Histone code pathway involving H3 S28 phosphorylation and K27 acetylation activates transcription and antagonizes polycomb silencing

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Histone H3 phosphorylation is a critical step that couples signal transduction pathways to gene regulation. To specifically assess the transcriptional regulatory functions of H3 phosphorylation, we developed an in vivo targeting approach and found that the H3 kinase MSK1 is a direct and potent transcriptional activator. Targeting of this H3 kinase to the endogenous *c-fos* promoter is sufficient to activate its expression without the need of upstream signaling. Moreover, targeting MSK1 to the α-globin promoter induces H3 S28 phosphorylation and reactivates expression of this polycomb-silenced gene. Importantly, we discovered a mechanism whereby H3 S28 phosphorylation not only displaces binding of the polycomb-repressive complexes, but it also induces a methyl-acetylation switch of the adjacent K27 residue. Our findings show that signal transduction activation can directly regulate polycomb silencing through a specific histone code-mediated mechanism.

chromatin | gene expression | phospho-acetylation | methylation

H3 phosphorylation is a downstream event for a number of signal transduction pathways in mammalian cells (reviewed in refs. 1 and 2). For example, rapid and transient phosphorylation of H3 at S10 (H3S10ph) is detected at the promoters and coding regions of immediate-early (IE) genes upon stimulation of the MAPK or p38 pathways, suggesting that this signaling-induced histone modification functions to activate transcription of IE genes. Thus far, multiple kinases, including RSK2, MSK1/2, PIM1, and IKKα, have been shown to directly phosphorylate H3. As a common target of diverse signaling cascades, H3 phosphorylation is thought to be a critical step translating signal transduction information to the chromatin/transcriptional regulatory machinery (3).

Currently, how H3 phosphorylation is mechanistically linked to the transcriptional process is still not fully understood. H3 S10 phosphorylation can be physically coupled to acetylation of nearby lysine residues (K9 or K14), suggesting that combinations of these modifications function together to activate transcription (4–6). Specific isoforms of 14-3-3 directly bind H3S10ph, and this interaction is greatly enhanced by additional acetylation of the nearby K14 residue, further illustrating the biological relevance of specific combinations of histone modifications (7, 8). Finally, recruitment of 14-3-3 through H3S10ph at the promoters of *HDAC1* and several IE genes is thought to facilitate transcription induction of these genes (7, 9, 10). Binding of 14-3-3 to H3S10ph at the *FOSL1* enhancer, which is located downstream of the transcription start site, initiates sequential recruitment of the histone acetyltransferase (HAT) males absent on the first (MOF), the bromodomain-containing protein 4 (BRD4), and the positive transcription elongation factor b (P-TEFb) (10). At least for this particular gene, H3 S10 phosphorylation leads to the release of the preinitiated but paused RNA polymerase II (RNAP II) and facilitates transcriptional elongation. In addition to S10, H3 is also phosphorylated at S28, but the link between this second phosphorylation site and transcriptional regulation is less well studied. 14-3-3 also binds strongly to H3S28ph peptides in pull-down assays, and nucleosomes at the promoters of several IE genes (*Jun* and *FOSL1*) are also phosphorylated at H3 S28 upon 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) induction (9). Interestingly, immunofluorescence and sequential immunoprecipitation experiments using phospho S10- and S28-specific antibodies suggest that these modifications occur on distinct populations of H3 (11, 12). At present, the functional differences between S10- and S28-phosphorylated H3 have not been explored.

The induction of multiple transcriptional activation programs by signal transduction cascades poses a significant complication in assessing the direct transcriptional function of H3 phosphorylation in vivo. For example, fibroblasts from MSK1/2 double-knockout mice no longer phosphorylate H3 in response to stimulation, but their IE genes are still activated, albeit with delayed kinetics (13). To circumvent this complication, we developed a method to directly target H3 kinases to reporter and endogenous genes and to determine the specific functions of H3 phosphorylation in the transcription process. Here, we show that MSK1 is a direct and potent transcription activator. Interestingly, our data suggest that H3 S10 phosphorylation alone is not sufficient to initiate transcription, whereas induction of H3 S28 phosphorylation correlated better with this process. In support of this observation, we found that targeting MSK1 to the polycomb-silenced α-globin gene reversed its silencing and reactivated its expression. Mechanistically, phosphorylation of H3 S28 displaces binding of polycomb group proteins and reduces the amount of H3K27me3 at the α-globin promoter. More importantly, targeting MSK1 also induces H3K27 acetylation, leading to the enrichment of K27ac/S28ph di-modified H3 at the α-globin promoter. This shows that H3 K27 acetylation and S28 phosphorylation are directly coupled and suggests that such combination can functionally antagonize polycomb silencing.

Results

H3 Kinase MSK1, Not RSK2, Activates Transcription of Reporter. To study the effects of H3 phosphorylation on transcriptional activation, we first asked whether H3 phosphorylation, through direct targeting of known H3 kinases, is sufficient to activate transcription of reporter genes. We fused constitutively active (CA) or kinase-dead (KD) versions of two well-studied H3 kinases, RSK2 and MSK1 (13, 14), to the Gal4 DNA-binding domain (DBD) sequences and transfected these constructs into 293T cells along with a luciferase reporter containing five Gal4-binding sites upstream of the SV40 promoter. Despite the equal expression levels of RSK2 and MSK1 kinases in the transfected cells (Fig. 1*A*, *Right*), only Gal4-CA-MSK1 strongly activated expression of the luciferase reporter (Fig. 1*A*, *Left*). Induction of

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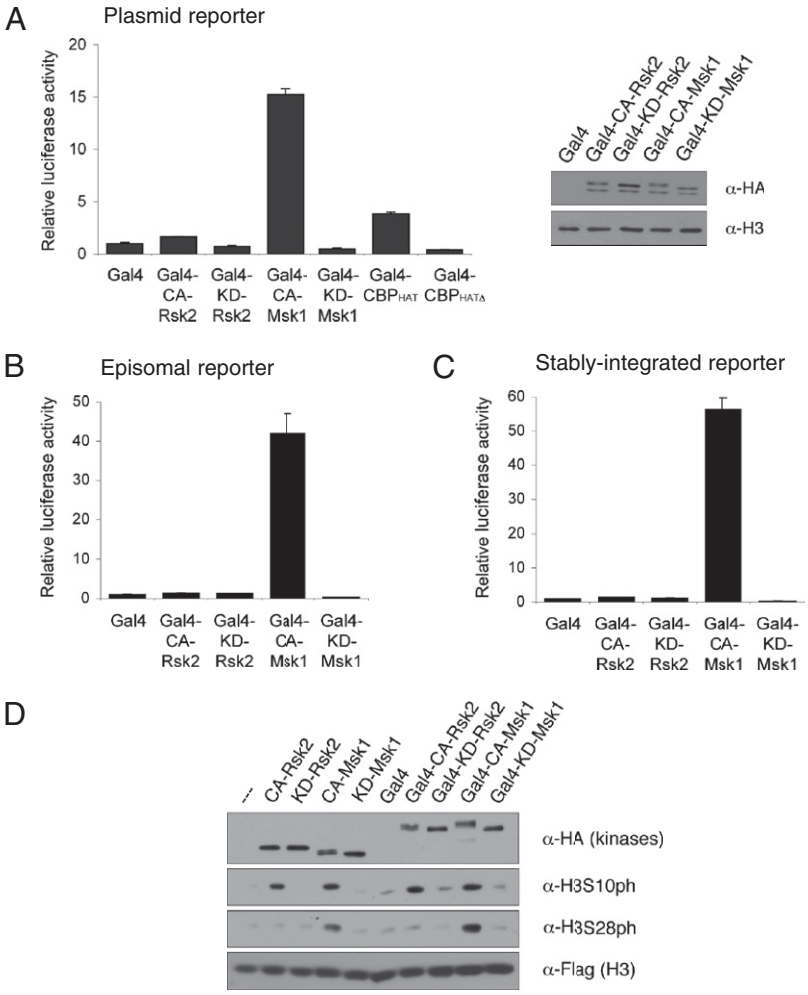

Fig. 1. MSK1, but not RSK2, phosphorylates H3 at serine 28 and activates transcription of reporter. (*A, Left*) Luciferase activities were measured 48 h after cotransfection of Gal4 kinase constructs and luciferase reporter into 293T cells. The transcriptional activator Gal4-CBP was used as a positive control. Luciferase levels were normalized to β-gal levels from a cotransfected β-gal plasmid, and data are represented as means ± SD from three independent experiments. (*Right*) Expression of HA-tagged Gal4 kinase fusion proteins was detected by Western blotting using an HA antibody. Total H3 was used as a loading control. (*B* and *C*) Luciferase assays were performed as in *A*, in cells harboring an episomal reporter (*B*) or in a 293T cell line containing a stably integrated reporter (*C*). (*D*) Flag-tagged H3 was cotransfected with RSK2 or MSK1 constructs into 293T cells. Phosphorylation levels of transfected H3 at S10 and S28 were examined by Western blotting. Total amount of transfected H3 was detected by using Flag antibody and was used as a loading control.

the reporter gene by MSK1 depended completely on a functional kinase domain (CA vs. KD constructs in Fig. 1*A*) and also on the presence of the Gal4-binding sites on the reporter (Fig. S1*A*). Moreover, the transactivation ability of Gal4-CA-MSK1 diminished proportionally when the Gal4-binding sites were systematically moved further upstream from the transcription start site of the luciferase gene, suggesting that promoter proximity of the kinase target is needed for maximal activation (Fig. S1*B*). We also performed this assay using luciferase reporters present on an episomal vector or stably integrated into the cellular genome. Again, in both cases, only Gal4-CA-MSK1 effectively activated transcription of the reporter gene (Fig. 1 *B* and *C*). Of note, induction of these more properly chromatinized reporter genes is significantly higher than the luciferase gene present on the nonepisomal plasmid (50- to 60-fold vs. ~20-fold induction, Fig. 1 *A*–*C*), supporting the conclusion that MSK1 activates transcription by phosphorylating a chromatin component such as H3.

One intriguing finding from this experiment is that only MSK1, but not RSK2, directly activated transcription of the reporter gene even though cotransfection of these kinases with H3 substrates showed that both kinases are equally efficient at phosphorylating H3 at S10 (Fig. 1*D*). Interestingly, only MSK1 effectively phosphorylates H3 at both S10 and S28, whereas RSK2 mainly phosphorylates H3 at S10 (Fig. 1*D*). Although many studies have strongly correlated H3S10ph with signaling-induced gene activation, our direct assay suggests that phosphorylation of H3 at S10 alone (in this case mediated by RSK2) is not sufficient for transcription initiation. Instead, it is H3 S28 phosphorylation, either alone or in combination with S10 phosphorylation, that correlates with this transcription step.

Targeting MSK1 to the Endogenous *c-fos* Promoter Activates Its Expression. To test whether H3 phosphorylation also directly activates endogenous IE genes, we took advantage of the nuclear factor 1 (NF1)–binding site present at the *c-fos* promoter and modified our targeting approach by fusing MSK1 to the DBD of NF1 (Fig. 2*A*). The addition of the NF1-DBD did not affect the activation of CA-MSK1, as indicated by the phosphorylation at the T-loop residue S212 of MSK1, a marker for the activated form of this kinase (Fig. 2*B*). However, addition of NF1-DBD to CA-MSK1 greatly enhanced phosphorylation of the endogenous H3 at S10 and S28 (Fig. 2*B*), suggesting that targeting this kinase to chromatin greatly increased its contact and phosphorylation of chromatin H3. Importantly, expression of the NF1-CA-MSK1 fusion proteins (in either orientation) also strongly activated transcription of the endogenous *c-fos* gene (Fig. 2*C*). Moreover, the NF1-KD-MSK1 controls did not activate *c-fos* transcription over background levels, confirming that this induction depends on the kinase activity of MSK1 and is not caused by cryptic transcriptional activation by the NF1-DBD. As additional controls, we also tested the effects of NF1-CA-MSK1 on two other endogenous genes, *c-jun* and *HSP70*. *c-jun* is another IE gene, but it does not have any NF1-binding sites in its promoter region, whereas the heat-shock protein *HSP70* gene contains an NF1-binding site at its promoter, and its expression has been associated with H3 phosphorylation (15). Quantitative RT-PCR analyses showed that NF1-CA-MSK1 induced expression of

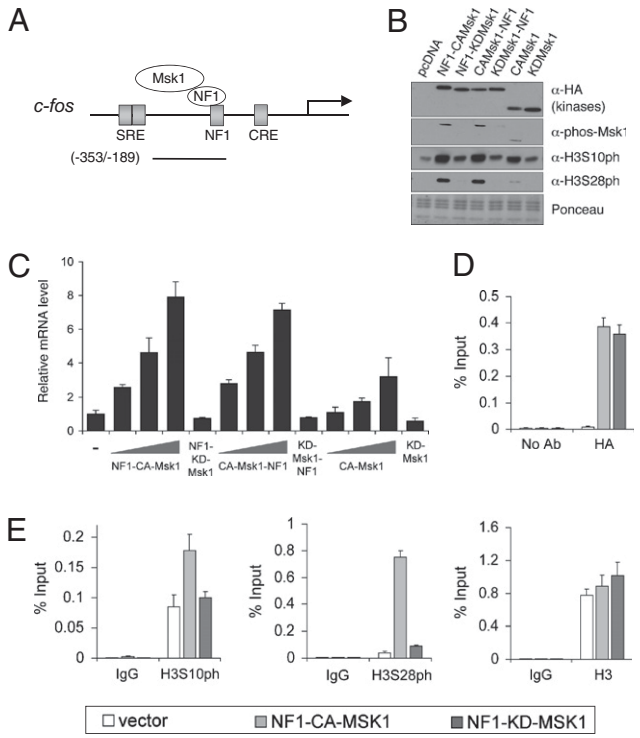

Fig. 2. Targeting MSK1 to the endogenous *c-fos* promoter induces H3 S10 and S28 phosphorylation and activates its expression. (*A*) Schematic diagram of the *c-fos* promoter showing the locations of *cis*-regulatory elements and primers used for ChIP analysis. CA- or KD-MSK1 was fused to the DBD of NF1 (residues 1–257) to directly target the H3 kinase to the endogenous *c-fos* promoter through its NF1-binding site. (*B*) Expression and kinase activity of different MSK1 constructs in 293T cells were examined by Western blotting using the indicated antibodies. Ponceau staining of core histones was used as loading control. (*C*) CA/KD-MSK1 constructs were transfected into 293T cells and their effects on expression of endogenous *c-fos* gene were determined by quantitative RT-PCR. *c-fos* mRNA levels were normalized to that of HPRT. (*D*) ChIP assays using an HA antibody showed that CA and KD versions of NF1-MSK1 fusions were both targeted to the promoter of endogenous *c-fos* gene. (*E*) ChIP analyses using antibodies against phosphorylated and total H3 showed induced H3 S10 and S28 phosphorylation at the *c-fos* promoter by NF1-CA-MSK1. Quantitative RT-PCR and ChIP–quantitative PCR results are represented as means ± SD ($n = 3$) and are representative of at least three independent experiments.

HSP70 but not *c-jun*. Together, these findings show that the engineered fusion kinase specifically activates genes that have NF1-binding sites (Fig. S2).

Using ChIP assays, we further confirmed that both NF1-CA-MSK1 and NF1-KD-MSK1 were targeted and enriched at the *c-fos* promoter with equal efficiencies (Fig. 2*D*). Consistent with their transcriptional activation abilities, only NF1-CA-MSK1, but not the KD version, increased both H3S10ph and H3S28ph levels at the *c-fos* promoter (Fig. 2*E*). We note that the induction of H3S28ph levels was much greater than the H3S10ph levels (30-fold vs. twofold increase), which supports our hypothesis that H3 S28 phosphorylation is more relevant to transcriptional activation compared with H3 S10 phosphorylation. Together, these results demonstrate that MSK1 can directly activate *c-fos* transcription and that targeting of this H3 kinase bypasses the requirement of upstream signaling to activate an IE gene.

MSK1 and H3 Phosphorylation Reactivate Expression of Polycomb-Silenced α-Globin in Nonerythroid Cells. Given our hypothesis that H3 S28 phosphorylation is important for transcriptional activation, we next asked whether there is functional interplay between this modification and the repressive mark on the adjacent K27 residue. Methylation of K27 on H3 is a key step in the polycomb-silencing pathway, which regulates a large number of genes critical for development and differentiation (reviewed in ref. 16). Previous studies have shown that H3 phosphorylation at S10 disrupts the binding of heterochromatin protein 1 (HP1) to the adjacent methylated K9 residue and functions to displace HP1 from condensed chromosomes during mitosis (17, 18). Given the highly analogous nature of H3 K9 methylation/S10 phosphorylation and K27 methylation/S28 phosphorylation, we wondered whether S28 phosphorylation also disrupts the H3 K27 methylation–mediated polycomb pathway.

By targeting MSK1 to an endogenous polycomb-silenced gene, we could directly test the impact of H3 S28 phosphorylation on this repression pathway. To that end, we have chosen the tissue-specific α-globin gene (*HBA1/2*) as a model polycomb regulated gene for several reasons. First, the α-globin gene is a developmentally regulated and tissue-specific gene expressed only in erythroid cells, and its repressed state in nonerythroid cells is mediated by H3 K27 methylation and polycomb repressive complex 2 (PRC2) (19). Second, the silenced gene can be reactivated in nonerythroid cells upon herpes simplex virus infection (20) or by treatment with the histone deacetylase inhibitor trichostatin A (19). Finally, a conserved NF1 recognition element is also present in the α-globin promoter (21). Similar to the previous results for the *c-fos* gene, ChIP assays showed that

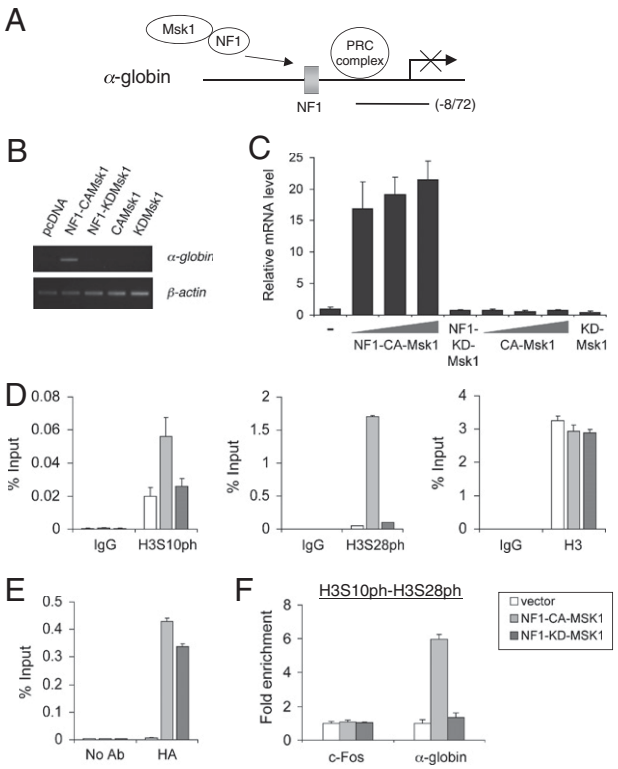

Fig. 3. MSK1 and H3 phosphorylation reactivate expression of polycomb-silenced α-globin gene in nonerythroid cells. (*A*) Schematic diagram of the α-globin promoter showing the targeting of MSK1 to the endogenous α-globin promoter through its NF1-binding site and the location of primers used for ChIP analysis. (*B* and *C*) Expression of α-globin in 293T cells expressing different MSK1 constructs was measured by semiquantitative (*B*) and quantitative (*C*) RT-PCR. (*D* and *E*) ChIP analyses using the indicated antibodies showed that both NF1-CA- and NF1-KD-MSK1 were targeted to the endogenous α-globin promoter, but only the active kinase induced H3 S10 and S28 phosphorylation. (*F*) Sequential ChIP assays were performed with antibodies against H3S10ph and H3S28ph. Coexistence of the two phospho marks in the same nucleosome was detected at the induced α-globin promoter but not the *c-fos* promoter.

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NF1-CA-MSK1 and NF1-KD-MSK1 were efficiently targeted to the α-globin promoter (Fig. 3*E*). In addition, only NF1-CA-MSK1 induced H3 S10 and S28 phosphorylation, and, again, the S28 site was the more significantly phosphorylated site (Fig. 3*D*). Finally, and most importantly, NF1-CA-MSK1 strongly activated transcription of the silenced α-globin gene (as measured by both semiquantitative PCR and quantitative real-time PCR, Fig. 3 *B* and *C*), whereas neither the NF1-KD-MSK1 nor the nontargeted CA-MSK1 could reactivate this gene. This observation strongly suggests that polycomb silencing on the α-globin promoter can be disrupted or reactivated by targeted H3 S28 phosphorylation.

Because we detected both H3S10ph and H3S28ph at the promoter regions of *c-fos* and α-globin, we further asked whether these two phospho marks exist in the same nucleosome by performing sequential ChIP assays. Previous studies have shown that TPA-induced phosphorylation of H3 S10 and S28 does not occur on the same nucleosome at IE gene promoters (9). Consistent with those results, we also observed that H3S10ph and H3S28ph do not coexist within the same nucleosome at the NF1-CA-MSK1–activated *c-fos* promoter (Fig. 3*F*). However, we did detect a strong co-occupancy of the two phospho H3 marks at the activated α-globin promoter, suggesting that these two marks can coexist. At present, we do not know what dictates whether H3S10ph and S28ph can coexist or not, but the underlying differences in the genomic context of *c-fos*, a gene that is poised for expression, and α-globin, a gene that is actively silenced, are likely determining factors.

H3 S28 Phosphorylation Disrupts PRC Recruitment and H3 K27 Methylation. To gain additional mechanistic insights into the reactivation of the α-globin gene, we used ChIP assays to examine the H3K27me3 status as well as recruitment of representative PRC components EZH2 and BMI1 at the reactivated α-globin promoter. We note that our initial Western blot analyses of total cell lysates showed that expression of NF1-CA-MSK1 strongly reduced H3K27me3 levels (Fig. S3*A*). However, more detailed tests revealed that the apparent loss of H3K27me3 was caused by epitope masking or occlusion whereby the H3K27me3 antibody does not recognize its epitope when the adjacent S28 residue is phosphorylated. Indeed, when we pretreated the lysates with lambda phosphatase, detection of the H3K27me3 epitope by the antibody was restored (Fig S3*A*). To eliminate this potential epitope occlusion problem in our ChIP analyses, we performed all of the H3K27me3, as well as H3K27ac, ChIP assays using phosphatase-treated chromatin. With such technical precautions, we found that the α-globin promoter in 293T cells is enriched for H3K27me3, and the enrichment level is similar to that of two other polycomb-silenced genes, *HoxD4* and *Myt1* (Fig. S3*B*). In addition, cells expressing NF1-CA-MSK1 consistently have reduced levels of H3K27me3, EZH2, and BMI1 at the α-globin promoter (Fig. 4*A*, all reduced by ~25–35% compared with the vector-alone control). The depletion of these polycomb-silencing hallmarks depends on the kinase activity of MSK1, suggesting that phosphorylation of H3 at S28 displaces binding of EZH2 and BMI1. Our findings show that a phos/methyl switch mechanism does exist in the case of H3S28ph whereby this modification functionally antagonizes the recruitment of PRC components. Moreover, even without complete loss of H3K27me3, strategic targeting of the MSK1 kinase is sufficient to reactivate the previously silenced α-globin gene.

Coupling of H3 S28 Phosphorylation and K27 Acetylation Antagonizes Polycomb Silencing. In many cases, activation of polycomb-regulated genes is not only associated with a loss of H3K27me3 but also is accompanied by a concomitant gain in H3 K27 acetylation (22). Therefore, we also examined the impact of NF1-CA-MSK1 on H3K27ac at the global and gene-specific levels. Remarkably, expression of NF1-CA-MSK1 not only enhanced H3 S10 and S28 phosphorylation but also induced a significant increase in H3 K27 acetylation (Fig. 4*B*). This increase depends on the kinase activity of MSK1, indicating that H3K27ac is functionally coupled to the S28 phosphorylation event. By ChIP

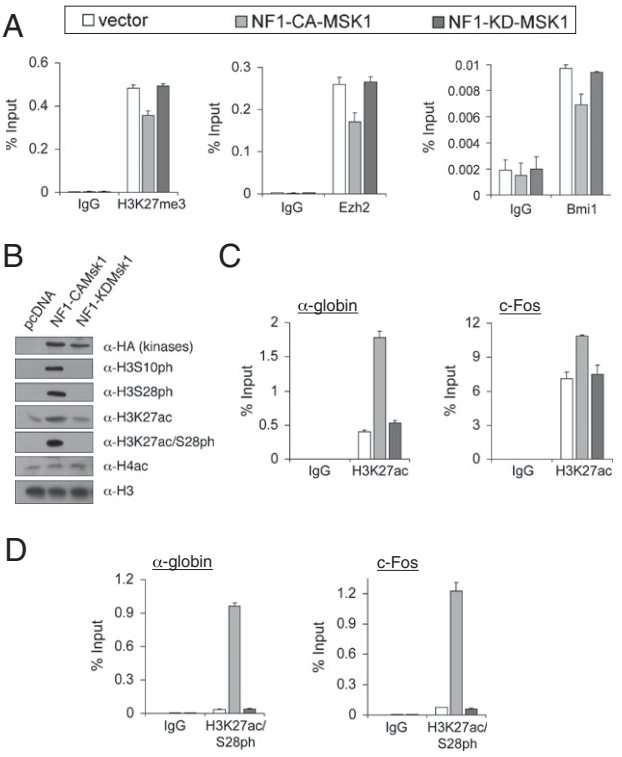

Fig. 4. Functional and physical coupling of H3 K27 acetylation and S28 phosphorylation antagonizes polycomb silencing. (*A*) ChIP assays were performed to examine the H3K27me3 status and recruitment of PRC components at the α-globin promoter. Cells expressing NF1-CA-MSK1 consistently have lower levels of H3K27me3 (*Left*) and reduced binding of PRC2 subunit Ezh2 (*Center*) and PRC1 subunit Bmi1 (*Right*) at the reactivated α-globin promoter. (*B*) Total cell lysates from transfected 293T cells were examined by Western blot analyses. Global changes in posttranslational modifications on H3 were determined by using the indicated antibodies. (*C*) Increase in H3K27 acetylation levels was detected by ChIP at the α-globin and *c-fos* promoter in NF1-CA-MSK1–transfected cells. (*D*) ChIP analyses using an antibody specific for the di-modified H3K27ac/S28ph mark showed that H3 K27 acetylation and H3 S28 phosphorylation are physically linked and coexist on the same H3 tail at the induced α-globin and *c-fos* promoters.

analyses, we found that NF1-CA-MSK1 targeting also increased the H3K27ac levels at the *c-fos* and α-globin promoters (Fig. 4*C*). The relative increase of H3K27ac at the *c-fos* promoter is not as high as that at the α-globin promoter because of the already high steady-state levels of H3K27ac associated with the *c-fos* gene.

A functional coupling between H3 K27 acetylation and S28 phosphorylation has not been reported before. To test whether these two modifications are also physically linked, we used an antibody that specifically recognizes the di-modified H3K27ac/S28ph epitope (Fig. S4), similar to the antibody we previously generated against H3S10ph/K14ac (4). Western blotting of total histones showed that expression of NF1-CA-MSK1 induced the phosphoacetylation of S28 and K27 on the same H3 molecule (Fig. 4*B*). More importantly, ChIP assays showed that such di-modified epitope is specifically increased at the NF1-CA-MSK1–activated α-globin promoter, indicating that the combination of these two modifications directly correlates with the reactivation of this silenced gene (Fig. 4*D*). Additional experiments further showed that activation of MSK1 through mitogen or stress stimulation also induces the H3K27ac/S28ph mark (Fig. S5). Together, these findings demonstrate that MSK1-mediated H3S28ph, in response to various upstream signaling conditions, enhances acetylation of the adjacent K27 residue on H3.

Both H3S28ph and H3K27ac/S28ph Are Directly Associated with the S5-Phosphorylated Form of RNA Polymerase II (RNAP II), RNAP-S5ph. Given our finding that the stimulated form of MSK1 directly activates transcription, and that this enzyme induces H3S10ph, S28ph, and H3K27ac/S28ph, we further tested whether all or some of these modified forms of H3 are specifically associated with transcription initiation. To do this, we performed sequential ChIP assays by first using antibodies against the aforementioned modified forms of H3 and followed by a second round of ChIP using an antibody against RNAP-S5ph, which represents the initiating form of RNAP II. By this assay, we found a strong association of H3S28ph, as well as H3K27ac/S28ph, with RNAP-S5ph at both NF1-CA-MSK1–activated *c-fos* and α-globin promoters (Fig. 5 *A–B*). In contrast, no co-occupancy of H3S10ph and RNAP-S5ph was observed at these loci in any of the transfected cells. Our data suggest that, at both the *c-fos* and α-globin promoters, the initiating form of RNAP II is directly and physically associated with H3S28ph and H3K27ac/S28ph but not with H3S10ph.

Discussion

H3 phosphorylation is mostly studied in the context of rapid activation of signal-inducible genes, such as the induction of IE genes upon mitogen or stress stimulation. However, recent studies showed that additional signaling pathways, such as the Toll-like receptor and retinoic acid signaling, also activate MSK1 to regulate non-IE genes (23, 24). Although many studies have provided excellent correlative data linking this histone modification to transcriptional regulation, they cannot distinguish between direct and indirect effects of H3 phosphorylation. Here we show that the H3 kinase MSK1 is a potent transcription activator when directly targeted to diverse promoters such as the luciferase reporter, the endogenous IE gene *c-fos*, and the polycomb-silenced α-globin gene. Most studies to date have focused on H3 S10 phosphorylation; however, our results suggest that phosphorylation of this site alone, mediated by the RSK2 kinase, is not sufficient to directly transactivate the luciferase reporter gene. Instead, it is the induction of H3 S28 phosphorylation that mirrors transcriptional activation. This finding is particularly evident in our ChIP analyses whereby we consistently observed much greater induction of H3S28ph, compared with H3S10ph, at the activated promoters. More importantly, sequential ChIP analyses showed that H3S28ph and H3K27ac/S28ph, but not H3S10ph, are directly associated with the transcription-initiating form of RNAP II.

Throughout our studies, we consistently observed a correlation between H3S28ph and transcriptional activation/initiation at the MSK1-targeted genes. However, previous studies also found that H3 S10 phosphorylation at the *FOSL1* enhancer (10) and *Drosophila* heat-shock genes (25) promotes the release of paused RNAP II from a promoter proximal site through the recruitment of the transcription elongation factor P-TEFb. It is possible that H3 S10 phosphorylation facilitates transcriptional elongation of genes that are regulated by polymerase pausing, whereas H3 S28 phosphorylation directly activates transcription at the initiation step. Further studies will be required to distinguish between the roles of H3 phosphorylation at these distinct sites in the transcriptional initiation and elongation steps.

To date, little is known about the link between H3 S28 phosphorylation and transcription. ChIP analyses showed that H3S28ph is enriched at IE gene promoters upon their activation (9). Interestingly, in chicken erythrocytes, H3 S28 phosphorylation preferentially occurs on the transcription-linked H3 variant H3.3 (26), supporting our hypothesis that S28 phosphorylation is functionally linked to transcriptional activation. Originally identified by a H3S10ph peptide pull-down assay, 14-3-3 actually has a much higher binding affinity for the H3S10ph/K14ac di-modified as well as the H3S28ph epitopes (7, 8). Given the general paradigm that histone modifications recruit modification–specific binding proteins to mediate downstream functions, it is likely that 14-3-3, or additional proteins, bind to the phospho S28 residue to facilitate transcriptional activation. In that regard, how acetylation of K27 might synergistically or antagonistically modulate recruitment of such S28ph binding protein represents yet another potential level of functional cross-talk between histone modifications. The mechanism that couples H3 K27 acetylation and S28 phosphorylation is currently unknown. It is possible that MSK1 and/or its kinase activity recruit an H3 K27 HAT to the promoter of target genes. Alternatively, H3 phosphorylated at S28 may be a better substrate for the H3 K27 HAT. In support of the first scenario, MSK1 was previously shown to coimmunoprecipitate with multiple HATs, including p300 and cAMP response element binding protein (CREB)-binding protein (CBP) (27), which are known to acetylate H3 at K27 (22). As for the second scenario, we and others have previous shown that the yeast HAT, Gcn5, preferentially acetylates H3S10ph peptides over the unmodified form (4). Therefore, H3 K27 HATs may also have a preference for S28-phosphorylated H3 as substrate. These two possibilities are not necessarily mutually exclusive, but further experiments will be required to test these hypotheses.

By using the tissue-specific α-globin gene as a model polycomb-regulated gene, our study identified a histone code pathway whereby H3 S28 phosphorylation induces a methyl-acetylation switch on the adjacent K27 residue (Fig. 6). More-

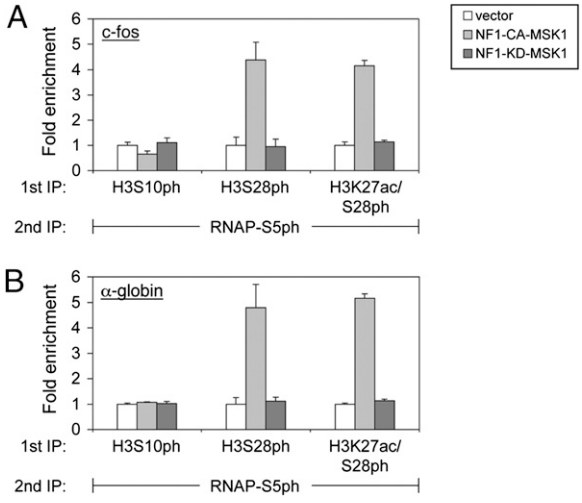

Fig. 5. H3S28ph and H3K27ac/S28ph marks correlate with transcription initiation at the activated *c-fos* and α-globin promoters. Sequential ChIP assays were performed on the promoters of *c-fos* (*A*) and α-globin (*B*). Antibody against H3S10ph (*Left*), H3S28ph (*Center*), or H3K27ac/S28ph (*Right*) was used in the first ChIP and then followed by a second round of ChIP with antibody against phospho S5 of RNAP II (RNAP-S5ph), which represents the initiating form of RNAP II. Fold enrichment represents percentage input expressed relative to the vector-alone control at the corresponding promoter.

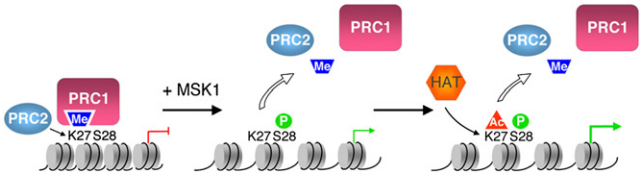

Fig. 6. H3 S28 phosphorylation initiates a unique histone code pathway by inducing a methyl-acetylation switch of the adjacent K27 residue. Our data support a model in which MSK1 and H3 S28 phosphorylation antagonize polycomb silencing through the displacement of PRCs and the removal of H3K27me3. Upon recruitment of a H3K27 HAT, the di-modified H3K27ac/S28ph mark is established, which can then recruit specific chromatin modifiers or transcription regulators to further modulate gene expression.

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over, this mechanism provides a direct link between signal transduction and polycomb regulation. Activation of polycomb target genes during differentiation is associated with displacement of polycomb group proteins and removal of H3K27me3; however, how this process is regulated is still poorly understood. Recent studies also showed that a switch from methylation to acetylation at H3 K27 often accompanies the activation of these genes. Our findings suggest that one way to regulate this switch could be through phosphorylation of the adjacent S28 residue. Given that genome-wide screens showed that many polycomb-regulated genes are downstream of diverse signaling pathways, our finding that H3 S28ph and K27ac are functionally coupled further raises the possibility that signal transduction pathways and activation of polycomb-regulated genes are directly linked through this unique histone code. If so, H3 S28 phosphorylation may have a yet-to-be appreciated function in modulating the epigenome during development and differentiation.

Materials and Methods

Plasmid Constructs. HA-tagged CA/KD-RSK2 and MSK1 have been previously described (28). Cloning of luciferase reporters and the Gal4 kinase and NF1 kinase fusions is described in *SI Materials and Methods*.

Cell Culture, Transfections, and Whole-Cell Lysis. 293T cells were grown in DMEM (Sigma) supplemented with 10% FBS. Stable cell line containing an integrated Gal4 luciferase reporter has been previously described (29). All transfections were performed with Lipofectamine 2000, and whole-cell lysates were prepared by directly lysing pelleted cells in boiling SDS sample buffer. Histones were resolved on 15% SDS/PAGE gels and analyzed by Western blotting using antibodies listed in Table S1.

Luciferase Reporter Assays. 293T cells transfected with Gal4 fusion constructs and luciferase reporters were harvested and lysed in cell culture lysis reagent (Promega) 48 h after transfection. Luciferase activities were measured with the Luciferase Assay System (Promega) per the manufacturer's instructions. Luciferase levels were normalized to β-gal levels from a cotransfected β-gal plasmid, and data are represented as means ± SD from three independent experiments.

RT-PCR. Total RNA was extracted with TRIzol Reagent (Invitrogen), and reverse transcription was carried out by using oligo(dT) primers and SuperScript II (Invitrogen) according to the manufacturer's instructions. Semiquantitative PCR was carried out with Taq polymerase (NEB), and PCR products were resolved on 2% agarose gel. β-Actin was used as loading control. Quantitative real-time PCR was performed with PerfeCTa SYBR Green SuperMix (Quanta BioSciences) on an Opticon 2 thermal cycler (BioRad). Relative mRNA levels were calculated with the GeneX program (BioRad), using hypoxanthine-guanine phosphoribosyltransferase (HPRT) as reference gene. Primer sequences are listed in Table S2.

ChIP and Sequential ChIP Assays. ChIP assays were performed as previously described (4), with minor modifications as detailed in *SI Materials and Methods*. Antibodies and primer sequences used are listed in Table S1 and Table S2.

Note Added in Proof. While this manuscript was under review, Gehani et al. (30) reported similar findings in that H3 S28 phosphorylation, mediated by MSK1/2, was important for activation of polycomb target genes in response to mitogenic, stress, and differentiation signals. Their conclusions are completely consistent with our findings. However, it is interesting to note that they identified a different posttranslational modification combination, H3K27me3/S28ph, which they interpret as important for activating polycomb-regulated genes. In contrast, our study showed that H3 K27 acetylation is both physically and functionally coupled to H3 S28 phosphorylation, and sequential ChIP assays confirmed that this di-modified H3 is directly associated with the initiating form of RNAP II. Further studies comparing the distribution and timing of these different dual modifications on H3 will help elucidate the functional differences and significances of the multiple combinations of H3 modifications in the polycomb regulation pathway.

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