

**All-trans Retinoic Acid and Sodium Butyrate Enhance Natriuretic Peptide Receptor A
Gene Transcription: Role of Histone Modification**

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Running Title: Modulation of *Npr1* gene expression

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Abbreviations: ANP, Atrial natriuretic peptide; ATRA, all-trans retinoic acid; BNP, brain natriuretic peptide; ChIP, chromatin immunoprecipitation assay; GC-A/NPRA, guanylyl cyclase/natriuretic peptide receptor-A; HAT, histone acetyltransferase; HDAC, histone deacetylase; H3-K9ac, acetyl histone H3-K9; H4-K12ac, acetyl histone H4-K12; H3-K9me2, dimethyl histone H3-K9; H3-K9me3, tri-methyl histone H3-K9; H3-K4me3, tri-methyl histone H3-K4; H3-K27me3, tri-methyl histone H3-K27; NaBu, sodium butyrate; PCAF, p300/CBP associated factor; RAR, retinoic acid receptor; RXR, retinoid X receptor; TBST, Tris-buffered saline-Tween 20; TSA, trichostatin A.

Abstract

The objective of the present study was to delineate the mechanisms of guanylyl cyclase/natriuretic peptide receptor-A (GC-A/NPRA) gene (*Npr1*) expression *in vivo*. We utilized all-trans retinoic acid (ATRA) and histone deacetylase (HDAC) inhibitor, sodium butyrate (NaBu) to examine the expression and function of *Npr1* using gene-disrupted heterozygous (1-copy; +/-), wild-type (2-copy; +/+), and gene-duplicated heterozygous (3-copy; +/+ +/+) mice. *Npr1*^{+/-} mice exhibited increased renal HDAC and reduced histone acetyltransferase (HAT) activity; on the contrary *Npr1*^{+/+} mice showed decreased HDAC and enhanced HAT activity compared with *Npr1*^{+/-} mice. ATRA and NaBu promoted global acetylation of histones H3-K9/14 and H4-K12, reduced methylation of H3-K9 and H3-K27, and enriched accumulation of active chromatin marks at the *Npr1* promoter. A combination of ATRA-NaBu promoted recruitment of activator-complex containing Ets-1, retinoic acid receptor α , and HATs (p300 and p300/CBP associated factor) at the *Npr1* promoter and significantly increased renal NPRA expression, GC activity, and cGMP levels. Untreated 1-copy mice showed significantly increased systolic blood pressure (SBP) and renal expression of α -smooth muscle actin (α -SMA) and proliferating cell nuclear antigen (PCNA) compared with 2-copy and 3-copy mice. Treatment with ATRA and NaBu synergistically attenuated the expression of α -SMA and PCNA and reduced SBP in *Npr1*^{+/-} mice. Our findings demonstrate that epigenetic upregulation of *Npr1* gene transcription by ATRA and NaBu leads to attenuation of renal fibrotic markers and SBP in mice with reduced *Npr1* gene copy number, which will have important implications in prevention and treatment of hypertension-related renal pathophysiological conditions.

Introduction

Cardiac hormones, atrial and brain natriuretic peptides (ANP and BNP) elicit natriuretic/diuretic, vasorelaxant, and antimitogenic responses, largely directed to the reduction of blood pressure and blood volume (de Bold, 1985; Levin et al., 1998; Pandey, 2008). Guanylyl cyclase/natriuretic peptide receptor-A (GC-A/NPRA) is one of the principal loci involved in the regulatory actions of ANP and BNP, which produces the intracellular second messenger cGMP in response to hormone binding (Drewett and Garbers, 1994; Pandey and Singh, 1990). The ANP/cGMP signaling through its downstream effector proteins, including cGMP-dependent protein kinases, phosphodiesterases, and cyclic nucleotide-gated ion channels mediates the cellular and physiological effects of NPRA (Garbers et al., 2006; Pandey, 2011). Gene-targeting and functional expression studies of *Npr1* gene (coding for GC-A/NPRA) have shown the hallmark significance of this receptor protein in providing the protective effects against renal and cardiac hypertrophic and fibrotic growth, extracellular matrix deposition, and cell proliferative responses (Das et al., 2010; Li et al., 2009; Vellaichamy et al., 2005). Previous studies have indicated an association of *Npr1* gene variants with left ventricular mass index and septal wall thickness in human essential hypertension (Pitzalis et al., 2003; Rubattu et al., 2006). Moreover, a longer thymidine adenine repeat unit in the spontaneously hypertensive rat model has been shown to regulate *Npr1* gene transcription, thus affecting diastolic blood pressure (Tremblay et al., 2003). The activity and expression of NPRA, assessed primarily through ANP-stimulated cGMP production are mediated by agents, including auto-regulation involving natriuretic peptides, osmotic sensors, and other regulatory hormones (Arise and Pandey, 2006; Chen et al., 2004; Garg and Pandey, 2003; 2005). However, the exact mechanisms of *Npr1* gene expression and regulation are not yet clearly understood.

Retinoic acid (RA) is a group of derivatives of vitamin A, including all-*trans* RA (ATRA), 9-*cis* RA, and 13-*cis* RA, which play critical regulatory roles in various physiological and pathophysiological conditions of numerous organ systems (Lee and Jeong, 2012; Zile, 2010). Considerable number of studies have shown that RA and its receptor agonists protect structure and function of kidney in numerous animal models of renal diseases, ascribing to its anti-inflammatory and anti-proliferative properties (Liu et al., 2008; Schaier et al., 2004). The physiological effects of retinoic acid is mediated by binding to retinoic acid receptors (RAR) and retinoid X receptors (RXR), which interact with multitude of co-regulators, chromatin modifiers, and transcription machinery (Bastien and Rochette-Egly, 2004; Wei, 2003). Chromatin remodeling by post-translational modifications of histone tails, including acetylation, methylation, and phosphorylation, play critical roles in regulating gene expression and function by controlling the availability of key regulatory elements to chromatin (Latham and Dent, 2007; Lennartsson and Ekwall, 2009).

Epigenetic mechanisms including changes in histone acetylation/deacetylation have been shown to be involved in altered gene expression under various pathological conditions (Lee et al., 2012; Marumo et al., 2010). Inhibition of histone deacetylases (HDAC) by sodium butyrate (NaBu), trichostatin A (TSA), and valproic acid, has shown improved cardiovascular and renal disease conditions (Bhaumik et al., 2007; Bush and McKinsey, 2010). Recently, it has been indicated that NaBu attenuated gentamicin-induced nephrotoxicity by enhancing renal antioxidant enzyme activity and expression of prohibitin protein (Sun et al., 2013). Moreover, hyaluronan mixed esters of butyric and retinoic acid have also been shown to enhance cardiac repair in infarcted rat and pig hearts (Simioniuc et al., 2011; Ventura et al., 2007). Previously, we have reported that ATRA induces *Npr1* gene transcription in cultured mouse mesangial cells

(MMCs) in association with Ets-1, Sp1, and histone acetylation (Kumar et al., 2010). However, the cross-talk between retinoid signaling and other regulatory factors including, chromatin modifiers that modulate the expression and regulation of *Npr1* gene under *in vivo* conditions is still unknown. In the present study, we examined the effect of ATRA- and NaBu-mediated regulation of *Npr1* gene transcription and expression in gene-targeted mutant mouse models. Furthermore, we have analyzed the epigenetic marks including histone H3 modifications, effected by ATRA and NaBu in controlling the *Npr1* gene transcription and expression, an important player in prevention of renal pathophysiology and hypertension.

Materials and Methods

Materials

Colorimetric histone acetylation and methylation quantification kits, global di-methyl histone H3-K9 (H3-K9me₂), tri-methyl histones H3-K9me₃, H3-K4me₃, and H3-K27me₃, and acetyl histones H3-K9ac and H4-K12ac, histone acetyltransferase (HAT) activity/inhibition assay kit, histone purification kit, and antibodies for p300 and H3-K9me₃ were purchased from Epigentek (Brooklyn, NY). RNeasy mini-kit for total RNA isolation, RT² First Strand cDNA kit, and RT² SYBR Green/ ROX master mix were obtained from Qiagen (Valencia, CA). The chromatin immunoprecipitation (ChIP)-IT Express enzymatic kit, HDAC assay kit, and H3-K4me₃ were purchased from Active Motif (Carlsbad, CA). All-trans retinoic acid and NaBu were purchased from Sigma Chemicals (St. Louis, MO) and ATRA-NaBu hybrid drug was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Generation of *Npr1* gene-targeted mice

Npr1 gene-disrupted and gene-duplicated mice were produced by homologous recombination in embryonic stem cells as described previously (Oliver et al., 1997; Oliver et al., 1998; Pandey et al., 1999). The mice were bred and maintained at the Tulane University Health Sciences Center animal facility. Animals were handled under protocols approved by the Institutional Animal Care and Use Committee. The mouse colonies were housed under 12-h light/dark cycles at 25 °C and fed regular chow (Purina Laboratory) and tap water *ad libitum*. All animals were littermate progeny of the C57/BL6 genetic background and were designated as *Npr1* gene-disrupted heterozygous (*Npr1*^{+/-}, 1-copy), wild-type (*Npr1*^{+/+}, 2-copy), and gene-duplicated heterozygous (*Npr1*^{+/+/+}, 3-copy) mice. All protocols were approved by the Institutional Animal Care and Use Committee at Tulane University Health Sciences Center.

ATRA and HDAC inhibitor treatment

The stock solutions of ATRA and ATRA-NaBu hybrid drug were prepared at 20 mg/ml and 10 mg/ml concentrations in DMSO, respectively, and stored at -70 °C. On the day of injection, the drugs were thawed, diluted with olive oil to appropriate concentrations, vortexed for 2 min at room temperature, and administered intraperitoneally. NaBu was prepared at 20 mg/ml in PBS (pH 7.4) and injected intraperitoneally. Control groups were injected with vehicle (DMSO and olive oil). Twenty-four week age-matched male adult 1-copy (n = 32), 2-copy (n = 32), and 3-copy (n = 32) mice were used in the following treatment groups: Group I (n = 8), vehicle-treated (control); Group II (n = 8), ATRA-treated (0.5 mg/kg/day); Group III (n = 8), NaBu-treated (0.5 mg/kg/day); Group IV (n = 8), ATRA-NaBu combination-treated (1 mg/kg/day). Animals were injected with the drugs intraperitoneally for 2-weeks and assays were performed.

Blood pressure analysis

Systolic blood pressures were measured by a noninvasive computerized tail-cuff method using Visitech 2000 and calculated as the average of three to five sessions per day for 5 consecutive days as described previously (Shi et al., 2001). All mice were first trained for 7 days (for acclimatization) and actual blood pressure was measured on and from 11th day till 15th day of the treatment.

Tissue Collection

Mice were euthanized by administration of a high concentration of CO₂. Kidney tissues were dissected, frozen in liquid nitrogen, and stored at -80 °C.

Cell Culture and Treatments

MMCs were isolated and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and insulin-transferrin-sodium selenite, as

described previously (Pandey et al., 2000). Cells were treated with ATRA-NaBu and stilbene-based retinoid TTNPB (4-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid), a RAR-specific agonist in DMEM containing 0.1% bovine serum albumin (BSA) for 24 h. All cultures were kept at 37 °C under an atmosphere of 5% CO₂ and 95% O₂.

Histone purification

Total histone was extracted from frozen kidney tissues using Epigentek's Epiquick extraction kit following manufacturer's protocol. Briefly, pooled kidney tissues from 3 animals were weighed and cut into small pieces and homogenized in 1 x pre-lysis buffer, transferred in a 2 ml tube, centrifuged at 10,000 rpm for 1 min at 4 °C. The supernatant was removed; tissue pellet was resuspended in 3 volumes of lysis buffer, incubated on ice for 30 min, and centrifuged at 12,000 rpm for 5 min at 4 °C. Balance-dithiothreitol (DTT) buffer (0.3 volumes) was added to the supernatant, which was stored at -80 °C. The protein concentration of the eluted histone was estimated using a Bradford protein detection kit (Bio-Rad) using BSA as a standard.

Quantification of global Histone H3 modifications

Histone extracts were prepared as described above. Quantification of global levels of lysine specific histone modifications was performed using EpiQuik global H3-K9/14ac, H4-K12ac, H3-K4me3, H3-K9me2, H3-K9me3, H3-K27me3 colorimetric kit following manufacturer's protocol. In the assay acetylated or methylated histones were captured with specific antibody and detected with a labeled detection antibody, followed by a color development reagent. Absorbance was read at 450 nm and results were calculated using a standard curve following manufacturer's instructions.

Cytosolic and nuclear extract preparation

Cytosolic and nuclear proteins were prepared from frozen kidney tissues as described previously (Das et al., 2010). The kidney tissues were homogenized in an ice-cold 10 mM Tris-HCl buffer (pH 8.0) containing 0.32 M sucrose, 3 mM calcium chloride (CaCl_2), 2 mM magnesium acetate (MgOAc), 0.1 mM EDTA, 0.5% nonidet P-40 (NP-40), 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM orthovanadate and 30 mM sodium fluoride (NaF) and 10 $\mu\text{g/ml}$ of leupeptin and aprotinin. The homogenate was centrifuged at $8000 \times g$ at 4 °C for 20 min and the supernatant was separated and stored as a cytosolic fraction. The pellet was washed in 3 x wash buffer (same as above except NP-40) by resuspending with a 20 gauge needle and centrifugation at $6000 \times g$. The pellet was resuspended in a low-salt buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl_2 , 20 mM KCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, and 0.5 mM PMSF), incubated on ice for 5 min, and mixed with an equal volume of high-salt buffer containing 20 mM HEPES (pH 7.9), 1.5 mM MgCl_2 , 800 mM KCl, 0.2 mM EDTA, 1% NP-40, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF, 1 mM orthovanadate and 30 mM NaF and 10 $\mu\text{g/ml}$ of leupeptin and aprotinin. The mixture was incubated on ice for 30 min and centrifuged at $14,000 \times g$ for 20 min. The supernatant was separated and stored as a nuclear fraction at -80 °C.

Western blot assay

Cytoplasmic fraction (40-60 μg) or nuclear extract (30-50 μg) was mixed with sample loading buffer and separated by using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) essentially as described earlier (Das et al., 2010). Proteins were electrotransferred onto a polyvinylidene difluoride (PVDF) membrane and the membrane was blocked with 1x Tris-buffered saline-Tween 20 (TBST; 25 mM Tris, 500 mM NaCl, and 0.05% Tween 20, pH 7.5) containing 5% fat-free milk for 1 h and incubated overnight in TBST containing 3% fat-free milk at 4 °C with primary antibodies (1:250 dilution). The membrane

was treated with corresponding secondary anti-rabbit or anti-mouse horseradish peroxidase-conjugated antibodies (1:5000 dilutions). Protein bands were visualized by enhanced chemiluminescence plus detection system with Alpha Innotech phosphoimager from Proteinsimple (Santa Clara, CA). The antibodies used in Western blot assay are listed in Supplemental Table 1.

Plasma Membrane Preparation and Guanylyl Cyclase Activity Assay

The plasma membranes from kidney tissues were prepared as described previously (Khurana and Pandey, 1996). Briefly, kidney tissues were homogenized in 5 volumes of 10 mM sodium phosphate buffer (pH 7.4) containing 250 mM sucrose, 150 mM NaCl, 1 mM PMSF, 5 mM benzamidine, 5 mM EDTA, and 10 $\mu\text{g/ml}$ each of leupeptin and aprotinin, centrifuged at $400 \times g$ for 10 min at 4 °C, and the supernatant collected was recentrifuged at $80,000 \times g$ for 1 h at 4 °C. The resultant supernatant was discarded, and the pellet was resuspended in 1 ml of 50 mM HEPES buffer (pH 7.4) containing 150 mM NaCl, 1 mM PMSF, 5 mM benzamidine, 5 mM EDTA, and 10 $\mu\text{g/ml}$ each of leupeptin and aprotinin and centrifuged at $80,000 \times g$ for 1 h at 4 °C. The final pellet was suspended in 200 μl of HEPES buffer (pH 7.4). GC activity was assayed as described by Leitman *et al.* (Leitman et al., 1988) with modifications (Khurana and Pandey, 1996). A 50 μg aliquot of plasma membrane was added to 100 μl of GC assay buffer containing 50 mM Tris-Cl buffer (pH 7.6), 4 mM MnCl_2 , 2 mM 3-isobutyl-1-methylxanthine, 1 mM BSA, 5 units creatinine phosphokinase, 7.5 mM creatine phosphate, and 0.5 mM GTP. The samples were incubated in a water bath at 37 °C for indicated time. Reaction was stopped by adding 900 μl of 55 mM sodium acetate (pH 6.2), and sample tubes were placed in boiling water bath for 5 min and then on ice for 15 min to stop the reaction. Samples were centrifuged at $13,000 \times g$ for 5

min, supernatant was collected, and the generated cGMP was determined using a direct cGMP immunoassay kit.

cGMP assay

Frozen kidney tissue samples were homogenized in 10 vol of 0.1 M HCl containing 1% Triton X-100. The homogenate was centrifuged at 10,000 rpm at 4 °C, and the supernatant was collected and used for cGMP assay. MMCs were treated with TTNPB and ATRA-NaBu for 24 h and stimulated with ANP at 37 °C for 15 min in the presence of 0.2 mM 3-isobutyl-1-methylxanthine, washed three times with PBS, and scraped into 0.5 N HCl. Cell suspension was subjected to five-cycles of freeze and thaw. The mixture was centrifuged at 10,000 rpm for 15 min. The supernatant thus collected was used for the cGMP assay. Cyclic GMP levels were determined using a direct cGMP immunoassay kit according to the manufacturer's protocol. The results are expressed as picomoles of cGMP/mg of protein.

HDAC and HAT activity assay

Total HDAC and HAT enzyme activities were measured in drug-treated and control kidney nuclear extracts using colorimetric ELISA assay kit from Active motif (Carlsbad, CA) and Epigentek (Farmingdale, NY), respectively. The HDAC activity was calculated by measuring the amount of HDAC-deacetylated product, which was directly proportional to the HDAC enzyme activity. For the HAT enzyme activity assay the amount of acetylated product was measured. Absorbance was read at 450 nm and results were calculated using a standard curve following manufacturer's instructions and expressed as ng/h/mg protein.

Quantitative Chromatin Immunoprecipitation Assay

Chromatin was prepared from frozen kidney tissue samples and ChIP was performed using ChIP-IT Express kit (Active Motif). Tissue samples were weighed, finely minced on ice, and 25

mg of tissue for each IP was used. Tissue was transferred to a 2 ml tube containing PBS and protease inhibitor cocktail and 37% formaldehyde (final concentration 1.5%) was added with rocking at room temperature for 20 min to crosslink protein to DNA. Cross-linking was stopped by adding 100 μ l of 10 x glycine, mixed for 5 min, and centrifuged at 1,500 rpm for 5 min at 4 °C. Pellet was washed with PBS, centrifuged at 1,500 rpm at 4 °C, and resuspended in PBS containing protease inhibitors. Pellet was disaggregated using Dounce's homogenizer, centrifuged at 1,500 rpm at 4 °C, and was used for nuclei preparation and chromatin digestion. The pellet was resuspended in 1 ml of digestion buffer and 50 μ l of enzymatic shearing mixture and incubated at 37 °C for 10 min. The reaction was stopped by adding 20 μ l of 0.5 M EDTA followed by chilling on ice for 10 min. Sheared DNA was centrifuged at 13,000 rpm at 4 °C for 10 min and supernatant was collected. Ten percent of supernatant was saved as input DNA and processed for further use as positive control. Immunoprecipitation was performed using protein G magnetic beads and 5 μ g of antibodies of H3-K9/14ac, H4-K12ac, H3-K4-me3, H3-K9me3, PCAF, and p300 or control IgG at 4 °C with overnight rotation. Beads were pelleted and washed sequentially once with ChIP-buffer 1 and twice with ChIP-buffer 2. After washing the magnetic beads, bound protein was eluted by gentle rotation for 25 min in elution buffer at 22 °C. Cross-linking of the protein/DNA complex was reversed at 65 °C overnight to release DNA. Immunoprecipitated DNA was sequentially treated with RNase A and proteinase K and then purified. The *Npr1* proximal promoter region containing Ets-1 and Sp1 transcription factor binding sites was PCR amplified using purified DNA as a template and the forward (5'-gaggggaggattcgtgc-3') and reverse (5'-ctaagaagagcgaggggagc-3') primers. The antibodies used in ChIP assay are listed in (Supplemental Table 1). For quantitative ChIP assay, real-time PCR

was performed with RT² real-time™ SYBR Green/ROX PCR Master Mix (SABiosciences).

Real-time RT-PCR Assay

Total RNA was extracted using RNeasy plus mini-kit (Qiagen). Thirty mg of kidney tissue was used to extract RNA. First-strand cDNA was synthesized from 1 µg of total RNA in a final volume 20 µl using RT² First Strand kit (SABiosciences). Primers for *Npr1* and β-actin were purchased from SABiosciences. The *Ets-1* gene was amplified using forward (5'-atcagctggacaggagattgc - 3') and reverse (5'-cgcgtatacgttagcgttg- 3') primers. Real-time RT-PCR was performed using the Mx3000P real-time PCR system and data were analyzed with MxPro software (Stratagene, La Jolla, CA). The reaction mixture without template cDNA was used as a negative control. The *Npr1* and *Ets-1* expression values were normalized to β-actin. Relative expression of the *Npr1* and *Ets-1* gene was determined by the comparative Ct value.

Immunohistochemistry of proliferating cell nuclear antigen (PCNA) and α-smooth muscle actin (α-SMA)

Immunohistochemical staining for PCNA and α-SMA proteins was done on 5 µm sections of paraffin-embedded kidney tissues as previously described (Das et al., 2010). Briefly, after dewaxing in xylene, the kidney sections were dehydrated by serial dilutions of alcohol (100%, 90%, and 70%) for 10 min each and washed in PBS for 5 min. Sections were incubated in 0.3% H₂O₂ prepared in methanol for 30 min to block the endogenous peroxidase activity. After washing in PBS for 20 min, antigen unmasking was done using warm 10 mM sodium citrate buffer (pH 6.0) and washed in PBS for 10 min. The sections were sequentially incubated at room temperature with normal blocking serum (goat serum) for 40 min, primary antibody (mouse monoclonal PCNA and α-SMA) diluted in PBST containing 1% BSA at 1:1000 dilution overnight at 4 °C, secondary antibody (biotinylated goat antimouse IgG) for 30 min, and avidin-

biotin horseradish peroxidase complex for 45 min, using the ABC staining kit (Santa Cruz). Peroxidase activity was visualized with 0.1% 3, 3'-diaminobenzidine. The slides were then washed in tap water, counterstained with hematoxylin, mounted using aqueous mounting medium, and coverslipped. Immunohistochemically stained sections were visualized and the percentage of PCNA and α -SMA-positive area to the total kidney were calculated using an Olympus BX51 camera and photographed with integrated Magnafire SP Digital Firewire camera software.

Statistical Analysis

The results are expressed as mean \pm S.E. The statistical significance was evaluated by one-way analysis of variance, followed by Dunnett's multiple comparison tests using PRISM software (GraphPad Software, San Diego, CA). Student's unpaired *t*-test with two-tailed analysis was also performed for comparison between groups. A *p* value of 0.05 was considered significant.

Results

The results showed that there was a significant reduction in SBP of *Npr1* gene-disrupted heterozygous 1-copy mice treated with ATRA and NaBu (114.6 ± 2.2 , $p < 0.01$ and 119.2 ± 1.7 , $p < 0.05$, respectively) versus vehicle-treated (128.2 ± 2.1) 1-copy mice (Table 1). The treatment with ATRA-NaBu hybrid also considerably lowered SBP in 1-copy mice (108.8 ± 4.3 , $p < 0.001$) compared with 1-copy control mice. Renal GC activity and cGMP levels were significantly reduced in 1-copy mice by 63% and 65%, respectively, and higher in gene-duplicated heterozygous 3-copy mice by 1.7- to 2-fold compared with wild-type 2-copy mice (Table 1). There was an increase in renal GC activity and cGMP levels in ATRA- and NaBu-treated *Npr1* mice with different gene copy numbers, however, treatment with ATRA-NaBu combination markedly augmented renal GC activity and cGMP levels in treated groups compared with their vehicle-treated control mice. The endogenous *Npr1* mRNA levels were considerably lower in 1-copy mice kidneys compared with 2-copy mice and higher in 3-copy mice (Fig. 1A). Treatment with ATRA and NaBu considerably increased the mRNA levels in 1-copy, 2-copy, and 3-copy mice compared with their vehicle-treated controls. On the other hand, ATRA-NaBu combination significantly augmented *Npr1* mRNA levels in 1-copy (9.5-fold), 2-copy (6-fold), and 3-copy (3.2-fold) mice compared with vehicle-treated respective controls. The Western blot analysis showed attenuated levels of NPRA protein in 1-copy mice kidney compared with 2-copy mice and higher in 3-copy mice (Fig. 1B). Treatment with ATRA-NaBu combination synergistically induced renal NPRA protein levels in all treated groups compared with their vehicle-treated controls. Retinoic acid-dependent induction in NPRA signaling was further confirmed under *in vitro* conditions utilizing a RAR agonist TTNPB and ATRA-NaBu combination drug in MMCs. There was a dose-dependent increase in NPRA protein expression

in cells treated with TTNPB and ATRA-NaBu combination drug compared with untreated controls (Fig. 1C). Treatment with TTNPB and ATRA-NaBu combination drug showed 90- and 145-fold stimulation in cGMP levels, respectively, in the presence of ANP compared with untreated controls (Fig. 1D).

In vivo quantitative ChIP assay was performed in kidney tissues to examine ATRA- and NaBu-mediated accumulation of acetylated and methylated histone marks at the *Npr1* proximal promoter region -120 to +73 (Fig. 2A). One-copy mice exhibited attenuated binding of active histone marks H4-K12ac and H3-K9/14ac with *Npr1* promoter, whereas 3-copy mice showed higher binding of acetylated histone H3 and H4 compared with 2-copy mice (Fig. 2, B and C). There was synergistic increase in H4-K12ac and H3-K9/14ac occupancy at the *Npr1* promoter in ATRA-NaBu combination-treated mice compared with their controls. ATRA and NaBu treatment significantly enhanced binding of H3-K4me3 at *Npr1* promoter in treated mice compared with controls (Fig. 2D). There was augmented binding of repressive histone code H3-K9me3 at *Npr1* promoter in 1-copy mice and attenuated binding in 3-copy mice compared with 2-copy mice (Fig. 2E). However, promoter occupancy of H3-K9me3 was markedly reduced in ATRA-NaBu combination-treated mice compared with their respective controls. Representative gel images have been generated from ChIP assays as analyzed by conventional PCR in drug-treated *Npr1* gene-targeted mice (Supplemental Figure 1).

The quantitative analyses of renal histone modifications showed lower levels of active histone marks H4-K12ac, H3-K9ac, and H3-K4me3 in 1-copy mice compared with 2-copy and 3-copy mice (Fig. 3, A-C). Treatment with ATRA-NaBu combination synergistically increased protein levels of H4-K12ac, H3-K9ac, and H3-K4me3 in treated mice compared with their controls. One-copy mice exhibited increased levels of repressive histone codes H3-K27me3, H3-

K9me2, and H3-K9me3 compared with 2-copy and 3-copy mice (Fig. 3, D-F). Treatment with ATRA-NaBu combination significantly attenuated H3-K27me3, H3-K9me2, and H3-K9me3 expression in treated mice compared with vehicle-treated controls. Western blot analysis further confirmed the global increase in expression levels of H4-K12ac, H3-K9/14ac, and H3-K4me3 histone marks and decrease in H3-K9me3 in renal tissues of ATRA- and NaBu-treated mice compared with their controls (Supplemental Figure 2, A-E). Quantitative ChIP assay demonstrated higher enrichment of RAR α to *Npr1* promoter in ATRA- and ATRA-NaBu combination-treated mice compared with their vehicle-treated controls (Fig. 4A). There was marked accumulation of Ets-1 protein at *Npr1* promoter in ATRA and ATRA-NaBu combination-treated 1-copy mice compared with vehicle-treated control mice (Fig. 4B). Furthermore, treatment with ATRA and ATRA-NaBu combination markedly induced renal Ets-1 mRNA and protein levels in treated mice compared with vehicle-treated controls (Fig. 4, C and D).

Renal HDAC activity was distinctly higher in 1-copy mice (2.5-fold) compared with 2-copy mice, but lower in 3-copy mice (Fig. 5A). However, the HDAC activity was reduced in 1-copy mice by 37% after treatment with NaBu and 66% in ATRA-NaBu combination-treated mice compared with vehicle-treated controls. Similarly, treatment with ATRA-NaBu combination noticeably attenuated HDAC activity in 2-copy and 3-copy mice compared with controls. On the other hand, HAT activity was significantly lower in 1-copy mice (50%) and higher in 3-copy mice (37%) compared with 2-copy mice (Fig. 5B). There was marked induction in HAT activity in 1-copy mice treated with ATRA and NaBu alone and 7.8-fold increase was observed in ATRA-NaBu combination-treated mice compared with controls. Likewise, ATRA-NaBu combination markedly increased HAT activity in 2-copy and 3-copy mice compared with

controls. Western blot and densitometric analysis of HATs namely; PCAF and p300 showed significant increases in PCAF and p300 protein levels in ATRA- and NaBu-treated 1-copy mice compared with vehicle-treated controls (Fig. 5, C and D). *In vivo* quantitative ChIP assay showed increased recruitment of PCAF at the *Npr1* promoter in ATRA- and NaBu-treated 1-copy mice and whereas ATRA-NaBu combination showed a synergistic effect compared with control mice (Fig. 5E). There was higher enrichment of p300 protein at *Npr1* promoter in ATRA-NaBu combination-treated experimental mice with pronounced effects observed in 1-copy mice compared with their respective control groups (Fig. 5F).

Immunohistochemical analyses for α -SMA showed significantly increased expression in tubules, arterioles, and interstitium of 1-copy mice compared with 2-copy and 3-copy mice (Fig. 6A). The α -SMA-positive stained cells were 6-fold higher in 1-copy mice compared with 2-copy and 3-copy mice (Fig. 6C). There was a significant attenuation in α -SMA expression in 1-copy mice treated with ATRA (42%, $p < 0.001$), NaBu (25%, $p < 0.001$), and ATRA-NaBu combination (70%, $p < 0.001$) compared with 1-copy control mice (Fig. 6A and Supplemental Figure 3A). The immunostaining of kidney sections for PCNA showed marked increase in PCNA-positive cells in the glomeruli of 1-copy mice compared with 2-copy and 3-copy mice (Fig. 6B). A substantial reduction in PCNA immunoexpression was observed in 1-copy mice-treated with ATRA (36%, $p < 0.001$), NaBu (16%, $p < 0.001$), and ATRA-NaBu hybrid (68 %, $p < 0.001$) (Fig. 6D and Supplemental Figure 3B). Western blot and densitometric analyses showed that treatment with ATRA and NaBu alone markedly reduced renal α -SMA and PCNA protein levels, whereas ATRA-NaBu hybrid synergistically decreased α -SMA and PCNA protein expression in 1-copy treated mice compared with 1-copy control mice (Fig. 6, E and F).

Discussion

The present results demonstrate that retinoic acid signaling and pharmacological inhibition of HDAC activity by NaBu markedly stimulated *Npr1* gene expression in *Npr1* gene-disrupted 1-copy mice. The increased *Npr1* gene expression was accompanied by modulation of HDAC and HAT activity resulting in global histone modifications, including, increased levels of H3-K9 and H4-K12 acetylation and decreased levels of H3-K9me2, H3-K9me3, and H3-K27me3. Previously, it has been reported that histone H3-K9/14 acetylation and H3-K4, K9, and K27 methylation are closely related with transcriptional status of the gene and are modulated by HDAC inhibitors such as TSA and NaBu to open the chromatin structure and enhance gene transcription (Bhaumik et al., 2007; Huang et al., 2011; Nightingale et al., 2007). Recently, it has been shown that treatment with valsartan in spontaneously hypertensive rats decreased local angiotensin-converting enzyme 1 mRNA and protein expressions, which were accompanied by higher levels of H3K9me2, as well as lower levels of H3ac and H3K4me3 (Lee et al., 2012). Furthermore, *in vitro* studies have shown that ATRA and HDAC inhibitors increase the binding of active histone codes (H3-K9ac and H3-K14ac) at the promoters of human pyruvate dehydrogenase kinase 4 and *neurogenin1* genes and attenuated the recruitment of repressive histone code H3-K27me3 (Kwon et al., 2006; Wu et al., 2009). Findings from our present study demonstrate that ATRA and NaBu significantly enhanced binding of H3-K9/14ac, H4-K12, and H3-K4me3 at the *Npr1* promoter and reduced binding of H3-K9me3 thereby modulating *Npr1* gene transcription.

Evidence suggests that ATRA treatment induces epigenetic modifications at the promoter level and as a consequence of histone acetylation and opened chromatin structure; transcription factors and the basal transcriptional machinery are recruited to the region, which activate gene

transcription (Kashyap and Gudas, 2010; Kwon et al., 2006). Our results show that ATRA and ATRA-NaBu combination significantly enhanced recruitment of RAR α and Ets-1 at the *Npr1* proximal promoter in 1-copy *Npr1* mice kidneys *in vivo*. Earlier studies have suggested that Ets-1 to be essential for normal development of mammalian kidneys and maintenance of glomerular integrity (Cederberg et al., 1999; Gomez and Norwood, 1999). The present data shows that increase in histone acetylation by ATRA and NaBu facilitates recruitment of transcription factors Ets-1 and RAR α at the *Npr1* proximal promoter, thereby mediating ATRA-induced *Npr1* gene transcription in intact animals *in vivo*.

The balance between HAT and HDAC is an essential regulatory switch of gene expression and an increase in histone acetylation can be achieved through the inhibition of its deacetylation process (Gaub et al., 2010; Peserico and Simone, 2011). Several classes of HDAC inhibitors including NaBu, TSA, vorinostat, and hydroxamic acid have been shown to inhibit HDAC activity and shift the overall balance in favor of enhanced HAT activity (Pascual et al., 2012; Yang and Seto, 2007). Consistent with those previous findings, the present data show that treatment with ATRA and NaBu significantly reduced HDAC activity and increased HAT activity in *Npr1* 1-copy mice. ATRA and NaBu significantly increased protein levels and accumulation of p300 and PCAF at the *Npr1* promoter in the mice kidney. Earlier studies have shown that ATRA treatment was able to enhance p300 and PCAF protein expression and their binding to gene promoters (Dietze et al., 2002; Gaub et al., 2010; Kim et al., 2010). In the absence of ligand, the retinoic acid receptors RAR/RXR heterodimers have been shown to bind gene promoters and interact directly with nuclear corepressor proteins, which recruit HDAC complexes and deacetylate histone tails and establish a “closed” chromatin tail (Kashyap and Gudas, 2010). Those previous findings indicated that ATRA binding to the promoters release the

corepressors and leads to the recruitment of coactivator proteins such as p300/CBP and PCAF complex, which mediate gene transcription (Kashyap and Gudas, 2010; Wei, 2003).

The present data suggest that ATRA and NaBu treatment elicited significant reduction in the percentage of glomeruli with PCNA-positive cells and attenuation in α -SMA immunoeexpression (a marker for epithelial mesenchymal transition) in 1-copy mice. Previously, it has been shown that ATRA exerts a beneficial effect in anti-glomerular basement membrane glomerulonephritis in rats and unilateral ureteral obstruction (UUO) mouse model via downregulation of PCNA, α -SMA, and collagen I expression and has been suggested as a therapeutic strategy for the treatment of progressive renal fibrosis in diseased kidneys (Kishimoto et al., 2011; Oseto et al., 2003). On the other hand, inhibition of HDAC activity attenuates renal fibroblast activation and interstitial fibrosis in obstructive nephropathy via downregulation of α -SMA and fibronectin and modulates proinflammatory and fibrotic changes in tubulointerstitial injury suggesting that HDACs can be a potential target for the treatment of fibrotic disorders (Marumo et al., 2010; Pang et al., 2009). Our previous studies have demonstrated that *Npr1* 1-copy mice exhibited increased levels of renal proinflammatory cytokines, fibrosis, and remodeling (Das et al., 2010; Zhao et al., 2013). We have also observed that a decrease in *Npr1* gene copy number increases renal PCNA and α -SMA protein expression leading to fibrosis and hypertrophic growth of the kidneys in *Npr1* 1-copy mice compared with 2-copy mice (Das et al., 2010). It has been suggested that different cross-talk mechanisms exist between histone acetylation and methylation networks, which constitute a complex framework for epigenetic control of gene transcription during biological or pathological developments (Baylin and Ohm, 2006; Latham and Dent, 2007).

The treatment of *Npr1* gene-disrupted 1-copy mice with ATRA-NaBu combination showed synergistic increase in renal NPRA protein expression, GC activity, and cGMP levels via modulation of HDAC and HAT activity, histone modifications, and recruitment of transcription factors to enhance *Npr1* gene transcription. The activation of NPRA/cGMP signaling in *Npr1* 1-copy mice inhibited renal fibrosis as evidenced by reduction in expression of renal fibrotic markers like α -SMA and PCNA. A schematic diagram of the mechanistic pathways of the ATRA/NaBu-dependent inhibition of HDAC and activation of HAT activities leading to enhanced *Npr1* gene transcription and signaling is depicted in Figure 7. An earlier study has also shown that the activation of endogenous NPRA system attenuates renal fibrosis in UUO mouse model and ANP pre-treatment significantly improved morphological changes with increase of renal cGMP levels (Nishikimi et al., 2009). Glucocorticoids have also been shown to improve renal responsiveness to ANP by upregulating NPRA expression accompanied by remarkable increase in renal cGMP levels in decompensated heart failure (Liu et al., 2011). Recently, it has been suggested that NaBu attenuated gentamicin-induced nephrotoxicity by enhancing renal antioxidant enzyme activity and expression of prohibitin protein (Sun et al., 2013). Our present data provide direct evidence that activation of NPRA/cGMP signaling by ATRA and NaBu via epigenetic mechanisms inhibits various renal fibrotic pathways and attenuates systolic blood pressure with decreasing *Npr1* gene copy number.

In summary, the present results provide the strong evidence that ATRA and NaBu inhibit HDAC activity and induce *Npr1* gene transcription and expression in the kidneys of 1-copy mice via histone acetylation and enhanced recruitment of transcription factors and HAT proteins to *Npr1* promoter. Our observations demonstrate that ATRA and NaBu-mediated chromatin remodeling is involved in transcriptional regulation of *Npr1* gene, which leads to an increased

renal NPRA/cGMP signaling and attenuation of renal fibrosis in *Npr1* gene-targeted 1-copy mice. Overall, the present study provides new insights into the epigenetic regulatory mechanisms of *Npr1* gene transcription, expression, and function, which are critical for the biological activity of NPRA for possible therapeutic targets in the diagnosis and treatment of renal disorders under reduced NPRA signaling.

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Authorship Contributions

Participated in research design: Kumar, Das, and Pandey.

Conducted experiments: Kumar, Periyasamy, Das, Neerukonda, Mani, and Pandey.

Contributed new reagents or analytic tools: Kumar, Periyasamy, Das, Mani, and Pandey.

Performed data analysis: Kumar, Periyasamy, and Pandey.

Wrote or contributed to the writing of the manuscript: Kumar and Pandey.

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Footnotes:

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Figure Legends

Figure 1. Effect of ATRA, NaBu, TTNPB on renal *Npr1* gene expression in gene-targeted mice and MMCs. (A) Relative mRNA expression of renal *Npr1* in drug-treated and control mice as determined by real-time RT-PCR, normalized to β -actin mRNA. (B) Western blot and densitometry analyses of renal NPRA protein expression in drug- or vehicle-treated *Npr1* gene-targeted mice. β -actin was used as loading control. (C) Western blot analysis of NPRA protein expression in cells stimulated with increasing concentrations of TTNPB and ATRA-NaBu and β -actin expression is shown as loading control. (D) Intracellular accumulation of cGMP in cells treated with increasing concentrations of TTNPB and ATRA-NaBu and induced with or without 100 nM ANP. Bar represents mean \pm SE of three independent experiments. WB indicates Western blot; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (vehicle-treated versus drug-treated same group); ## $p < 0.01$, ### $p < 0.001$ (1-copy or 3-copy versus 2-copy); $n=8$ mice per group.

Figure 2. Effect of ATRA and NaBu on binding of acetylated and methylated histones at the proximal promoter of *Npr1* gene. (A) Schematic representation of *Npr1* proximal promoter region (-120 to +73) amplified for ChIP assay having Ets-1, Sp1, and p300 transcription factor binding sites. Recruitment of (B) H4-K12ac, (C) H3-K9ac, (D) H3-K4me3, and (E) H3-K9me3 to the *Npr1* promoter in kidneys of ATRA- and Nabu-treated 1-copy, 2-copy, and 3-copy mice. Values are expressed as mean \pm SE of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (vehicle-treated versus drug-treated same group); # $p < 0.05$, ## $p < 0.01$ (1-copy or 3-copy versus 2-copy); $n=7$ mice per group.

Figure 3. Quantitative analysis of renal H3 modifications in ATRA- and NaBu-treated *Npr1* gene-targeted mice. Renal protein expression of H4 and H3 modifications (A) H4-K12ac, (B) H3-K9ac, (C) H3-K4me3, (D) H3-K27me3, (E) H3-K9me2, and (F) H3-K9me3 at specific lysines was done by EpiQuik quantification kit from EpiGentek. Bar represents mean \pm SE of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (vehicle-treated versus drug-treated same group); # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ (1-copy or 3-copy versus 2-copy); $n=7$ mice per group.

Figure 4. Effect of ATRA and NaBu on RAR α and Ets-1 binding at the proximal promoter region of *Npr1* gene. (A) Schematic representation of *Npr1* proximal promoter region (-120 to +73) amplified for ChIP assay having Ets-1, Sp1, and p300 transcription factor binding sites. Recruitment of RAR α to the *Npr1* promoter in kidneys of drug-treated and control mice. (B) Ets-1 recruitment to the *Npr1* promoter in response to ATRA and NaBu treatment. (C) Relative mRNA expression of renal Ets-1 in drug-treated and control mice as determined by real-time RT-PCR, normalized to β -actin mRNA. (D) Western analysis of renal Ets-1 protein expression in drug- or vehicle-treated *Npr1* gene-targeted mice. β -actin was used as loading control. Pooled samples from 6 mice in each group were used for experiments. Values are expressed as mean \pm SE of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (vehicle-treated versus drug-treated same group).

Figure 5. Modulation of renal HDAC and HAT activity and expression of PCAF and p300 by ATRA and NaBu. (A) HDAC and (B) HAT activity were measured in nuclear extracts of drug-treated and vehicle-treated mice kidney tissues. (C and D) Western blot and densitometry

analysis of renal protein levels of PCAF and p300 in *Npr1* 1-copy, 2-copy, and 3-copy mice treated with ATRA and NaBu. TBP was used as loading control. Quantitative ChIP assay demonstrating *in vivo* recruitment of (E) PCAF and (F) p300 to the *Npr1* promoter in ATRA- and NaBu-treated and vehicle-treated mice. Bar represents mean \pm SE. WB represents Western blot; * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$; (vehicle-treated versus drug-treated same group); # $p < 0.05$, ## $p < 0.01$ (1-copy or 3-copy versus 2-copy); $n=6$ mice per group.

Figure 6. Renal immunoexpression of α -SMA and PCNA in drug-treated and vehicle-treated *Npr1* gene-targeted mice. Immunohistochemistry showing renal expression of α -SMA (A) and PCNA (B) in drug-treated and vehicle-treated 1-copy, 2-copy, and 3-copy mice. (C and D) Quantitative analysis of α -SMA and PCNA in drug-treated and vehicle-treated mice groups. Western blot and densitometric analyses of α -SMA (E) and PCNA (F) in drug-treated and vehicle-treated mice. β -actin was used as loading control. Values are expressed as mean \pm SE of three independent experiments. WB indicates Western blot; * $p < 0.05$; ** $p < 0.01$ (vehicle-treated versus drug-treated same group). In each group, five mice were used.

Figure 7. Schematic presentation of proposed epigenetic signaling during ATRA and NaBu-mediated *Npr1* gene regulation *in vivo*. The proposed diagram indicates that under non-permissive chromatin state, the repressive histone marks (H3-K9me3, H3-K9me2, and H3-K27me3) are associated with *Npr1* gene promoter. HDACs, class I and II, form a corepressor complex, associates with retinoic acid receptors, and ensure that H3 remains hypoacetylated. Treatment with ATRA and NaBu inhibits HDAC activity and induces HAT activity with increased expression and recruitment of HATs (p300 and PCAF) leading to hyperacetylated H3-

K9/14 and H4-K12 in the proximity of *Npr1* promoter. Increased promoter occupancy of active histone marks (H3-K9/14ac, H4-K12ac, and H3-K4me3) leads to localized unwinding of DNA and allows transcription factors (Ets-1 and RAR α) to bind in the promoter region and enhance *Npr1* gene expression. Enhanced NPRA/cGMP signaling reduces expression of renal fibrotic markers α -SMA and PCNA in 1-copy mice. The open downward arrow indicates decrease in HDAC activity and fibrosis; whereas the bold upward arrows indicate increase in HAT activity and *Npr1* gene transcription and expression.

Table I: Comparisons of blood pressure, kidney guanylyl cyclase (GC) activity, and cGMP levels among *Npr1* genotypes treated with ATRA and NaBu.

Treatments/Parameters	<i>Npr1</i> genotype		
	1-copy	2-copy	3-copy
<i>Systolic Blood Pressure (mm Hg)</i>			
Vehicle	128.2 ± 2.1 ^{###}	103.4 ± 1.8	94.7 ± 1.1 [#]
ATRA	114.6 ± 2.2 ^{**}	100.0 ± 1.9	92.8 ± 0.9
NaBu	119.2 ± 1.7 [*]	101.9 ± 3.8	92.4 ± 1.6
ATRA+NaBu	108.8 ± 3.1 ^{***}	97.9 ± 1.3	88.43 ± 2.1
<i>Guanylyl cyclase activity (pmol cGMP/mg protein/30 min)</i>			
Vehicle	24.8 ± 2.1 ^{##}	68.6 ± 3.3	116.2 ± 4.5 [#]
ATRA	40.7 ± 7.4 [*]	91.4 ± 3.5 ^{**}	145.7 ± 6.3 [*]
NaBu	31.8 ± 1.8 [*]	79.9 ± 8.8 [*]	133.3 ± 11.4 [*]
ATRA+NaBu	58.5 ± 2.5 ^{**}	106.1 ± 5.6 ^{**}	195.2 ± 7.5 ^{**}
<i>cGMP (pmol/mg protein)</i>			
Vehicle	11.8 ± 0.9 [#]	34.3 ± 1.8	82.3 ± 6.8 [#]
ATRA	21.9 ± 1.3 [*]	57.4 ± 5.1 ^{**}	121.7 ± 10.1 [*]
NaBu	19.4 ± 0.7 [*]	46.5 ± 5.9 [*]	101.5 ± 5.3 [*]
ATRA+NaBu	36.3 ± 1.8 ^{**}	84.4 ± 7.6 ^{**}	160.2 ± 12.5 ^{**}

Measurement of systolic blood pressure (SBP), renal GC activity, and cGMP levels in ATRA- and NaBu-treated mice. Blood pressure was measured by computerized tail-cuff method in *Npr1* gene-targeted mice. Kidney GC activity and cGMP levels were quantitated by ELISA as described in 'Methods' section. Values represent the mean \pm SE of three independent experiments in triplicate. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (vehicle-treated control vs. drug-treated same gene copy number); # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ (vehicle-treated 1-copy and 3-copy vs. vehicle-treated 2-copy); $n = 6$ in each group.

Figure 1

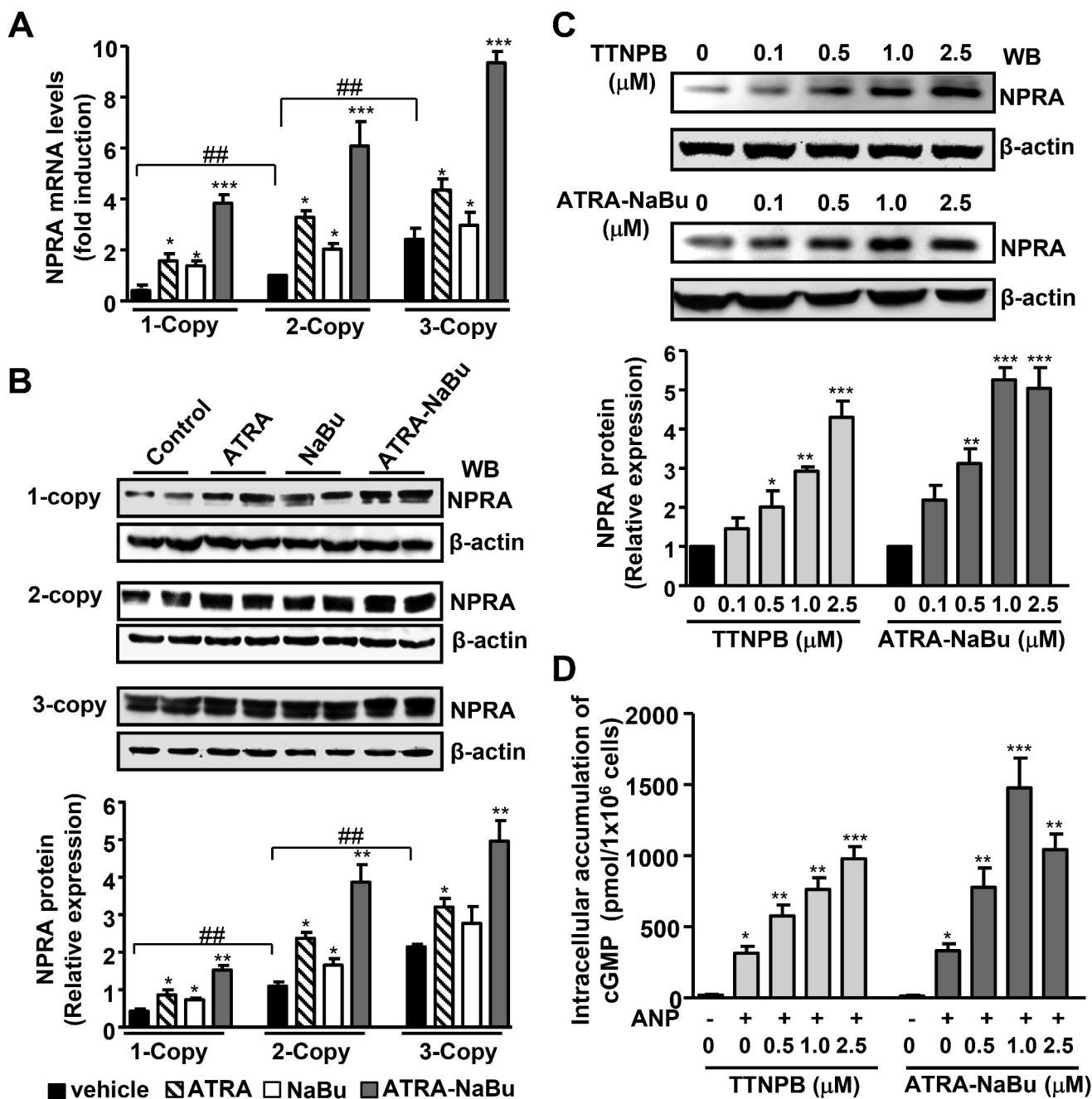


Figure 2

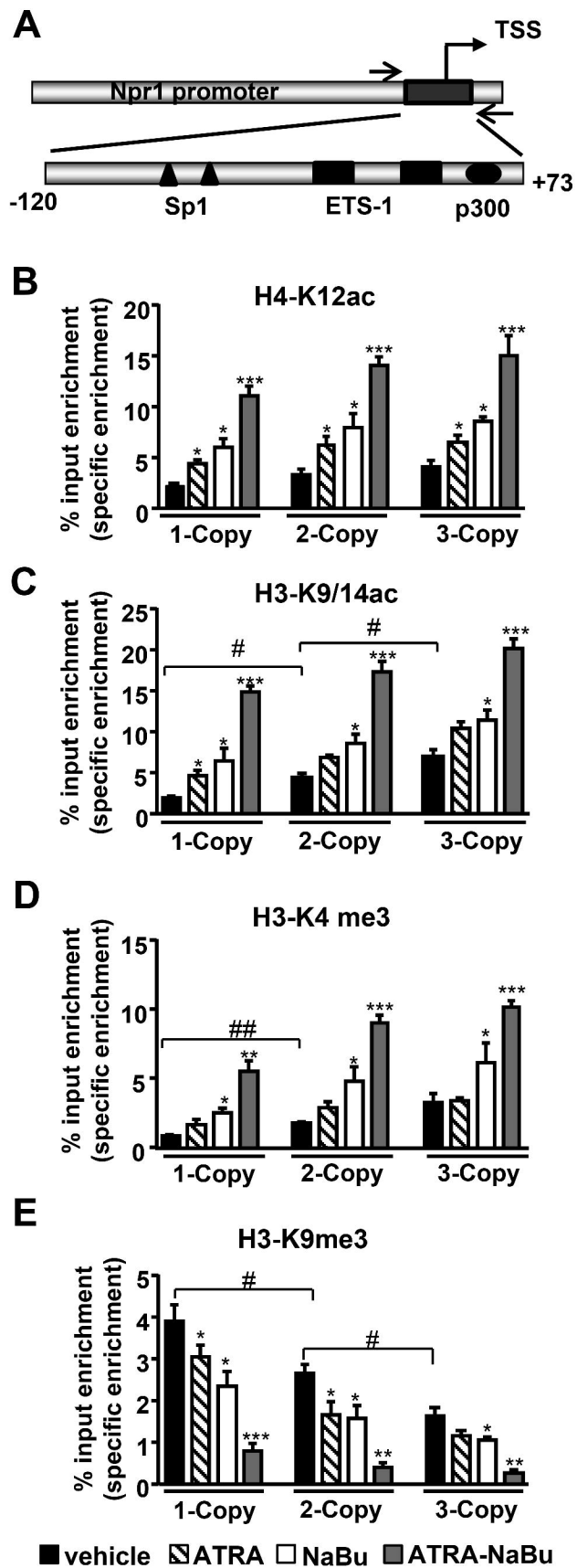


Figure 3

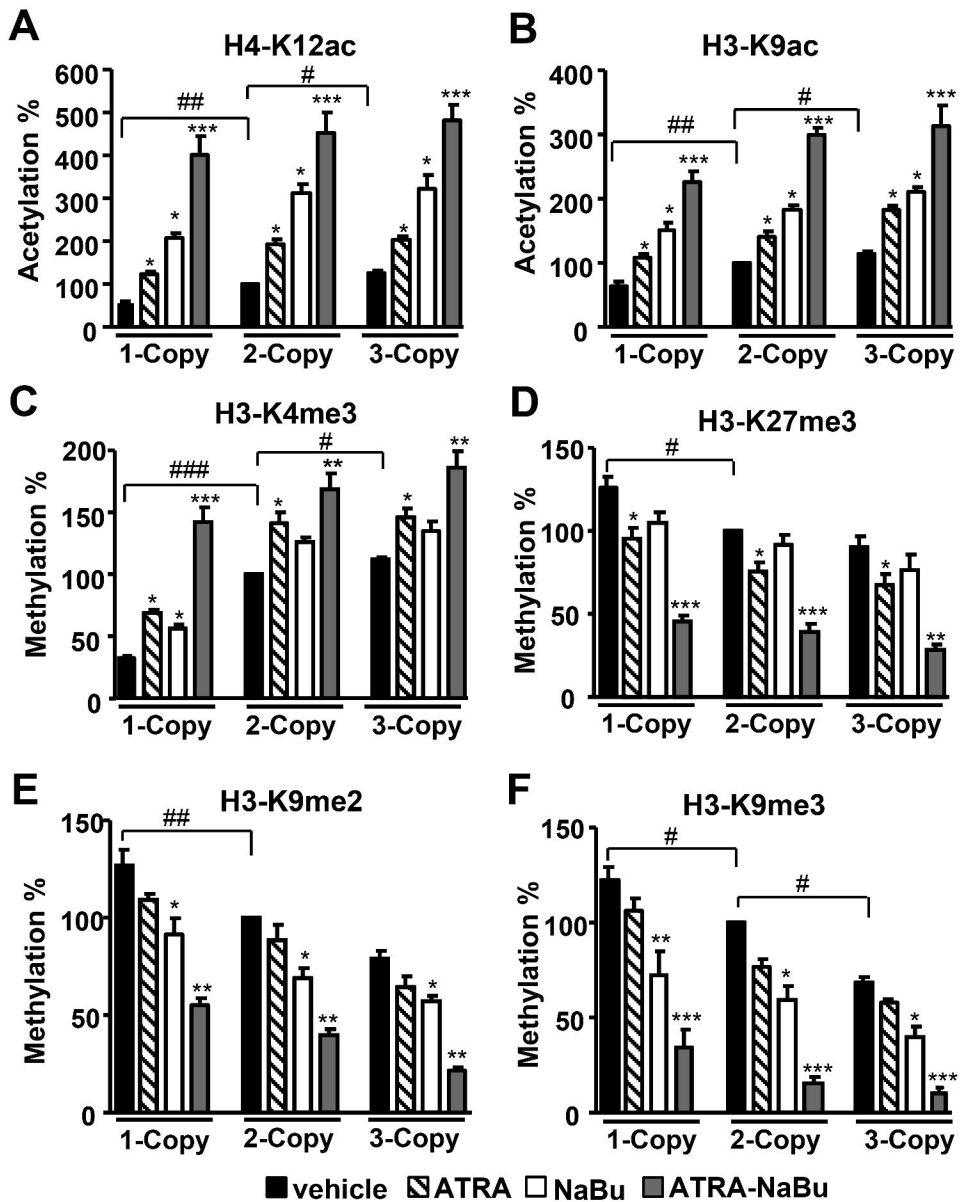


Figure 4

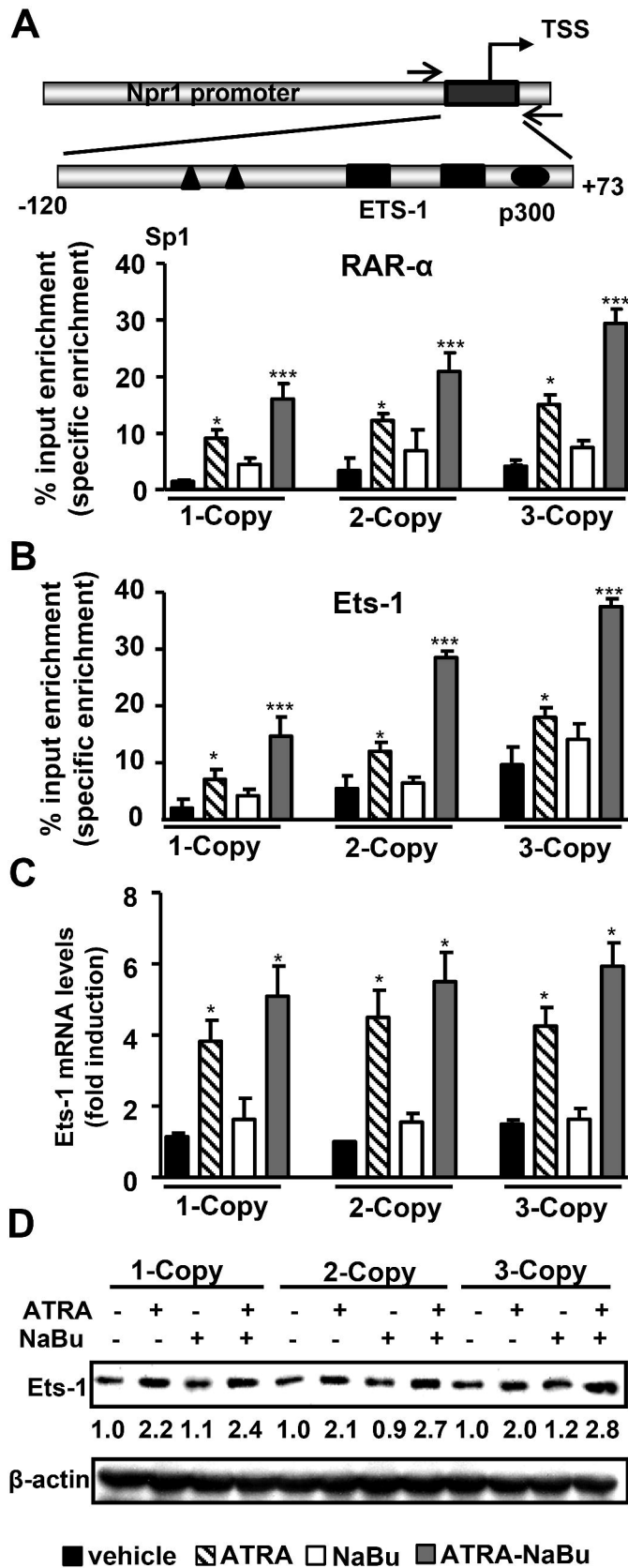


Figure 5

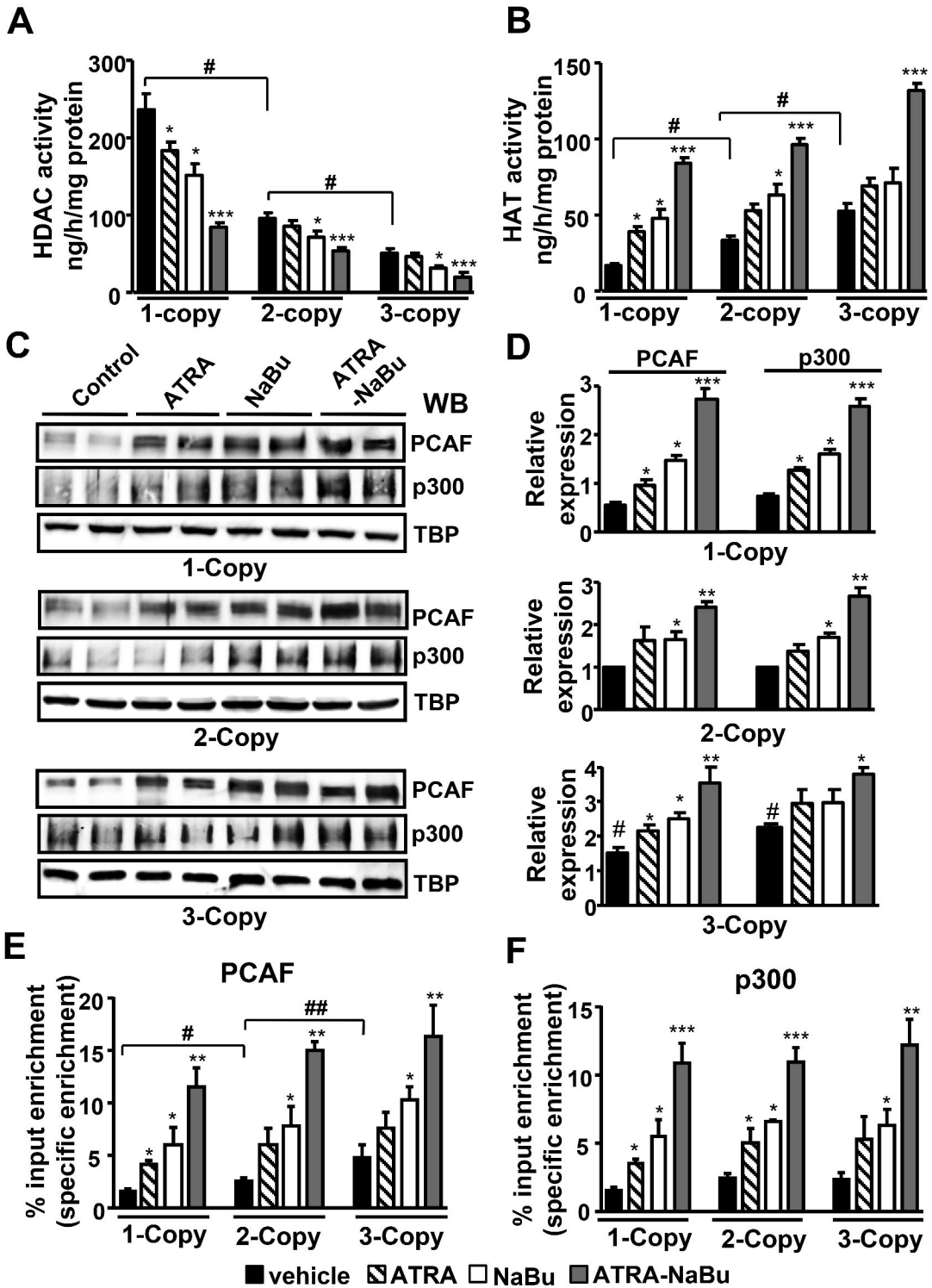


Figure 6

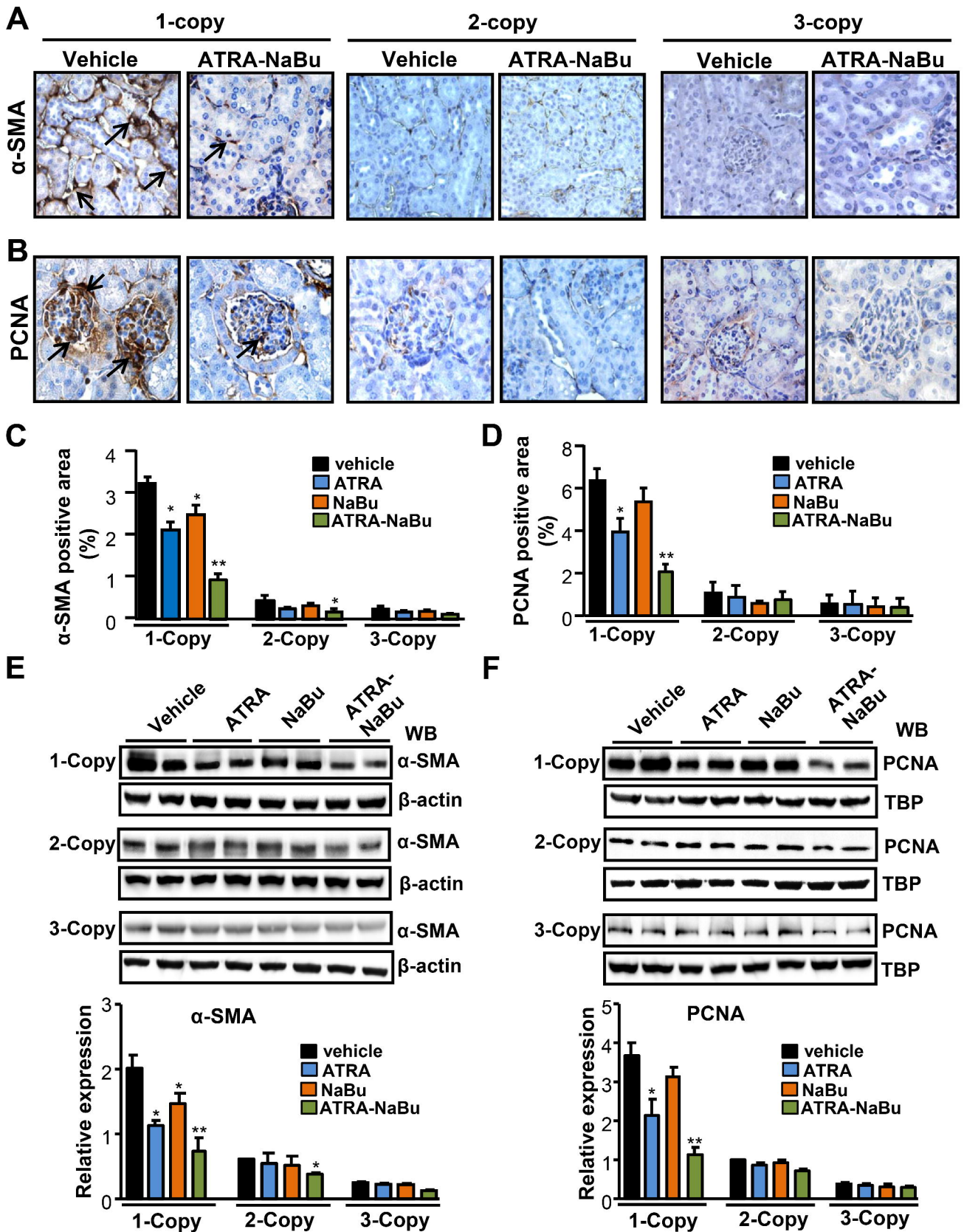
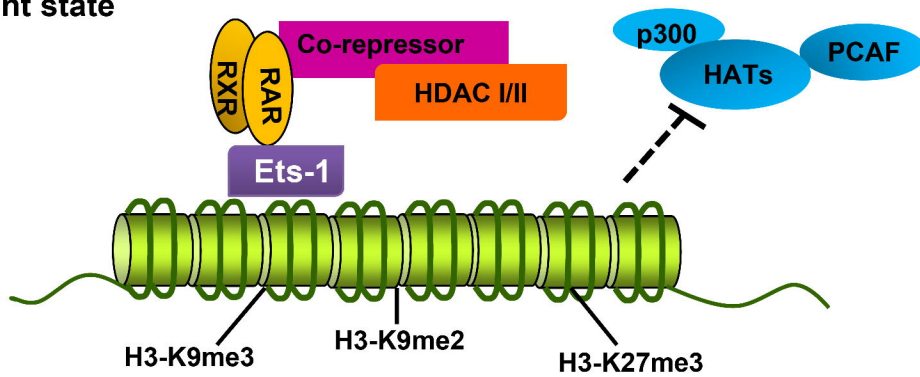


Figure 7

A

Silent state



B

Active state

