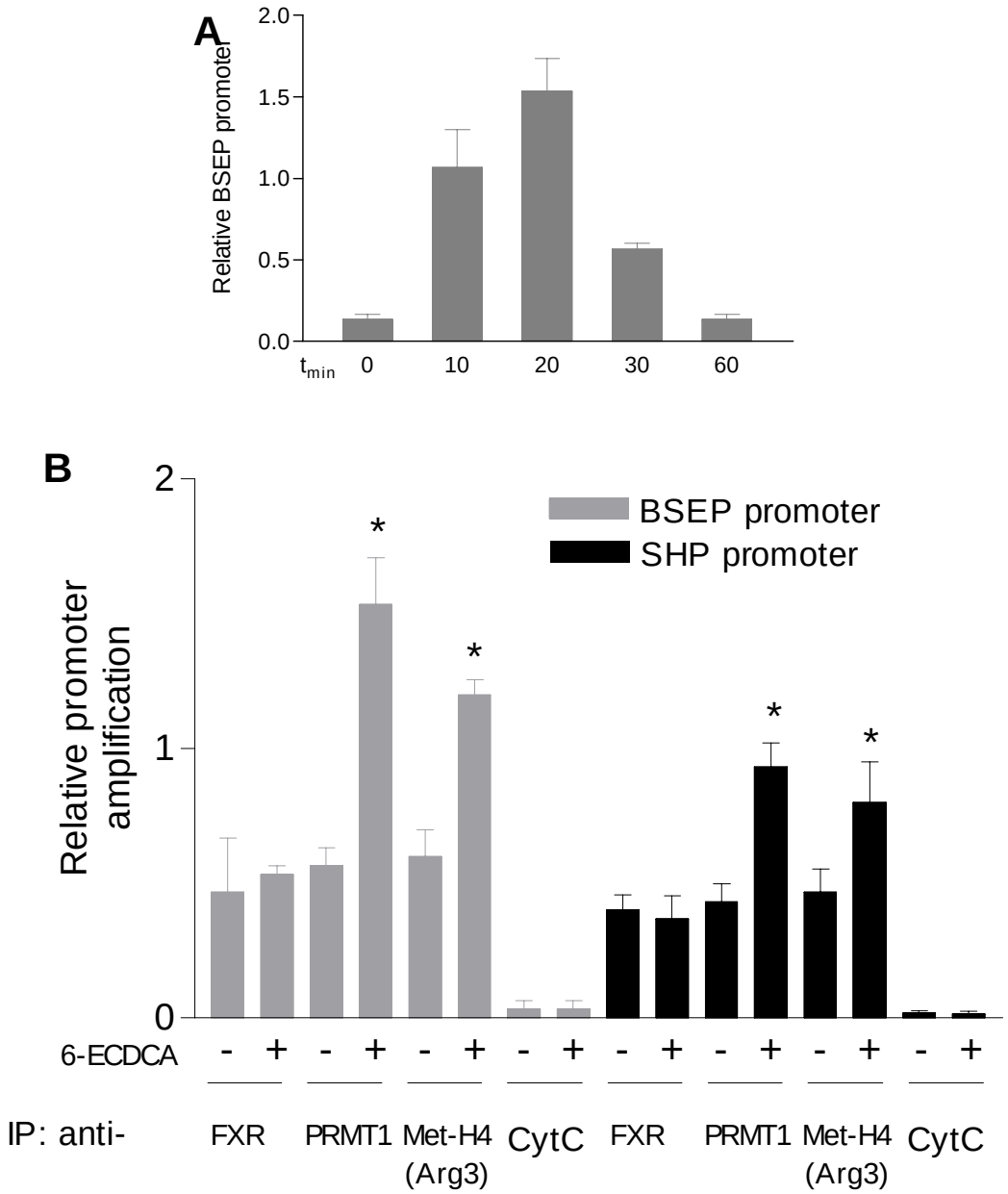

Figure 6

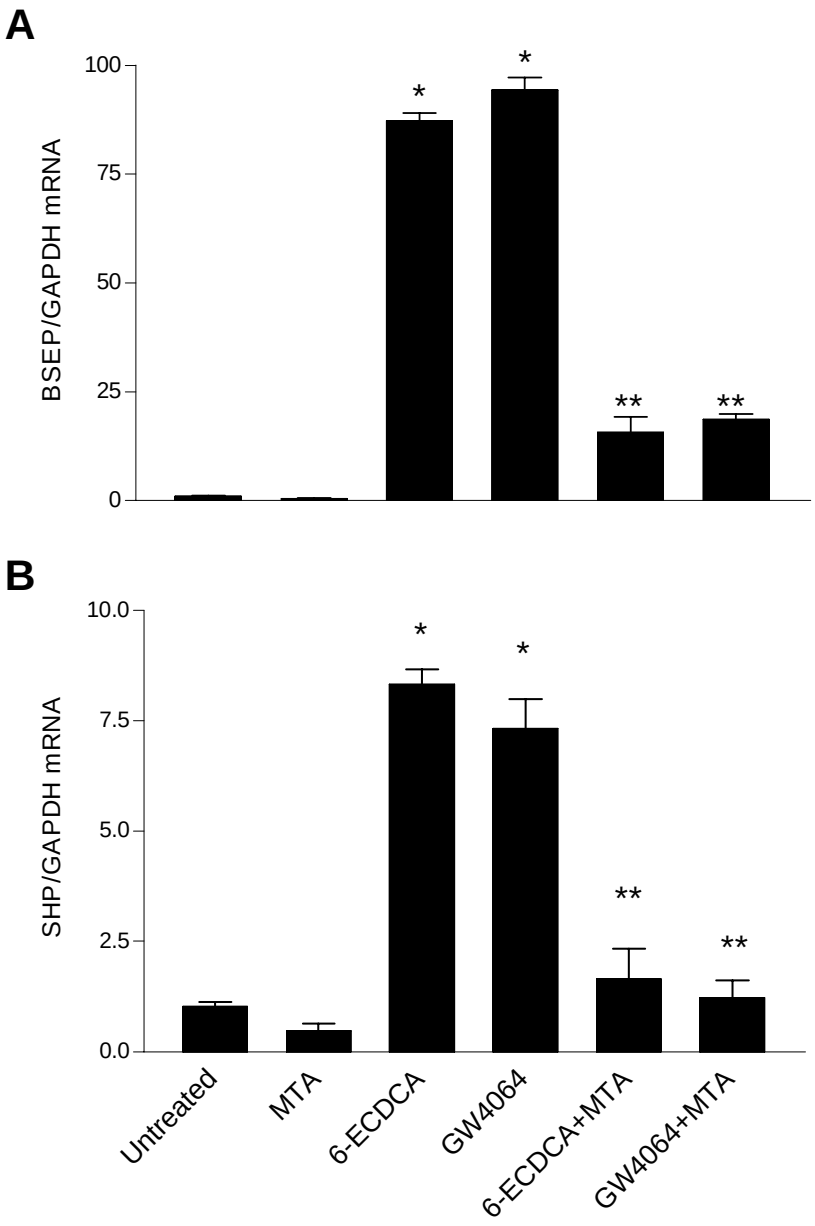

Figure 7