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Promoter chromatin remodeling of immediate-early genes is mediated through H3 phosphorylation at either serine 28 or 10 by the MSK1 multi-protein complex

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ABSTRACT

Upon activation of the ERK and p38 MAPK pathways, the MSK1/2-mediated nucleosomal response, including H3 phosphorylation at serine 28 or 10, is coupled with the induction of immediate-early (IE) gene transcription. The outcome of this response, varying with the stimuli and cellular contexts, ranges from neoplastic transformation to neuronal synaptic plasticity. Here, we used sequential co-immunoprecipitation assays and sequential chromatin immunoprecipitation (ChIP) assays on mouse fibroblast 10T1/2 and MSK1 knockdown 10T1/2 cells to show that H3 serine 28 and 10 phosphorylation leads to promoter remodeling. MSK1, in complexes with phosphoserine adaptor 14-3-3 proteins and BRG1 the ATPase subunit of the SWI/SNF remodeler, is recruited to the promoter of target genes by transcription factors such as Elk-1 or NF-κB. Following MSK1-mediated H3 phosphorylation, BRG1 associates with the promoter of target genes via 14-3-3 proteins, which act as scaffolds. The recruited SWI/SNF remodels nucleosomes at the promoter of IE genes enabling the binding of transcription factors like JUN and the onset of transcription.

INTRODUCTION

In mammalian cells, histone H3 phosphorylation at serine 10 or 28 occurs in association with the induction of immediate-early (IE) genes, and is part of the nucleosomal response downstream of the activation of the ERK1/2 or p38 MAPK pathways. Mitogen and stress activated protein kinases 1 and 2 (MSK1 and MSK2) are activated by either MAPK pathway and have been identified as the kinases mediating the nucleosomal response (1). As a downstream target of MAPK signaling pathways, H3 phosphorylation is a response to a vast array of extracellular stimuli including growth factors, stressors such as UV light, alcohol and neurotransmitters (2–5). Increased MSK1 activation resulting in elevated steady-state levels of H3 phosphorylation correlates with tumorigenesis and metastasis in fibroblast and epidermal cells (6–12). In postmitotic neurons, MSK1/2-mediated H3 phosphorylation plays a role in synaptic plasticity and long-term potentiation (5,13–18), as well as phase resetting of the circadian clock (19). More particularly, in the dentate gyrus of the hippocampus, the nucleosomal response is relevant to the physiology of stress-related memory formation (20), while in the striatum, it is linked to the long-term effects of drugs of abuse and physiological reward-controlled learning (21).

The involvement of H3 serine 10 phosphorylation in the induction of IE genes downstream of the MAPK pathways is not only suggested by the temporal parallelism of the two events, but has also been directly demonstrated, in different cell types, by chromatin immunoprecipitation (ChIP) assays (6,13,17,22–26). Much less studied than H3 phosphorylated at serine 10 (H3S10ph), H3S28ph was only recently shown by ChIP assay to associate with the promoter region of the IE *Jun* gene upon its induction, providing direct evidence that this histone post-translational modification is coupled with

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transcriptionally active genes (12). These recent data also imply that H3 phosphorylation events on S10 and S28 are not promoter-specific, as both modifications are associated with the *Jun* promoter region. Yet, sequential immunoprecipitation studies and high-resolution fluorescence microscopy analysis have revealed that H3S10ph and H3S28ph are present in distinct pools in the nucleus (9,27). In view of these results, it appears but remains to be shown that phosphorylation events on S10 or S28 do not happen together on the same promoter.

There is no doubt that MSK-mediated H3 phosphorylation is a crucial intermediate step between signaling at cell-surface receptors and transcriptional reprogramming, and H3 phosphorylation has been suggested to lead to chromatin remodeling, giving transcription factors access to regulatory DNA sequences. Consistent with this hypothesis, chromatin remodeling of the MMTV promoter via SWI/SNF takes place after recruitment of MSK1 and H3 S10 phosphorylation (28). However, the mechanisms involved in the recruitment of the SWI/SNF remodeler have not yet been elucidated. The 14-3-3 proteins, particularly the isoforms ε and ζ, bind to H3S10ph and H3S28ph, with the binding affinity being the highest for H3S28ph independently of its acetylation status, while the binding affinity for H3S10phK14Ac is higher than for H3S10ph (29,30). Importantly, knockdown of 14-3-3ζ prevented the activation of the HDAC1 gene transcription by stimulation of the p38 pathway and HDAC inhibition (31). However, the role of 14-3-3 proteins in the induction of IE genes is currently unknown. Furthermore, the distribution of H3 phosphorylation along induced genes has not been systematically explored, leaving unanswered the question whether H3 phosphorylation plays a role in transcriptional elongation. In this study, we provide evidence that H3S10ph and H3S28ph have functions primarily in promoter remodeling, acting at separate promoters, and we present a model illustrating the role of 14-3-3 in this promoter remodeling.

MATERIALS AND METHODS

Cell culture

Mouse fibroblast 10T1/2 cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 in α-MEM medium supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin G (100 U/ml), streptomycin sulfate (100 μg/ml) and amphotericin B (250 ng/ml). Human embryonic kidney (HEK) 293 cells were grown in DMEM medium supplemented with 10% (v/v) FBS. To induce Ras-MAPK signaling, 90–100% confluent 10T1/2 cells were serum starved for 24 h in α-MEM medium supplemented with 0.5% FBS and treated with 100 nM 12-*O*-tetradecanoyl-phorbol-13-acetate, (TPA) (Sigma) for 15 or 30 min. When required, 10T1/2 cells were pre-treated with 10 μM H89 (Calbiochem) for 30 min or with 100 nM Rp-cAMP (Sigma) for 1 h prior to TPA treatment.

Generation and maintenance of MSK1 stable knockdown mouse fibroblasts

Empty GIPZ lentiviral vector and the GIPZ Lentiviral shRNAmir clones for mouse MSK1 (clone V2LMM_54318- SENSE 2240, Thermo Scientific Open Biosystems, USA) were obtained from the Biomedical Functionality Resource at University of Manitoba. To produce replication-incompetent lentiviral vector particles for mouse fibroblasts transduction, we transfected the GIPZ vector, the packaging vector and the VSV-G env-expressing plasmid into HEK293T cells using the BD CalPhos^{TM} Mammalian Transfection kit, as described previously (32). To generate stable MSK1 knockdown 10T1/2 mouse fibroblasts, we seeded 5×10^4 cells in a 24-well plate 24 h before transduction. Medium was aspirated and replaced with 250 μl of the lentiviral supernatant (at 1×10^6 TU) and 8 μg/ml of Polybrene. After incubation for 2 h at 37°C in 5% CO_2, the culture medium that contained the lentiviral vectors was removed. The transduced cells were cultured in 1 ml of fresh media for 3 days before they were selected for puromycin resistance (at a final concentration of 8 μg/ml). Freshly prepared selective media was added every 2–3 days and the cells were monitored for cell survival by light microscopy, and/or for the TurboGFP expression by flow cytometry using BD FACSCalibur. After 10 days, puromycin-resistant cells were expanded in puromycin-containing media until the desired confluency was reached.

ChIP/reChIP assay

10T1/2 cells were treated with 1% formaldehyde for 10 min at room temperature. After harvesting, the cells were pelleted and resuspended in cell lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM NaF, 1.0 μg/ml leupeptin, 1 μg/ml aprotinin and 25 mM β-glycerophosphate). After 10 min rotation at 4°C the cellular material was spun at $2000 \times g$ for 10 min to obtain the nuclei. The nuclear pellet was resuspended in MNase digestion buffer (10 nM Tris–HCl pH 7.5, 0.25 M sucrose, 75 mM NaCl, plus above indicated phosphatase/protease inhibitors) and A_{260} was measured. In order to obtain ~150 bp DNA fragments, 2.5 U of MNase per A_{260} of nuclear suspension were added in the presence of 3 mM $CaCl_2$ and incubated at 37°C for 20 min. The MNase reaction was stopped by the addition of EDTA pH 8.0 (5 mM final concentration). In order to release nuclear material, the samples were adjusted to 0.5% SDS and rotated for 1 h at room temperature. Insoluble material was pelleted at $2000 \times g$ for 10 min and the soluble material was diluted to 0.1% SDS with radio immuno precipitation assay (RIPA) buffer along with above-mentioned phosphatase/protease inhibitors. Twelve A_{260} of protein G-Sepharose (Pierce) pre-cleared 10T1/2 lysate was incubated with 12 μl of anti-MSK1 (Sigma, M5437) or anti-H3S10ph (Santa Cruz, sc-8656-R), anti-H3S28ph (Milipore, 07–145), anti-H3K9acK14ac (Milipore, 06–599), anti-14-3-3ε (Santa Cruz, sc-1020), anti-14-3-3ζ (Santa Cruz,

sc-1019), anti-BRG1 (Milipore, 07–478), anti-PCAF (AbCam, ab12188-50), anti-JUN (Santa Cruz, sc-1694), or anti-RNAPII S5ph (AbCam, ab5131-50) overnight at 4°C. Magnetic protein G Dynabeads (Invitrogen) were added for 2 h at 4°C. The specificity of the antibodies against H3S10ph and H3S28ph was determined in several ways. In immunoblot experiments, both antibodies only detected H3. Using commercial (Abcam) H3 peptides with S10ph or S28ph, these antibodies only recognized one or the other peptide. With regards to acetylation, we previously reported that these antibodies detected S10ph or S28ph when H3 was hyperacetylated (9). With regards to the MSK1 antibody, in immunoblot experiments, we found that the antibody only detected one band. We have also shown that in MSK1 knockdown experiments, the MSK1 signals detected in immunoblot experiments, were greatly diminished. For reChIP assays, after the elution of first ChIP, the samples were diluted 10 times with dilution buffer (15 mM Tris–HCl pH 8.1, 1% Triton X-100, 1 mM EDTA, 150 mM NaCl) and subjected to the ChIP procedure again. Negative control included performing ChIP/reChIP assays without adding antibody. DNA–protein fragments were processed as previously described (33). Input and ChIP/reChIP DNAs were quantified using PicoGreen assay. Equal amounts of input, ChIP (0.1 ng) or reChIP (0.05 ng) DNA were used to perform SYBR Green real time PCR on iCycler IQ5 (BioRad). The enrichment was calculated as follows: Fold enrichment = $R^{(Ct\ input\ -\ Ct\ ChIP)}$, where R is the rate of amplification. Values for each time point were normalized to time 0 values. Primer sequences were designed using Oligo5 software and are shown in Supplementary Table S1.

Preparation of cell extracts and immunoprecipitations

Cells were harvested and lysed in 400 µl of ice-cold Nonidet P40 (NP-40) buffer (150 mM NaCl, 50 mM Tris–HCl pH 8.0, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM NaF, 1.0 µg/ml leupeptin, 1.0 µg/ml aprotinin, 25 mM β-glycerophosphate). Cell extracts were subjected to centrifugation at 11 950 × *g* for 10 min at 4°C, and the supernatant was saved. Protein concentration of the supernatant was determined using the BCA protein assay (BioRad). For immunoprecipitations in HEK293 cells, 500 µg of total cell extract were incubated with 20 µg of anti-MSK1 antibodies overnight at 4°C. Twenty microliters of protein G-Sepharose beads were added the following day and incubated for 2 h at 4°C. The beads were washed with ice-cold NP-40 buffer two times. Input (20 µg), bound, un-bound and non-specific (no antibody IP) fractions were resolved on SDS–10%-PAGE and immunoblotted with indicated antibodies. In some instances, non-specific antibodies (anti-FLAG) were used in control immunoprecipitations. For immunoprecipitations in cycling 10T1/2 cells, 20 µg of anti-MSK1 antibodies were incubated with 1 mg of total cell extract overnight at 4°C. Further processing of the samples was as described above. For sequential immunoprecipitations, 30 min TPA-treated 10T1/2 cells were treated with membrane-permeable protein cross-linking reagent dimethyl 3.3′-dithiobisproprionamidate-2-HCl (DTBP, 2 mM, Pierce) for 30 min at 37°C. The reaction was stopped with ice-cold 0.1 M Tris–HCl (pH 7.5) and the cells were lysed and incubated with anti-MSK1 antibodies as described above. The immunoprecipitated proteins were eluted with 0.2 M glycine (pH 2.6) for 10 min at room temperature and re-immunoprecipitated with antibodies against BRG1.

RNA isolation and real-time RT–PCR analysis

Total RNA from untreated and treated 10T1/2 cells was isolated using RNeasy Mini Kit (QIAGEN). RNA (10 ng/µl) in 80 µl volume was used for cDNA conversion (Invitrogen). Real-time PCR reactions were performed on iCycler IQ5 (BioRad) using SYBR Green for labeling. Primer sequences are shown in Supplementary Table S1. Fold change was normalized to GAPDH levels in untreated and treated samples.

RESULTS

MSK1 recruitment and H3 phosphoacetylation at the regulatory regions of induced genes in response to ERK-MAPK signaling

Stimulation of quiescent mouse fibroblast 10T1/2 cells with phorbol esters such as tumor promoter 12-O tetradecanoylphorbol-13-acetate (TPA) activates the ERK1/2 MAPK pathway and triggers the downstream MSK1/2-mediated nucleosomal response associated with transcriptional induction (1). Among the induced genes are those coding for cyclooxygenase-2 (COX-2) and members of the AP-1 transcription factor family [*Fos*, *Jun*, FOS-like antigen 1 (*Fosl1*) also known as FOS related antigen 1 (*Fra-1*)] which have roles in tumorigenesis and synaptic signaling (18,34–36). Figure 1A shows the genomic structure and regulatory sites of the murine *Jun*, *Cox-2* and *Fosl1* genes. Following treatment of quiescent 10T1/2 cells with TPA for 0, 15 or 30 min, the distribution of MSK1 along the regulatory and coding regions of these genes was determined, using a high-resolution ChIP assay, in which nuclei isolated from formaldehyde treated cells were digested with micrococcal nuclease such that the chromatin was processed down to mononucleosomes. The regions chosen for analysis are displayed in Figure 1A. Upon TPA induction, MSK1 associated with both upstream regions of the *Jun* gene, the 5′ distal region (−711) containing a putative binding site for the Elk-1 transcription factor and the 5′ proximal region (−146) with binding sites to several transcription factors including JUN (Figure 1A and B). There was no TPA-induced association of MSK1 with the *Jun* coding region (+129, +1115 and +2953). TPA treatment of 10T1/2 cells also resulted in the binding of MSK1 to the 5′ distal (−493) and 5′ proximal (−111) upstream regions of the *Cox-2* gene, but not to the coding region (+903, +1982 and +4255) (Figure 1A and B). For the *Fosl1* gene, TPA treatment induced the occupancy by

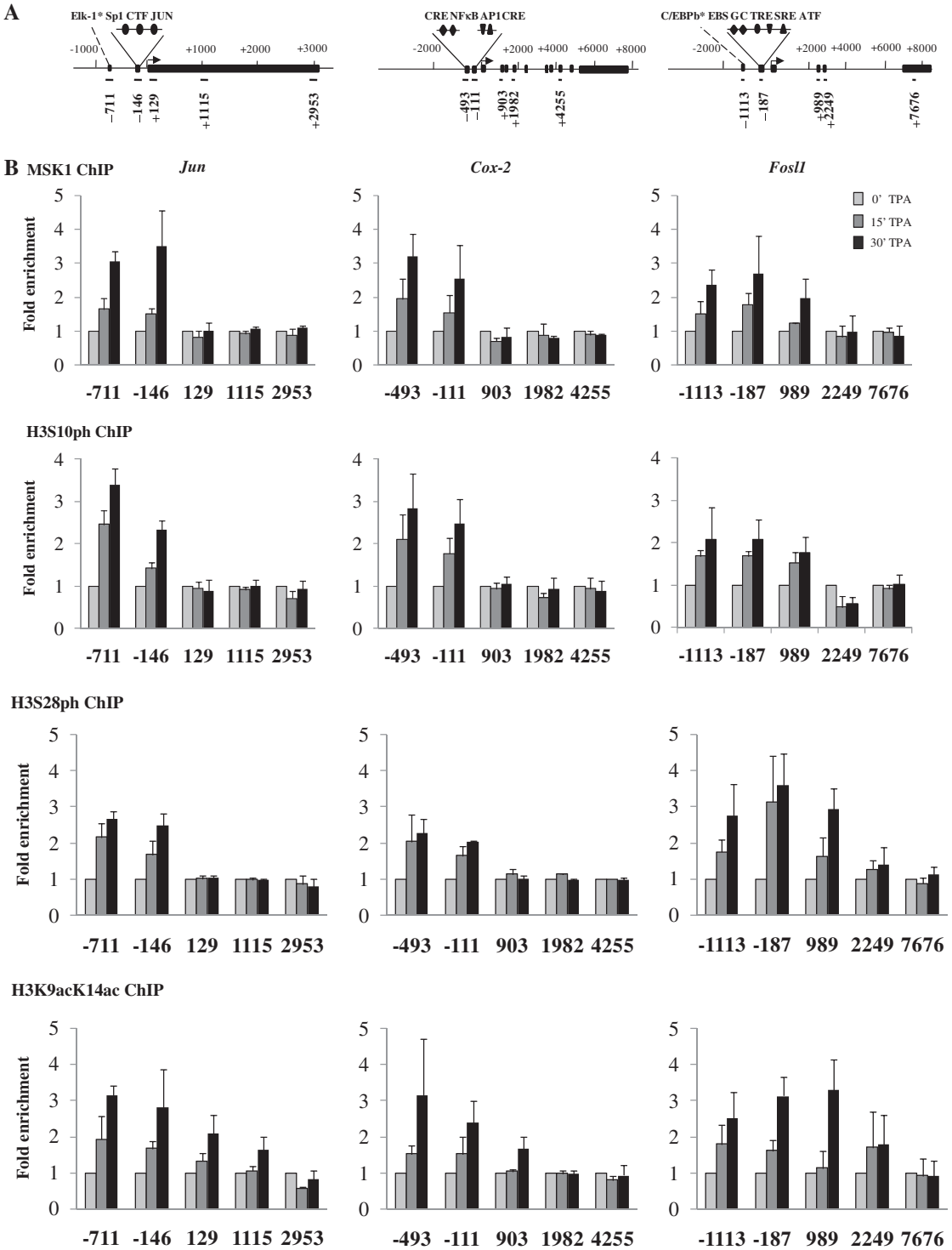

Figure 1. TPA-induced MSK1 recruitment and H3 phosphoacetylation at the *Jun*, *Cox-2* and *Fosl1* regulatory regions. (**A**) Schematic representation of *Jun*, *Cox-2* and *Fosl1* genes showing regions amplified in the ChIP assays. Each region is labeled according to the 5′ position of the forward primer relative to the transcription start site. Exons are represented by boxes, and binding sites of relevant transcription factors located in the amplified regions are displayed. CTF, CCAAT-box-binding protein (also known as NF1, nuclear factor 1); CRE, cyclic-AMP responsive element; C/EBP, CCAAT-enhancer binding protein; EBS, Ets binding site; GC, GC box which is a binding site for the Sp family transcription factors; TRE, TPA-responsive element; SRE, serum-responsive element. AP-1 constitutes a combination of dimers formed of members of the JUN, FOS and ATF families of transcription factors. Asterisk indicates a putative binding site. (**B**) ChIP experiments were performed using antibodies against MSK1, H3S10ph, H3S28ph or H3K9acK14ac, on formaldehyde-crosslinked mononucleosomes prepared from serum-starved 10T1/2 cells treated with TPA for 0, 15 or 30 min. Equal amounts of input and immunoprecipitated DNA were quantified by real-time quantitative PCR. Enrichment values are the mean of three independent experiments, and the error bars represent the standard deviation.

MSK1 of three regions, the 5′ distal region (−1113) containing a putative binding site for the C/EBPb protein, the 5′ proximal region (−187) with multiple responsive elements and the region (+989) located in intron 1 which has an AP-1 site (Figure 1A and B). Thus, in response to ERK1/2 MAPK signaling, MSK1 was recruited to the regulatory regions of responsive genes, likely by transcription factors such as Elk-1, NF-κB and AP-1 (25,37,38).

As H3 is a substrate of MSK1, antibodies against H3S10ph or H3S28ph were used to immunoprecipitate formaldehyde-crosslinked mononucleosomes from quiescent 10T1/2 cells treated with TPA for 0, 15 or 30 min. Figure 1B shows that the positioning of H3S10ph and H3S28ph at the regulatory regions upstream of the genes, and in the first intron in the case of *Fosl1*, mirrors that of MSK1. On the other hand, ChIP experiments with antibodies raised against di-acetylated H3 (H3K9acK14ac) revealed that TPA-induced acetylation of H3 extended from the regulatory regions of the *Jun*, *Cox-2* and *Fosl1* genes into their coding region, although with progressively decreasing levels (Figure 1B).

Recruitment of 14-3-3 proteins and chromatin remodelers/modifiers to regulatory regions of induced genes in response to ERK-MAPK signaling

As 14-3-3 isoforms were reported to be effectors of H3 phosphorylation at inducible genes (29), we performed ChIP assays to determine the distribution of 14-3-3ε or 14-3-3ζ along the *Jun*, *Cox*-2 and *Fosl1* genes before and after their induction with TPA. Figure 2 shows that, upon TPA-stimulation of quiescent 10T1/2 cells, 14-3-3ε and 14-3-3ζ associated with the same regulatory regions of the *Jun*, *Cox*-2 and *Fosl1* genes as did H3S10ph and H3S28ph. BRG1, the ATPase subunit of the SWI/SNF remodeling complex, and PCAF, an H3 K-acetyltransferase (also known as KAT2B) also associated with the same regulatory regions. In the case of the *Cox-2* gene, BRG1 and PCAF, to a lesser extent, associated with the 5′ distal region (−493) upon TPA stimulation, while the TPA-induced association of BRG1 with the 5′ proximal region (−111) was weaker, and TPA-induced binding of PCAF to the −111 region was negligible (Figure 2). Yet, TPA-induced H3 acetylation was evident at both regulatory regions (−493 and −111) of the *Cox-2* gene (Figure 1B).

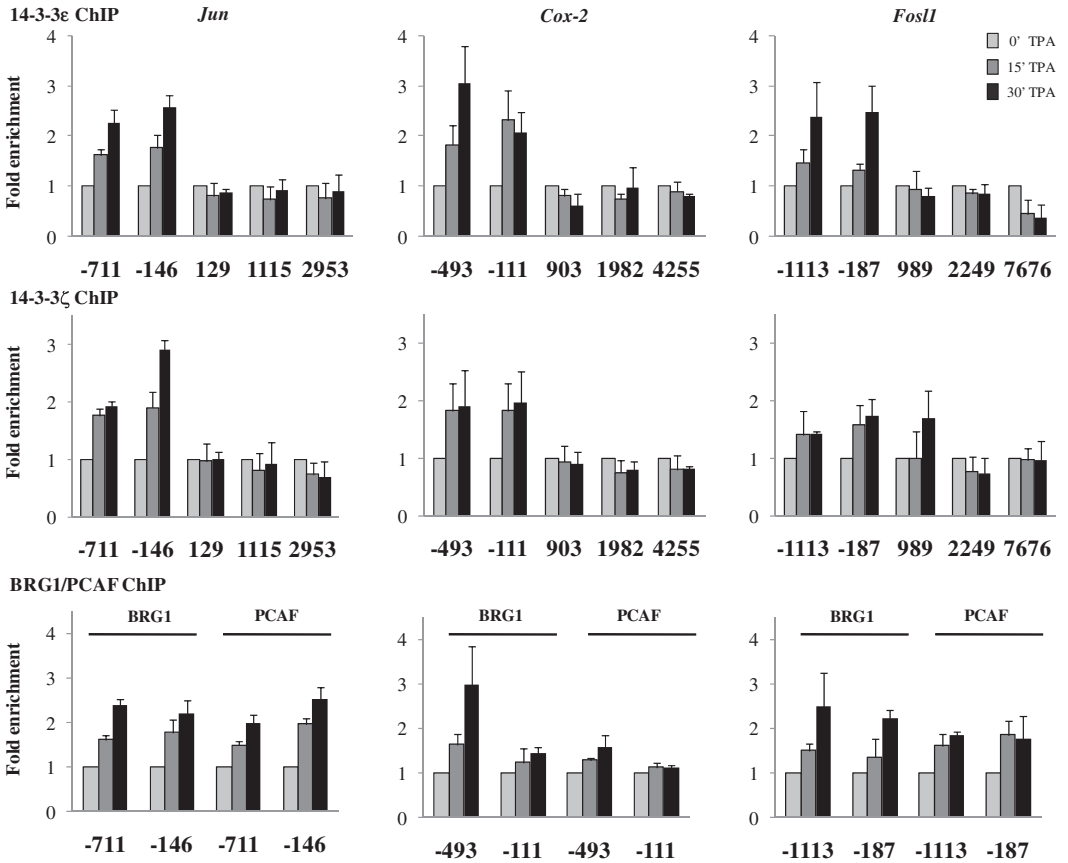

Figure 2. TPA-induced recruitment of 14-3-3 proteins and chromatin remodelers/modifiers to *Jun*, *Cox-2* and *Fosl1* regulatory regions. ChIP experiments were performed using antibodies against 14-3-3ε, 14-3-3ζ, BRG1 or PCAF on formaldehyde-crosslinked mononucleosomes prepared from serum-starved 10T1/2 cells treated with TPA for 0, 15 or 30 min. Enrichment values, obtained by real-time quantitative PCR assays on equal amounts of input and immunoprecipitated DNA, are the mean of three independent experiments, and the error bars represent the standard deviation.

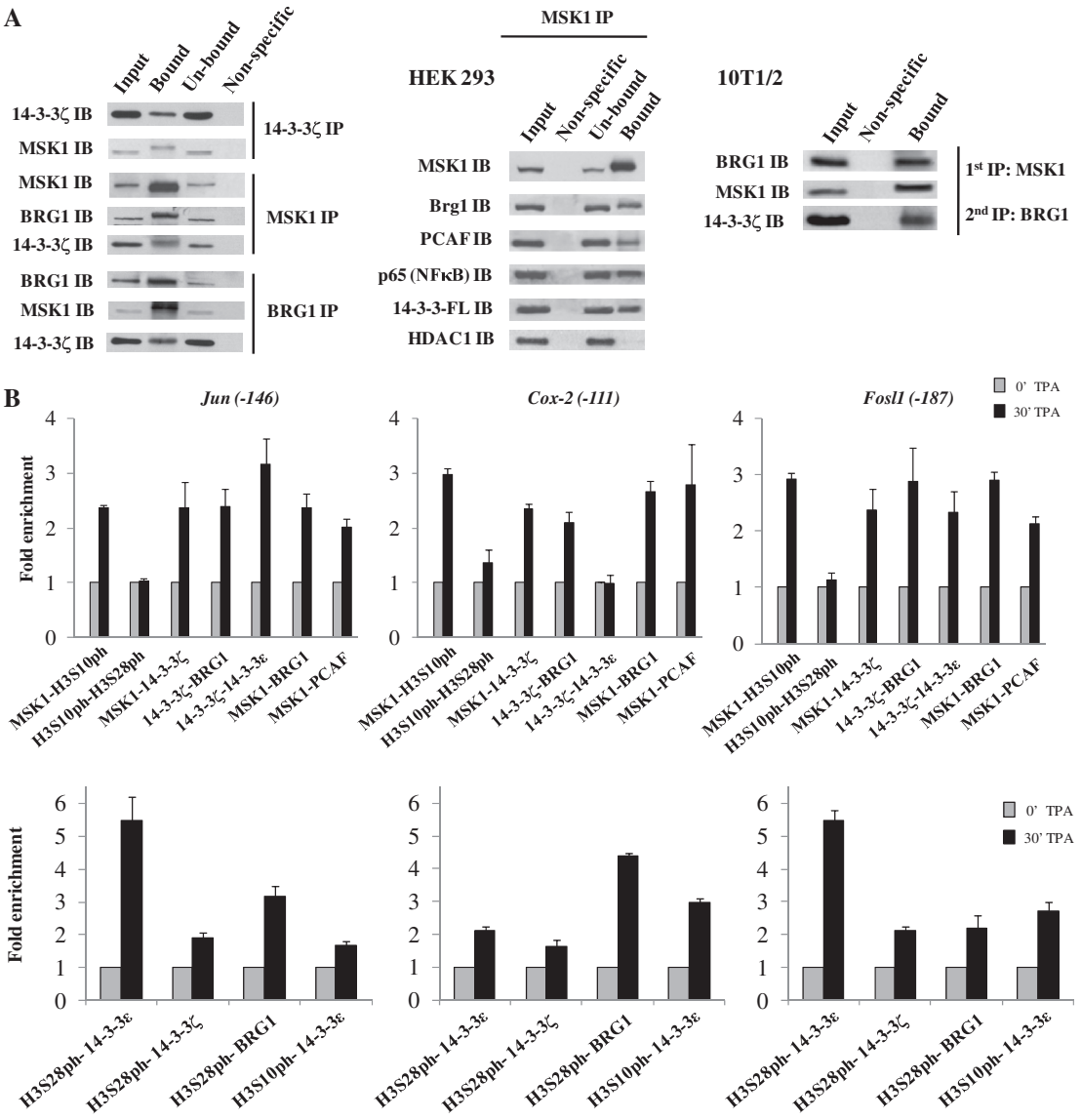

Figure 3. MSK1, 14-3-3 proteins and BRG1 complex formation and TPA-induced co-occupancy of MSK1, 14-3-3 proteins and chromatin remodelers/modifiers to *Jun*, *Cox-2* and *Fosl1* regulatory regions. (**A**) Aliquots of 1 mg 10T 1/2 (left panel) or 500 µg HEK293 (middle panel) total cell extracts were incubated with anti-14-3-3ζ, anti-MSK1 or anti-BRG1 antibodies. Alternatively, 1 mg total cell extract from serum-starved 10T1/2 cells stimulated with TPA for 30 min and treated with DTBP was sequentially incubated with anti-MSK1 and anti-BRG1 antibodies (right panel). The whole 'Bound' and 'Non-specific' fractions and equivalent volumes of 'Input' and 'Un-bound' fractions, corresponding to 25 µg of total cell extracts were resolved on SDS–10%-PAGE and immunoblotted with indicated antibodies (IB). (**B**) Re-ChIP experiments were performed on formaldehyde-crosslinked mononucleosomes prepared from serum-starved 10T1/2 cells treated with TPA for 0 or 30 min. The antibodies were used as indicated. Equal amounts of input and immunoprecipitated DNA were quantified by real-time quantitative PCR. The enrichment values of the 5′ proximal sequences of *Jun* (−146), *Cox-2* (−111) and *Fosl1* (−187) genes are the mean of three independent experiments, and the error bars represent the standard deviation.

14-3-3-Mediated interactions between MSK1 and chromatin remodelers/modifiers at regulatory regions of induced genes in response to ERK-MAPK signaling

The function of 14-3-3 proteins in IE gene expression is currently unknown. Since they exist as homodimers and heterodimers, it is possible that 14-3-3 proteins act as scaffolds to anchor proteins at the promoter of IE genes (39). For example, one 14-3-3 site would bind to H3S10ph or H3S28ph, while the other site would bind to a component of a nucleosome remodeling complex. To test this hypothesis, we checked if MSK1 could be co-immunoprecipitated with antibodies raised against 14-3-3. Figure 3A shows that MSK1 was present in the anti-14-3-3ζ immunoprecipitate from a 10T1/2 cell lysate. Reciprocally, anti-MSK1 antibodies co-precipitated BRG1 and 14-3-3ζ, while anti-BRG1 antibodies co-precipitated MSK1 and 14-3-3ζ (Figure 3A). Thus, in 10T1/2 cells, 14-3-3ζ is associated with BRG1 and MSK1. These interactions were also detected in HEK293 cells, where 14-3-3 proteins and BRG1 were co-immunoprecipitated with antibodies against MSK1 (Figure 3A). PCAF and NF-κB p65 transcription factor,

but not histone deacetylase 1 (HDAC1), were also co-immunoprecipitated with anti-MSK1 antibodies (Figure 3A). To determine if a ternary complex is formed between MSK1, 14-3-3 and BRG1, cell lysates from TPA-stimulated 10T1/2 cells were subjected to sequential immunoprecipitations first with antibodies against MSK1, and second with antibodies against BRG1. Figure 3A shows 14-3-3ζ is part of a complex including MSK1 and BRG1. To determine if these interactions occur at the promoter region of IE genes, re-ChIP assays were performed. Formaldehyde-crosslinked mononucleosomes from 10T1/2 cells treated or not for 30 min with TPA were subjected to the ChIP procedure with anti-MSK1 antibodies and again precipitated using anti-H3S10ph antibodies. Figure 3B shows that the MSK1-H3S10ph re-ChIP assay resulted in a marked increase in DNA precipitation of the proximal regulatory regions from the *Jun* (−146), *Cox-2* (−111) and *Fosl1* (−187) genes when cells were treated with TPA. Conversely, the H3S10ph–H3S28ph re-ChIP assay did not result in a significant TPA-induced increase of immunoprecipitation of the proximal regulatory regions from the *Jun* and *Fosl1* genes, and only showed a slight increase in the case of *Cox-2*. These data demonstrate that, upon TPA stimulation, MSK1 and H3S10ph are found together on the same proximal regulatory region from the *Jun*, *Cox-2* and *Fosl1* genes, while a nucleosome from the *Jun* or *Fosl1* promoter region has either H3S10ph or H3S28ph. In the case of the *Cox-2* promoter region, a low level of nucleosomes including both H3S10ph and H3S28ph was detected. Other re-ChIP experiments performed on the same crosslinked mononucleosome preparations demonstrated that following TPA treatment of 10T1/2 cells, there was an increase in co-occupancy of the proximal regulatory regions from the *Jun*, *Cox-2* and *Fosl1* genes by MSK1 and 14-3-3ζ, 14-3-3ζ and BRG1, MSK1 and BRG1, as well as MSK1 and PCAF (Figure 3B). On the other hand, a TPA-induced increase in co-occupancy of the proximal regulatory region by 14-3-3ζ and 14-3-3ε was detected for the *Jun* and *Fosl1* genes, but not for the *Cox-2* gene (Figure 3B).

While it is well known that H3S10ph is associated with the induction of IE genes in different cell types, so far there has only been one study reporting the association of H3S28ph with the *Jun* IE gene promoter region (12,40). To elaborate on the contribution of H3S28ph in the remodeling of IE gene promoter regions, we performed sequential ChIP assays to determine whether there would be co-recruitment of H3S28ph and 14-3-3ε, 14-3-3ζ or BRG1 to the proximal regulatory regions from the *Jun* (−146), *Cox-2* (−111) and *Fosl1* (−187) genes when cells were treated with TPA. Figure 3B shows that 14-3-3ε, 14-3-3ζ and BRG1 were associated with H3S28ph at the promoter region of these IE genes upon TPA induction. Figure 3B also demonstrates an association of 14-3-3ε with H3S10ph. These data demonstrate that either MSK-induced phosphorylation of H3 S10 or S28 of IE gene promoter region nucleosomes is involved in the events leading to the chromatin remodeling of IE gene promoter regions.

Inhibition by MSK inhibitor H89 of IE gene transcriptional induction in response to ERK-MAPK signaling

At a concentration of 10 μM, the MSK inhibitor H89 has been shown to selectively inhibit the nucleosomal response (41). Supplementary Figure S1A shows that the TPA-induced expression of the *Jun*, *Cox-2* and *Fosl1* genes was abolished when quiescent 10T1/2 cells were exposed to H89 prior to TPA treatment. Conversely, the GAPDH expression levels were not affected by H89 pre-treatment [data not shown and (7)]. As H89 is an equally potent inhibitor of another H3 kinase, protein kinase A (PKA) (42), we analyzed the effects of Rp-cAMP, a specific PKA inhibitor, on the induction of IE genes. We found that prior exposure of quiescent 10T1/2 cells to Rp-cAMP did not affect the TPA-induced transcription of IE genes (Supplementary Figure S2), demonstrating that the effects of H89 were due to the inhibition of MSK. As revealed by ChIP assays, RNA polymerase II phosphorylated at serine 5 (RNAPII S5ph), the initiation-engaged form of RNAPII (43), was not associated with the promoter region of *Jun*, *Cox-2* and *Fosl1*, upon TPA stimulation of H89 pre-treated 10T1/2 cells (Supplementary Figure S1B). These results suggest that the formation of an initiation complex at the 5′ end of IE genes requires a chromatin remodeling step which is dependent on the MSK-mediated nucleosomal response. To test this hypothesis, we analyzed by ChIP assays the effect of H89 treatment of quiescent 10T1/2 cells on the TPA-induced MSK1 recruitment, nucleosomal response and 14-3-3ε, 14-3-3ζ, BRG1 and PCAF binding to the upstream regulatory regions of the *Jun*, *Cox-2* and *Fosl1* genes. Results for the proximal 5′ regions of the three genes [*Jun* (−146), *Cox-2* (−111) and *Fosl1* (−187)] demonstrated that the TPA-induced MSK1 recruitment and ensuing H3 S10 and S28 phosphorylation were abolished, when 10T1/2 cells were exposed to H89 before TPA treatment (Figure 4A). Similarly, TPA-induced binding of 14-3-3ε, 14-3-3ζ, BRG1 and PCAF, as well as TPA-induced H3 acetylation at the 5′ proximal region of the three genes [*Jun* (−146), *Cox-2* (−111) and *Fosl1* (−187)] were markedly reduced following H89 pre-treatment of cells (Figure 4A). Although results on BRG1 and PCAF occupancy of the *Cox-2* promoter region (−111) were less clear, TPA-induced H3 acetylation was reduced by prior exposure of 10T1/2 cells to H89 (Figure 4A). Analysis of the immunoprecipitated DNA for the 5′ distal regulatory regions of the three genes [*Jun* (−711), *Cox-2* (−493) and *Fosl1* (−1113)] showed that TPA-induced MSK1 recruitment, H3 phosphoacetylation and binding of 14-3-3ε, 14-3-3ζ, BRG1 and PCAF were similarly affected at these upstream regulatory regions by H89 pre-treatment of cells (Supplementary Figure S3).

The lack of MSK1 recruitment to the promoter regions of IE genes following H89 pre-treatment of cells suggests that the recruitment of MSK1 is dependent on its kinase activity, which is required to activate the complex such that it is recruited to the IE gene promoter regions. An alternative explanation could be that H89 interferes with

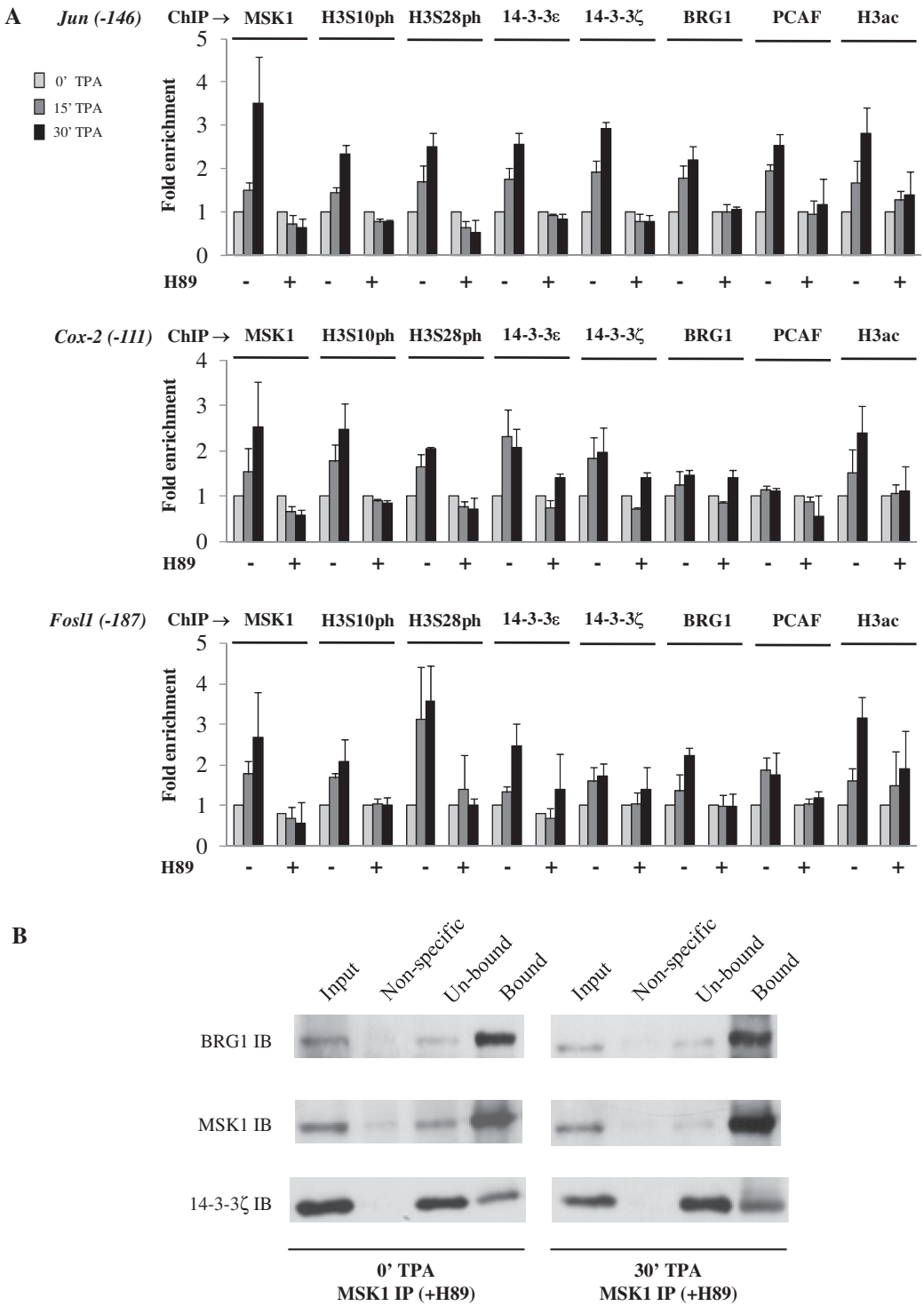

Figure 4. Inhibition by H89 of TPA-induced nucleosomal response and chromatin remodeler/modifier recruitment to the *Jun*, *Cox-2* and *Fosl1* 5′ proximal regulatory regions. (**A**) Serum-starved 10T1/2 cells were pre-treated or not with H89 prior to TPA stimulation for 0, 15 or 30 min. Formaldehyde crosslinked mononucleosomes were prepared and used in ChIP assays with antibodies against MSK1, H3S10ph, H3S28ph, 14-3-3ε, 14-3-3ζ, BRG1, PCAF or H3K9acK14ac. Equal amounts of input and immunoprecipitated DNAs were quantified by real-time quantitative PCR. The enrichment values of the 5′ proximal sequences of *Jun* (−146), *Cox-2* (−111) and *Fosl1* (−187) genes are the mean of three independent experiments, and the error bars represent the standard deviation. (**B**) Serum-starved 10T1/2 cells were pre-treated with H89 prior to TPA stimulation for 0 or 30 min. Aliquots of 1 mg total cell extract were incubated with anti-MSK1 antibodies. The whole 'Bound' and 'Non-specific' fractions and equivalent volumes of 'Input' and 'Un-bound' fractions, corresponding to 25 μg of total cell extracts were resolved on SDS–10%-PAGE and immunoblotted with indicated antibodies (IB).

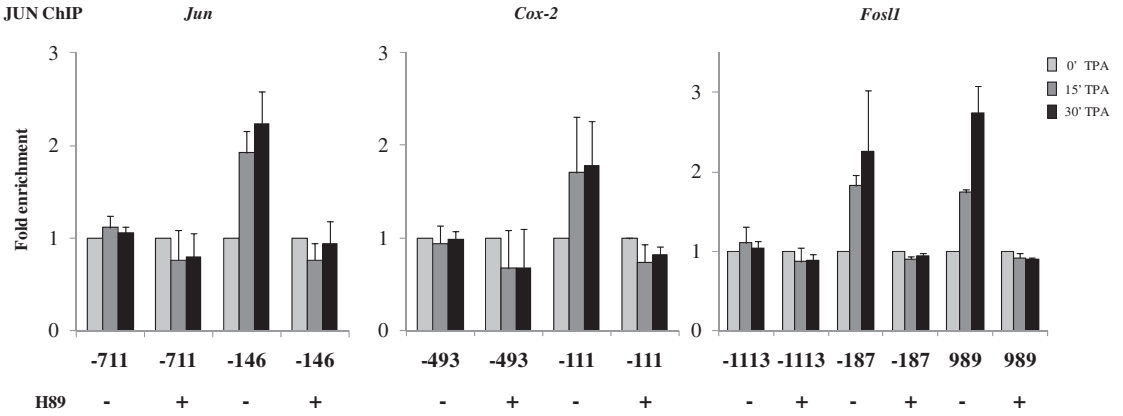

Figure 5. Inhibition by H89 of TPA-induced JUN recruitment to the *Jun*, *Cox-2* and *Fosl1* regulatory regions. Serum-starved 10T1/2 cells were pre-treated or not with H89 prior to TPA stimulation for 0, 15 or 30 min. Formaldehyde crosslinked mononucleosomes were prepared and used in ChIP assays with anti-JUN antibodies. Enrichment values, obtained by real-time quantitative PCR assays on equal amounts of input and immunoprecipitated DNAs, are the mean of three independent experiments, and the error bars represent the standard deviation.

the formation of the MSK1–14-3-3–SWI/SNF complex. To explore this latter possibility, we tested the ability of anti-MSK1 antibodies to co-immunoprecipitate 14-3-3ζ or BRG1 when quiescent 10T1/2 cells were exposed to H89 prior to TPA treatment. Figure 4B shows that BRG1 and 14-3-3ζ formed complexes with MSK1 in 10T1/2 cells pre-treated with H89 and TPA-stimulated for 0 or 30 min. Thus, H89 did not prevent the formation of MSK1 complexes, and its negative effect on promoter region remodeling and IE gene induction is most likely due to the inhibition of MSK1 kinase activity.

The AP-1 transcription factor is a homodimer or heterodimer consisting of proteins belonging to the JUN, FOS (including FRA) and ATF families, and is involved in the regulation of IE genes, including genes coding for its components. We investigated the TPA-induced association of JUN with the regulatory regions of the *Jun*, *Cox-2* and *Fosl1* by ChIP assay. Figure 5 shows that, upon TPA stimulation, JUN binds to the 5′ proximal, but not distal, regulatory regions of *Jun*, *Cox-2* and *Folsl1*, as well as the regulatory region located in the first intron of *Folsl1* (989). These results are in agreement with the mapping of known JUN and AP-1 binding sites and TPA-responsive element (TRE). Moreover, Figure 5 shows that, when 10T1/2 cells were treated with H89 prior to TPA stimulation, JUN association with its DNA binding sites was abolished for each of the three genes. This indicates that the binding of JUN to its cognate DNA sequences in the three investigated genes was dependent on the TPA-induced nucleosomal response.

Reduced nucleosomal response and promoter chromatin remodeling of IE genes in MSK1 knockdown 10T1/2 cells

To further evaluate the importance of MSK1 in the remodeling of the IE gene promoter regions following induction, we used a lentiviral vector system stably expressing short hairpin RNA (shRNA) to generate a MSK1 stable knockdown 10T1/2 cell line. The transfection of an empty lentiviral vector provided a negative control cell line. Immunoblot analysis demonstrated that the level of MSK1 in MSK1 knockdown cells was reduced by 74%, compared with the control cells (Supplementary Figure S4A), while the levels of *Jun*, *Cox-2* and *Fosl1* mRNAs in MSK1 knockdown cells compared to control cells were reduced by 59, 66 and 70%, respectively (Supplementary Figure S4B). Our high-resolution ChIP experiments were performed on quiescent control and MSK1 knockdown 10T1/2 cells that were either not stimulated or stimulated with TPA for 30 min, using antibodies against H3S10ph, H3S28ph, 14-3-3ε, 14-3-3ζ, BRG1 or JUN. Figure 6A shows that the association of all these proteins with the *Jun*, *Cox-2* and *Fosl1* 5′ proximal regulatory regions was reduced by half in MSK1 knockdown cells compared to control cells. A similar reduction in the association of 14-3-3ζ and BRG1 to the three gene proximal regulatory regions was observed in re-ChIP experiments performed on mononucleosomes from quiescent control and MSK1 knockdown 10T1/2 cells that were either not stimulated or stimulated with TPA for 30 min (Figure 6B). Thus, MSK1 is required for the TPA-induced nucleosomal response and chromatin remodeling taking place at the promoter regions of IE genes.

DISCUSSION

In this study, we provide direct evidence that the MSK1-mediated nucleosomal response plays a crucial role in the chromatin remodeling and transcription initiation of IE genes in response to MAPK signaling. Our data show the exclusive positioning of H3S10ph or H3S28ph at the regulatory regions of genes, which indicates that these H3 post-translational modifications have functions primarily in promoter remodeling and transcription initiation but not in elongation. Figure 7 presents our model, where the SWI/SNF remodeler is recruited to nucleosomes displaying H3S10ph or H3S28ph via 14-3-3 proteins. This model is supported by co-immunoprecipitation data showing that MSK1 and BRG1, the ATPase subunit of SWI/SNF, are associated with 14-3-3, by re-ChIP assays demonstrating co-occupancy of IE gene regulatory regions

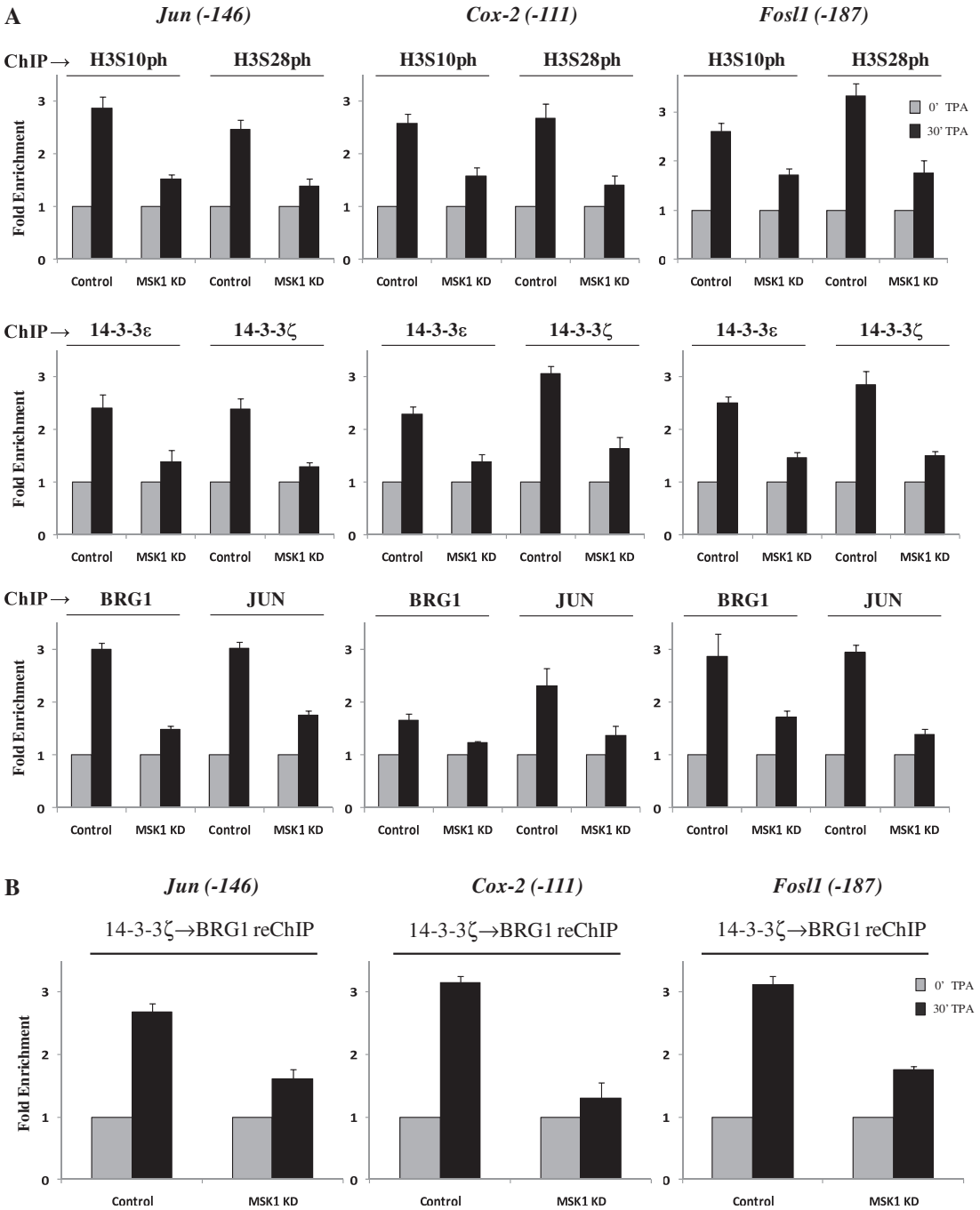

Figure 6. MSK1 is required for the nucleosomal response and the recruitment of 14-3-3 isoforms, chromatin remodelers and transcription factors at the regulatory regions of *Jun*, *Cox-2* and *Fosl1*. ChIP experiments using antibodies againstH3S10ph, H3S28ph, 14-3-3ε, 14-3-3ζ, BRG1 or JUN (**A**) and re-ChIP experiments using antibodies as indicated (**B**) were performed on formaldehyde-crosslinked mononucleosomes prepared from serum-starved MSK1 knockdown and control 10T1/2 cells treated with TPA for 0 or 30 min. Equal amounts of input and immunoprecipitated DNA were quantified by real-time quantitative PCR. The enrichment values of the 5′ proximal sequences of *Jun* (−146), *Cox-2* (−111) and *Fosl1* (−187) genes are the mean of three independent experiments, and the error bars represent the standard deviation.

by MSK1, 14-3-3 and BRG1, and by the demonstration that in MSK1 knockdown 10T1/2 cells, all the events such as H3 S10 and S28 phosphorylation, the recruitment of 14-3-3, BRG1 and JUN to the IE gene promoter regions, and the expression of the IE genes were reduced. These experiments established the importance of MSK1 in the remodeling of the IE gene promoter regions following TPA induction. Thus we propose a model in which one 14-3-3 site binds to H3S10ph or S28ph, while the other site binds to the SWI/SNF complex. The recruited SWI/SNF remodels nucleosomes at the promoter of IE genes, enabling the binding of transcription factors and transcription initiation. This model is also validated by the lack of TPA-induced association of 14-3-3, BRG1 and

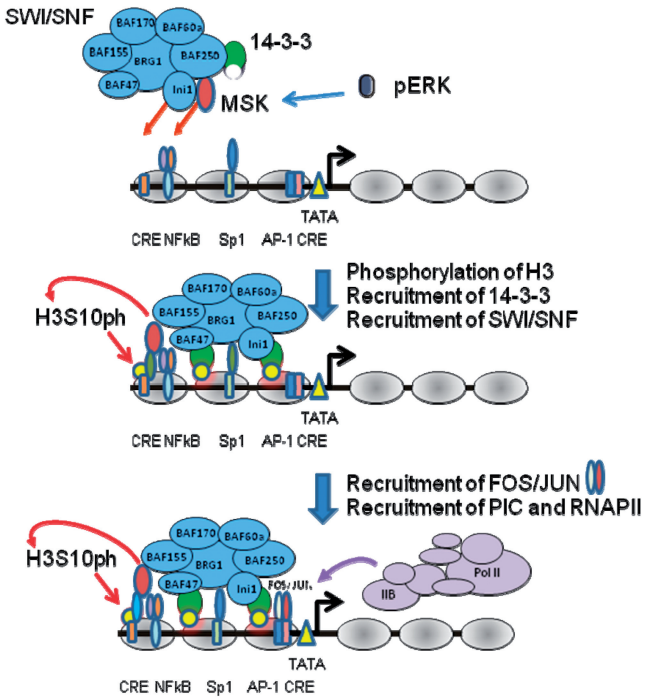

Figure 7. Schematic model representing the role of MSK1 and 14-3-3 in IE gene remodeling and induction in response to MAPK signaling. MSK shown as a large red oval, and 14-3-3 as a green partial oval are in complex with the SWI/SNF chromatin remodeler. The grey ovals along the DNA represent nucleosomes, the NF-κB transcription factor (p65/p50 dimer) is shown as a purple/orange pair of ovals binding to the NF-κB binding site (blue oval). In response to the activation of the ERK and p38 MAPK pathways, the MSK1 multiprotein complex is recruited to the regulatory regions of IE genes by transcription factors such as Elk1, NF-κB or C/EBPb. MSK1 phosphorylates either H3 S10 or H3 S28, with H3S10ph being shown (yellow circles). H3S10ph and H3S28ph recruit 14-3-3 proteins, which mediate the recruitment of the chromatin remodeler SWI/SNF, with BRG1 being the ATPase subunit. The ensuing remodeling of promoter nucleosomes allows the access of transcription factors such as AP-1 to the promoter target sequences. The pre-initiation complex is recruited at the TATA box (yellow triangle), and initiation of transcription follows as is made apparent by the presence of RNAPII S5ph.

PCAF to the regulatory regions of IE genes following the selective inhibition of the nucleosomal response by H89.

In agreement with a previous report that MSK1 was recruited to human *FOS* and *EGR-1* promoters by transcription factor Elk-1 (38), we observed the TPA-induced recruitment of MSK1 to the *Jun* 5′ distal regulatory region (−711), which exhibits a putative Elk-1 binding site. We found that MSK1 is in complex with 14-3-3, BRG1, PCAF and p65 subunit of NF-κB. Transcription factor NF-κB in the MSK1 complex may recruit MSK to specific promoters (e.g. inflammatory promoters, TFF1) (25,44,45). The presence of 14-3-3 and BRG1 in the MSK1 complex facilitates the recruitment of the phospho-serine adaptor and chromatin remodeler to the phosphorylated H3 tails of promoter nucleosomes.

Our study demonstrates that H3S10ph and H3S28ph do not typically exist in the same nucleosome associated with the promoters of IE genes. The observations that H3S10ph and H3S28ph are not present together in a string of two to three nucleosomes and that anisomycin/TPA-induced H3S10ph and H3S28ph foci are distinct provide evidence that IE gene promoters are associated with either H3S10ph or H3S28ph (9,27). It is conceivable that H3S10ph is associated with a given promoter region in one cell, while in another cell H3S28ph is bound to that promoter region. Alternatively, H3S10ph and H3S28ph association may be allele specific.

This work provided a detailed picture of the events taking place at IE gene promoter regions following simulation of the signal transduction pathway, a process leading to the activation of IE gene expression and subsequent gene expression programming events involved in wound healing, metastasis, stress-related memory response and food reward-driven learning and cocaine addiction. Although MSK1/2 knockout mice are viable, upon closer examination these animals were found to be defective in stress-related learning and memory processes, with the NMDA-R-mediated H3S10ph and associated *Fos* gene induction being abolished in the dentate granule neurons (5,20). Interestingly, the molecular defect in a mouse model for Huntington's disease, a neurodegenerative disease, was a deficiency in MSK1 expression resulting in the lack of induced H3S10ph and down-regulation of *Fos* transcription in the striatum (46). MSK1 is also involved in the regulation of clock gene expression in the suprachiasmatic nucleus (47) and in the regulation of inflammatory genes, including interleukin-1, -8 and *COX-2* (44,45). It is likely that the MSK-mediated nucleosomal response plays a role in chromatin remodeling and transcriptional reprogramming in many physiological processes.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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