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Epigenetic activation of mu opioid receptor (MOR) gene via increased expression and function of mitogen- and stress-activated protein kinase 1 (MSK1)

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Running title: MSK1 expression activates mu opioid receptor transcription

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Abbreviations: ATP, adenosine triphosphate; BCA, bicinchoninic acid; Brg1, Brm-related gene 1; BSA, bovine serum albumin; CREB, cAMP response element binding protein; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehydes 3-phosphate dehydrogenase; HAT, histone acetyltransferase, HDAC, histone deacetylase; HMG-A1, high mobility group at-hook 1; IgG, immunoglobulin G; I κ B α , I-kappa-B-alpha; JNK, c-jun NH₂-terminal kinase, MAPK, mitogen-activated protein kinase; MeCP2, methyl-CpG-binding protein 2; MOR, mu opioid receptor; MSK, mitogen-and stress-activated protein kinase; MyoD, myogenic differentiation; NaB, sodium butyrate; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; NRSF, neuron-restrictive silencer factor; Oct-1, octamer-1; PARP, poly ADP ribose polymerase; PCBP, poly(rC)-binding protein; PDTTC, pyrrolidine dithiocarbonate; PI-3K, phosphatidylinositide 3-kinase; PKA, protein kinase A; QNZ, 6-amino-4-(4-phenoxyphenylethylamino)quinazoline; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; SAM, S-adenosylmethionine; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole; SDS, sodium dodecyl sulfate; SP600125, anthra(1,9-cd)pyrazol-6(2H)-one; SP1, specificity protein 1; STAT, signal transducer and activator of transcription; SWI/SNF, SWItch/sucrose nonfermentable; TSA, trichostatin A; U0126, 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene; QNZ, 6-amino-4-(4-phenoxyphenylethylamino)quinazoline; VPA, valproic acid

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ABSTRACT

Since the discovery of mu opioid receptor (MOR) gene two decades ago, various regulatory factors have been shown to interact with the MOR promoter and modulate transcript levels. However, the majority of early transcriptional studies on MOR gene have not addressed how intracellular signaling pathways mediate extracellular modulators. In this study, we demonstrate that MOR epigenetic regulation requires multiple co-ordinated signals converged at the MOR promoter, involving mitogen-activated protein kinase (MAPK) activation and mitogen-and stress-activated protein kinase (MSK1) - similar ranges of intracellular signaling pathways that are activated by opioid agonists. Inhibiting p38 MAPK or ERK 1/2 MAPK (upstream activators of MSK1) reduced MOR expression levels, and accordingly, the functional role of MSK1, but not MSK2, was demonstrated using genetic approaches. However, for maximal MSK1 effect, an open chromatin configuration was required, because *in-vitro* CpG methylation of the MOR promoter abolished MSK1 activity. Finally, endogenous MSK1 levels concomitantly increased to regulate MOR gene expression during neuronal differentiation of P19 cells, suggesting a conserved role of this kinase in the epigenic activation of MOR in neurons. Taken together, our findings indicate that the expression of MOR gene requires the activity of intracellular signaling pathways that have been implicated in the behavioral outcomes of opioid drugs, which suggests that an auto-regulatory mechanism may function in opioid systems.

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Introduction

Opioid drugs act through one of the four different G-protein coupled opioid receptor systems: mu, delta, kappa, and opioid receptor like-1 (ORL1) (Al-Hasani and Bruchas, 2011). These receptors are widely expressed in the central nervous system with varying densities among the nociceptive neural circuitry as well as critical brain regions involved in reward and emotion-related responses (Hwang et al., 2009). Since its discovery two decades ago, the genes encoding mu opioid receptor (OPRM1 and Oprm1) (MOR) has been extensively characterized at the cellular and molecular levels, and transgenic mouse models have been generated to demonstrate that the analgesic response to the prototypical opioid, morphine, depends on MOR expression levels (Sora et al., 1997) (Loh et al., 1998). Accordingly, transcriptional regulation of MOR gene in various cellular contexts has been reported, and several positive (e.g., Interleukin-4 response element, SOX, PCBP, SP1, NF- κ B, CREB) and negative regulatory factors (e.g., NRSF, Oct-1, PU.1, SP3, PARP1) that affect MOR transcription have been identified (for review see (Wei and Loh, 2011)). However, the majority of these early studies on MOR gene transcription were heavily *in-silico* based, and applied genetic approaches to analyze whether various factors were bound to putative recognition site(s) within the MOR promoter to modulate expression. Nonetheless, a number of recent studies have shown that MOR gene transcription activates in response to various extracellular stimuli, and follows multiple co-ordinated intracellular signals that converge at the MOR promoter (Kim do et al., 2011) (Wagley et al., 2013). Quite interestingly, MOR gene expression follows a unique spatial and epigenetic regulation in different mouse brain regions where opioid drugs act (Hwang et al., 2009). As such, stimulation of P19 embryonic carcinoma cells with epigenetic modifiers such as a histone deacetylase inhibitor or a demethylating agent has been shown to increase de novo transcription of MOR gene in a

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tightly regulated event that repositions nucleosomes to mount the transcription machinery (Hwang et al., 2007) (Hwang et al., 2010). Yet, the precise role of signal transduction events during this critical chromatin directed events remain largely elusive.

Histone deacetylase (HDAC) inhibitors results in the accumulation of hyperacetylated core histones in nucleosomes leading to transcriptional reprogramming, which is considered to enhance the therapeutic benefits of HDAC inhibitors on several diseases such as cancers, cardiovascular diseases, neurodegenerative disorders, and pulmonary diseases (Delcuve et al., 2012) (Glaser et al., 2003) (Johnstone, 2002) (Chueh et al., 2014). Even such, only a small proportion (2-10%) of expressed genes in transformed cells are truly modulated by HDAC inhibitors, which suggests that the net transcriptional outcome largely depends on functions of the regulatory proteins that can interact with the reconfigured nucleosomes (Dokmanovic et al., 2007). Indeed, phylogenetic analyses have suggested that HDACs preceded the evolution of histones (Dokmanovic et al., 2007), and can target the activity of non-histone, regulatory proteins to determine the transcriptional outcome of various genes such as p21, γ -globin, and myostatin (Sangerman et al., 2006; Zhong et al., 2003) (Rivero and Adunyah, 1996) (Han et al., 2010) (Simboeck et al., 2010). For example, treatment with the classical HDAC inhibitor, Trichostatin A (TSA), resulted in mitogen-activated protein kinase (MAPK) signaling pathway mediated increase of histone H3 serine 10 phosphorylation, that was crucial for acetylation of lysine 14 and recruitment of activated RNA polymerase II at the p21 promoter (Simboeck et al., 2010). Additionally, sodium butyrate (NaB) have been shown to directly activate p38 MAPK and ERK-1 in myocytes and lymphoid cells (Sangerman et al., 2006) (Rivero and Adunyah, 1996) (Han et al., 2010) (Zhong et al., 2003), and in some cellular models, TSA stimulation have been shown to increase EGFR tyrosine phosphorylation and phosphatidyl inositol-3-kinase (PI-3K)/AKT activation to modulate

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survivin expression levels (Zhou et al., 2006).

In this study, we utilized classical HDAC inhibitors as a tool to investigate whether intracellular signaling cascades regulate the de novo transcriptional activation of MOR gene in P19 embryonic carcinoma cells. Interestingly, we observed that HDAC inhibition of P19 cells led to MAPK activation, and an increase in expression (and function) of downstream mitogen- and stress-activated kinase (MSK1) to affect MOR gene transcription. Analogous increase in MSK1 expression (and function) occurred alongside MOR gene transcription during retinoic acid mediated neuronal differentiation of P19 cells, which indicates a central regulatory role of MAPK/MSK1 cascade in the epigenetic activation of MOR gene.

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Materials and Methods

Materials. Trichostatin A (TSA), Valproic acid (VPA), Sodium butyrate (NaB), retinoic acid, H89 and SP600125 (SP) were purchased from Sigma (St Louis, MO). 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) imidazole (SB203580 (SB)), and 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (U0126) were purchased from Cell Signaling Technology (Beverly, MA). Anti-phospho-ERK 1/2, anti-phospho-p38 MAPK, anti-ERK 1/2, anti-p38 MAPK alpha, anti-GAPDH, and anti-MSK1 antibodies were obtained from Cell Signaling Technology (Beverly, CA). Anti-phospho- phospho histone H3 (Ser 28), anti-p65, anti-p50, anti- β -III tubulin, and anti- β -actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CREB, anti-p38 MAPK beta-2, anti-acetyl histone H3, anti-phosphoacetyl histone H3 (phospho-Ser10-acetyl-Lys14) were obtained from Millipore (Billerica, MA). Anti-phospho-histone H3 (Ser 10), anti-phospho-MSK1 (Ser 360), and anti-MSK2, were obtained from Abcam (Cambridge, MA). Alkaline phosphatase-conjugated goat anti-rabbit, goat anti-mouse, and rabbit anti-goat IgG were supplied by BioRad (Hercules, CA). Restriction enzymes and buffers were obtained from New England Biolabs (Ipswich, MA). Supplies for cell cultures were from Gibco. Other reagents for molecular studies were supplied by Sigma Chemicals (St. Louis, MO).

Cells and stimulations. P19 cells were used throughout this study because epigenetic activation of MOR gene (upon stimulation with TSA or retinoic acid mediated neuronal differentiation (see below)) has been demonstrated (Hwang et al., 2007), which makes this an excellent model to investigate the role of intracellular signaling cascades. For cell stimulations, the stock solutions of TSA (10 μ M) and retinoic acid (1.0 mM) were prepared in Ethanol. Stock solutions of NaB (0.5 M), VPA (1.0 M), H89 (10 mM) were prepared in sterile distilled water and used within two months of preparation. Stock solutions of SB (25 mM),

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SP (25 mM) and U0126 (10 mM) were prepared in cell-culture grade DMSO (Sigma). All stock solutions for cell stimulation were stored at -20°C in the dark. One day before treatment, 3×10^5 cells were seeded into each wells of a 6-well plate. On the treatment day, cells were washed twice with serum free medium and cultured further for 4 h before stimulating with TSA (25 nM), VPA (10 μ M), NaB (5 mM) for various lengths of time. The final concentration of the vehicles (Ethanol or DMSO) used for cell stimulation were between 0.1-0.2%, which do not affect MOR gene expression in P19 cells (Hwang et al., 2007). At the end of stimulation, cells were washed twice with ice-cold PBS, and total RNA or protein extracts were prepared as required for analysis.

Neuronal differentiation of P19 cells. P19 embryonic carcinoma cells were cultured and differentiated into neuronal cells as previously described (Hwang et al., 2007) (Wagley et al., 2013) with minor modifications. Briefly, for induction of differentiation, 5×10^6 cells were cultured in differentiation medium (alpha-MEM (Life Technologies) supplemented with 5% FBS (HyClone)) in the presence of 0.5 μ M retinoic acid in bacteriological grade petri dishes for 4 days to induce aggregation. On the fourth day, cell aggregates were extensively washed with serum free alpha-MEM, and dissociated using 0.1% Trypsin-EDTA and 50 μ g/ml Dnase I. The dissociated cells were seeded on tissue culture dishes that were treated with 0.1 mg/ml poly-L-ornithine. Cell stimulations and transfection of the neuronally differentiating cells were carried out 4-8 h after seeding, and designated as day 1. The differentiating cells received fresh media with or without inhibitor every 48 h after plating.

Preparation of acid-soluble proteins- Acid-soluble histone proteins were extracted as described previously (Zhong et al., 2001) with minor modifications. Briefly, 1×10^6 P19 cells stimulated with HDAC inhibitors for various lengths of time were collected and resuspended in 180 μ l of cold PBS. 20 μ l of 2N HCl was added to lyse the cells. Acid-soluble

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supernatant fractions were collected after centrifugation at 12,000 rpm/10 min and treated with a final concentration of 25% trichloroacetic acid on ice for 30 min to precipitate the proteins. The protein pellets were collected by centrifugation at 12,000rpm/10 min at 4 °C, and washed twice with ice-cold acetone. Finally, the protein pellets were air-dried for 5 minutes at room temperature and solubilized using 1% SDS. The protein concentration was determined using a BCA protein assay as described by the manufacturer (Thermo Scientific, Rockford, IL), and 500 ng-1 µg of the preparation was used to perform immunoblotting with various anti-histone H3 antibodies.

RT-PCR and real-time quantitative RT-PCR (qRT-PCR)- Total RNA was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH) and analyzed by RT-PCR using the MOR gene-specific primers 5'- CATCAAAGCACTGATCACGATTCC-3' (mMOR-S) and 5'- TAGGGCAATGGAGCAGTTTCTGC-3' (mMOR-AS) (Hwang et al., 2007) (Wagley et al., 2013). Semi-quantitative RT-PCR was performed in 500 ng of total RNA using a Qiagen OneStep RT-PCR kit (Valencia, CA). Similar reactions were performed using β-actin as an internal control (Hwang et al., 2007). qRT-PCR was performed as previously described (Hwang et al., 2010) using the Quantitect SYBR Green RT-PCR kit (Valencia, CA). To calculate relative mRNA gene expression, amplification curves of test sample and standard samples that contained 10¹ to 10⁶ molecules of the MOR gene (using pcDNA3.1-mMOR, constructed in our laboratory) were monitored, and the number of target molecule in the test sample was analyzed using qCalculator version 1.0 software (<http://www.gene-quantification.de/download.html#qcalculator>) based on the mathematical model of Pfaffl (Pfaffl, 2001). The numbers of target molecules was normalized against that obtained for β-actin, which was used as an internal control (Hwang et al., 2007). The specificity of qRT-PCR reaction was determined using a melt curve after the amplification

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and the PCR products were also verified on an agarose gel. The RT-PCR and qRT-PCR experiments were performed in triplicate and each experiment was repeated with at least three different biological replicates. Results from at least two separate experiments were combined and presented as mean \pm S.E.M.

Immunoblotting- Immunoblotting was performed as described previously (Wagley et al., 2013). Briefly, 3×10^5 cells were seeded into each well of 6-well dishes and treated as required. After washing the monolayer $3 \times$ with ice-cold phosphate buffered saline (PBS), lysis buffer composed of 50 mM Tris-Cl, 150 mM NaCl, 0.1 % SDS, 1% NP-40, 0.5 % sodium deoxycholate, protease inhibitor cocktail, and phosphatase inhibitors were added. Cell lysates were collected, vortexed vigorously, and clarified by centrifugation. The protein concentrations in the supernatant were determined using BCA protein assay. 30 μ g of each lysate was loaded into SDS-polyacrylamide gels and electrotransferred onto polyvinyl difluoride membranes. Membranes were blocked for 30 min in 3% BSA solution in T-TBS (Tris buffered saline containing 0.05 % Tween-20), and then incubated overnight at 4 °C using primary antibody dilutions as suggested by the manufacturers. Membranes were washed $3 \times$ with T-TBS and incubated with alkaline-phosphatase conjugated secondary antibodies for 1 h at room temperature. Data were collected on a Storm 860 PhosphorImager Fluorescent Scanner (Molecular Dynamics) with appropriate settings for each antibody. Band densities were determined using the ImageQuant software (GE Healthcare life sciences) and statistical analysis was performed using GraphPad Prism (version 5.04).

Preparation of cytosolic and nuclear protein fractions- Nuclear and cytosolic protein preparations were carried out as described previously (Wagley et al., 2013). At the end of treatment, cells were washed $3 \times$ in ice-cold PBS. Cytosolic protein fraction was prepared by directly adding hypotonic lysis buffer (10 mM HEPES, 10 mM KCl, 0.1 mM

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EDTA, 0.5 % NP-40, 1 mM DTT, 0.5 mM PMSF, and protease inhibitor cocktail) to the monolayer and stored for 5 minutes on ice. Cell lysates were collected by scraping and following centrifugation, supernatants were collected as cytosolic protein fractions. Nuclear pellets were washed twice by centrifugation with hypotonic lysis buffer and resuspended in hypertonic buffer solution (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and protease inhibitor cocktail). Nuclear membrane was disrupted by vigorous vortexing, and stored on ice for 30 min. Following the removal of insoluble particles by centrifugation, the supernatant was collected as nuclear fraction. A BCA protein assay was performed to determine the protein yield. 10-20 μ g proteins from each fraction were used for immunoblot analysis as described above.

Transient Transfection Analysis. Approximately 1.5×10^6 P19 cells were seeded in 60- mm dishes one day before transient transfection. Cells were transfected using a complex containing 10 μ l of LipofectAMINE 2000, 300 ng MOR promoter construct (pGL450), 1.0 μ g pCMV5-constitutively active-MSK1 (S376D, T581D, T700D) (CA-MSK1) (received from Dr. James C. Hastie, University of Dundee), and 50 ng of pCH110- β -galactosidase per transfection sample diluted in 500 μ l of Opti-MEM (Life Technologies). Forty eight hours after transfection, cells were washed with serum free medium and treated with HDAC inhibitors for 8 h, and cell lysates were analyzed for firefly luciferase activity and β -galactosidase activity as described by the manufacturer's protocol (Promega and Tropix, respectively). Results were expressed as relative luciferase activity compared to the control cells (Hwang et al., 2003) (Wagley et al., 2013).

RNA Interference. The gene-specific effect on MOR transcription was analyzed by transfecting small-interfering RNA (siRNA) against p38 MAPK alpha or p38 MAPK beta (Cell Signaling Technology), and MSK1 or MSK2 (Satna Cruz Biotechnology). For

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transfection, approximately 1×10^5 P19 cells/well in 1 ml of P19 growth medium (alpha-MEM supplemented with 7.5% newborn calf serum and 2.5% fetal bovin serum) were plated into 12-well plates. Cells were allowed to attach for 4- 8 h and transfected with 50 nM each of control siRNA (Cell Signaling Technology) or gene specific siRNAs using 3 μ l of LipofectAMINE 2000 in a transfection mixture prepared in 200 μ l OPTI-MEM. Twelve hours after transfection, 1 ml fresh growth medium was added to the cells and continued to culture for another 24 h. At the end of transfection, cells were stimulated with HDAC inhibitors as required and harvested for qRT-PCR and immunoblot assays.

CpG methylation of MOR promoter- CpG Methylation of MOR promoter construct pGL450 was carried out using *SssI* methylase as described by the manufacturer. Briefly, 2 μ g of MOR promoter construct was added to a reaction mixture containing 640 μ M of S-adenosylmethionine (SAM) buffer and 4 units of *SssI* methylase in a final reaction volume of 20 μ l. The reaction was allowed to continue for 3 h at 37°C. At the end of incubation period, the reaction was terminated by heating at 65°C for 30 minutes. Parallel reactions that lacked *SssI* enzyme was carried out as control. For analyses, one fourth (approximately 500 ng) of the methylated or sham-methylated MOR promoter was co-transfected with 1 μ g of MSK1 expression construct and 50 ng of pCH110- β -galactosidase for 48 h as described above. At the end of transfection, cells lysates were prepared and assayed for luciferase and β -galactosidase activity, and presented as relative activity compared to control.

Statistical analysis- All data presented herein were performed in at least three different biological replicates. Numerical values were presented as the mean \pm S.E.M. For comparison between two samples, t-test analysis was performed. For multiple comparisons, analysis of variance with Bonferroni's post hoc test was used. All statistical analyses were performed in GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA). p values

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representing significances of <0.05 , 0.01 are denoted with symbols *, **, whereas significances <0.05 , 0.01 among various treatment groups are represented with †, ††, respectively.

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Results

Time delay between histone acetylation and mu opioid receptor (MOR) gene expression- Although it is known that the de novo transcription of mu opioid receptor (MOR) gene increases upon histone deacetylase (HDAC) inhibition and requires an extensive chromatin remodeling at the MOR promoter (Kraus, 2012; Lin et al., 2008), it is largely unknown how signal transduction cascades influence changes at the MOR promoter. A kinetic analysis of MOR gene expression induced by a classical HDAC inhibitor, Trichostatin A (TSA) in MOR-negative P19 embryonic carcinoma cells (P19 cells) was performed. As shown by quantitative real-time RT-PCR (qRT-PCR) analysis in Fig. 1A, TSA stimulations of P19 cells increased MOR gene expression in a time-dependent fashion, which appeared 1 h after the treatment (~2-fold), dramatically increased at 2 h (**, $p < 0.01$; ~6-fold), and continued further (~10-fold) by 8 h treatment. Quite interestingly, immunoblot analysis (Fig. 1B) of the acid-soluble histone proteins prepared from similarly stimulated cells (0-4 h) showed that the increase in global histone H3 acetylation had already reached high significance (**, $p < 0.01$ at $t = 1$ h) (Fig. 1B, upper histogram) compared to the dramatic increase in MOR gene expression (Fig. 1 A, from ~2-fold to ~6-fold between 1 and 2 h), indicating a time delay in between the two events. Further experiments were performed to investigate whether sodium butyrate (NaB), another HDAC inhibitor, also demonstrates a similar pattern of MOR gene transcription and histone acetylation kinetics. As shown in Fig. 1C, qRT-PCR analysis showed that NaB stimulation of P19 cells also significantly increases MOR gene expression by 2 h (~3.8 fold, **, $p < 0.01$), which dramatically increases further to reach ~12 fold by 4 h. As observed with TSA stimulation (Fig. 1B), NaB stimulation of P19 cells also led to an similar histone H3 acetylation kinetics (Fig. 1D) with maximum acetyl H3 achieved at 1 h (**, $p < 0.01$) (Fig. 1D, upper histogram). Taken together, there exists a similar

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time delay between the dramatic increase in MOR gene expression (starting 2 h after stimulation) and significant histone H3 acetylation (starting 1 h after stimulation) with two different HDAC inhibitors (TSA and NaB), which suggests that HDAC effects on MOR transcription are mediated by biochemical changes unique to histone acetylation.

p38 MAPK and ERK 1/2 regulates HDAC inhibition mediated MOR gene expression- As mentioned previously, HDAC inhibitor mediated transcription is a combinatorial outcome of histone modifications and functions of proteins of the signal transduction cascade that directs sequence specific transcription factors and components of the basal transcription machinery to the responsive promoter (Dokmanovic et al., 2007). Thus, we next examined if HDAC inhibitor mediated increase of MOR gene expression is dependent on the activity of classical mitogen-activated protein kinase (MAPK) components such as p38 MAPK, c-jun N-terminal kinase (JNK), or extracellular signal regulated kinase (ERK). For this purpose, P19 cells were pretreated with pharmacological inhibitors of p38 MAPK (SB203580, SB), JNK (SP600125, SP), and MEK/ERK 1/2 (U0126, U0) for 1 h and stimulated with TSA or NaB for a further 8 h. Total RNA were extracted from stimulated cells, and analyzed for MOR expression. Fig. 2A and 2B shows that SB and U0 each significantly blocked the MOR gene expression induced by TSA or NaB, suggesting the involvement of MAPK activities. Intriguingly, JNK inhibitor (SP) showed a synergistic effect, and further potentiated MOR expression levels (~3 fold increase in MOR expression levels compared to TSA or NaB stimulation alone) (Fig. 2 A and 2B). As a control, we have previously demonstrated that SP increases MOR gene expression via a p38 MAPK dependent mechanism in P19 cells whereas SB or U0 treatment has minimal effects (Wagley et al., 2013).

Since inhibition of either p38 MAPK or ERK 1/2 reduced MOR gene expression

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induced by TSA or NaB stimulation, we determined if p38 MAPK and ERK 1/2 phosphorylation levels are increased in the stimulated cells. As shown in Fig. 2C, the levels of phosphorylated p38 MAPK increased after 0.5 h of TSA stimulation and continued further to reach maximum levels by 2 h (**, $p < 0.01$) (Fig. 2C, lanes 3-5). As a control, the levels of total p38 MAPK were analyzed, and found to be minimally changed. Interestingly, unlike p38 MAPK phosphorylation kinetics, which started to decline after reaching maximum levels at 2 h, increases in the levels of phosphorylated ERK 1/2 (but not total ERK 1/2) were sustained, and continued throughout the 4 h stimulation period (Fig. 2C, c.f. lanes 1-6). Similar analyses in NaB stimulated P19 cells (Fig. 2D) revealed analogous increase of ERK 1/2 phosphorylation that sustained over the 4 h stimulation period (Fig. 2D, c.f. lanes 1 and 5), whereas p38 MAPK phosphorylation followed a slightly different course, and showed maximum levels at 1 h after stimulation (compared to 2 h for TSA stimulation). Collectively, these results demonstrate that HDAC inhibition increases p38 MAPK and ERK 1/2 phosphorylation in P19 cells, and that pharmacological inhibition of these kinase reduces MOR gene expression levels induced by the same HDAC inhibition.

MSK1 expression increases by HDAC inhibition- Depending upon the stimulus, activation of MAPKs, specifically p38 MAPK and ERK 1/2, can lead to the phosphorylation and activation of downstream kinase- mitogen and stress-activated protein kinase 1 (MSK1), which affects transcription by increasing the phosphorylation of histone proteins to remodel transcriptionally inactive chromatin into a more active configuration, and/or by increasing the phosphorylation and down-stream activity of transcription factors such as ER81, ATF1, NF- κ B, CREB, HMG14 etc. (Keum et al., 2013; Zhong et al., 2003). Thus, MSK1 activation was examined by immunoblot analyses of total cell lysates prepared from TSA and NaB stimulated P19 cells (0-4 h). As shown in Fig. 3A, MSK1 phosphorylation at serine 360 (a

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common substrate of p38 MAPK and ERK 1/2 within the linker domain of MSK1 (Arthur, 2008)) increased upon either TSA or NaB stimulation in a time-dependent fashion that started 1 h after the stimulation and continued further up to 4 h (cf. lanes 1-4 and 5-8). Unexpectedly, analyses of total MSK1 also showed increased expression levels that were clearly evident by stimulations with TSA and NaB after 4 h (Fig. 3A, c.f. lanes 1, 4 and 5, 8). Meanwhile, densitometric analyses (Fig. 3A lower graphs) showed that the ratio of phosphorylated MSK1 to that of total MSK1 increased nominally between 1-2 h of TSA stimulation (Fig. 3 A, lower panel, left graph) but remained fairly indistinguishable throughout NaB stimulation (Fig. 3A, lower panel, right graph). Further analyses at time points earlier than 1 h (0, 15 min, and 30 min) upon TSA or NaB stimulations were also carried out and showed minimal changes in both forms of MSK1 (data not shown). In order to rule out the possibility of general increase of cellular protein levels upon stimulation, immunoblot analyses of β -actin levels were carried out, which showed minimal changes (Fig. 3A). Additionally, as already demonstrated, since the changes in the levels of total p38 MAPK and ERK 1/2 also remained minimal after TSA or NaB stimulation (Fig. 2C and Fig. 2D), the increase of MSK1 levels appears to be a specific effect of HDAC inhibition.

Since increased MSK1 expression and phosphorylation (Fig. 3A) occurred simultaneously with increases in MOR expression levels upon TSA or NaB stimulation (Fig. 1A and Fig. 1C), we investigated whether MSK1 activity is involved by using a small molecule PKA/MSK1 inhibitor, H89. Therefore, P19 cells were stimulated with TSA or NaB in presence of a range of H89 (0-10 μ M), and MOR expression levels were determined by qRT-PCR analyses. As presented in Fig. 3B, MOR gene expression induced by both HDAC inhibitors reduced in a dose-dependent fashion by H89 treatment (~40% and ~65% reduction compared to control for TSA, and ~32% and ~47% reduction compared to control for NaB

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stimulation by 5 μ M or 10 μ M H89 respectively), which suggests that MSK1 activity is involved. In further experiments, we determined if VPA (valproic acid, a different class of HDAC inhibitor used for the treatment of bipolar disorders), shares a common mechanism to regulate MOR gene transcription involving increased expression (and activity thereof) of MSK1 proteins. As suspected, and as shown in Fig. 3C (upper gel image), MSK1 expression (and phosphorylation) indeed increased in a time dependent fashion (Fig. 3C, c.f. 1, 3 and 4) upon VPA stimulation of P19 cells. Further qRT-PCR analyses showed that MOR gene expression too increased in a similar time-dependent fashion (data not shown), and that H89 treatment exerted a dose-dependent antagonistic effect on those MOR expression levels (Fig. 3C, lower histogram).

p38 MAPK beta regulates MOR gene transcription but minimally affects MSK1 expression- Although ~ 60% homologous in their amino acid sequences, each of the four mammalian p38 MAPK isoforms (alpha, beta, gamma, and delta) differ considerably in their tissue specific expression patterns, substrate specificities, and susceptibility to chemical inhibitors (Cuenda and Rousseau, 2007) (Coulthard et al., 2009). Of note, only the activities of p38 MAPK alpha and p38 MAPK beta are inhibited by the chemical compound SB (Cuenda and Rousseau, 2007). As demonstrated earlier (Fig. 2A and Fig. 2B), since SB inhibited MOR gene expression induced by TSA (~65% reduction compared to control) and NaB (~60% reduction compared to control); and given the fact that activation of p38 MAPK can lead to the phosphorylation and activation of downstream MSK1, we next determined which p38 MAPK isoform (alpha and/or beta) regulates MSK1 phosphorylation in response to HDAC inhibition. P19 cells were transfected with siRNA against p38 MAPK alpha or p38 MAPK beta for 36 h, and further stimulated with TSA or NaB for 4 h to analyze changes in MSK1 expression and phosphorylation levels. The 4 h time point was chosen because both

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the expression and phosphorylation levels of MSK1 are clearly increased at this time point (Fig. 3A). As shown in Fig 4A and Fig. 4B, immunoblot analyses, quite unexpectedly, revealed that although each p38 MAPK siRNAs specifically and significantly decreased the expression levels of their respective targets, neither phosphorylation nor the expression of MSK1 levels decreased under stimulated conditions. In an attempt to determine whether both p38 MAPK activities simultaneously regulate MSK1 phosphorylation, further analyses using the pharmacological inhibitor SB were carried out, which also proved insignificant (data not shown). Taken together, these results demonstrate that HDAC inhibitors do not utilize p38 MAPK pathway to increase the phosphorylation and expression of MSK1 proteins in P19 cells.

In further experiments, we examined whether MOR expression levels are affected by each p38 MAPK isoform. Thus, P19 cells were transfected with p38 MAPK alpha or p38 MAPK beta specific siRNAs for 36 h and stimulated with HDAC inhibitors (TSA, NaB, and VPA) for further 8 h. As shown in Fig. 4C, qRT-PCR analyses showed that only p38 MAPK beta siRNA transfection significantly reduced MOR expression levels upon TSA (~35% reduction compared to control siRNA) or VPA (~50% reduction compared to control siRNA) stimulation; but, both siRNAs effectively reduced MOR expression levels induced by NaB (~50% reduction by each siRNA compared to control). Since the role of p38 MAPK beta appeared to be conserved equally across three different types of HDAC inhibitors, we further tested the effect of these siRNAs across two more stimuli that are known to increase MOR expression via a p38 MAPK dependent mechanism: protein synthesis inhibitor cycloheximide (CHX) and JNK inhibitor (SP) (Kim do et al., 2011) (Wagley et al., 2013). As shown in Fig. 4D, although both p38 MAPK siRNAs significantly reduced MOR gene expression induced by either stimulus, the effects were more pronounced with p38 MAPK beta siRNA (~60-70%

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reduction in MOR expression levels compared to ~50% reduction by p38 MAPK alpha siRNA). As a control, immunoblot analyses were performed (Fig. 4D, lower panel) and revealed that each siRNAs specifically and significantly decreased the expression levels of their respective p38 MAPK targets, without affecting ERK 1/2, JNK, or AKT levels. Collectively these results suggest that p38 MAPK beta has a variable MOR regulatory function, dependent largely on the type of stimulus and whether or not p38 MAPK alpha is also involved.

Next, we investigated whether ERK 1/2 mediates MSK1 phosphorylation and expression because a sustained and a time-dependent increase were observed for both the kinases after TSA or NaB stimulation (Fig. 2C, Fig. 2D, Fig. 3A). P19 cells were pretreated with U0 for 1 h to inhibit MEK/ERK activities, and further stimulated with TSA, NaB or VPA for 4 h. As shown by immunoblot analysis (Fig. 4E), although U0 significantly blocked the phosphorylation of MSK1 (Fig. 4E, c.f. lanes 1-4 and 5-8) induced by HDAC inhibition, the expression levels of MSK1 proteins remained unchanged. As a control, ERK 1/2 phosphorylation levels were analyzed and found to be reduced by U0 pretreatment. In further experiments, we used SB and U0 together to completely block upstream MAPK signals, and then exposed the cells to HDAC inhibitors to analyze the expression and phosphorylation levels of MSK1 proteins. As expected, basal as well as the HDAC inhibitor mediated MSK1 phosphorylation levels were completely abolished by SB and U0 pretreatment (Fig. 4E, lanes 9-12), which indicate that complete inhibition of the MSK1 phosphorylation requires a simultaneous inhibition of both upstream kinase activities (p38 MAPK and ERK 1/2) under either stimulated or non-stimulated conditions. Nonetheless, despite complete lack of p38 MAPK and ERK 1/2 activities, MSK1 expression levels were still increased by HDAC inhibition. Collectively, these results indicate that HDAC inhibitors mediated increase in

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MSK1 expression levels is independent of MAPK activities; yet, MAPKs activities do act upon the MSK1 proteins to increase its phosphorylation status.

MSK1 expression increases and HDAC inhibitors potentiate MOR promoter activities- The core MOR promoter contains binding sites for two of the transcription factors known to be activated by MSK1- NF- κ B p65 and CREB (Wei and Loh, 2011). We examined whether forced MSK1 expression potentiates MOR promoter activities. Thus, cell lysates were prepared from P19 cells co-transfected with MOR promoter reporter (pGL450) and constitutively-active-MSK1 (CA-MSK1) expression constructs and further stimulated with HDAC inhibitors. As shown in Fig. 5A, upper histogram, CA-MSK1 expression increased the luciferase activity (~3.3 fold compared to vector transfected cells), and upon HDAC inhibition, it was further potentiated (~2 fold for TSA, ~3-fold each for NaB and VPA stimulations). As an internal control, CA-MSK1 expression was analyzed by immunoblotting, and showed correct expression (Fig. 5A, lower image). Since, the increase in MOR promoter activity by CA-MSK1 over-expression (~3.3 fold) alone was comparable to that induced by HDAC inhibitors (TSA ~2.6 fold, NaB ~2.7 fold, VPA – 2.4 fold); we determined whether MOR expression levels are also increased. Thus total RNA were prepared from P19 cells transfected with a varying amounts of CA-MSK1 for 48 h, and MOR expression levels were determined. As shown in Fig. 5B, both the expression levels of CA-MSK1 (upper gel image) and MOR mRNA (lower histogram) increased in a dose-dependent fashion. In additional experiments, the effect of HDAC inhibition on MOR mRNA expression was analyzed by qRT-PCR in CA-MSK1 expressing cells. As shown in Fig. 5C, TSA stimulation of CA-MSK1 expressing cells showed a significant increase in the MOR expression levels (~10 fold in vector transfected cells compared to ~16 fold in CA-MSK1 transfected cells). Additional qRT-PCR analyses performed with NaB and VPA stimulations under conditions expressing

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CA-MSK1 also showed increased MOR expression levels (data not shown).

Since, MSK1 and MOR gene expression kinetics was similar upon HDAC inhibition, inhibition of PKA/MSK1 activity by a small molecule inhibitor, H89 abrogated the MOR gene expression, and MSK transfection increased and HDAC inhibitor potentiated MOR promoter activities, we used siRNA strategy to directly confirm MSK1's role in MOR gene expression. P19 cells were transfected with control siRNA or siRNA specific for MSK1 or MSK2 (a closely related protein to MSK1) for 36 h, and stimulated for 8 h with HDAC inhibitors (TSA, NaB or VPA). As shown by qRT-PCR analysis in Fig. 5D, although MSK1 siRNA transfection did not completely abolish MOR expression induced by HDAC inhibitors, the levels of MOR expression were significantly decreased (~50% for TSA, ~25% for NaB, and ~45% for VPA). On the other hand, MSK2 siRNA transfection showed minimal changes and MOR expression levels remained comparable to that obtained with control siRNA transfection. In order to confirm the specificity of each siRNAs, immunoblot analyses of the total cell lysates prepared at the end of transfection period showed that (Fig. 5E, upper image and quantification in lower histograms), each siRNA significantly reduced the expression levels of their respective target proteins, MSK1 and MSK2, but not vice versa. Collectively, these results demonstrate that HDAC inhibition mediated MOR gene expression depends on the activity of MSK1.

CpG methylation abolishes MSK1 mediated increase in MOR promoter activity- As reported earlier from our laboratory (Hwang et al., 2010), the CG residues of the endogenous MOR promoter in P19 cells are heavily methylated, and are recognized by MeCP2 proteins which recruits repressive Brm proteins under unstimulated conditions. However, upon neuronal differentiation of P19 cells or stimulation with TSA, these repressive chromatin marks are removed, which allows the binding of activating transcription factors such as SP1.

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As demonstrated above, MSK1 strongly activated the MOR promoter (and activity was further potentiated by HDAC inhibition), thus we were interested to determine if *in-vitro* methylation of the MOR promoter construct affects MSK1 activity. P19 cells were co-transfected with *in-vitro* methylated pGL450 promoter construct and the ability of CA-MSK1 to increase the MOR promoter activities were analyzed. As shown in Fig. 6 (upper histogram), CA-MSK1 mediated luciferase reporter activities from the methylated MOR promoter constructs were significantly reduced (~47%) compared to the activities obtained from sham-methylated constructs. As an internal control, immunoblot analyses of the same cell lysates (Fig. 6, lower panel) showed that MSK1 expressions are comparable. Collectively, these results indicate that the repressive CpG methylation mark in the endogenous MOR promoter hinders efficient MSK1 action during the transcriptional activation of MOR gene.

MSK1 expression increases and regulates MOR gene expression in differentiating neurons- P19 cells can be induced to differentiate into neuronal cells by retinoic acid (RA), a process that closely resembles mammalian neurogenesis *in-vivo* (Monzo et al., 2012). As reported earlier, MOR gene expression starts to increase within 2 days after the induction of differentiation, and continues further as the cells fully differentiate into neuronal subtypes (Hwang et al., 2010). Interestingly, phosphorylation of all three major MAPKs (p38 MAPK, ERK 1/2, and JNK) also increase during neuronal differentiation of P19 cells, and follows MOR expression kinetics, among which pharmacological inhibition of p38 MAPK abrogates MOR gene expression (Wagley et al., 2013). Thus, to determine whether MSK1 is also activated alongside MOR expression, total cell lysates and total RNA samples were prepared in parallel from neuronally differentiating P19 cells each day for a total of 5 days, and analyzed by immunoblotting and qRT-PCR analyses. As shown in Fig. 7A, upper histogram, and as reported previously, qRT-PCR analysis showed that MOR expression levels start to

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increase 2 days after the induction of differentiation and dramatically increases up to the fifth day when the cells completely differentiate into the neuronal types (the MOR levels on first day of neuronal differentiation was indistinguishable with parent P19 cells and were not included in the analysis). Quite interestingly, as shown in Fig.7A, lower panel gel images, a biphasic MSK1 phosphorylation kinetics was observed; a first reduction phase which lasted till the cells started to differentiate into neuronal types (Fig. 7A, lanes 1, 2 and 3, and data not shown); however, the levels gradually increased and surpassed the levels seen with non-differentiating cells within 3 days after the induction of neuronal differentiation (Fig. 7A, c.f lanes 1, 4, 5, 6). Corresponding analyses with total MSK1 also followed a biphasic course, but unlike the phosphorylated form, its levels followed a sustained increase (~1.3 - ~2.0 fold; Fig. 7A, lower) in its expression levels until the cells reached maximum neuronal differentiation at day 4, after which it slightly declined (~1.5 fold in day 5, Fig. 7A, cf. lanes 1-5 and 6). As an internal control for neuronal differentiation, immunoblot analyses of neuron specific β -III tubulin levels were performed, which showed a continued increase and sustained its levels after 4 days. Further analyses to detect the expression changes in levels of β -actin and GAPDH (total p38 MAPK, ERK 1/2, NF- κ B p65 etc, data not shown) showed minimal changes, and thus, suggested that the changes in MSK1 levels are specific events observed during neuronal differentiation of P19 cells.

Next, we determined whether increase in MSK1 expression (and phosphorylation) during neuronal differentiation of P19 cells is functionally relevant to increase MOR gene expression. Thus, qRT-PCR analysis was performed with total RNA extracted from neuronally differentiating P19 cells cultured in the presence of varying concentration of PKA/MSK1 inhibitor, H89 (0-10 μ M) for 3 days. Fig. 7B, upper histogram shows that H89 blocked the MOR expression in a dose-dependent fashion (~40%--~70% reduction by 5-10

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μ M of H89), which indicates that MSK1 activity is required for MOR expression in differentiating neurons. In following set of experiments, we analyzed whether H89 decreased MOR expression levels due to a general inhibition of neuronal differentiation. Thus, the levels of the neuron specific β -III tubulin protein levels were immuno-detected in total cell lysates prepared from neuronally differentiating P19 cells cultured in presence of H89 as above. As shown in the lower gel image (Fig. 7B), β -III tubulin levels remained comparable amid H89, and suggested that PKA/MSK1 inhibition in general, minimally affects the neuronal differentiation process.

As mentioned previously, depending on the stimulus, the phosphorylation and activation of MSK1 is regulated by either one or both of the upstream MAPKs-p38 MAPK or ERK 1/2. Also, in our previous report, we have demonstrated that the pharmacological inhibition of p38 MAPK or NF- κ B, but not ERK 1/2 abolishes MOR gene expression during neuronal differentiation of P19 cells (Wagley et al., 2013). Thus, to determine whether/which p38 MAPK isoform regulates MSK1 phosphorylation during neuronal differentiation of P19 cells, we transfected differentiating cells with p38 MAPK alpha or p38 MAPK beta siRNA and cell lysates were analyzed. As shown in Fig. 7C, top panel gel image, transfection with each p38 MAPK siRNAs showed a specific and significant reduction of their respective targets, and ~30% reduction in the levels of phosphorylated MSK1 (Ser 360) by each siRNA (Fig 7C, gel image and quantification in middle panel histograms). However, the overall changes in neuronal differentiation were insignificant because the expression levels of the neuron specific β -III tubulin protein changed minimally by p38 MAPK knock-down. In further experiments, MOR expression levels were determined by qRT-PCR analysis of similarly transfected cells, and demonstrated that each of these siRNAs, by themselves, significantly reduced MOR expression levels (~40% and ~60% reduction by p38 MAPK

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alpha and p38 MAPK beta siRNA respectively compared to control siRNA), which suggest a co-ordinate p38MAPK/MSK1 pathway may be functioning to regulate MOR expression during neuronal differentiation of P19 cells. Additional qRT-PCR analysis using simultaneous transfections of both siRNA did not produce additive inhibition of MSK1 phosphorylation levels or MOR expression levels, and was identical to the data obtained with p38 MAPK beta siRNA (data not shown).

To finally prove MSK1's role in MOR gene regulation during neuronal differentiation of P19 cells, a specific siRNA targeting MSK1 was introduced into the differentiating cells, and 3 days later, MOR expression level was determined by qRT-PCR analysis. As expected, MOR expression level was significantly reduced in MSK1 siRNA transfected cells (~50% reduction compared to control siRNA) (Fig. 7D, left panel) which was analogous with the inhibition levels obtained with p38 MAPK siRNA (Fig. 7C, qRT-PCR histograms). Corresponding immunoblot analysis (Fig. 7D, right panel) showed the specificity of the siRNA and showed that MSK1 expression was reduced in the transfected cells. Collectively, these results identify a regulatory role of MAPK/MSK1 cascade on MOR gene expression during neuronal differentiation of P19 cells.

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Discussion

The transcriptional activation of mu opioid receptor (MOR) gene is a complex process and requires extensive chromatin remodeling that allows the binding of various transcription factors to the MOR promoter (for review see (Wei and Loh, 2011)). Here, we provide evidence that HDAC inhibition activates components of the mitogen activated protein kinase (MAPK) cascade thereby augmenting MOR transcription through regulatory signals that converge at the MOR promoter. Stimulation of P19 cells with classical HDAC inhibitors, Trichostatin A (TSA) or sodium butyrate (NaB) increased MOR expression after p38 MAPK and ERK 1/2 were phosphorylated; and inhibiting the activities of either of these MAPKs decreased MOR expression levels (Fig. 1 and Fig. 2). The expression levels of their common downstream effector kinase, MSK1, also increased upon HDAC inhibition, and regulated MOR transcript levels (Fig. 3). In further analyses, although inhibition of p38 MAPK activities failed to modulate MSK1 levels (Fig. 4A and Fig. 4B), MOR expression decreased in cells transfected with p38 MAPK beta siRNA or MSK1 siRNA (Fig. 4C and Fig. 5D). On the other hand, ERK 1/2 activities was found to regulate the phosphorylation but not the expression of MSK1 proteins (Fig. 4E), suggesting different modes of MSK1 expression and functional regulation. Likewise, the expression and function of MSK1 proteins increased to regulate MOR expression during neuronal differentiation of P19 cells (Fig. 7A and Fig. 7B), suggesting a central functional role of this kinase in epigenetic regulation of MOR gene (Fig. 8).

Although most of the transcriptional effects of HDAC inhibitors such as TSA or NaB, have been attributed to direct chromatin remodeling by histone acetylation (Zhong et al., 2003), HDAC inhibitors only modulate a small proportion (2-10%) of expressed genes in transformed cells, suggesting that the net effect on gene transcription is a combinatorial

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outcome of histone modifications and functions of regulatory proteins such as components of signal transduction cascade and basal transcriptional machinery, which specifically recognize the modified histone via protein domains specialized for such purpose (Dokmanovic et al., 2007). Indeed, the data presented in this study also supports the active role of such specialized proteins: specifically the components of the MAPK cascades, because p38 MAPK and ERK 1/2 were activated concurrently with a dramatic increase in MOR gene expression induced by TSA or NaB (Fig. 1 and Fig. 2). In agreement to this observation, a number of studies have shown that HDAC inhibitors can directly activate ERK 1/2 and p38 MAPK pathways in erythroid cells and lymphoid cells (Witt et al., 2002) (Xie et al., 2010), and lead to increased transcription of genes such as p21, γ -globin, and myostatin (Zhong et al., 2003) (Sangerman et al., 2006) (Rivero and Adunyah, 1996) (Han et al., 2010) (Simboeck et al., 2010). Arguably, in some instances HDAC inhibitors have been shown to inhibit MAPK activity; possibly by increased activities of MAPK phosphatase due to acetylation (Cao et al., 2008). Therefore, it appears that the net outcome of HDAC inhibition on MAPK depends on whether the MAPK kinase or MAPK phosphatase activities are affected. Nonetheless, p38 MAPK and ERK 1/2 activities were important for TSA or NaB mediated MOR transcription, because inhibition of either MAPK blunted MOR levels (Fig. 2A and Fig. 2B). Further, HDAC inhibitor induced MOR expression was potentiated in the presence of JNK inhibitor (SP) (Fig. 2A and Fig. 2B). Since, MOR gene expression upon JNK inhibition, protein synthesis inhibition (cycloheximide, puromycin), or neuronal differentiation of P19 cells (see below) have already been shown to depend on p38 MAPK activities (Wagley et al., 2013) (Kim do et al., 2011); and that p38 MAPK beta siRNA transfection reduced MOR expression induced with all these stimuli (Fig. 4C, Fig. 4D, and Fig. 7C), p38 MAPK beta appears to be essential for MOR gene regulation.

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The regulation of MOR gene expression upon HDAC inhibition or neuronal differentiation of P19 was found to be a conserved mechanism because MOR gene expression occurred concurrently with increases in MSK1 expression and activation (Fig. 3 and Fig. 7A). Although both p38 MAPK and ERK1/2 are known to effectively activate MSK1; different stimuli have been shown to require at least one or a combination of both (p38 and ERK1/2) MAPK modules to efficiently activate MSK1 (McCoy et al., 2005). As mentioned above, and as shown in this study, HDAC inhibitors utilized ERK 1/2 as the major upstream kinase (Fig. 4E), whereas p38 MAPK was equally functional during neuronal differentiation of P19 cells (Fig. 7C). Nevertheless, the definitive role of MSK1 in MOR gene expression is supported, because pharmacological inhibition of PKA/MSK1 with H89 or MSK1 siRNA transfection abrogated the increases in MOR expression levels (Fig. 5D and Fig. 7D). Yet, MSK1 activity appeared to play a regulatory role rather than an absolute turn on/off switch because only a modest increase in the MOR mRNA levels were observed after the expression of constitutively active-MSK1 (CA-MSK1) construct (Fig. 5B and Fig. 5C), and that CA-MSK1 failed to efficiently activate the *in-vitro* methylated MOR promoter constructs (Fig. 6). These results indicate that MSK1 requires epigenetic modification at the MOR promoter and probably increases MOR transcription via activation of the transcription factors such as NF- κ B or CREB; each of which contains binding sites in the functional MOR promoter (Wei and Loh, 2011). In support to this hypothesis, we have observed that the levels of MSK1 and its downstream transcription factor NF- κ B p65 both increases in the nucleus of TSA stimulated P19 cells or P19 cells undergoing neuronal differentiation, and pharmacological inhibition of NF- κ B activity using QNZ, reduces the MOR promoter activity in HDAC inhibitor stimulated cells (unpublished observation). The role of RSK 1/2 (another group of MAPK effector kinase) is dubious in all these processes, since p38 MAPK

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pathway (involved in both models of MOR gene transcription presented here) does not activate RSK 1/2 (Healy et al., 2012), whereas the effect of ERK 1/2 was partial and only affected MOR expression levels induced by HDAC inhibitors. Regardless, it is clear that, other intracellular pathways may also play a functional role in MOR expression mechanism because the increase in total MSK1 levels were sustained even after simultaneous inhibition of p38 MAPK and ERK 1/2 pathways (Fig. 4E).

An earlier study from our laboratory has shown that TSA treatment shifts the border of the mononucleosome surrounding the MOR minimal promoter from -345 to -262, which suggests chromatin modification in this region (Hwang et al., 2010). With regards to this observation, the role of MAPK cascade in MOR gene expression involving the MOR minimal promoter region involves two steps, both chromatin modifications and transcription factor activation. Quite obviously, although the data presented and discussed in this study supports MAPK/MSK1 dependent transcription factor activation; further studies on chromatin remodeling are also warranted because increasing evidence suggests MAPK activation regulates epigenetic changes in multiple ways (see below) and this was not fully addressed in this study. For example, during smooth muscle cell differentiation, p38 MAPK is directly recruited to the myogenic loci by SWI-SNF chromatin remodeling complexes; and forced activation of p38 MAPK in myoblast by expression of a constitutively active MKK6 promotes unscheduled SWI-SNF recruitment to the myogenin promoter (Simone et al., 2004). In recent years, MSK-mediated histone phosphorylation is regarded to be the critical event that directs extracellular cues to nucleosomal responses, and transcriptional activation (Healy et al., 2012). According to this model, MSK1-mediated histone H3 phosphorylation allows recruitment of the 14-3-3 proteins and Brg1, component of the ATP-dependent chromatin remodeling SWI-SNF complex, to the promoter of the target genes by transcription factors

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such as Elk-1 or NF- κ B (Drobic et al., 2010) (Gehani et al., 2010). Indeed, Brg1 has been shown to be recruited to the MOR promoter (Hwang et al., 2010), and that NF- κ B activity directly affects MOR expression during the neuronal differentiation of P19 cells (Wagley et al., 2013), it would be interesting to determine how MAPK/MSK1 activity links the chromatin remodeling events with transcriptional initiation at the MOR promoter.

In recent years, accumulating evidences has pointed out that opioid agonists can stabilize the same receptor in multiple active conformations to propagate differential intracellular signals (ERK 1/2, JNK, protein kinase A or C pathways) that determines the behavioral plasticity associated with these drugs (Al-Hasani and Bruchas, 2011) (Hofford et al., 2009) (Melief et al., 2010). Quite interestingly, HDAC inhibitors also modulate behavioral outcomes of opioid drugs such as acute morphine induced hyperactivity, opioid dependence, the development of behavioral sensitization and the precipitation of morphine withdrawal syndrome *in vivo* (Jing et al., 2011) (Sanchis-Segura et al., 2009) (Rehni et al., 2012). In addition, emerging studies suggest that HDAC inhibitors facilitate the extinction of rewarding memory of drug taking (Wang et al., 2012), and drug induced conditioned place preference (Wang et al., 2010). In these contexts, it is valuable to understand that the same range of intracellular signaling cascades elicited by opioid drugs to modulate behavioral outcomes can also be modulated by HDAC inhibitors (which are increasingly used in clinical trials for the treatment of various disorders) to regulate MOR expression levels (Fig. 8), which suggest that the intracellular signaling cascades might play an auto-regulatory role in pharmacological outcomes of opioid drugs.

In conclusion, we demonstrate that HDAC inhibitor stimulation or retinoic acid mediated neuronal differentiation of P19 cells activate co-ordinated intracellular signals that converge at the MOR promoter via increased expression and MAPK dependent function of

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MSK1 proteins to regulate MOR transcription (Fig. 8).

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Authorship contributions

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Wrote or contributed to the writing of the manuscript: Wagley, Law, Wei, and Loh

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References

- Al-Hasani R and Bruchas MR (2011) Molecular mechanisms of opioid receptor-dependent signaling and behavior. *Anesthesiology* **115**(6):1363-1381.
- Arthur JS (2008) MSK activation and physiological roles. *Front Biosci* **13**:5866-5879.
- Cao W, Bao C, Padalko E and Lowenstein CJ (2008) Acetylation of mitogen-activated protein kinase phosphatase-1 inhibits Toll-like receptor signaling. *J Exp Med* **205**(6):1491-1503.
- Chueh AC, Tse JW, Togel L and Mariadason JM (2014) Mechanisms of Histone Deacetylase Inhibitor-Regulated Gene Expression in Cancer Cells. *Antioxid Redox Signal*.
- Coulthard LR, White DE, Jones DL, McDermott MF and Burchill SA (2009) p38(MAPK): stress responses from molecular mechanisms to therapeutics. *Trends Mol Med* **15**(8):369-379.
- Cuenda A and Rousseau S (2007) p38 MAP-kinases pathway regulation, function and role in human diseases. *Biochim Biophys Acta* **1773**(8):1358-1375.
- Delcuve GP, Khan DH and Davie JR (2012) Roles of histone deacetylases in epigenetic regulation: emerging paradigms from studies with inhibitors. *Clin Epigenetics* **4**(1):5.
- Dokmanovic M, Clarke C and Marks PA (2007) Histone deacetylase inhibitors: overview and perspectives. *Mol Cancer Res* **5**(10):981-989.
- Drobic B, Perez-Cadahia B, Yu J, Kung SK and Davie JR (2010) Promoter chromatin remodeling of immediate-early genes is mediated through H3 phosphorylation at either serine 28 or 10 by the MSK1 multi-protein complex. *Nucleic Acids Res* **38**(10):3196-3208.
- Gehani SS, Agrawal-Singh S, Dietrich N, Christophersen NS, Helin K and Hansen K (2010) Polycomb group protein displacement and gene activation through MSK-dependent

MOL #106567

H3K27me3S28 phosphorylation. *Mol Cell* **39**(6):886-900.

Glaser KB, Staver MJ, Waring JF, Stender J, Ulrich RG and Davidsen SK (2003) Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: defining a common gene set produced by HDAC inhibition in T24 and MDA carcinoma cell lines. *Mol Cancer Ther* **2**(2):151-163.

Han DS, Huang HP, Wang TG, Hung MY, Ke JY, Chang KT, Chang HY, Ho YP, Hsieh WY and Yang WS (2010) Transcription activation of myostatin by trichostatin A in differentiated C2C12 myocytes via ASK1-MKK3/4/6-JNK and p38 mitogen-activated protein kinase pathways. *J Cell Biochem* **111**(3):564-573.

Healy S, Khan P, He S and Davie JR (2012) Histone H3 phosphorylation, immediate-early gene expression, and the nucleosomal response: a historical perspective. *Biochem Cell Biol* **90**(1):39-54.

Hofford RS, Hodgson SR, Roberts KW, Bryant CD, Evans CJ and Eitan S (2009) Extracellular signal-regulated kinase activation in the amygdala mediates elevated plus maze behavior during opioid withdrawal. *Behav Pharmacol* **20**(7):576-583.

Hwang CK, Kim CS, Kim do K, Law PY, Wei LN and Loh HH (2010) Up-regulation of the mu-opioid receptor gene is mediated through chromatin remodeling and transcriptional factors in differentiated neuronal cells. *Mol Pharmacol* **78**(1):58-68.

Hwang CK, Song KY, Kim CS, Choi HS, Guo XH, Law PY, Wei LN and Loh HH (2007) Evidence of endogenous mu opioid receptor regulation by epigenetic control of the promoters. *Mol Cell Biol* **27**(13):4720-4736.

Hwang CK, Song KY, Kim CS, Choi HS, Guo XH, Law PY, Wei LN and Loh HH (2009) Epigenetic programming of mu-opioid receptor gene in mouse brain is regulated by MeCP2 and Brg1 chromatin remodelling factor. *J Cell Mol Med* **13**(9B):3591-3615.

MOL #106567

- Hwang CK, Wu X, Wang G, Kim CS and Loh HH (2003) Mouse mu opioid receptor distal promoter transcriptional regulation by SOX proteins. *J Biol Chem* **278**(6):3742-3750.
- Jing L, Luo J, Zhang M, Qin WJ, Li YL, Liu Q, Wang YT, Lawrence AJ and Liang JH (2011) Effect of the histone deacetylase inhibitors on behavioural sensitization to a single morphine exposure in mice. *Neurosci Lett* **494**(2):169-173.
- Johnstone RW (2002) Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. *Nat Rev Drug Discov* **1**(4):287-299.
- Keum YS, Kim HG, Bode AM, Surh YJ and Dong Z (2013) UVB-induced COX-2 expression requires histone H3 phosphorylation at Ser10 and Ser28. *Oncogene* **32**(4):444-452.
- Kim do K, Hwang CK, Wagley Y, Law PY, Wei LN and Loh HH (2011) p38 mitogen-activated protein kinase and PI3-kinase are involved in up-regulation of mu opioid receptor transcription induced by cycloheximide. *J Neurochem* **116**(6):1077-1087.
- Kraus J (2012) Expression and functions of mu-opioid receptors and cannabinoid receptors type 1 in T lymphocytes. *Ann N Y Acad Sci* **1261**:1-6.
- Lin YC, Flock KE, Cook RJ, Hunkele AJ, Loh HH and Ko JL (2008) Effects of trichostatin A on neuronal mu-opioid receptor gene expression. *Brain Res* **1246**:1-10.
- Loh HH, Liu HC, Cavalli A, Yang W, Chen YF and Wei LN (1998) mu Opioid receptor knockout in mice: effects on ligand-induced analgesia and morphine lethality. *Brain Res Mol Brain Res* **54**(2):321-326.
- McCoy CE, Campbell DG, Deak M, Bloomberg GB and Arthur JS (2005) MSK1 activity is controlled by multiple phosphorylation sites. *Biochem J* **387**(Pt 2):507-517.
- Melief EJ, Miyatake M, Bruchas MR and Chavkin C (2010) Ligand-directed c-Jun N-terminal kinase activation disrupts opioid receptor signaling. *Proc Natl Acad Sci U S A* **107**(25):11608-11613.

MOL #106567

- Monzo HJ, Park TI, Montgomery JM, Faull RL, Dragunow M and Curtis MA (2012) A method for generating high-yield enriched neuronal cultures from P19 embryonal carcinoma cells. *J Neurosci Methods* **204**(1):87-103.
- Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**(9):e45.
- Rehni AK, Singh N, Rachamalla M and Tikoo K (2012) Modulation of histone deacetylase attenuates naloxone-precipitated opioid withdrawal syndrome. *Naunyn Schmiedebergs Arch Pharmacol* **385**(6):605-619.
- Rivero JA and Adunyah SE (1996) Sodium butyrate induces tyrosine phosphorylation and activation of MAP kinase (ERK-1) in human K562 cells. *Biochem Biophys Res Commun* **224**(3):796-801.
- Sanchis-Segura C, Lopez-Atalaya JP and Barco A (2009) Selective boosting of transcriptional and behavioral responses to drugs of abuse by histone deacetylase inhibition. *Neuropsychopharmacology* **34**(13):2642-2654.
- Sangerman J, Lee MS, Yao X, Oteng E, Hsiao CH, Li W, Zein S, Ofori-Acquah SF and Pace BS (2006) Mechanism for fetal hemoglobin induction by histone deacetylase inhibitors involves gamma-globin activation by CREB1 and ATF-2. *Blood* **108**(10):3590-3599.
- Simboeck E, Sawicka A, Zupkovitz G, Senese S, Winter S, Dequiedt F, Ogris E, Di Croce L, Chiocca S and Seiser C (2010) A phosphorylation switch regulates the transcriptional activation of cell cycle regulator p21 by histone deacetylase inhibitors. *J Biol Chem* **285**(52):41062-41073.
- Simone C, Forcales SV, Hill DA, Imbalzano AN, Latella L and Puri PL (2004) p38 pathway targets SWI-SNF chromatin-remodeling complex to muscle-specific loci. *Nat Genet*

MOL #106567

36(7):738-743.

Sora I, Takahashi N, Funada M, Ujike H, Revay RS, Donovan DM, Miner LL and Uhl GR

(1997) Opiate receptor knockout mice define mu receptor roles in endogenous nociceptive responses and morphine-induced analgesia. *Proc Natl Acad Sci U S A* **94(4):1544-1549.**

Wagley Y, Hwang CK, Lin HY, Kam AF, Law PY, Loh HH and Wei LN (2013) Inhibition of

c-Jun NH2-terminal kinase stimulates mu opioid receptor expression via p38 MAPK-mediated nuclear NF-kappaB activation in neuronal and non-neuronal cells. *Biochim Biophys Acta* **1833(6):1476-1488.**

Wang R, Zhang Y, Qing H, Liu M and Yang P (2010) The extinction of morphine-induced

conditioned place preference by histone deacetylase inhibition. *Neurosci Lett* **483(2):137-142.**

Wang WS, Kang S, Liu WT, Li M, Liu Y, Yu C, Chen J, Chi ZQ, He L and Liu JG (2012)

Extinction of aversive memories associated with morphine withdrawal requires ERK-mediated epigenetic regulation of brain-derived neurotrophic factor transcription in the rat ventromedial prefrontal cortex. *J Neurosci* **32(40):13763-13775.**

Wei LN and Loh HH (2011) Transcriptional and epigenetic regulation of opioid receptor

genes: present and future. *Annu Rev Pharmacol Toxicol* **51:75-97.**

Witt O, Monkemeyer S, Kanbach K and Pekrun A (2002) Induction of fetal hemoglobin

synthesis by valproate: modulation of MAP kinase pathways. *Am J Hematol* **71(1):45-46.**

Xie N, Wang C, Lin Y, Li H, Chen L, Zhang T, Sun Y, Zhang Y, Yin D and Chi Z (2010) The

role of p38 MAPK in valproic acid induced microglia apoptosis. *Neurosci Lett* **482(1):51-56.**

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Zhong S, Goto H, Inagaki M and Dong Z (2003) Phosphorylation at serine 28 and acetylation at lysine 9 of histone H3 induced by trichostatin A. *Oncogene* **22**(34):5291-5297.

Zhong S, Zhang Y, Jansen C, Goto H, Inagaki M and Dong Z (2001) MAP kinases mediate UVB-induced phosphorylation of histone H3 at serine 28. *J Biol Chem* **276**(16):12932-12937.

Zhou C, Qiu L, Sun Y, Healey S, Wanebo H, Kouttab N, Di W, Yan B and Wan Y (2006) Inhibition of EGFR/PI3K/AKT cell survival pathway promotes TSA's effect on cell death and migration in human ovarian cancer cells. *Int J Oncol* **29**(1):269-278.

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Footnotes

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Legends for Figures

Fig. 1. HDAC inhibitors increase MOR transcription in a time-dependent fashion. (A) P19 cells were stimulated with TSA (25 ng/ml) for 0-8 h as indicated, and total RNA was extracted. MOR expression levels were determined from total RNA samples by quantitative real-time RT-PCR (qRT-PCR) analysis and presented as relative expression as described in materials and methods (* $p < 0.05$, ** $p < 0.01$, $n = 4$). (B) Gel image: P19 cells were stimulated with TSA (0- 4 h) as indicated, and acid-soluble protein fractions were prepared. A representative immunoblot that shows changes in the levels of acetylated histone H3 (Acetyl H3) is presented. The levels of total histone H3 were monitored as internal control. (histogram) The pixel densities obtained for acetyl H3 and total H3 were measured for each time-point and presented as relative change compared to control (* $p < 0.05$, ** $p < 0.01$, $n = 3$). (C) P19 cells were treated with NaB (5 mM) for (0-8 h) as indicated and qRT-PCR analysis to determine relative MOR expression was performed as described in (A) (* $p < 0.05$, ** $p < 0.01$, $n = 5$). (D) Gel image: P19 cells were stimulated with NaB (0-4 h) as indicated, and the levels of acetyl H3 and H3 in the acid-soluble protein fractions were determined by immunoblot analysis. (histogram) The pixel densities for acetyl H3 and total H3 were measured for each sample and presented as relative change compared to control (* $p < 0.05$, $n = 5$).

Fig. 2. HDAC inhibition activates p38 MAPK and ERK 1/2 to regulate MOR mRNA expression. (A) P19 cells were pre-treated for 1 h with 25 μ M SB (p38 MAPK inhibitor), 25 μ M SP (JNK inhibitor) or 10 μ M U0 (MEK/ERK inhibitor) and then treated for 8 h with 25 ng/ml TSA. Total RNA was extracted, reverse transcribed, and analyzed by quantitative real-time RT-PCR to determine changes in MOR expression levels. Results were normalized using the levels of β -actin as internal control ($F(4, 15) = 167.8$, ** $p < 0.01$ versus control; $\dagger\dagger p < 0.01$,

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† $p < 0.05$ versus TSA stimulation). (B) P19 cells were pre-treated for 1 h with SB, SP or UO as in (A) and then treated for 8 h with 5 mM NaB. Total RNA was extracted, and changes in MOR expression levels were determined as explained above ($F(4, 14) = 74.07$, ** $p < 0.01$ versus control; †† $p < 0.01$, † $p < 0.05$ versus NaB stimulation). (C) (gel) P19 cells were treated with 25ng/ml TSA for various lengths of time as indicated (0-4 h), and total cell lysates were analyzed by immunoblotting with anti-phospho-p38 MAPK, anti-p38 MAPK alpha, anti-phospho-ERK 1/2, and anti-ERK 1/2 antibodies. (graph) The pixel densities obtained for phospho-p38 MAPK and phospho-ERK 1/2 was normalized against the pixel densities obtained for p38 MAPK and ERK 1/2 respectively. Data from three independent results were combined to present as relative change compared to non-treated control (* $p < 0.05$, ** $p < 0.01$). (D) (gel) P19 cells were treated with 5 mM NaB (0-4 h) as indicated and the levels of phospho-p38 MAPK, p38 MAPK, phospho-ERK 1/2, and ERK 1/2 were determined in the total cell lysates as above. (graph) The pixel densities obtained for phospho-p38 MAPK and phospho-ERK 1/2 were plotted against the pixel densities obtained for p38 MAPK and ERK 1/2 respectively as in (C) (* $p < 0.05$, ** $p < 0.01$).

Fig. 3. HDAC inhibitors increase MSK1 protein levels in P19 cells. (A) (gel) P19 cells were treated with 25 ng/ml TSA or 5 mM NaB for 0-4 h as indicated and total cell lysates were prepared. Immunoblotting was performed to determine the expression levels of phospho-MSK1 and MSK1. Each blot was then reprobbed with anti- β -actin as control. (graph) The pixel density values for phospho-MSK1, MSK1 and β -actin were measured for each time point, and the relative changes in the ratio of phospho-MSK1/MSK1 and MSK1/ β -actin was presented (TSA left graph, and NaB right graph; * $p < 0.05$, ** $p < 0.01$, $n = 4$). (B) P19 cells were pre-treated for 1 h with various concentrations of H89 (0, 5 or 10 μ M) followed by TSA

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(25 ng/ml) or NaB (5 mM) stimulation for 6 h. Total RNA was prepared and analyzed by quantitative real-time RT-PCR to determine changes in MOR expression levels as described previously (* $p < 0.05$, ** $p < 0.01$, $n = 4-8$). (C) (gel) P19 cells were treated with 10 μM VPA for 0-4 h as indicated and total cell lysates were analyzed by immunoblotting with phospho-MSK1, MSK1 and β -actin antibodies. (histogram) P19 cells were pre-treated for 1 h with various concentrations of H89 (0-10 μM) followed by VPA treatment for 6 h. MOR mRNA levels were determined by quantitative real-time RT-PCR as described in Fig. 1. Error bars represents the range of standard errors, and asterisks represent statistically significant findings (* $p < 0.05$; **, $p < 0.01$, $n = 3$).

Fig. 4. HDAC inhibition mediated MAPK activity does not affect MSK1 expression. (A) (gel) P19 cells were transfected with control siRNA, p38 MAPK alpha siRNA or p38 MAPK beta siRNA for 36 h. At the end of transfection, cells were left untreated or treated with TSA (25 ng/ml) for 4 h, and changes in the expression levels of phospho-MSK1 and MSK1 were analyzed by immunoblotting. The levels of p38 MAPK alpha and p38 MAPK beta were analyzed to show specificity of the siRNA transfection, and β -actin levels were analyzed as internal control. (histogram) The pixel densities obtained for MSK1 was normalized against the pixel densities obtained for β -actin values and presented as relative change compared to control (** $p < 0.01$, $n = 4$). (B) P19 cells transfected with control siRNA or siRNA specific for p38 MAPK alpha and beta isoforms for 36 h were treated with NaB (5 mM) for a further 4 h, and immunoblot analysis (gel) and MSK1 level quantification (histogram) were performed as described above (** $p < 0.01$, $n = 4$). (C) Total RNA extracted after 6 h stimulation with TSA (25 ng/ml), NaB (5 mM) or VPA (10 μM) in P19 cells transfected as above were analyzed by quantitative real-time RT-PCR to determine changes in MOR mRNA

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levels. Results were normalized against the levels of β -actin and presented as relative change compared to control (** $p < 0.01$, $n = 7-10$). (D) (histogram) Quantitative real-time RT-PCR analysis to determine changes in MOR mRNA levels were performed as above from cells transfected as in (B) but stimulated with cycloheximide (CHX, 10 $\mu\text{g/ml}$) or SP (25 μM) (** $p < 0.01$, $n = 3$). (gel) Immunoblot analysis to detect changes in p38 MAPK alpha, p38 MAPK beta, p42/p44 MAPK. SAPK/JNK, AKT and β -actin in the siRNA transfected cells used for quantitative real-time RT-PCR is shown. (E) (gel) P19 cells were pre-treated for 1 h with 10 μM U0 (lanes 5-8) or 10 μM U0 plus 25 μM SB (lanes 9-12), and then treated for 4 h with TSA (25 ng/ml), NaB (5 mM) or VPA (10 μM). Total cell lysates were prepared and analyzed by immunoblotting to determine changes in the levels of phospho-MSK1, and MSK1. The same blots were reprobed with phospho-ERK 1/2 to show specificity of U0 treatment and β -actin levels were analyzed as internal control. (histogram) The pixel densities obtained for phospho-MSK1 were measured and plotted against the values obtained for β -actin, which was used as internal control. Error bars represents the range of standard errors from three independent experiments.

Fig. 5. MSK1 regulates MOR gene expression induced by HDAC inhibitors. (A) (histogram) P19 cells were co-transfected with the MOR promoter construct (pGL 450, 300 ng) and expression plasmids for FLAG-tagged constitutively active-MSK1 (CA-MSK1) (1 μg) for 48 h followed by stimulation with TSA, NaB or VPA for a further 8 h. Luciferase activities in the cell lysates were measured and expressed as relative activity compared to control. (gel) A representative immunoblot analysis to demonstrate the expression of CA-MSK1 in the samples used for luciferase assay is presented (** $p < 0.01$, $n = 4-6$). (B) (gel) Immunoblot analysis to detect MSK1 expression was performed with total cell lysates prepared from P19

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cells transfected with increasing amounts of CA-MSK1 as indicated (0-2.0 μg) for 48 h. The levels of β -actin were monitored as internal control. (histogram) Quantitative real-time RT-PCR analyses were performed to determine changes in MOR mRNA levels from cells transfected as above (* $p < 0.05$, ** $p < 0.01$, $n = 5$). (C) P19 cells were transfected with 1 μg CA-MSK1 for 48 h and further stimulated with TSA for 8 h. Total RNA was extracted and quantitative real-time RT-PCR was performed to determine changes in MOR mRNA expression levels as described previously (** $p < 0.01$, $n = 6$). (D) P19 cells were transfected with control siRNA, MSK1 siRNA or MSK2 siRNA for 36 h. At the end of transfection, cells were treated with TSA (25 ng/ml), NaB (5 mM) or VPA (10 μM) for a further 8 h and total RNA was analyzed by quantitative real-time RT-PCR to determine MOR mRNA levels. Results were normalized against the levels of β -actin as internal control, and presented as relative change compared to control (** $p < 0.01$, $n = 3-6$). (E) (gel) Immunoblot analysis to detect changes in MSK1, MSK2 and β -actin in the siRNA transfected cells used for quantitative real-time RT-PCR in (D) is shown. (histogram) The pixel densities obtained for MSK1 and MSK2 were calculated, normalized against the values obtained for β -actin and plotted as relative expression compared to control. Error bars represents the range of standard errors, and asterisks represent statistically significant findings (** $p < 0.01$, $n = 3-4$).

Fig. 6. CA-MSK1 fails to activate MOR promoter upon CpG methylation. (histogram) MOR promoter construct pGL450 was mock methylated or *SssI* methylated and co-transfected into P19 cells with 1 μg FLAG-tagged CA-MSK1 for 48 h. Luciferase activities were measured in the cell lysates and normalized against pCH110 β -galactosidase activity to calculate relative activity compared to control. The data represents the mean \pm S.E.M of three independent experiments with at least two different plasmid preparations (*, $p < 0.05$; **, $p < 0.01$). (gel) A

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representative immunoblot for CA-MSK1 expression in the cell lysates used for one of the luciferase assays is shown. The levels of β -actin were monitored as internal control.

Fig. 7. MSK1 level increases and regulates MOR expression in differentiating neurons. (A) (histogram) P19 cells were induced to differentiate in presence of 0.5 μ M retinoic acid for 4 days in bacteriological petri dishes. Cell aggregates were treated with trypsin-EDTA to get homogenous cell suspension and replated onto poly-l-ornithine coated tissue culture plates. Total RNA was extracted each day for 5 days after plating, and quantitative real-time RT-PCR were performed to determine relative MOR mRNA expression levels compared to day 1 (** $p < 0.05$, $n = 3$). (gel) Total cell lysates were collected each day for 5 days from P19 cells undergoing neuronal differentiation as above. Immunoblotting was performed with anti-phospho-MSK1, and anti-MSK1 as indicated. β -III tubulin (a neuron specific protein) immunoblot was included to show correct neuronal differentiation of the P19 cells. The levels of β -actin and GAPDH were analyzed as internal control to show comparable protein loading across the samples. (B) (histogram) Neuronally differentiating P19 cells on day 1 were treated with a range of H89 (0-10 μ M) for two days. RNA was extracted and MOR expression was determined by quantitative real-time RT-PCR as explained previously (** $p < 0.01$, $n = 3$). (gel) Total cell lysates from neuronally differentiating P19 cells treated as above were analyzed by immunoblotting with neuron specific anti- β -III tubulin antibodies. The levels of β -actin were monitored as internal control. (C) (gel) Neuronally differentiating P19 cells on day 1 were transfected with control siRNA, p38 MAPK alpha siRNA or p38 MAPK beta siRNA for a further 2 days. At the end of transfection, total cell lysates were prepared and changes in the levels of p38 MAPK alpha, p38 MAPK beta, phospho-MSK1, MSK1, β -III tubulin and β -actin were determined by immunoblotting. (middle panel

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histogram) The pixel densities of phospho-MSK1 and β -III tubulin were measured from the siRNA transfected cells and normalized against the values for β -actin levels to plot as relative change compared to control (** $p < 0.01$ versus control siRNA, $n = 3$). (lower histogram) The changes in the MOR mRNA expression levels were determined from the siRNA transfected cells as described previously (** $p < 0.01$ versus control siRNA, $n = 3$). (D) Neuronally differentiating P19 cells (day 1) were transfected with MSK1 siRNA for 2 days. MOR mRNA expression (histogram, quantitative real-time RT-PCR) and MSK1 expression levels (gel, immunoblotting) were determined as described previously. Results are representative of three separate experiments and error bars represent the range of standard errors. Asterisks represent statistically significant finding compared to control siRNA (**, $p < 0.01$).

Fig. 8. Schematic representation of events during MOR gene transcription after stimulation of P19 cells with HDAC inhibitors or retinoic acid mediated neuronal differentiation. HDAC inhibitors stimulation or neuronal differentiation of P19 cells leads to a general increase in the expression of MSK1 proteins, which are concurrently activated by upstream ERK 1/2 or p38 MAPK. Upon activation, MSK1 increases the activity of transcription factors that bind to the remodeled MOR proximal promoter to increase transcription of MOR gene.

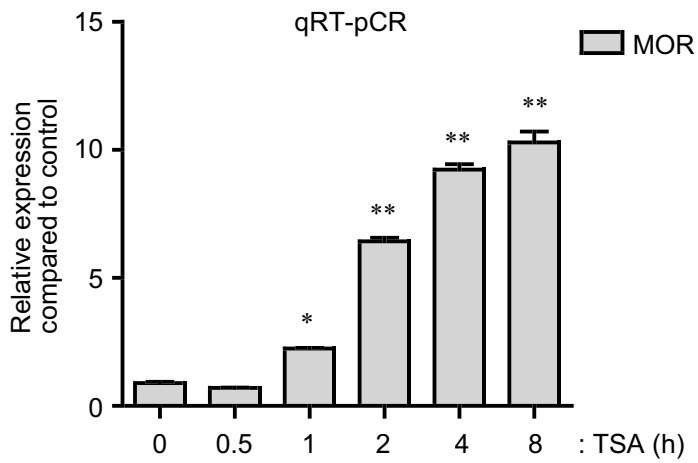
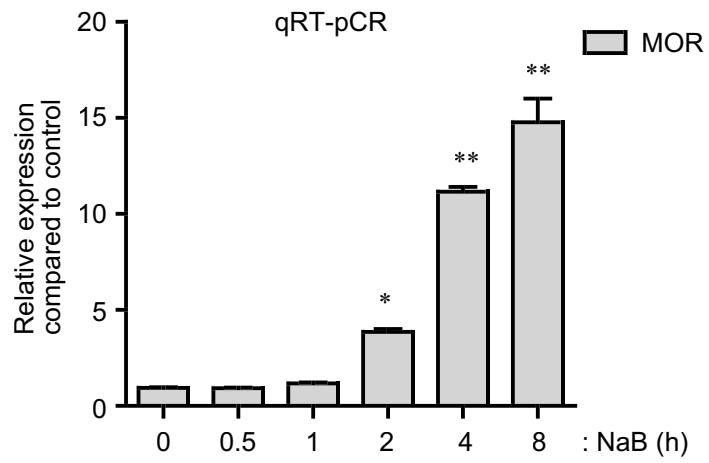
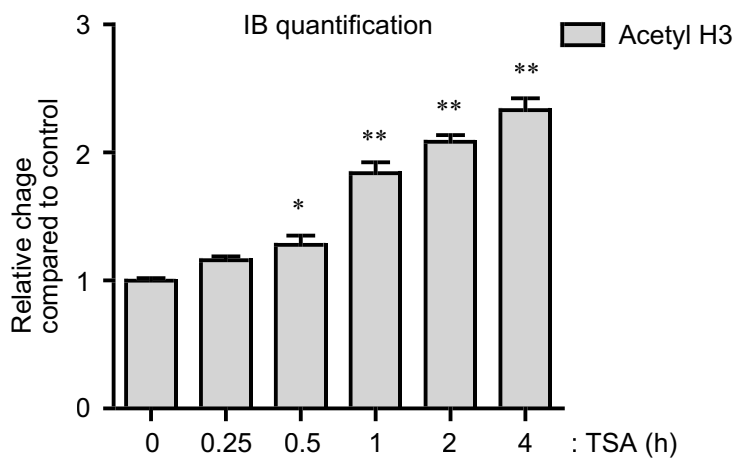
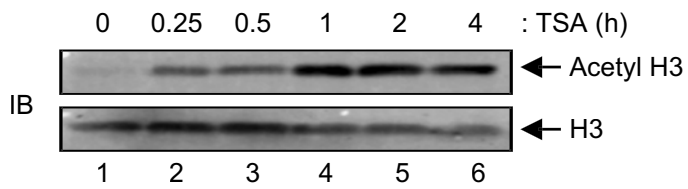
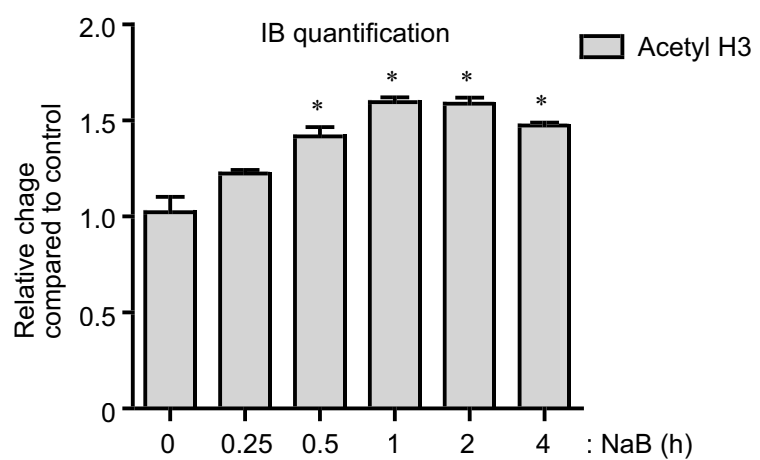
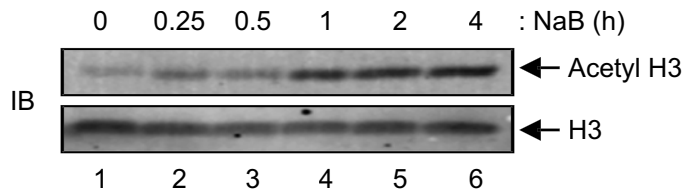
FIG. 1**A****C****B****D**

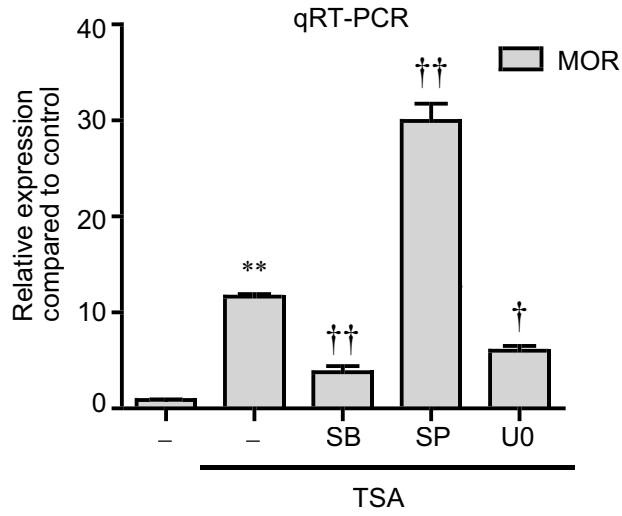
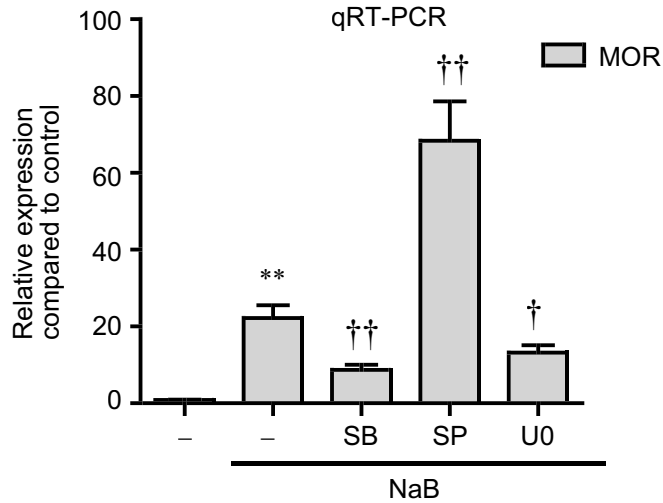
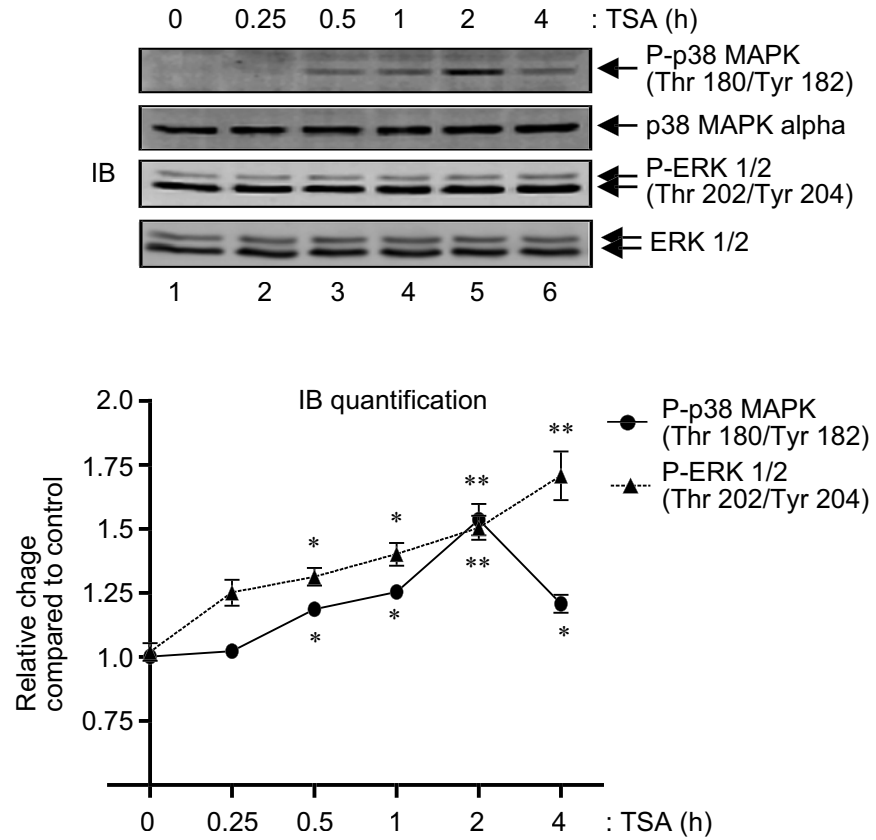
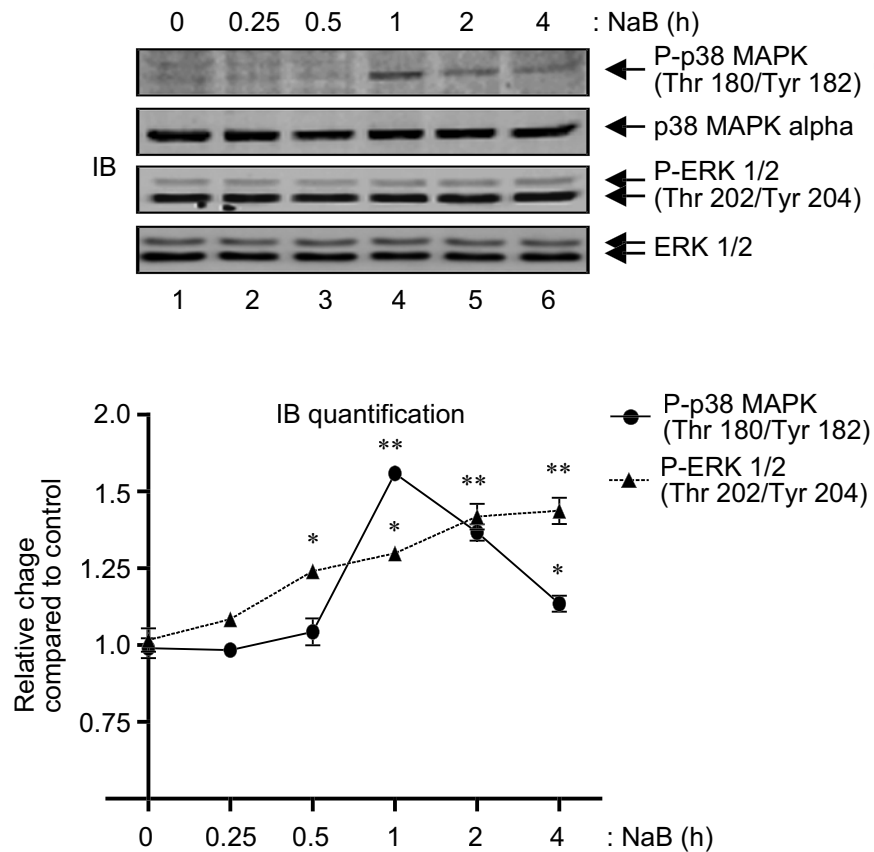
FIG. 2**A****B****C****D**

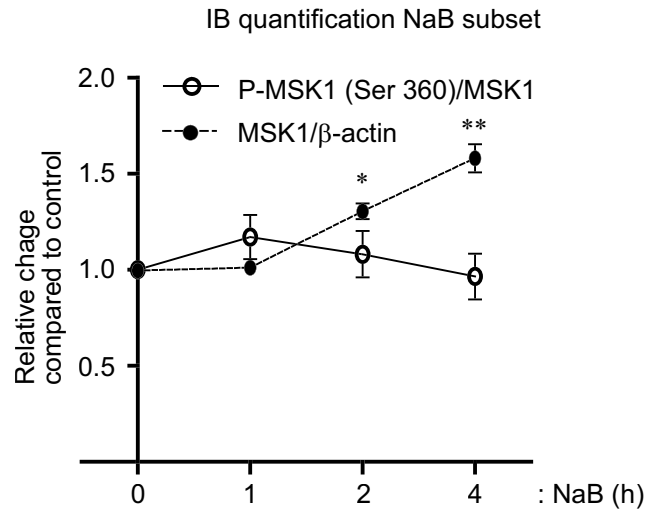
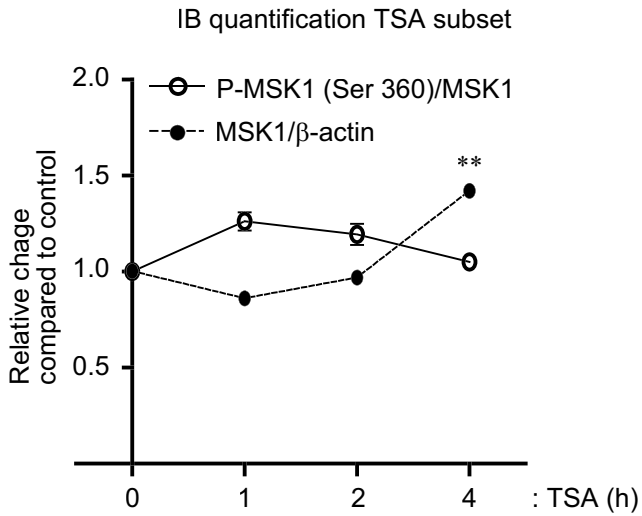
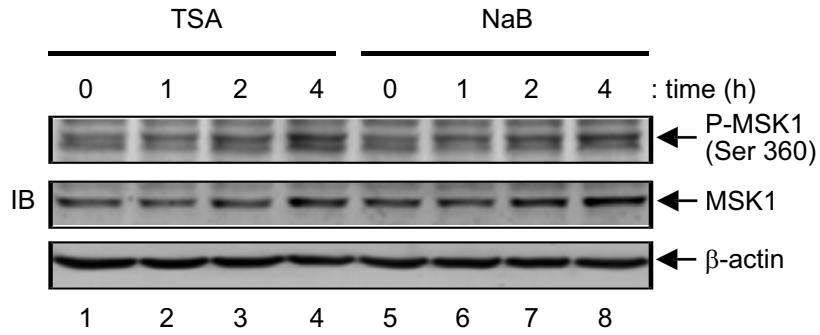
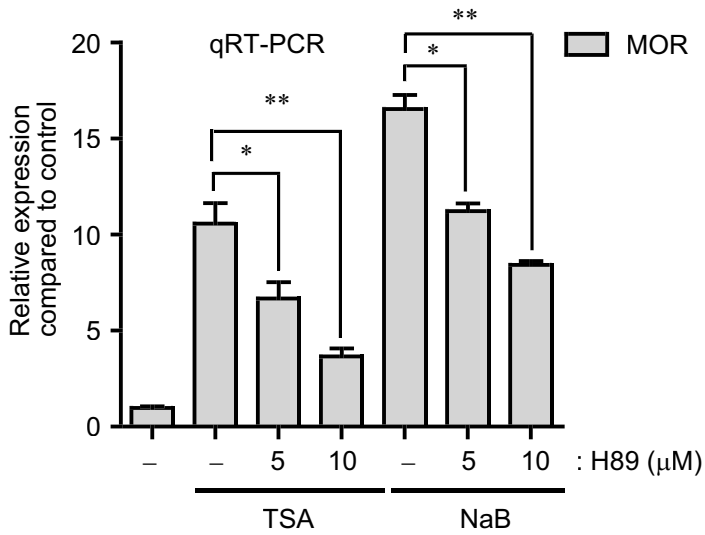
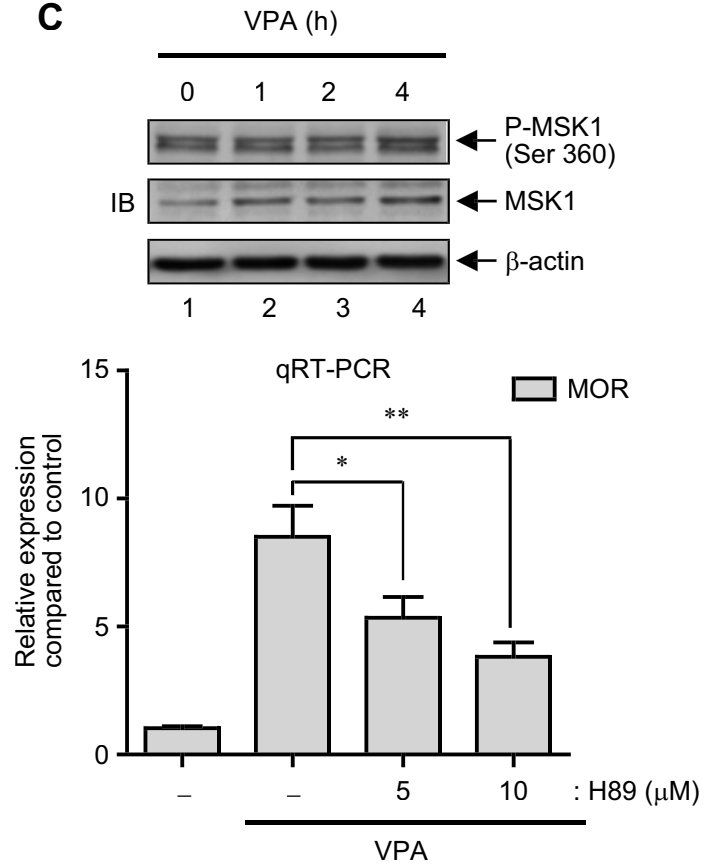
FIG. 3**A****B****C**

FIG. 4

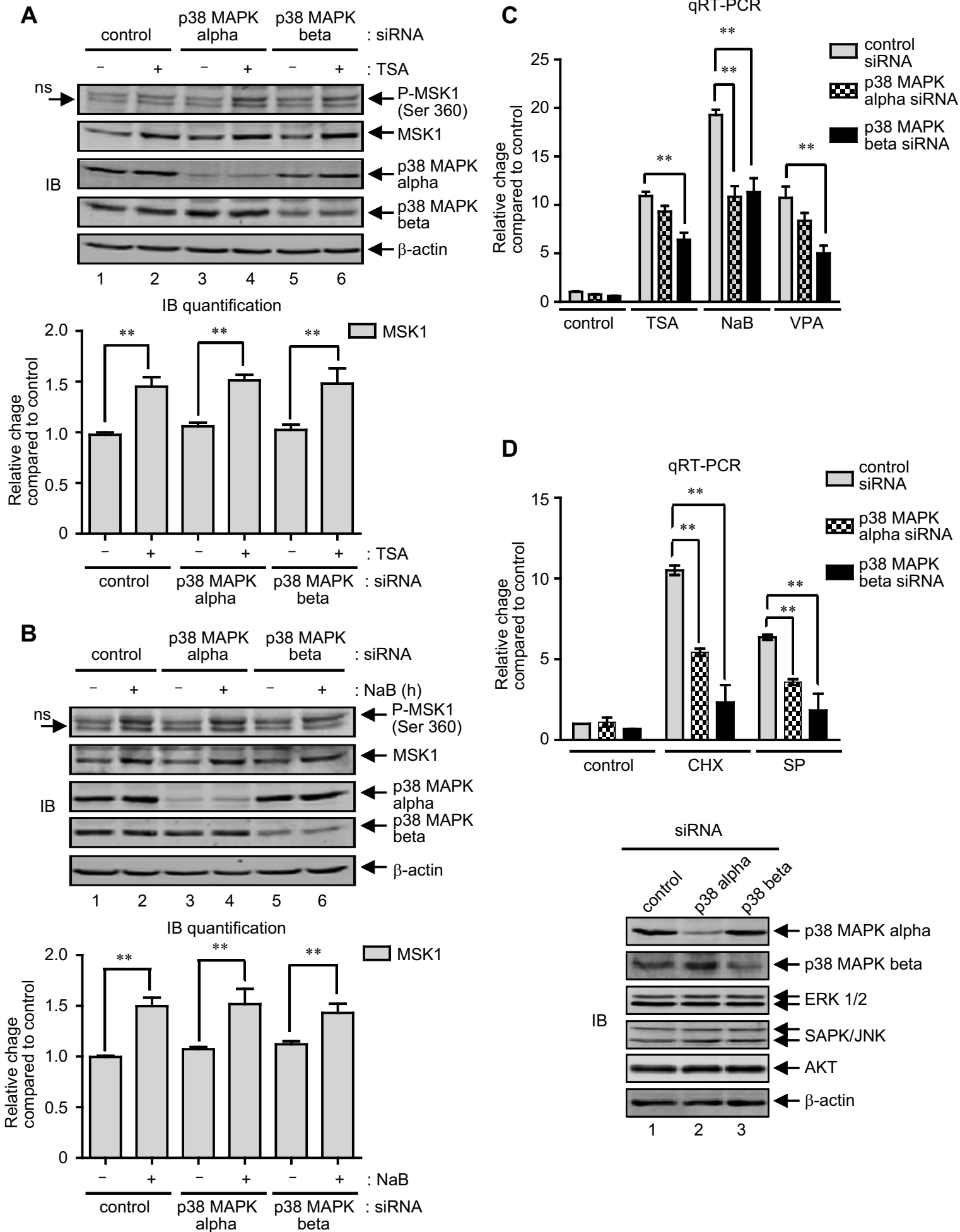


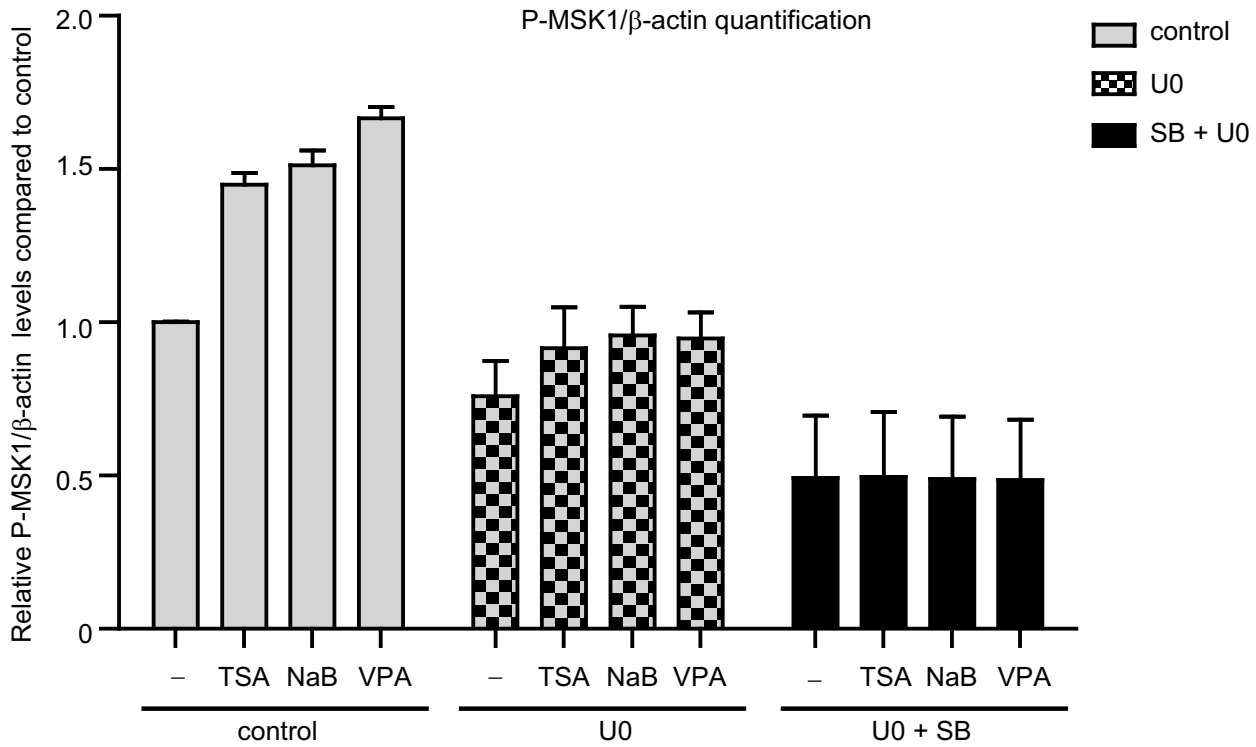
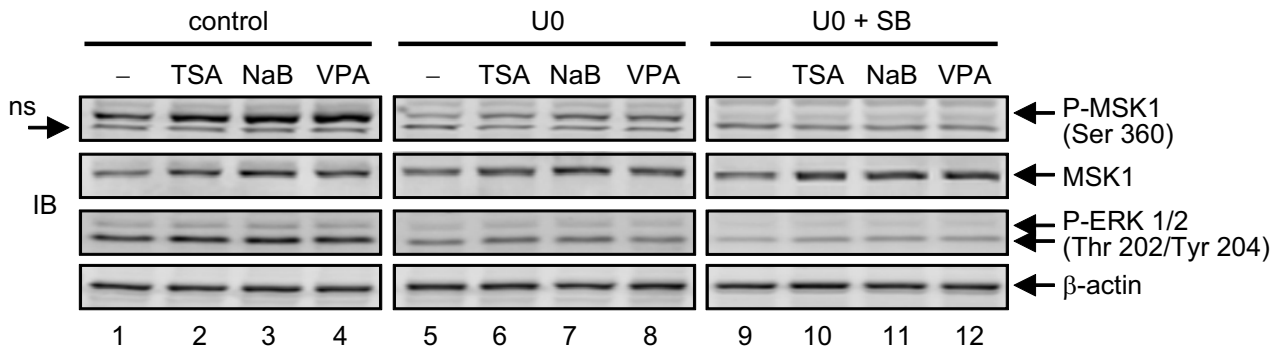
FIG. 4**E**

FIG. 5

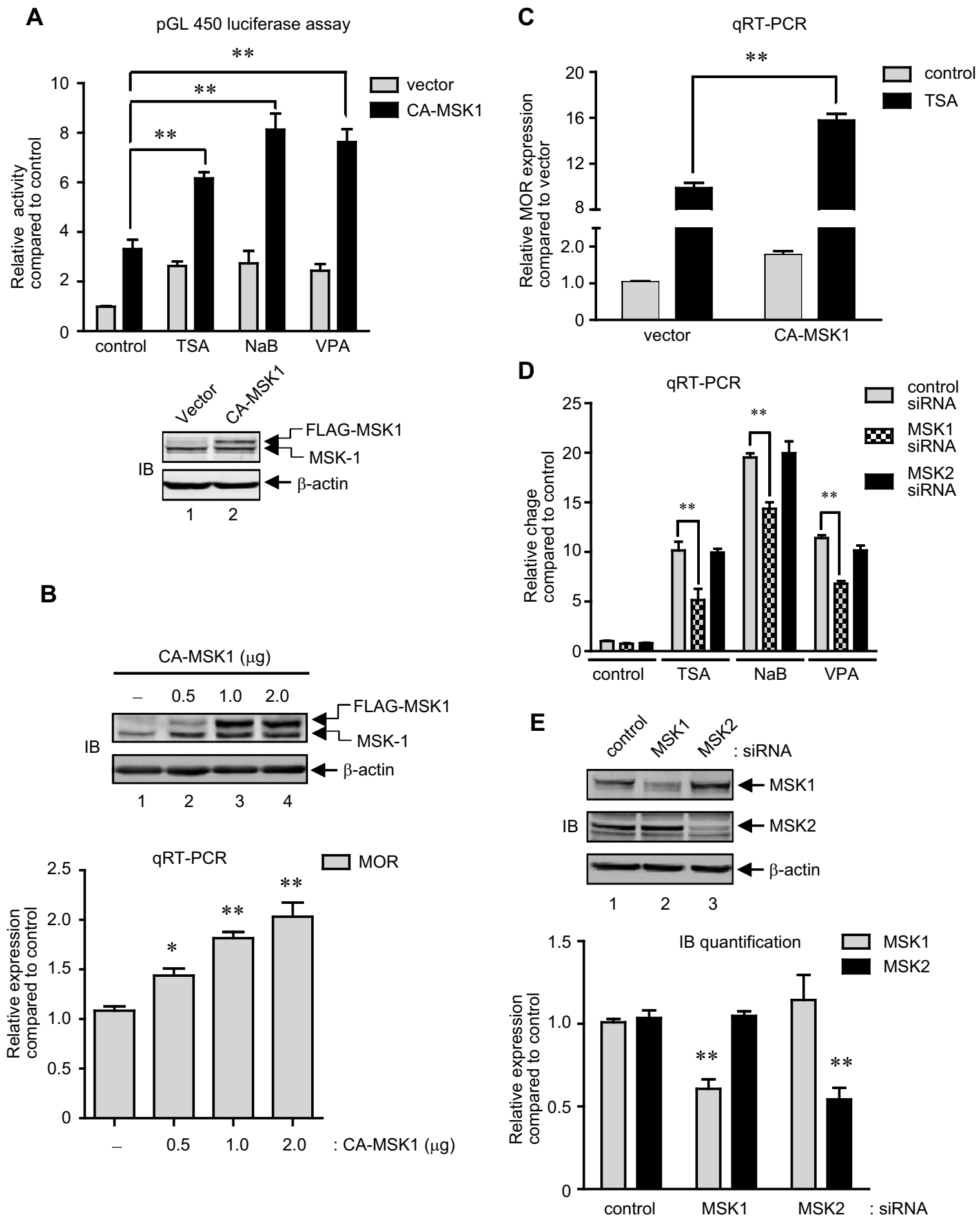


FIG. 6

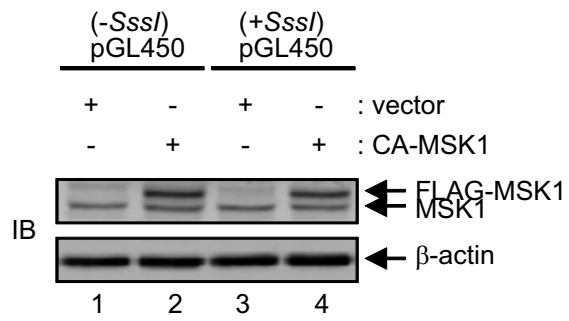
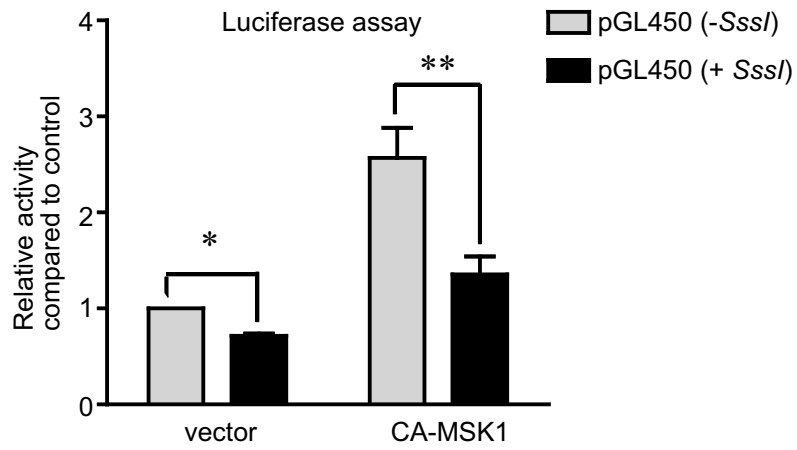


FIG. 7

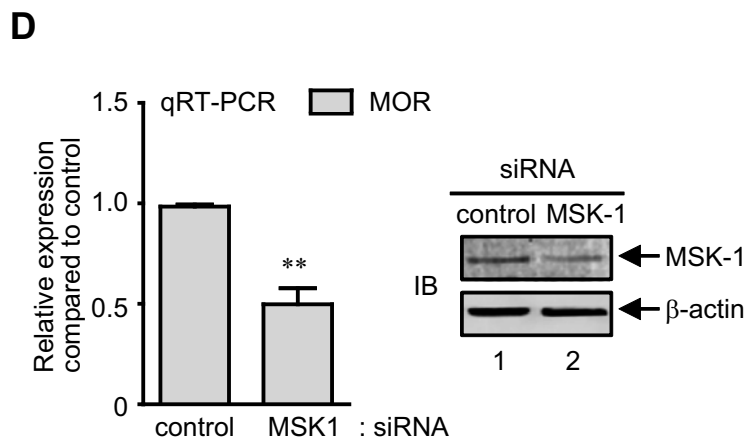
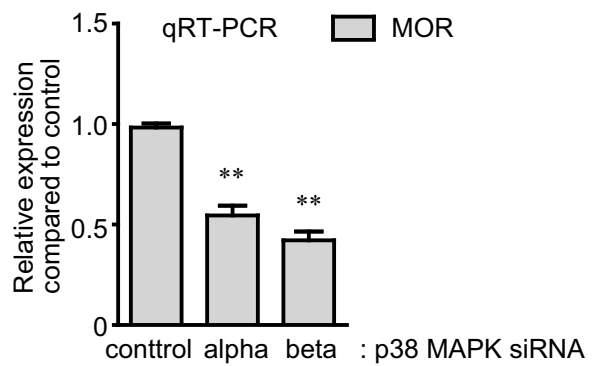
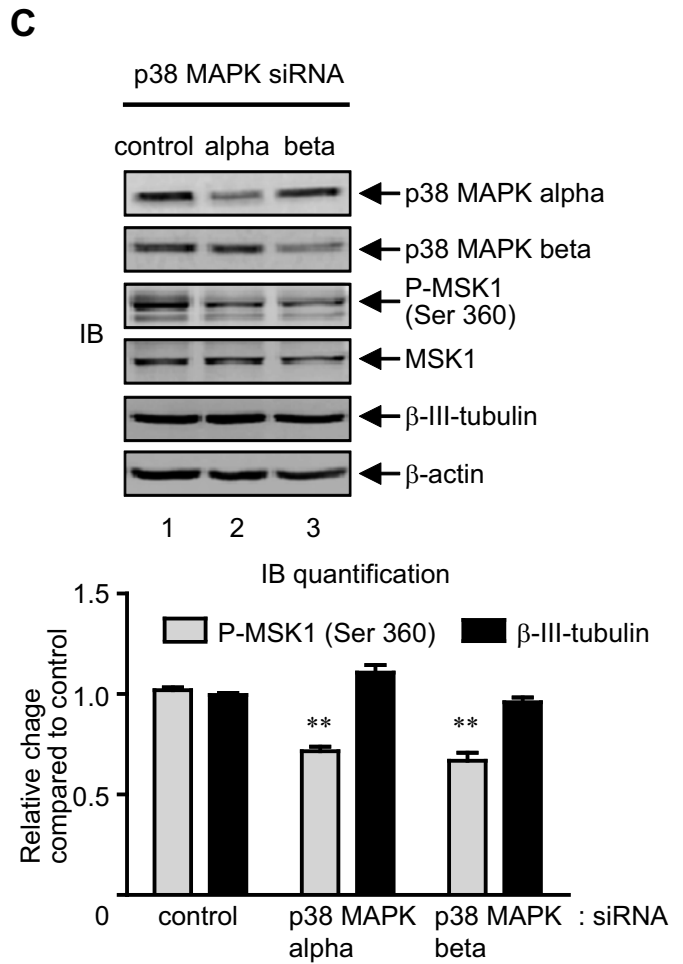
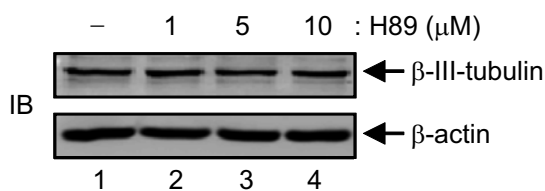
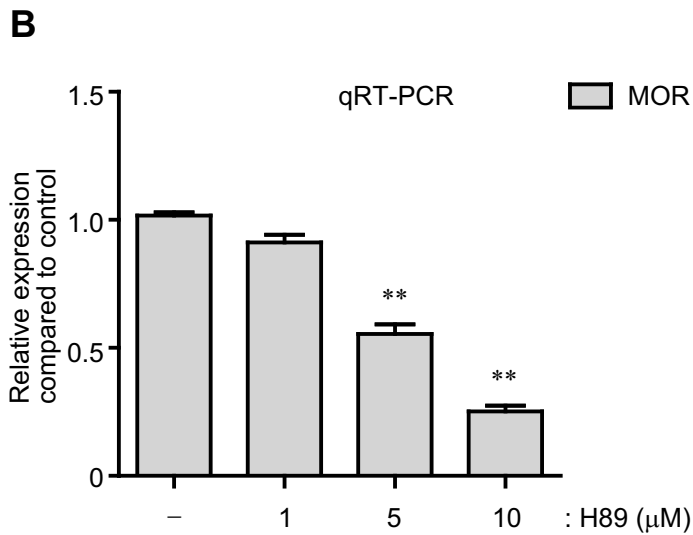
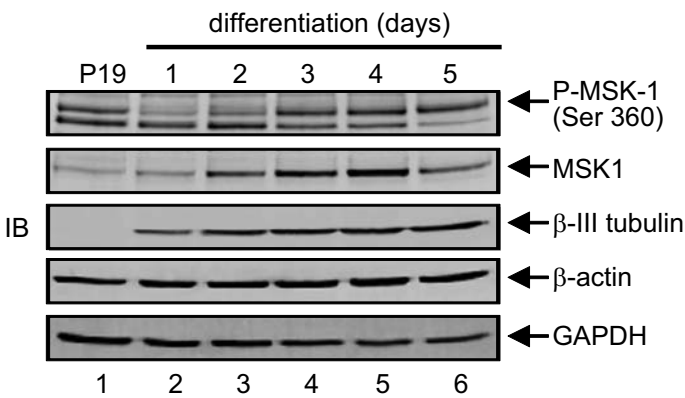
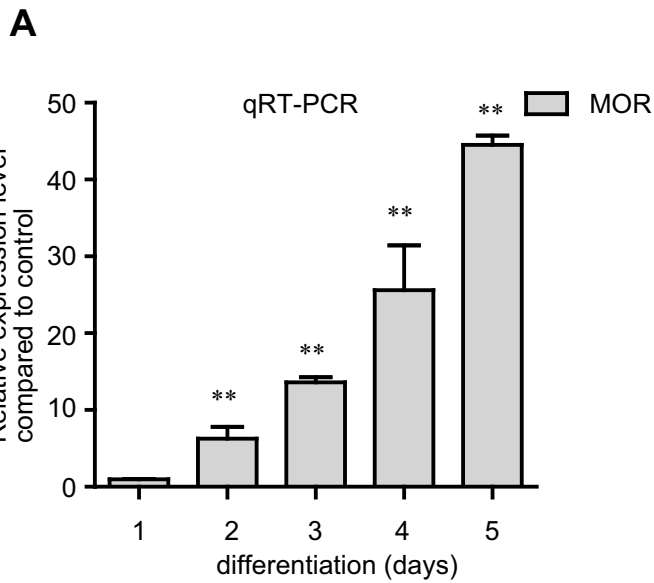


FIG. 8

