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Elucidating combinatorial histone modifications and crosstalks by coupling histone-modifying enzyme with biotin ligase activity

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ABSTRACT

Histone post-translational modifications (PTMs) often form complex patterns of combinations and cooperate to specify downstream biological processes. In order to systemically analyse combinatorial PTMs and crosstalks among histone PTMs, we have developed a novel nucleosome purification method called Biotinylation-assisted Isolation of CO-modified Nucleosomes (BICON). This technique is based on physical coupling of the enzymatic activity of a histone-modifying enzyme with *in vivo* biotinylation by the biotin ligase BirA, and using streptavidin to purify the co-modified nucleosomes. Analysing the nucleosomes isolated by BICON allows the identification of PTM combinations that are enriched on the modified nucleosomes and function together within the nucleosome context. We used this new approach to study MSK1-mediated H3 phosphorylation and found that MSK1 not only directly phosphorylated H3, but also induced hyperacetylation of both histone H3 and H4 within the nucleosome. Moreover, we identified a novel crosstalk pathway between H3 phosphorylation and H4 acetylation on K12. Involvement of these acetyl marks in MSK1-mediated transcription was further confirmed by chromatin immunoprecipitation assays, thus validating the biological relevance of the BICON results. These studies serve as proof-of-principle for this new technical approach, and demonstrate that BICON can be further adapted to study PTMs and crosstalks associated with other histone-modifying enzymes.

INTRODUCTION

Histones are subjected to a variety of post-translational modifications (PTMs) including acetylation, methylation, phosphorylation, ubiquitylation and sumoylation (1). Histone-modifying enzymes, and their resultant PTMs, can be viewed as an extension of signal transduction networks. They function to transmit signals to chromatin, which then translates external stimuli into the appropriate nuclear responses (2,3). Moreover, signaling cascades also occur on histones, whereby one PTM on a histone can positively or negatively influence the deposition of other downstream PTMs (4). Such crosstalk can occur within the same histone tail (*cis* crosstalk) or between different histones (*trans* crosstalk). One of the earliest examples of histone PTM crosstalk is the direct coupling of phosphorylation and acetylation on H3 during gene activation, whereby phosphorylation of S10 on H3 facilitates subsequent acetylation on the neighboring K14 by the Gcn5 acetyltransferase (5,6). The *trans*-tail crosstalk pathways are best exemplified by the functional links between H2B ubiquitylation and H3 methylation. More specifically, in yeast and human cells, mono-ubiquitylation of H2B at the C-terminus is an absolute pre-requisite for H3K4 methylation by Set1 and H3K79 methylation by Dot1 (7,8). This complex interplay between PTMs dictates the legitimate combinations of histone modifications that can occur together on nucleosomes, and coordinates their functions to elicit specific outcomes (9,10).

One well-established mechanism by which histone PTMs mediate downstream functions is through the recruitment and direct binding of downstream effector proteins to chromatin. This docking of effectors to modified histones is mediated through conserved protein modules that recognize histone PTMs in modification-specific and site-specific manners (11). For example, bromodomains and chromodomains are conserved

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structural motifs that, respectively, bind acetylated and methylated lysine residues. Increasing evidence also suggests that multiple histone-binding modules function in concert, and coordinately engage multiple PTMs within a nucleosome (12). Specific combinations of histone PTMs can therefore serve as multivalent docking sites for stabilizing the contacts between chromatin and the recruited proteins or complexes. By doing so, they collectively coordinate the recruitment (or repulsion) of effector-binding proteins to direct biological outcomes. Although most studies to date focus on individual histone PTMs, elucidating the combinatorial patterns of histone PTMs and how they cross regulate one another represents the next important frontier of this field.

H3 phosphorylation was first observed on mitotic chromosomes, but it is also associated with transcriptional activation in interphase cells (2). In response to stress or growth factor stimulation, H3 is rapidly and transiently phosphorylated on S10 and/or S28 by signaling kinases such as MSK1 (13,14). We previously found that MSK1 is a potent transcriptional activator. Targeting MSK1 to the immediate-early gene (IEG) *c-fos*, as well as the polycomb-silenced α-globin gene, not only efficiently phosphorylated H3 at the gene promoters, but also strongly induced their transcription in the absence of other external stimuli (15). It is currently unclear if phosphorylation of S10 and S28 has differential effects on gene expression regulation. Previous studies suggest that these modifications occur on distinct pools of H3 and are present on separate H3 tails at IEG promoters (16–18). Interestingly, we detected co-occupancy of H3S10ph and H3S28ph marks at the reactivated α-globin (but not *c-fos*) promoter, suggesting that these two marks can co-exist within the same nucleosome depending on the genes and context (15). Even though the functional differences between phosphorylation of these two different sites are still largely unknown, it is clear that both H3S10ph and H3S28ph can mediate multiple crosstalks with other histone modifications. For example, our early work showed that H3S10ph is physically coupled to H3K14ac during mitogen-induced activation of IEG (5). Importantly, acetylation of H3 K14 enhances the interaction between H3S10ph and 14-3-3, suggesting that the dual modifications on H3 S10 and K14 cooperate to recruit downstream effector proteins (19,20). At the *FOSL1* enhancer, phosphorylation of H3S10 by PIM1 kinase not only recruits 14-3-3, but also induces acetylation on H4 K16, ultimately leading to transcription elongation (21). Besides recruiting 14-3-3 and other downstream chromatin modifiers, H3 phosphorylation can also disrupt binding of chromodomain-containing proteins to methylated H3. During mitosis and transcriptional activation, phosphorylation of H3 S10 displaces HP1 from H3K9me3 (22–24). Such a 'phospho/methyl' switch also occurs on H3K27me3/H3S28ph, with H3S28ph displacing polycomb-group proteins from polycomb-silenced genes (15,25). Moreover, we found that phosphorylation of H3 S28 by H3 kinase MSK1 is functionally and physically coupled to K27 acetylation, and this dual modification correlates with reactivation of polycomb-silenced α-globin gene in non-erythroid cells (15). All these findings indicate that H3 phosphorylation cooperates with PTMs on multiple histone sites and together they regulate binding of effector proteins and downstream biological processes.

To extend these studies, we sought to develop an unbiased method to identify histone PTMs that occur together with MSK1-mediated H3 phosphorylation. To that end, we developed an original affinity purification approach, which we termed Biotinylation-assisted Isolation of CO-modified Nucleosomes (BICON) to capture and study phospho-H3-containing nucleosomes. This method involves the coupling of *in vivo* biotinylation mediated by the *Escherichia coli* BirA enzyme (26) and phosphorylation of H3 by MSK1, and using streptavidin-coupled beads to isolate MSK1-modified nucleosomes. Analysing the spectrum of histone PTMs on these nucleosomes, we not only found that their H3 are hyperphosphorylated, but specific residues on H3 and H4 are also hyperacetylated. This suggests that crosstalk between phosphorylation and acetylation occurs both *in cis* and *in trans* within the nucleosome. Importantly, chromatin immunoprecipitation (ChIP) assays examining MSK1-target genes confirmed that these specific combinations of histone modifications are induced upon gene activation. Therefore, these studies showed that the BICON method not only revealed combinatorial histone PTMs and new histone crosstalks, but also illustrated the potential usefulness of this technique.

MATERIALS AND METHODS

Plasmid constructs

HA-tagged CA-MSK1 and KD-MSK1 in pMT2 were provided by Dr Morten Frodin (University of Copenhagen, Denmark). For Avi-Flag tagging, a tandem Avi-tag followed by a Flag-tag was fused in frame to the 3′-end of the H3.3 coding sequence. The Avi-tag refers to a 15 amino acid sequence (GLNDIFEAQKIEWHE) that contains a biotinylation site for the *E. coli* biotin ligase BirA. BirA expression construct was provided by Dr John Strouboulis (Alexander Fleming Biomedical Sciences Research Center, Greece). BirA coding sequence was PCR-amplified and fused in frame to the N-terminal side of CA- or KD-MSK1 to generate the BirA-MSK1 fusion constructs in pcDNA3.1+. NF1-CA/KD-MSK1 constructs have been previously described (15).

Cell culture, transfections, TPA and H89 treatment

293T cells were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum. All transfections were performed using Lipofectamine 2000. For 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA, Sigma) stimulation, 293T cells were switched to serum-free media for 20 h prior to stimulation. Serum-starved cells were mock-treated with DMSO or stimulated with 400 ng/ml TPA for 0–90 min and then harvested for ChIP assays. To inhibit MSK1 activity, 10 μM H89 (Sigma) was added to cells for 30 min before stimulation with TPA.

Harvesting of protein samples, gel electrophoresis, Western blot analyses and antibodies

Whole cell lysates were prepared by directly lysing pelleted cells in boiling SDS sample buffer. For acid extraction of histones, histones were extracted with 0.4 N H_2SO_4 and then precipitated with 20% trichloroacetic acid (TCA). Acid-extracted histones were analysed on an acid-urea (AU) gel system as described in (5). Whole cell lysates and isolated mono-nucleosomes were resolved on SDS-PAGE gels and transferred to PVDF membranes for Western blotting analyses. Antibodies used in this study are as follows: H3 (ab1791) and H3K27ac (ab4729) from Abcam, H3S10ph (sc-8656R) and 14-3-3ζ (sc-1019) from Santa Cruz, H3S28ph (H9908), Flag (F1804) and avidin-horseradish peroxidase (Avi-HRP, A3151) from Sigma-Aldrich, H3K14ac (06-911), H3ac (06-599), H4ac (06-946), H4K12ac (07-595), H4K16ac (07-329), H4 (05-858) and Rabbit IgG (12-370) from Millipore, HA.11 (MMS-101R) from Covance, H2AZ (39113) from Active Motif, and H3K27ac/S28ph antibody was described in (15).

Nucleosome purification with streptavidin beads

293T cells were co-transfected with H3.3-AviFlag and pcDNA vector alone or constructs that express NLS-BirA, BirA-CA-MSK1 or BirA-KD-MSK1. 38 h after transfection, transfected cells were trypsinized and washed twice with PBS. Cell pellets were resuspended in buffer A (20 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM dithiothreitol and protease inhibitors) containing 0.2% Triton X-100, and then incubated on ice for 5 min. Nuclei were pelleted by centrifugation at 600*g*, and washed once in MNase Cutting Buffer (10 mM HEPES, pH 7.9, 60 mM KCl, 10 mM NaCl). Nuclei were then resuspended in MNase Cutting Buffer containing 3 mM $CaCl_2$ and 2 mM *p*-chloromercuri-phenylsulfonic acid (pCMPS, Toronto Research Labs; as phosphatase inhibitor), and digested with micrococcal nuclease (MNase, Worthington) at 37°C for 30 min at a concentration of $10U/10^7$ cells. Digestion of chromatin by MNase was stopped by addition of EGTA to a final concentration of 5 mM, and equal volume of 2 x Lysis Buffer (30 mM HEPES, pH 7.9, 220 mM KCl, 3 mM $MgCl_2$, 2 mM EDTA, 0.4% Triton X-100, 20% glycerol) was added to the digested chromatin. Samples were centrifuged at maximum speed for 5 min to remove cell debris, and the resulting supernatant containing mono-nucleosomes was used as input material for affinity purification. For isolation of biotinylated nucleosomes, input chromatin was incubated with streptavidin-agarose beads (Sigma S1638; 20 µl bed volume/sample) overnight at 4°C. Beads were washed three times in WB1 (20 mM HEPES, pH 7.9, 140 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EGTA, 0.2% Triton X-100 and 10% glycerol), following by two times in WB2 (20 mM HEPES, pH 7.9, 300 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EGTA, 0.5% Triton X-100 and 10% glycerol) and once in WB1 again. Bound materials were eluted in 2 x SDS-sample buffer without reducing agent (100 mM Tris, pH 6.8, 4% SDS and 20% glycerol), and boiled for 10 min. Eluted nucleosomes were resolved on 15% SDS-PAGE gels and analysed by Western blotting according to standard practices.

ChIP assays

ChIP assays were performed as previously described (5,15). Briefly, for ChIP assays involving NF1-MSK1, 293T cells were transfected with pcDNA vector alone or expression vectors for NF1-CA-MSK1 or NF1-KD-MSK1 and were harvested 30 h after transfection. These transfected cells or DMSO/TPA-stimulated cells were fixed in 1% formaldehyde for 8 min at room temperature. After swelling and lysis, chromatin was sonicated to an average of ~500 bp with a Branson Sonifier 450. Antibodies were prebound to protein A/G magnetic Dynabeads (Invitrogen) for at least 3 h and rabbit IgG were used as negative controls. Sonicated lysates were precleared with Dynabeads for 2 h and then incubated with antibody-bound beads overnight at 4°C. After successive washes, immunoprecipitated chromatin was eluted in elution buffer (1% SDS, 100 mM $NaHCO_3$) and then reverse-crosslinked overnight at 65°C. Following RNase A and Proteinase K treatment, DNA was extracted with phenol/chloroform and analysed by quantitative PCR. Input and immunoprecipitated material were analysed in parallel using PerfeCTa SYBR Green SuperMix (Quanta) on a 7900HT fast real-time PCR system (Applied Biosystems). Reactions were performed in triplicate and presented as % of precipitated material relative to the amount in the input sample (% input) and have been normalized to H3 levels to adjust for nucleosome density. Signals from specific antibodies were at least 30-fold above rabbit IgG controls (not shown). Error bars indicate means ± standard deviation ($n = 3$), and are representative of at least three independent experiments. The following primers were used for ChIP-qPCR analyses: α-globin promoter, forward 5′-GGGCCGGCA CTCTTCTG-3′, reverse 5′-GGCCTTGACGTTGGTCTT GT-3′; control region (upstream of α-globin), forward 5′-GAGATGCTGGAGTCAGGACCAT-3′, reverse 5′-AG GAGTCAGGAGCAGCAGTCA-3′; c-fos promoter, forward 5′- GAGCAGTTCCCGTCAATCC-3′, reverse 5′-GCATTTCGCAGTTCCTGTCT-′.

RESULTS

Coupling of *in vivo* biotinylation and MSK1 phosphorylation

Our previous study showed that phosphorylation of H3S28 by MSK1 can enhance acetylation of the adjacent K27 residue (15). This generates a di-modified H3K27acS28ph mark, which correlates with transcriptional activation. To extend our studies, we aimed to further dissect the PTMs that co-exist and functionally synergize with H3 phosphorylation, and to examine the crosstalk pathways initiated by the MSK1 kinase. To do this, we needed to specifically isolate phospho-H3-containing nucleosomes for analyses. One major obstacle to this goal is that only a small fraction of total H3 is phosphorylated in response to mitogen or stress

stimulation (27). To overcome this limitation, we developed a new approach, which we termed BICON, to isolate and enrich for MSK1-modified H3 and its associated nucleosomes (Figure 1A and 2A). This method is based on an *in vivo* biotinylation system, which utilizes the *E. coli* biotin ligase BirA's ability to recognize and biotinylate a short 15 amino acid peptide called Avi-tag (26). Because there are no endogenous mammalian proteins that contain this BirA recognition sequence, only introduced substrates containing the Avi-tag will be biotinylated. Therefore, this allows very specific isolation of the BirA-targeted substrate using avidin/streptavidin-coupled beads. For our purpose, we note that H3.3 is the transcription-associated isoform of H3 (28), and that MSK1 has a 2-fold preference in phosphorylating H3.3 over canonical H3.1 (Supplementary Figure S1). Therefore, we chose this variant as the targeted substrate in our studies. This *in vivo* biotinylation system has been successfully employed to study protein–protein interactions and protein complexes in a variety of cell types (26). It has also been adapted for ChIP assays to map chromosomal targets of transcription factors and histone-modifying enzymes (29). More importantly, this system has been used to biochemically purify an assembly complex for *Drosophila* centromeric H3, CID, and also has been applied to H3.3 for epigenomic profiling of this H3 variant in *Caenorhabditis elegans* (30,31), indicating that biotinylation of Avi-tagged histones does not alter their biological properties.

To enrich for MSK1-phosphorylated H3.3, we fused BirA to constitutively active (CA)-MSK1 to generate a BirA-CA-MSK1 fusion protein, such that biotinylation and phosphorylation can occur together on the same H3.3 molecule (Figure 1A). We reasoned that such co-modification will allow preferential enrichment of phosphorylated-H3.3 nucleosomes upon incubation with streptavidin beads, and allow us to study the global PTMs on these nucleosomes. A kinase-dead version, BirA-KD-MSK1, was also generated and used as a control to generate biotinylated, but not phosphorylated, H3.3 for comparison. When the BirA-MSK1 fusion enzymes and the Avi-tagged H3.3 were co-expressed in 293T cells, there were no observable differences in the growth of these cells compared to the untransfected control cells. To assess the ability of the BirA-MSK1 fusions to biotinylate and phosphorylate H3.3, biotinylation and phosphorylation levels of the tagged H3.3 were detected on Western blots using avidin-horseradish peroxidase (Avi-HRP) conjugate and phospho-H3 specific antibodies, respectively. These analyses confirmed that biotinylation of the histone substrate is specific and dependent on the presence of BirA (Figure 1B). Background histone biotinylation level is negligible and, therefore, is not a concern in this purification scheme. In addition, BirA-CA-MSK1, but not KD, strongly phosphorylated H3.3 on S10 and S28 (Figure 1B), further confirming that the BirA-MSK1 fusions are functional *in vivo* and modify the transfected H3.3 as intended; however, it is currently unclear whether BirA or MSK1 is responsible for targeting the BirA-MSK1 fusion to its substrates.

We further verified our co-modification approach by analysing the Avi-Flag-tagged H3.3 on an AU gel system, which separates proteins based on size and charge (Figure 1C). Addition of a phosphate or acetyl group alters the overall charge on histones and will cause an electrophoretic mobility shift on an AU gel (32). Using an anti-Flag antibody to detect the total amount of transfected H3.3, we observed no obvious change in the banding pattern between different samples (Figure 1C, top). This suggests that only a small fraction of transfected H3.3 is hyperphosphorylated. However, detection by Avi-HRP showed that the biotinylated fraction of H3.3 is hyper-shifted on the AU gel, indicating that they are specifically phosphorylated and/or acetylated in an active MSK1-dependent manner (Figure 1C, bottom). Collectively, these results demonstrate that our approach of coupling biotinylation and phosphorylation of Avi-tagged H3.3 is feasible, and that purification of biotinylated H3.3 will also enrich for MSK1-modified H3.3.

Affinity purification of BirA-biotinylated and MSK1-modified nucleosomes

To gain further insight into the links among MSK1, histone modifications and transcriptional activation, we sought to identify other histone modifications that are present on MSK1-phosphorylated H3.3 and its associated nucleosomes. We preferentially enriched for MSK1-modified H3.3 nucleosomes by capturing the co-modified (MSK1-phosphoryated and BirA-biotinylated) H3.3 and its associated nucleosomes onto streptavidin beads. The general scheme of BICON is shown in Figure 2A. Briefly, we co-expressed Avi-Flag-tagged H3.3 and BirA-MSK1 (CA or KD) fusions in 293T cells. Nuclei from these transfected cells were isolated by hypotonic lysis, followed by digestion with micrococcal nuclease (MNase) to liberate mono-nucleosomes. To ensure that we are only analysing PTMs within the same H3.3 nucleosome, and not neighboring ones, complete digestion of chromatin to mono-nucleosomes is essential. DNA isolated from input chromatin was analysed on agarose gels to ensure that our digest yielded mainly mono-nucleosomes (~150 bp DNA fragments, Figure 2B). These soluble mono-nucleosomes were then used as starting material for affinity pull-down with streptavidin-coupled beads. Following extensive and high stringency washes, bound nucleosomes were eluted and separated on SDS-PAGE gels. PTMs on these MSK1-modified H3.3 nucleosomes were then analysed by Western blotting and modification-specific antibodies.

Analysis of the purified material confirmed that the association of biotinylated H3.3 with streptavidin beads was specific. In the pcDNA vector alone sample, which only contained non-biotinylated H3.3, we did not detect any H3.3-AviFlag or other histones in the eluate (Figure 2C). As seen by coomassie blue staining, the isolated H3.3 co-purified with other core histones (H2A, H2B, H4) in an equimolar stoichiometry, indicating the presence of nucleosomes in the pull-down material (Figure 2C, right). In addition, we detected an association

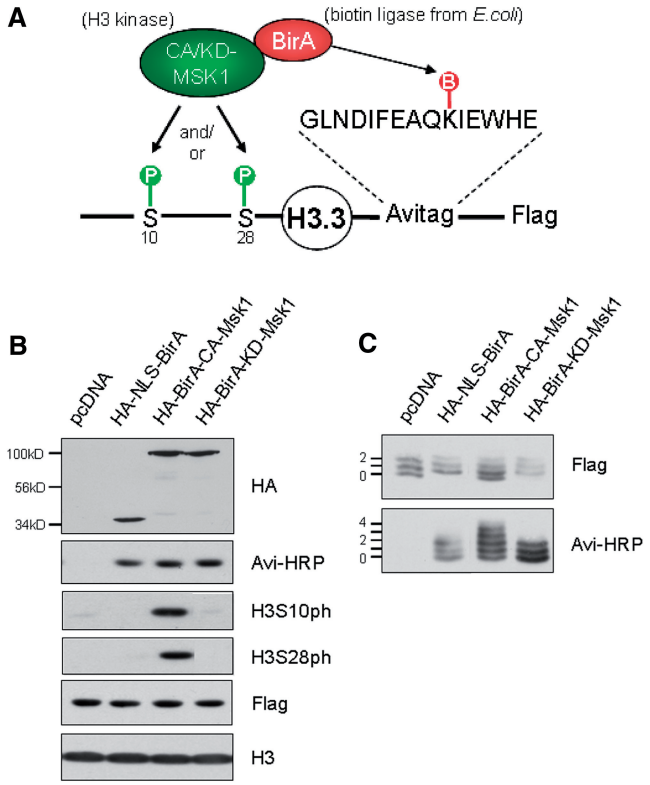

Figure 1. *In vivo* biotinylation and phosphorylation of Avi-tagged H3.3. (**A**) Schematic of H3.3-AviFlag and BirA-Msk1 fusion constructs. H3.3-AviFlag contains a tandem Avi-Flag tag at its C-terminus. The 15 amino acid Avi-tag encompasses a recognition sequence for the *E. coli* biotin ligase BirA, which catalyses the addition of biotin to the lysine residue on the tag. Also shown are the two serine phosphorylation sites (S10, S28) at the N-terminus of H3. (P, phosphorylation; B, biotinylation) (**B**) Vector alone (pcDNA), BirA or BirA-MSK1 (CA or KD) constructs were co-transfected with H3.3-AviFlag into 293T cells. Total cell lysates were resolved on SDS-PAGE gels. Expression and enzymatic activities of BirA-MSK1 fusions were examined by Western blotting using the indicated antibodies. (**C**) Acid-extracted histones from transfected 293T cells were resolved on AU gel, followed by Western blot analyses. Blots were probed with α-Flag-antibody to detect total amount of H3.3-AviFlag and Avi-HRP to detect biotinylated H3.3-AviFlag.

with H2A variant H2A.Z (Figure 2C, bottom), which is consistent with the known occurrence of H3.3/H2A.Z nucleosomes (33). Taken together, these results strongly suggest that Avi-tagged H3.3 is properly incorporated into chromatin and that the modified H3.3-containing nucleosomes can be isolated by streptavidin pull-down.

MSK1-phosphorylated nucleosomes are enriched for 14-3-3ζ and H3/H4 acetylation

Using nucleosomes isolated by BICON, we tested which other histone PTMs are linked to H3.3 phosphorylation by probing these nucleosomes with a panel of histone modification-specific antibodies. As expected, we detected strong phosphorylation signals (H3S10ph and H3S28ph) on BirA-CA-MSK1-modified nucleosomes, confirming that they are hyperphosphorylated relative to the BirA- or BirA-KD-MSK1-modified nucleosomes (Figure 3A). Direct comparison of input material and streptavidin pull-downs further showed that the levels of phosphorylated H3 were higher on pull-down nucleosomes than in the input, suggesting that our BICON approach enriched for MSK1-phosphorylated nucleosomes as intended (Supplementary Figure S2). Moreover, we observed a preferential binding of 14-3-3ζ to these phosphorylated-H3.3 nucleosomes (Figure 3B). To date, only two phospho-H3 binding proteins have been identified, 14-3-3ε and 14-3-3ζ, which both belonged to the 14-3-3 family (34). Previous studies on the interaction between 14-3-3 and phosphorylated H3 were based on *in vitro* binding assays or involved formaldehyde-crosslinked samples in ChIP assays (18,19,35). Therefore, our system not only recapitulates the previously reported binding of 14-3-3 to H3S10ph and H3S28ph, but it is also the first demonstration of 14-3-3ζ binding to phospho-H3 in a native *in vivo* setting. This suggests that BICON may also be useful for studying the interactions of PTM-binding proteins with their cognate PTMs within a nucleosome context.

Due to the well-established roles of histone phosphorylation and acetylation in transcriptional activation, we examined the acetylation levels of H3.3 and H4 on MSK1-phosphorylated H3.3 nucleosomes. Using site-specific α-acetyl antibodies and comparing the nucleosomes that were pulled down from cells transfected with BirA, BirA-CA-MSK1 or BirA-KD-MSK1, we were able to dissect the specific PTM patterns on MSK1-phosphorylated nucleosomes. In agreement with our published data (15), we found that H3K27ac and H3K27ac/S28ph levels were significantly higher on BirA-CA-MSK1-modified nucleosomes (Figure 3C, left). We also detected an increase in H3K14ac and H3ac (H3K9/14ac) levels on these BirA-CA-MSK1-modified nucleosomes. This is consistent with our earlier finding that H3S10 phosphorylation is synergistically coupled to H3 K14 acetylation during the activation of IEG *c-fos* (5). In addition to analysing crosstalks between PTMs on the same H3.3 tail, one advantage of our experimental approach of isolating mono-nucleosomes is that we can also examine coupling of epigenetic marks on other histones within the same nucleosome. Indeed, we found that CA-MSK1-modified nucleosomes have increased H4 acetylation (H4ac) levels, and this increase was dependent on the kinase activity of MSK1 (Figure 3C, right). Using site-specific H4ac antibodies, we found that an increase in H4K12ac, but not H4K16ac, was associated with phosphorylated H3.3. These data therefore illustrate that MSK1-mediated H3.3 phosphorylation initiates a series of PTM crosstalks both in *cis* on H3.3 and in *trans* on H4.

H4 K12 acetylation is associated with activation of MSK1-target genes

Based on our biochemical pull-down results, we found that a number of H3ac and H4ac marks were induced by MSK1 and H3 phosphorylation. To validate the biological relevance of the crosstalk between H3 phosphorylation and H3/H4 acetylation, we next asked whether any of these acetyl marks are involved in transcriptional activation of MSK1-responsive genes. We performed ChIP

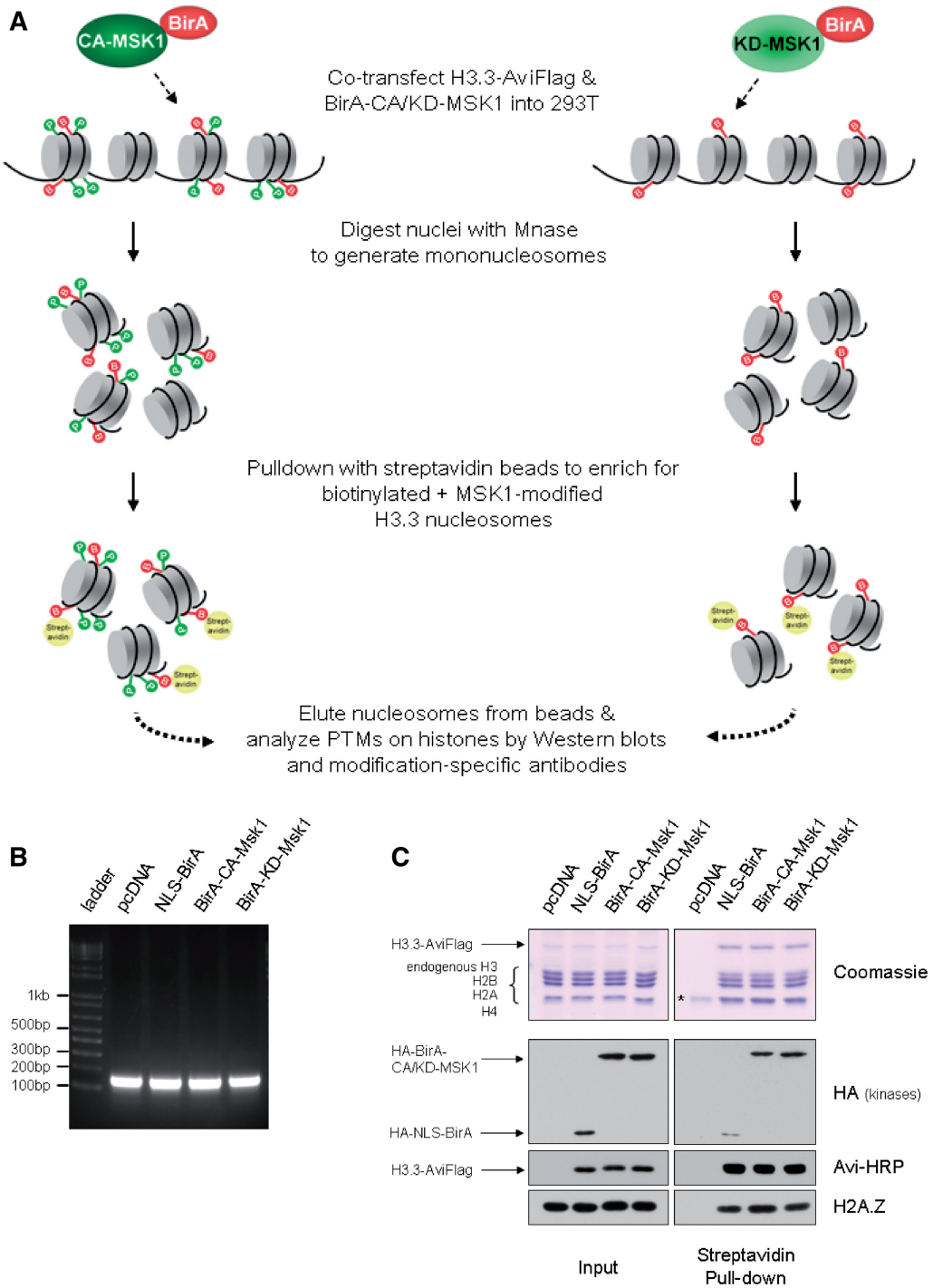

Figure 2. Affinity purification of BirA-biotinylated and MSK1-modified nucleosomes. (**A**) Workflow of the affinity purification method used for isolation of co-modified nucleosomes. (**B**) DNA was extracted from MNase-digested input chromatin, resolved on a 2% agarose gel and visualized with ethidium bromide. (**C**) Mono-nucleosomes containing H3.3-AviFlag were purified as described in (A). Input chromatin (left) and pull-down material (right) were resolved on SDS-PAGE gels and visualized by coomassie blue staining (top) or analysed by Western blotting using the indicated antibodies (bottom). * streptavidin monomer.

assays using antibodies specific to these marks on two independent MSK1-target gene systems. First, we made use of our previously described NF1-targeting system, whereby we target MSK1 to endogenous NF1-responsive genes by fusing the DNA-binding domain of NF1 to MSK1 (15). Targeting MSK1 to the polycomb-silenced α-globin gene through this method reactivated its expression in non-erythroid cells, and this reactivation was associated with an increase in H3K27ac and H3K27ac/S28ph (15). As shown in Figure 3C, these modifications were also enriched on MSK1-modified H3.3 nucleosomes isolated by BICON, thus confirming the consistency

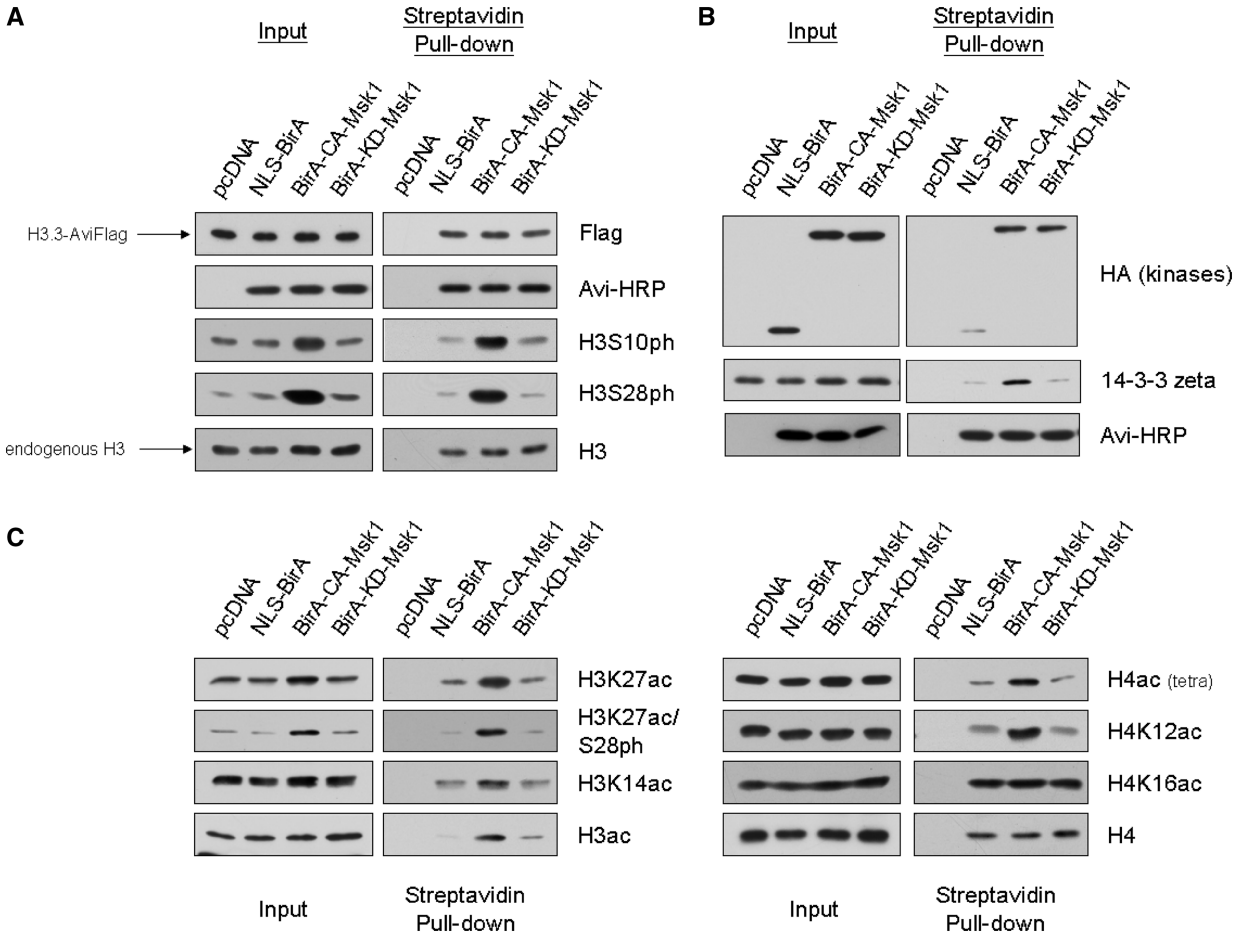

Figure 3. MSK1-modified nucleosomes are enriched for H3 acetylation and H4 acetylation. (**A**) MSK1-modified nucleosomes were purified as outlined in Figure 2A. Input and affinity-purified mono-nucleosomes were resolved on 15% SDS-PAGE and analysed by Western blotting. Total amount of transfected H3.3 was detected using α-Flag antibody. Endogenous H3 was detected using α-H3 antibody and was used as a loading control. Biotinylation and phosphorylation levels on H3.3-AviFlag were detected using Avi-HRP and phospho-specific antibodies (α-H3S10ph, α-H3S28ph), respectively. (**B**) MSK1-modified nucleosomes and its associated proteins were purified as in Figure 2A. Association of 14-3-3ζ with biotinylated H3.3 nucleosomes were analysed using α-14-3-3ζ antibody. (**C**) Acetylation levels on ectopic H3.3 and endogenous H4 were analysed using site-specific α-acetyl antibodies.

between the biochemical purification and *in vivo* ChIP results. We then asked whether H4 acetylation is involved in this MSK1-mediated reactivation process. Specifically, we used ChIP assays to examine the levels of H4ac at the α-globin promoter. Consistent with our biochemical findings, we indeed found that activation of the α-globin gene by NF1-CA-MSK1 targeting is accompanied by increased H4ac and H4K12ac levels at the promoter of the activated gene (Figure 4A, top). This increase was specific to the reactivated α-globin promoter, since no change in H4ac and H4K12ac levels was observed in an upstream control region (Figure 4A, bottom). Finally, it is interesting to note that we did not observe any change in H4K16ac level at the MSK1-activated α-globin promoter. This suggests that the MSK1-H4K12ac link is distinct from the previously discovered link between PIM1-mediated H3S10 phosphorylation and H4K16 acetylation (21).

As a second test, we examined the activation of IEG *c-fos* by phorbol ester 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA). Activation of IEGs by growth factors or phorbol esters is a classic model for studying H3 phosphorylation and gene activation (13,14). Upon TPA stimulation, IEGs such as *c-fos* are strongly induced and this is associated with an increase in H3 phosphorylation by MSK1 (18,36,37). TPA also induced H3 acetylation (H3K9/K14ac) at the regulatory regions of several IEGs, and this increase is dependent on the kinase activity of MSK1 (18). We therefore used this as a second model system to futher test the biological relevance of our observed link between H3 phosphorylation and H4 acetylation. Similar to results at the α-globin gene, we found an increase in H4ac and H4K12ac, but not H4K16ac, levels at the *c-fos* promoter upon TPA stimulation (Figure 4B). More importantly, pre-treatment with H89, which inhibits MSK1 activity, greatly compromised TPA-induced H4 acetylation (H4ac and H4K12ac). This was especially evident at the earlier times points (15 min and 30 min), suggesting that H4K12 acetylation is downstream of H3 phosphorylation. This reduction in H4 acetylation also correlated well with the reported reduction in *c-fos* gene expression in the H89-treated cells and MSK1/2 knockout mouse fibroblasts

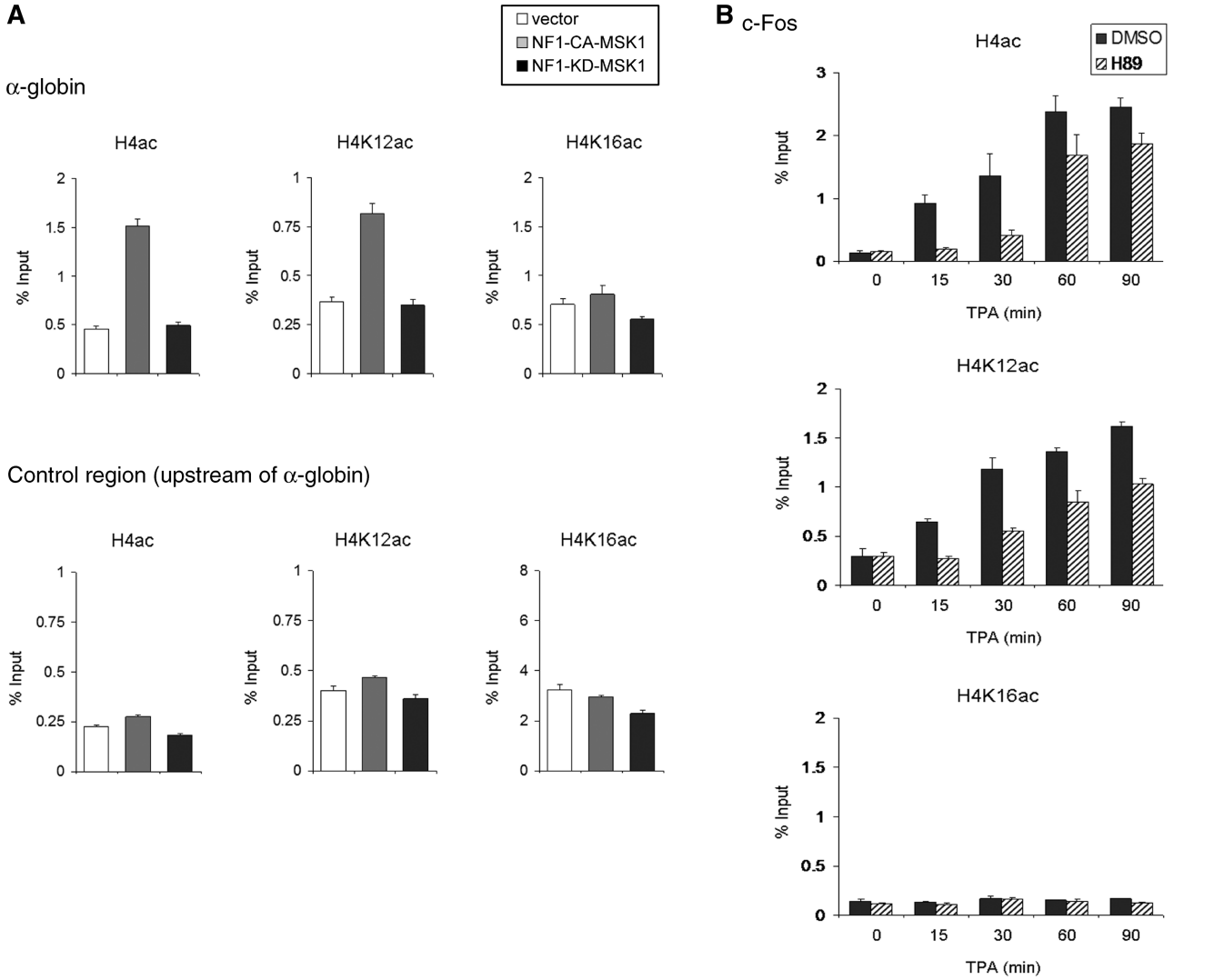

Figure 4. H4K12, but not H4K16, acetylation is induced upon activation of MSK1-target genes. (**A**) ChIP assays were performed on 293T cells transfected with vector or NF1-MSK1 (CA or KD). H4 acetylation levels on the α-globin promoter (top) and a control upstream region (bottom) were examined using antibodies against H4ac (left), H4K12ac (middle) and H4K16ac (right). The increase in H4ac and H4K12ac is dependent on the kinase activity of MSK1 and correlates with reactivation of the α-globin gene by NF1-CA-MSK1. (**B**) Serum-starved 293T cells were pre-treated with DMSO (mock treatment) or H89 (MSK1 inhibitor) for 30 min before stimulation with 400 ng/ml TPA. H4 acetylation levels on the *c-fos* promoter were examined by ChIP assays. TPA-induced H4 acetylation (H4ac, H4K12ac) was greatly compromised upon treatment with kinase inhibitor H89. ChIP-qPCR data are presented as % of precipitated material relative to the amount in the input sample (% input) and have been normalized to H3 levels to adjust for nucleosome density. Error bars indicate means ± SD ($n = 3$).

(36,37). As the duration of TPA stimulation increased, we observed an increase in H4 acetylation, albeit to a lesser extent, in H89-treated cells. This points to a role for H3 phosphorylation in expediting acetylation at the IEG promoter, but an independent mechanism involving acetylation may also be involved as the stimulation continues (38). All together, these ChIP assays further validated our biochemical pull-down results and confirmed the biological relevance of our newly identified *trans*-tail crosstalk between MSK1 and H4K12 acetylation.

DISCUSSION

Here, we describe a new approach, which we termed BICON, to isolate nucleosomes specifically modified by a histone-modifying enzyme of choice. Importantly, this method allowed us to study the crosstalks among histone PTMs initiated by the enzyme of interest. In line with our interest in H3 phosphorylation, we have chosen to study the H3 kinase MSK1 and H3.3 phosphorylation in this pilot study, and found that MSK1 initiates extensive crosstalks linking histone phosphorylation and acetylation. These data represent the first demonstration of a histone-modifying enzyme being sufficient to initiate a series of PTM crosstalks among the histones within a nucleosome. Increasing evidence shows that individual histone PTMs do not just function in isolation; it is therefore important to identify the combinatorial marks that function together and determine how these patterns are established on chromatin. To our knowledge, this is the first method specifically

designed to identify the 'nucleosome-code' initiated by a specific histone-modifying enzyme. This potentially paves the way for additional studies investigating other histone-modifying enzymes (such as histone acetyltransferases, methyltransferases, E3 ligases). Expansion of this method will allow systematic elucidation of crosstalk pathways associated with the ever-growing list of histone-modifying enzymes and their corresponding PTMs.

We and others have previously proposed that chromatin can act as a signaling platform that receives signals from upstream activated signaling cascades and transmits them to downstream effectors (2,3). This idea is particularly well illustrated by the collective studies on MSK1-mediated H3 phosphorylation. In response to mitogen or stress stimulation, MSK1 is activated and phosphorylates H3 on S10 and/or S28 at specific target genes and induces their expression. Our findings clearly showed that MSK1 not only induces H3 phosphorylation, but also initiates a series of downstream events on chromatin, including the binding of 14-3-3 to MSK1-phosphorylated H3, and other PTM changes within the nucleosome. Using the BICON approach, we discovered that the crosstalks not only occur *in cis* on the same histone (H3.3), but also extend *in trans* to H4 within the same nucleosome. Therefore, H3 phosphorylation can be seen as a means to integrate cellular stimuli and signal this information to the transcriptional machinery through multiple chromatin modifications.

Our study revealed a novel *trans*-tail crosstalk between MSK1-mediated H3 phosphorylation and H4K12 acetylation. However, it is currently unclear how such crosstalk is established. It is possible that MSK1 physically associates with the H4K12 acetyltransferase (HAT), and thus both enzymes are recruited to co-modify nucleosomes on MSK1 target genes. The H4 HAT could also be recruited indirectly through phospho-H3 binding proteins, such as through 14-3-3 at the *FOSL1* enhancer (21). In either case, identifying the HAT responsible for H4K12ac, as well as its mode of recruitment to the target genes, would help elucidate the mechanistic details of this crosstalk pathway. Alternatively, phosphorylated H3 may have a negative effect on the binding or activity of histone deacetylases (HDAC), leading to an increase in overall histone acetylation. It is of interest to note that the previously reported link between H3S10 phosphorylation and H4K16 acetylation was initiated by a different H3 kinase, PIM1 (21). This crosstalk occurs at the *FOSL1* enhancer and is associated with transcriptional elongation. In light of our observed coupling of MSK1-mediated H3 phosphorylation and H4K12 (but not K16) acetylation, this raises the possibility that different H3 kinases activate different crosstalk pathways, which may then lead to different downstream functions.

In this study, we focused on analysing combinatorial PTMs on nucleosomes, but BICON can potentially be extended to study the interaction of PTM-specific binders to chromatin. Using our co-modification approach, we are the first to demonstrate binding of 14-3-3ζ to phosphorylated H3 nucleosomes in non-crosslinked samples *in vivo*. Previously, this interaction has only been characterized by *in vitro* binding assays and its association with target genes by ChIP assays (18,19,35). Unlike the commonly used peptide-based assays, a major strength of BICON is that it allows the identification of PTM-binding proteins under physiological conditions and in a more relevant chromatin context, thus providing insights into PTM-dependent interactions *in vivo*. It is now clear that binding of effectors is sensitive to the presence of PTMs on neighboring residues, and multivalent recognition of chromatin marks allows for cooperative and stronger binding (39). Therefore, further adaption of this technique can complement existing *in vitro* binding assays and will be highly useful for identifying factors that bind nucleosomes through multivalent interactions.

In conclusion, we have successfully established the feasibility of BICON as a method for probing combinatorial PTMs within a nucleosome, and also revealed potential future applications of this nucleosome purification system. Our proof-of-principle study with H3 kinase MSK1 and H3 phosphorylation lays the foundation for more extensive examination of crosstalks between different histone PTMs, and suggests that BICON can be widely applicable to enzymes involving other key histone PTMs. As such, this new technique could be an invaluable tool to help advance our understanding of these combinatorial histone marks and further elucidate their functions in various cellular processes.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–2.

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