

# A Novel Mammalian Complex Containing Sin3B Mitigates Histone Acetylation and RNA Polymerase II Progression within Transcribed Loci<sup>∇</sup>

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**Transcription requires the progression of RNA polymerase II (RNAP II) through a permissive chromatin structure. Recent studies of *Saccharomyces cerevisiae* have demonstrated that the yeast Sin3 protein contributes to the restoration of the repressed chromatin structure at actively transcribed loci. Yet, the mechanisms underlying the restoration of the repressive chromatin structure at transcribed loci and its significance in gene expression have not been investigated in mammals. We report here the identification of a mammalian complex containing the corepressor Sin3B, the histone deacetylase HDAC1, Mrg15, and the PHD finger-containing Pfl1 and show that this complex plays important roles in regulation of transcription. We demonstrate that this complex localizes at discrete loci approximately 1 kb downstream of the transcription start site of transcribed genes, and this localization requires both Pfl1's and Mrg15's interaction with chromatin. Inactivation of this mammalian complex promotes increased RNAP II progression within transcribed regions and subsequent increased transcription. Our results define a novel mammalian complex that contributes to the regulation of transcription and point to divergent uses of the Sin3 protein homologues throughout evolution in the modulation of transcription.**

Regulation of gene expression impacts virtually all cellular processes and relies primarily on accurate regulation of transcription. It has become increasingly clear that transcription itself is regulated not only at the level of initiation, but also during elongation and termination. While the molecular events underlying transcription initiation have been in part elucidated in the recent past, how transcription elongation and transcription termination modulate gene expression in mammals remains largely elusive.

In eukaryotes, the presence of nucleosomes interspersed along the chromatin fiber is believed to represent a major barrier for RNA polymerase II (RNAP II) entry and progression (30). To allow the recruitment of the transcription machinery and transcriptional initiation, the transcriptional start sites (TSS) of active genes are, for the most part, devoid of nucleosomes. In contrast, downstream transcribed regions are tightly packed with nucleosomes, which are likely to prevent aberrant entry of the transcription machinery within coding regions, but they can also hinder progression of the polymerase. In order for RNAP II to progress through the transcribed region, the generation of a permissive chromatin structure is needed and involves the removal and redeposition of nucleosomes along transcribed regions. Such a mechanism is believed to be controlled in part by posttranslational modifications of histones, such as by acetylation and methylation, which are actively involved in regulation of transcriptional elongation (2, 18, 20). Among these modifications, histone acetylation has long been recognized to be associated with transcriptional ac-

tivation. However, its function in transcription and its regulation have been studied mostly at promoters, where the presence of acetylated histones correlates almost invariably with active transcription (14). More recently, it has been suggested that a dynamic histone acetylation/deacetylation cycle mediated by chromatin remodeling complexes facilitates the displacement of nucleosomes and allows the progression of RNAP II, while keeping previously transcribed regions refractory to the aberrant recruitment of transcription factors (20, 33). Like histone acetylation, the presence of histone methylation throughout coding regions has also been investigated as it relates to transcriptional activation. At actively transcribed genes, histone trimethylated at lysine 4 (H3K4me3) is highly enriched around the promoters, while H3K36me3 is absent from promoters but found within the 3' moiety of coding regions, in a transcription-dependent manner. Recently, how these different molecular events are coordinated and their precise functions in the regulation of transcription elongation were partly elucidated through studies of *Saccharomyces cerevisiae* (4, 16, 17, 23).

In these studies, Rpd3S, a yeast complex composed of the histone deacetylase Rpd3, the transcription factor Sin3, and two additional proteins, Eaf3 and Rco1, was found to be required to prevent cryptic initiation of transcription in actively transcribed regions. This function is believed to be achieved through Rpd3-mediated deacetylation of histones downstream of transcriptional promoter region, thus resulting in a chromatin environment incompatible with initiation of transcription. The recruitment of Rpd3S in the coding regions depends on the direct binding of the Eaf3 chromodomain to the H3K36me3-containing nucleosomes. In addition, Rco1, a PHD (*plant homeodomain*) finger-containing protein, is also essential for tethering the complex to chromatin, although the precise molecular basis for this requirement remains unknown

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(21). Components of the yeast Rpd3S complex have well-defined mammalian counterparts; two highly homologous mammalian Sin3 proteins, Sin3A and Sin3B, have been initially identified through their interaction with the sequence-specific transcriptional repressors Mad1 and Mxi1 (1, 28). They were subsequently found to associate with histone deacetylases 1 and 2 (HDAC1 and HDAC2) (32). Numerous studies have demonstrated that mammalian Sin3 proteins can serve as recruitment interfaces between an HDAC-containing complex and sequence-specific transcription factors (9, 22, 24, 35, 42). In addition, we have previously demonstrated that an integral mammalian Sin3 complex is required for proper pericentric heterochromatinization, suggesting that the Sin3 complex can also be targeted to loci independently of sequence-specific transcription factors (8). Although mammalian Sin3 proteins are commonly thought of as corepressors and are found associated with promoters of silent genes, recent evidence indicates that mammalian Sin3 proteins are also present on loci corresponding to active genes, downstream of the transcriptional start site (39). How they are tethered to these specific regions and their function at actively transcribed loci remain unknown. Mammalian Mrg15, like its yeast homologue Eaf3, possesses a chromodomain that specifically binds H3K36me3 (45). Like its yeast counterpart, it has been identified as a stable component of both histone acetyltransferase (HAT) and HDAC complexes (3, 44), suggesting a function as a modulator of histone acetylation. Mrg15 was found to associate with both Sin3A (44) and Sin3B (13). However, its role in regulation of active genes has not been addressed.

Here, we present evidence that a mammalian Sin3B/HDAC1 complex regulates the level of histone acetylation and transcription of active genes through its recruitment downstream of the transcription start site. We identify mammalian Pf1, a PHD finger protein, as a homologue of Rco1, and show that all four components, Sin3B, HDAC1, Mrg15, and Pf1, can form a stable complex, which is recruited downstream of the transcriptional start site through complex interactions with histones. In addition, depletion of Sin3B or Pf1 leads to increased levels of transcription of constitutively active genes. Therefore, we propose an unsuspected role of the HDAC1/Sin3B/Pf1/Mrg15 tetrameric complex in fine-tuning gene expression downstream of TSS in constitutively active genes in mammals.

#### MATERIALS AND METHODS

**Cell culture, plasmids, and transfections.** Human embryonic kidney HEK293T cells and human cervical carcinoma HeLa cells were cultured in Dulbecco's modification of Eagle's medium (DMEM; catalog number 10-013-CV; Cellgro) supplemented with 10% newborn calf serum. Cells were maintained at 37°C in an atmosphere containing 5% CO<sub>2</sub> and 100% humidity. Sin3B and Pf1 wild type (WT) and mutants used in coimmunoprecipitation (co-IP) assays were cloned in a pBabe Puro vector. For complex reconstitution assays, Sin3B and Pf1 WT and mutants were cloned in a pcDNA3.1(+) vector. Detailed cloning and mutational strategies are available upon request. Transient-transfection assays with Ca-phosphate were performed using a standard procedure. The amount of plasmid used in the transfection mixtures was 2 µg unless otherwise stated. Stable transfections with shRNAs in HeLa cells were performed according to the manufacturer's recommendations (OpenBiosystems).

**Protein-protein interaction assays, micrococcal nucleosome extraction, and antibodies.** Endogenous coimmunoprecipitation was performed as follows. The nuclear extracts were prepared from HEK293T cells. Per co-IP mixture, one 10-cm dish was used. The cells were washed twice in ice-cold phosphate-buffered

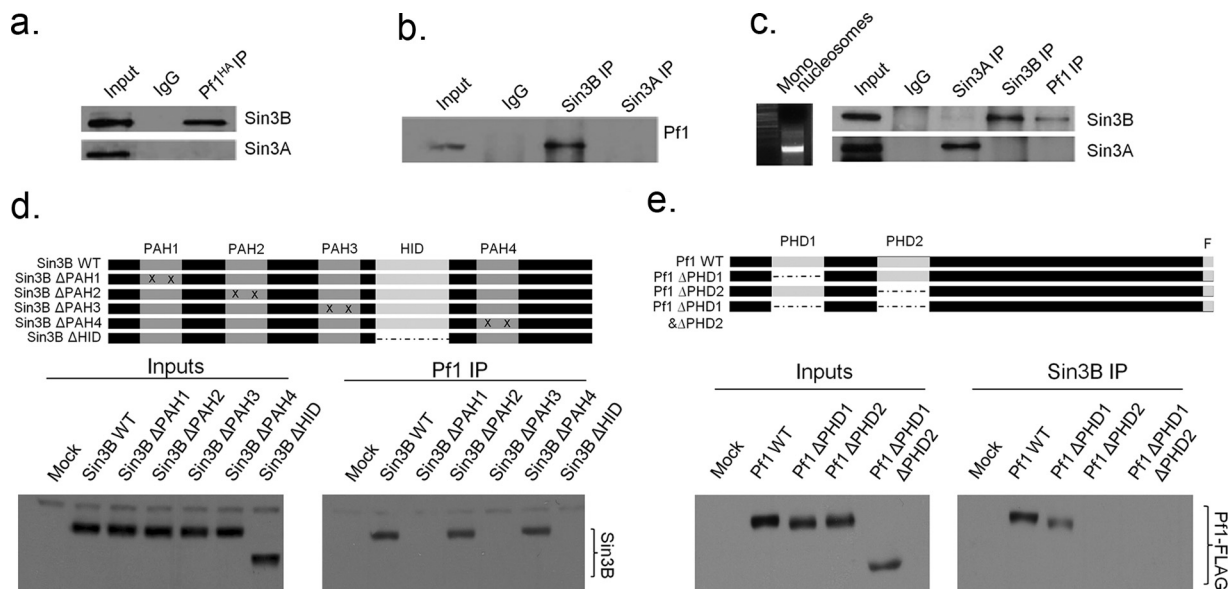
saline (PBS). One milliliter of reticulocyte standard buffer (10 mM Tris [pH 7.5], 10 mM KCl, 3 mM MgCl<sub>2</sub>; with fresh protease cocktail inhibitor [PIC] added) (RSB) was added, and cells were scraped into an Eppendorf tube and spun at 2,000 × g, and 100 µl RSB-G40 (10 mM Tris [pH 7.5], 10 mM KCl, 3 mM MgCl<sub>2</sub>, 10% glycerol, 0.5% NP-40; with fresh PICs added) was added. The samples were spun at 2,000 × g to pellet the nuclei. The nuclei were washed with 100 µl RSB-G (10 mM Tris [pH 7.5], 10 mM KCl, 3 mM MgCl<sub>2</sub>, 10% glycerol), and nuclei were lysed with 50 µl NE buffer (20 mM HEPES [pH 7.9], 420 mM NaCl, 1.5 MgCl<sub>2</sub>, 0.2 mM EDTA, 25% glycerol; with fresh 0.5 mM dithiothreitol [DTT] and PICs added). After 30 min of incubation on ice the samples were spun at maximum speed and the supernatant was placed in a separate tube. One milliliter of PBS was supplemented with 0.5% NP-40 and PICs, and 2 µg of immunoprecipitating antibody was added and incubated at 4°C overnight. The next day, 10 µl pre-washed protein A/G Plus-agarose (sc-2003) was added, and the reaction was incubated for 4 h. After three washes in PBS supplemented with 0.5% NP-40, the samples were boiled in SDS-protein loading buffer. The proteins were then separated on SDS-PAGE and detected with Western blotting with the corresponding antibodies.

Nucleosome extraction was done using a standard procedure. The cells were washed twice in PBS followed by adding 300 µl buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.34 M sucrose, 10% glycerol, 0.1% Triton X-100, with freshly added 1 mM DTT and PICs). After 8 min of incubation on ice, the samples were spun for 5 min at 2,000 × g, and pellets were washed once with buffer A without Triton X-100. Pellets were then resuspended in 100 µl buffer B (3 mM EDTA, 0.2 mM EGTA, with freshly added 1 mM DTT and PICs) and incubated on ice for 20 min. The samples were spun for 5 min at 2,500 × g, and 50 µl buffer C was added (10 mM Tris, 10 mM KCl, 1 mM CaCl<sub>2</sub>). This was followed by addition of 1 U micrococcal nuclease for 2 min at 37°C. The reaction was put on ice, and 1 mM EGTA was added to neutralize the enzyme. The sample was spun at 3,000 × g, and the supernatant with nucleosomes was placed in a separate tube. Five microliters was used for checking on a 2% DNA agarose gel.

For nucleosome coimmunoprecipitation assay purposes, prior to nucleosome extraction as described above, HeLa cells were cross-linked with 1% formaldehyde for 10 min at room temperature. Fifty microliters of nucleosomes was then diluted in 1 ml IP buffer (100 mM Tris [pH 7.5], 150 mM NaCl, 0.1% NP-40, 5% glycerol, with freshly added 1 mM DTT and PICs), and 2 µg of corresponding antibody was added. The reaction mixture was then incubated overnight at 4°C. The following day 10 µl pre-washed protein A/G plus-agarose (sc-2003) was added, and the reaction mixture was further incubated for 2 h at 4°C. This was followed by five washes in immunoprecipitation (IP) buffer and boiling in SDS-protein loading buffer. The proteins were then separated on SDS-PAGE and detected with Western blotting with the corresponding antibodies. Antibodies used for IPs and Western blotting were the following: Sin3B (Santa Cruz Biotechnology AK-12), Pf1 (Bethyl Labs BL6317), Sin3A (Santa Cruz Biotechnology AK-12), FLAG (m2 monoclonal Sigma F3165).

**Complex reconstitution assay and interaction with nucleosomes.** A complex reconstitution assay was performed as follows. Each component of the complex was coexpressed in HEK293T cells by transient Ca-phosphate transfection. Only immunoprecipitating protein was FLAG tagged. Two days after transfection, proteins were extracted with 1 ml KCl lysis buffer (20 mM HEPES [pH 7.9], 150 mM KCl, 5% glycerol, 1 mM DTT, 100 µM Zn-acetate, 2 mM MgCl<sub>2</sub>, 2 mM EDTA, 0.2% NP-40, with freshly added PICs) per 10-cm dish. One-half hour after incubation in lysis buffer, the samples were spun for 5 min at maximum speed. The supernatant was then placed in a separate tube, and the extracts were incubated overnight with pre-washed FLAG-Sepharose beads (Sigma F2426). The following day the complexes were washed five times with high-salt buffer (20 mM HEPES [pH 7.9], 300 mM KCl, 5% glycerol, 1 mM DTT, 100 µM Zn-acetate, 2 mM MgCl<sub>2</sub>, 2 mM EDTA, 0.1% NP-40), boiled in SDS-protein loading buffer, separated by SDS-PAGE, and stained with Coomassie brilliant blue. The complex-nucleosome interaction assay was performed as follows. The complex was purified using the standard procedure described above. After the washing step, the complex was additionally washed once in nucleosome interaction buffer (25 mM HEPES [pH 7.5], 100 mM KCl, 10% glycerol, 100 µg/ml bovine serum albumin, 0.1% NP-40, 0.5 mM DTT, with freshly added PICs) and subsequently incubated overnight in the same buffer supplemented with 10 µg (measured by Bradford assay) of nucleosomes extracted from HEK293T cells as described above. The nucleosomes were then detected by Western blotting with histone H3 antibodies.

**RNA extraction, cDNA synthesis, and Northern blotting.** RNA extraction was done as previously described (5). For cDNA synthesis, 500 ng RNA was brought to a volume of 8 µl diethyl pyrocarbonate-treated water. One microliter of RNase-free DNase was added, and the reaction mixture was incubated for 30



**FIG. 1. Sin3B interacts with Pf1.** (a) Western blot of coimmunoprecipitation assay products, showing ectopic hemagglutinin-tagged Pf1 pulling down endogenous Sin3B but not Sin3A. (b) Western blot of *in vivo* coimmunoprecipitation assay products, showing endogenous Sin3B, but not Sin3A, pulls down endogenous Pf1. (c) Nucleosome coimmunoprecipitation assay showing *in vivo* association of Pf1 and Sin3B, but not Sin3A. The right panel shows nucleosomes visualized on a DNA agarose gel with ethidium bromide. The left panel shows Pf1-mononucleosome-coimmunoprecipitated Sin3B detected by Western blotting. Nucleosomes were extracted from formaldehyde-cross-linked HeLa cells and immunoprecipitated with either Sin3A, Sin3B, or Pf1 antibody. (d and e) The Sin3B and Pf1 association is dependent on multiple interaction domains. A schematic representation of Sin3B, Pf1, and corresponding mutants is shown. Gray boxes represent PAH (paired amphipathic helix), HID, PHD, and the FLAG tag (F). The Xs indicated point mutants (each X represents a point mutation from leucine to proline), and dashes indicate deletion mutants. The mutants and wild-type proteins were overexpressed in HEK293T cells and coimmunoprecipitated with endogenous Pf1 or Sin3B. The interacting proteins were then detected by Western blotting using Sin3B (for Pf1 IP) or FLAG (for Sin3B IP) antibody.

min at 37°C. After adding 1  $\mu$ l STOP solution, the samples were further incubated at 65°C for 10 min and subsequently left on ice for 2 min. Then, oligo(dT) mix (Promega) was added, and cDNA synthesis was performed according to the manufacturer's instructions. Northern blot studies were done according to a standard procedure (27). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and RPL13a probes were labeled using [ $\alpha$ - $^{32}$ P]dCTP according to the manufacturer's instructions (Amersham RPN1633).

**ChIP assays.** Chromatin immunoprecipitation assays (ChIPs) were performed as previously described (34). The primers used in this study are available upon request. Antibodies used in the ChIPs were as follows: Sin3B (Santa Cruz Biotechnology AK-12), Pf1 (Bethyl Labs BL6317), Sin3A (Santa Cruz Biotechnology AK-12), H4Ac (Upstate Biotechnology 06-866); H3 (ab1791), RNAP II (Sigma 8wg16). Normal rabbit or mouse serum was used as an IgG control.

## RESULTS

**Sin3B, but not Sin3A, interacts with Pf1.** To investigate whether mammalian Sin3 proteins, like their yeast counterparts, participate in the restoration of the repressive chromatin structure at transcribed loci, we first sought to identify the mammalian homologues of each subunit of the yeast Rpd3S complex. By sequence comparison and limited functional characterization, the homologues of Rpd3, Sin3, and Eaf3 had previously been identified as HDAC1/2, Sin3A/B, and Mrg15, respectively (1, 28, 36, 45). In contrast, the identity of the mammalian homologue of Rco1, the fourth component of the yeast Rpd3S complex, remains elusive. Recently, the purification of the Mrg15-interacting proteins led to the isolation of Sin3B, HDAC1, and most notably a PHD-containing protein, Pf1, also known as Phf12 (13). Pf1, like Rco1, contains two PHD fingers in its N terminus, both of which share the highest homology with Rco1 PHD fingers (13, 43) (data not shown).

Along with Pf1's reported ability to interact with the mammalian Sin3 proteins HDAC1/2 and Mrg15, this observation suggests that Pf1 is likely to represent the mammalian homologue of Rco1. While Pf1 was originally reported to associate with Sin3A (43), surprisingly only Sin3B was identified in Mrg15-interacting proteins along with Pf1 (13). To clarify whether Pf1 interacts with Sin3A, Sin3B, or both, we performed co-IP assays (Fig. 1). As shown in Fig. 1a, ectopically expressed Pf1 associates with endogenous Sin3B but not endogenous Sin3A. The specificity of the Pf1-Sin3B interaction was confirmed in a co-IP assay with endogenous Pf1, where endogenous Pf1 was detected in Sin3B immunoprecipitate but not in Sin3A immunoprecipitate, indicating a specific interaction between Sin3B and Pf1 (Fig. 1b). Since Sin3B is known to associate with chromatin (11), we then asked whether the association between Sin3B and Pf1 occurs on chromatin. Briefly, HeLa cells were subjected to chemical cross-linking to ensure that protein interactions remained intact, and the insoluble nuclear fraction containing nucleosomes was digested with micrococcal nuclease. Using antibodies raised against the Sin3B, Pf1, or Sin3A proteins, we then immunoprecipitated the chromatin fraction associated with each protein. The presence of nucleosomes in the different immunoprecipitates was confirmed by Western blotting using a histone H3 antibody (data not shown). As shown in Fig. 1c, Pf1-associated chromatin contains Sin3B specifically, indicating that the interaction between Pf1 and Sin3B occurs on chromatin.

To further understand the nature of the Sin3B-Pf1 association and define which particular protein interaction domains



mediate the interaction, we generated mutants of Sin3B and Pf1 and tested their abilities to interact by co-IP (Fig. 1d and e). Sin3B has several conserved protein interaction domains, including four PAHs (*paired amphipathic helices*) and one HID (*HDAC interaction domain*), consistent with Sin3B's putative function as a scaffold protein for complex formation (28). Within each PAH, a double point mutation was introduced to convert two leucines, previously reported to be essential for the PAH tertiary structure, into two prolines (26). Since the identity of the amino acids important for HID tertiary structure is unknown, we elected to generate a mutant deleted for the HID region, Sin3B $\Delta$ HID. Following overexpression of each mutant, IP experiments were performed with an anti-Pf1 antibody, and the Pf1-interacting Sin3B proteins were detected with anti-Sin3B antibody (Fig. 1d). Of note, all mutants were stably expressed and detected by the Sin3B antibody (Fig. 1d, left panel). These experiments indicate that the interaction between Sin3B and Pf1 requires multiple domains within Sin3B, namely, PAH1, PAH3, and HID, suggesting that the interaction with Pf1 may necessitate a specific conformation of the Sin3B protein.

As mentioned above, Pf1 contains two PHD fingers. Interestingly, PHDs can function as protein-protein interaction domains, and some PHDs have recently been identified as either modified or nonmodified histone-binding modules (25, 29, 41). We therefore generated Pf1 deletion mutants for each of the PHD fingers, as well as a mutant resulting from the deletion of both fingers (Fig. 1d). Co-IP experiments between endogenous Sin3B and overexpressed wild-type or mutant Pf1 indicated that the interaction with Sin3B requires PHD2<sup>Pf1</sup>, while PHD1<sup>Pf1</sup> is largely dispensable. Altogether, these results suggest that PHD2<sup>Pf1</sup> mediates a specific interaction with Sin3B, but not with Sin3A. It is interesting that the disruption of PHD1<sup>Pf1</sup> does not affect the interaction with Sin3B, consistent with what was reported for Rco1 in yeast (21).

**Sin3B, Pf1, Mrg15, and HDAC1 associate in a stable complex that binds H3K4me3/H3K36me3-enriched nucleosomes.** It has been shown that Rpd3, Sin3, Eaf3, and Rco1 associate in a stable tetrameric complex (4). We therefore tested the hypothesis that all four mammalian homologues of these proteins, namely, HDAC1, Sin3B, Pf1, and Mrg15, can also stably associate in a tetrameric complex. We overexpressed the different components to limit the dependence on endogenous proteins. For each experiment, a single component was FLAG tagged, and its associated proteins were affinity purified and visualized by staining with Coomassie brilliant blue. As shown in Fig. 2a, immunoprecipitation with a FLAG antibody of either Sin3B-, Pf1-, or Mrg15-associated proteins leads to the detection of the tetrameric complex composed of Sin3B, Pf1, Mrg15, and HDAC1 (Fig. 2a, lanes 3, 6, and 9). These results strongly suggest that all four proteins can form a stable complex. Notably, the complex formation between Pf1, Sin3B, and Mrg15 remained intact upon omission of HDAC1 (Fig. 2a, lanes 2, 5, and 8), suggesting that HDAC1 does not mediate the interactions detected between the other components of the complex. Although unlikely based on the high overexpression levels for each component in this assay, it remains possible that endogenous HDAC1/2 can compensate for the absence of exogenous HDAC1 in this assay. To better understand the interdependence between all components in the formation of

the complex, we used the same assay but omitted either Pf1 or Mrg15 (Fig. 2b). To ensure that the observed bands corresponded to the overexpressed proteins, Pf1 and Sin3B were expressed and purified as single components (Fig. 2b, lanes 1 and 2). While Pf1 was able to bind to Sin3B/HDAC1 in the absence of Mrg15 overexpression (lane 4), Mrg15 did not associate with Sin3B/HDAC1 in the absence of Pf1 overexpression (lane 3 and lane 5), suggesting that Pf1 mediates the interaction between Mrg15 and Sin3B/HDAC1. Next, we co-expressed Pf1-FLAG with Mrg15 and HDAC1 (Fig. 2c) and either Sin3B WT (lane 1), Sin3B $\Delta$ HID (lane 2), or Sin3B $\Delta$ PAH4 (lane 3). Consistent with our previous data (Fig. 1d), the Sin3B $\Delta$ HID mutant was unable to associate with Pf1, while Sin3B WT and Sin3B $\Delta$ PAH4 efficiently associated with Pf1. Together, these results suggest that (i) all four components can associate within a stable complex, (ii) Mrg15 does not bind Sin3B directly, and (iii) Pf1 serves as a bridging partner between Sin3B/HDAC1 and Mrg15 in a manner dependent on the Sin3B HID.

Since we demonstrated that the interaction between Sin3B and Pf1 occurs on chromatin (Fig. 1c), we then asked whether the tetrameric complex, reconstituted as presented above, associates with purified nucleosomes. Additionally, we also investigated whether specific histone modifications are found enriched in the complex-bound nucleosomes. Of note, in the experiments presented in Fig. 2a to c, no histones were visualized with Coomassie staining; however, association of the complex with exogenous nucleosomes was detected by Western blotting with anti-histone H3 antibody, in the presence of Mrg15, as shown in Fig. 2d. Consistent with this observation, and in view of the established ability of Mrg15 to interact with methylated H3K36-containing nucleosomes (45), we first tested the presence of H3K36me3-modified histones in the complex-interacting nucleosomes. As anticipated, the complex efficiently interacted with H3K36me3 nucleosomes (Fig. 2e, lane 3). This interaction was largely dependent on the presence of Mrg15, as a complex composed of Pf1, Sin3B, and HDAC1 only was unable to associate with H3K36me3 nucleosomes (Fig. 2e, lane 2). Surprisingly, nucleosomes associated with a complex devoid of Mrg15 (Fig. 2e), indicating that Mrg15 is unlikely to be the only component within the complex that is able to interact with nucleosomes. Importantly, while H3K4me1-containing nucleosomes did not interact with the complex (Fig. 2e), H3K4me3 nucleosomes coprecipitated efficiently with a full tetrameric complex. Strikingly, a complex devoid of Mrg15 was almost as equally able to bind to H3K4me3-containing nucleosomes as a full tetrameric complex (Fig. 2e). As neither Sin3B nor HDAC1 is known to possess histone interaction domains, we speculated that this Mrg15-independent binding may be due to the presence of Pf1. Notably, while Pf1 by itself was not able to bind histone H3 (Fig. 2d, lane 5), the presence of Sin3B and HDAC1 promoted the interaction between Pf1 and nucleosomes (Fig. 2d and f), the molecular basis of which remains unclear. While the full tetrameric complex associates with both H3K4me3- and H3K36me3-enriched nucleosomes (Fig. 2f, lane 5), the Sin3B/HDAC1 heterodimer does not bind to nucleosomes (lane 2). Importantly, the omission of Pf1 abolished the binding to any nucleosome tested in this assay, as revealed by the absence of H3, H3K4me3, or H3K36me3 in the pulled-down nucleosomes

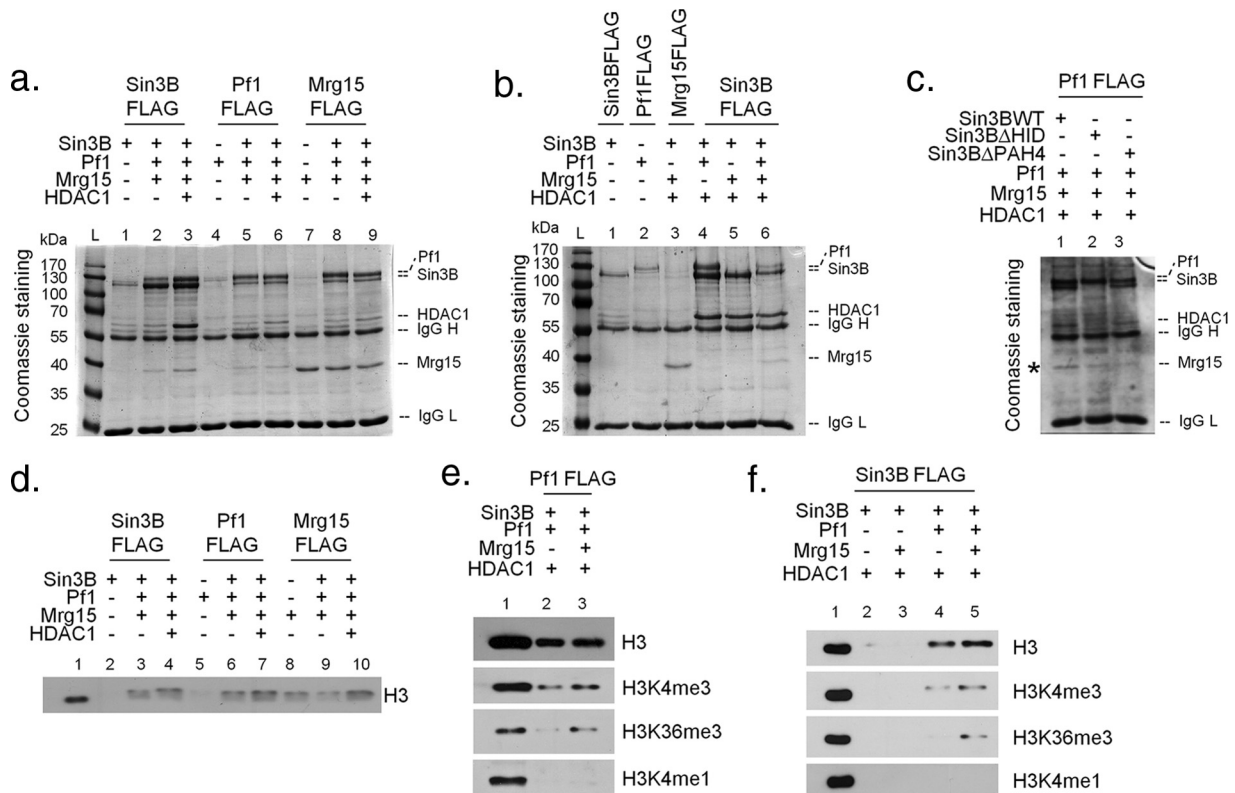


FIG. 2. Sin3B, Pf1, Mrg15, and HDAC1 associate within a stable complex that binds H3K4me3/H3K36me3-enriched nucleosomes. (a to c) Complex reconstitution assays. Each component of the complex was coexpressed in HEK293T cells (indicated by +), followed by lysate extraction and immunoprecipitation with FLAG antibody. Only immunoprecipitating protein was FLAG tagged, as indicated above the panels. Complexes were resolved on SDS-PAGE and visualized by Coomassie brilliant blue staining. IgG H and L indicate heavy and light chains, respectively. (a) Reciprocal complex reconstitution assays showing stable formation of the tetrameric complex (HDAC1/Sin3B/Pf1/Mrg15). Note the formation of complex in all three permutations (lanes 3, 6, and 9). (b) Sin3B acts as a bridging protein between HDAC1 and Pf1, while Pf1 is a scaffold between Sin3B and Mrg15. Lanes 3 and 5 show the lack of interaction between Sin3B and Mrg15 in the absence of Pf1. (c) Complex formation with Sin3B mutants. Sin3B<sup>ΔHID</sup> cannot form a complex with Pf1 (lane 2), unlike Sin3B<sup>ΔPAH4</sup> (lane 3). The asterisk indicates a band corresponding to Mrg15. (d to f) Complex-nucleosome interaction assay. Complexes were purified as described for panels a to c. Complexes, immobilized on the beads, were incubated with nucleosomes extracted from HeLa cells; nucleosomes were then separated on SDS-PAGE, and histones and histone modifications were detected by Western blotting with the antibodies indicated on the right side of the panels. In all figures lane 1 corresponds to 1% input. (d) The complex associates with nucleosomes in the presence of Mrg15. (e) Mrg15 tethers the complex to H3K36me3-enriched nucleosomes. (f) The complex association with H3K4me3-enriched nucleosomes is dependent on Pf1.

(Fig. 2f, compare lane 3 and lane 4). Of note, the lack of association between Sin3B and H3K36me3 nucleosomes in the absence of Pf1 is consistent with our results suggesting that Pf1 is required for Sin3B to associate with Mrg15. Collectively, these results indicate that Pf1 mediates the interaction between the tetrameric complex and H3K4me3-enriched nucleosomes in an Mrg15-independent manner. We cannot rule out the possibility that H3K4me3-enriched nucleosomes are found associated with the complex via an interaction between Pf1 and a yet-unknown nucleosome modification that segregates with H3K4 methylation.

**The complex associates with transcribed loci.** Having established that HDAC1, Sin3B, Pf1, and Mrg15 form a stable complex that associates with H3K4me3- and H3K36me3-enriched chromatin, we sought to determine the *in vivo* localization of the complex on chromatin. Since both H3K4me3 and H3K36me3 are hallmarks of active transcription, we first tested the possibility that this complex associates with the *GAPDH* and *RPL13a* loci, two prototypical constitutively transcribed

genes in HeLa cells. As controls, we included *CD4* and *INS* (insulin), transcriptionally silent genes in HeLa cells (Fig. 3a). A ChIP assay with antibodies specific to endogenous Sin3B or Pf1 demonstrated that both proteins accumulate in the region immediately downstream of the TSS of the active genes *GAPDH* and *RPL13a* (Fig. 3b, top panel). In contrast, neither protein was significantly enriched throughout the *CD4* or *INS* loci. Importantly, no accumulation of Sin3A within any regions of these transcribed genes was detected (Fig. 4b and data not shown), which was consistent with our previous observation that Sin3A is not a part of the Pf1-containing complex (Fig. 1). In agreement with previous reports investigating the distribution of histone modifications throughout the coding region of transcriptionally active genes (20, 38), H3K4me3 levels decline downstream of the TSS, while H3K36me3 levels increase along *RPL13a* (Fig. 3c). Notably, both Pf1 and Sin3B are found to be significantly enriched at the very point of transition from H3K4me3- to H3K36me3-enriched nucleosomes. Together with our nucleosome interaction data (Fig. 2e and f), these results

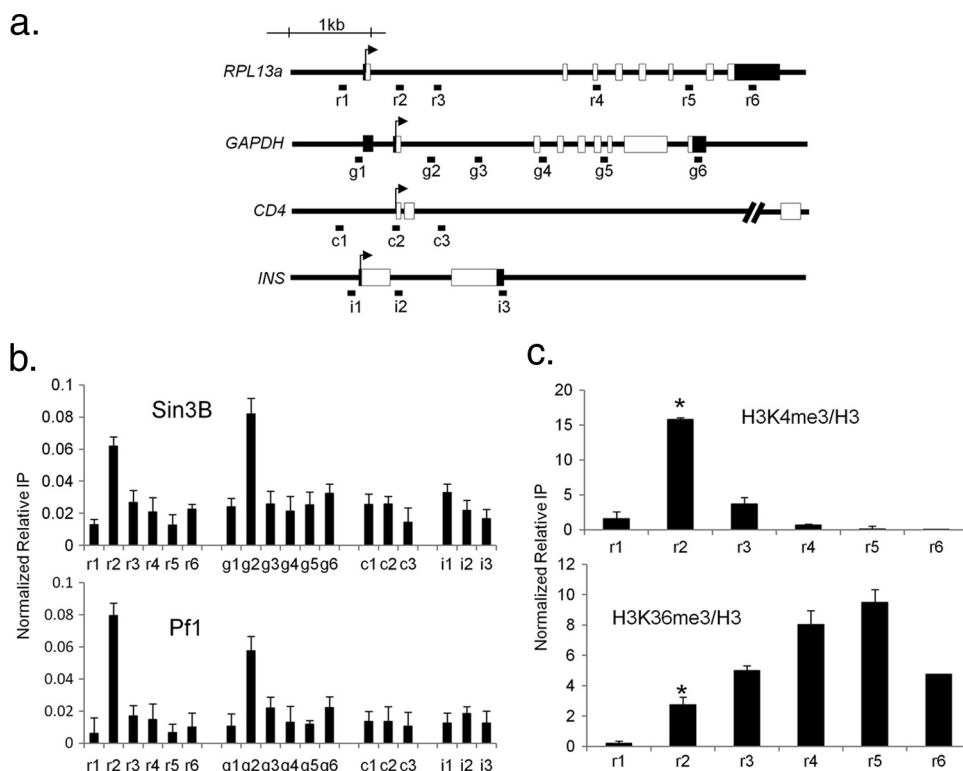


FIG. 3. The complex is localized downstream of the TSS within the active genes. (a) Schematic diagrams of the *RPL13a*, *GAPDH*, *CD4*, and *INS* genes. Untranslated regions are indicated as black boxes and open reading frames are shown as white boxes. The regions amplified by real-time PCR are indicated below the diagram. ATG start sites are marked by arrows. (b) ChIP analysis of the distribution of Sin3B and Pf1 within *RPL13a* (r1 to r6), *GAPDH* (g1 to g6), *CD4* (c1 to c3), and *INS* (i1 to i3). (c) ChIP analysis of the distribution of H3K4me3 and H3K36me3 along the *RPL13a* gene. Asterisks in panel c indicate the site of Pf1 and Mrg15 binding. In both panels b and c, the enrichment was analyzed by real-time PCR. Values represent the ratio of precipitated DNA to input DNA. The positions of amplified regions are indicated on the x axis. Error bars represent standard deviations of two independent replicates.

indicate that the Pf1/Sin3B-containing complex is recruited at discrete sites within actively transcribed loci, likely through its interaction with H3K4me3/H3K36me3-enriched chromatin.

Our *in vitro* results point to an Mrg15- and Pf1-dependent association between Sin3B and modified nucleosomes. Therefore, we asked whether Pf1 and Mrg15 also contribute to the specific localization of Sin3B within transcribed regions. Consistent with our hypothesis, the recruitment of Sin3B within the region downstream of the *RPL13a* TSS is strongly affected by shRNA-mediated knockdown of either Pf1 or Mrg15 levels (Fig. 4a and b). Importantly, decreasing either Mrg15 or Sin3B levels prevented Pf1 from associating downstream of the TSS, suggesting that an integral Sin3B/Pf1/Mrg15 complex may be required to allow the tethering to chromatin. Knockdown of Pf1, Sin3B, or Mrg15 did not significantly affect the expression levels of the other components of the complex (Fig. 4a). Notably, no significant change in Sin3A recruitment was observed upon the loss of Sin3B, suggesting that Sin3A cannot compensate for the loss of Sin3B in this context (Fig. 4b, bottom panel).

**The tetrameric complex modulates levels of gene expression.** Having established that a Sin3B-containing complex is recruited downstream of the TSS at transcribed loci, we then asked whether this complex has any regulatory function in gene expression. As shown in Fig. 5a, Sin3B or Pf1 knockdown resulted in a significant increase in the abundance of *GAPDH*

and *RPL13a* transcripts (2.6- and 2.2-fold increases, respectively) related to 18S transcripts. This increase was confirmed by Northern blotting (Fig. 5b). Notably, a similar increase in expression was detected for several other constitutively active genes, including *GPI*, *STK19*, *Actin*, *CYCL1*, and *CCND* (Fig. 5c), suggesting that Sin3B and Pf1 may modulate the transcription of constitutively active genes in a general manner. Of note, no transcripts of aberrant size were detected upon Sin3B and Pf1 knockdown, as revealed by Northern blot analysis with probes corresponding to the 5' and the 3' portion of the *GAPDH* and *RPL13a* transcripts (data not shown), indicating the authenticity of the transcripts. No change in expression was observed for the constitutively silenced *CD4* gene upon Sin3B and Pf1 knockdown (data not shown).

As we found that the histone deacetylase HDAC1 is an integral component of the Sin3B-containing complex, we hypothesized that the molecular mechanism by which the tetrameric complex affects gene expression might involve the modulation of histone acetylation levels within the region downstream of the TSS. Indeed, HDAC1 accumulates within the same region as Sin3B and Pf1, downstream of the TSS of *RPL13a*, and this accumulation is dependent on both Pf1 and Sin3B (Fig. 5d, top panel). Upon RNA interference-mediated depletion of Pf1 or Sin3B, the levels of histone H4 acetylation increased in the 5' part of the coding region, consistent with

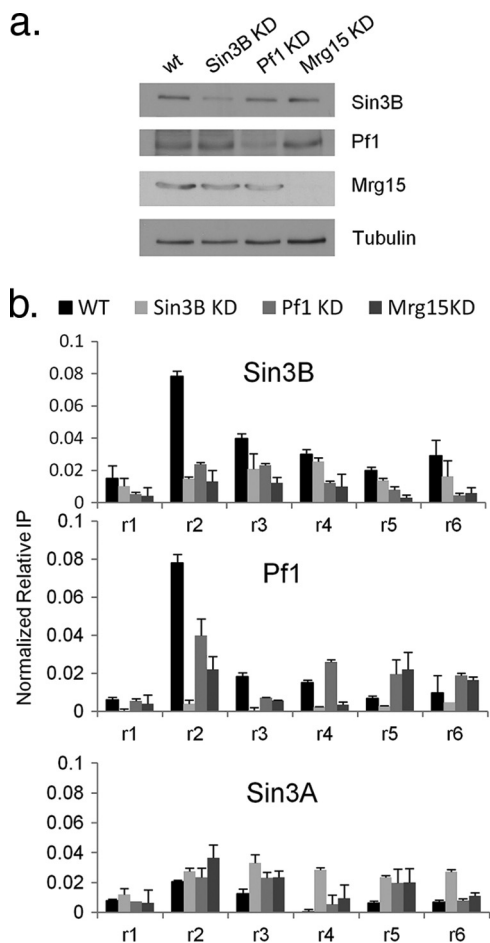


FIG. 4. The complex disassociates from the chromatin upon Pf1, Mrg15, or Sin3B knockdown. (a) Knockdown of Sin3B, Pf1, and Mrg15 in HeLa cells. The endogenous levels of proteins were analyzed by Western blotting using the corresponding antibodies. (b) Chromatin immunoprecipitation testing of the distribution of Sin3B, Pf1, and Sin3A within the *RPL13a* gene (r1 to r6) in WT and knockdown HeLa cells. The enrichment was analyzed by real-time PCR. Values represent the ratios of precipitated DNA to input DNA. The positions of amplified regions are indicated on the x axis. Error bars represent standard deviations of two independent replicates.

the loss of HDAC1 recruitment (Fig. 5d, middle panel). Concurrently, RNAP II levels increased significantly upon Pf1 or Sin3B knockdown along the *RPL13a* transcribed region. Notably, this increase was not restricted to the 5' end of the coding region but also occurred within and downstream of the TSS region (Fig. 5d, bottom panel). Altogether, these results strongly suggest that the Sin3B-containing complex is present downstream of the TSS of transcribed regions, preventing histone acetylation from spreading within the coding region and mitigating RNAP II progression downstream of the TSS.

## DISCUSSION

In this study, we have described a previously unidentified mammalian complex comprising Sin3B, HDAC1, Mrg15, and Pf1 (here referred to as the SHMP complex for discussion purposes), which localizes to constitutively active genes

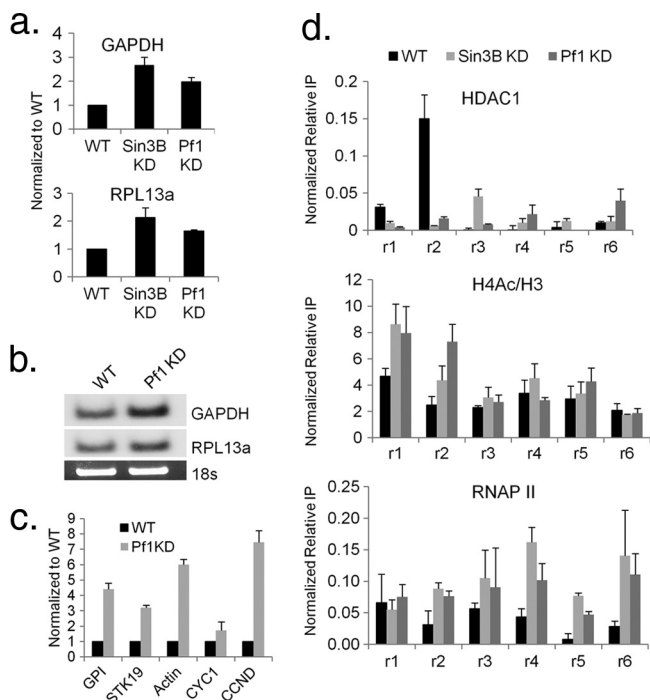


FIG. 5. Pf1 and Sin3B knockdown results in increased *RPL13a* and *GAPDH* transcription. (a) Quantitative real-time PCR measuring the expression levels of *RPL13a* and *GAPDH* in Sin3B and Pf1 knockdown HeLa cells compared to the WT. The values were normalized to the 18S rRNA levels. Error bars represent standard deviations of two independent replicates. (b) Northern blot analysis of *RPL13a* and *GAPDH* RNA expression levels in WT and Pf1 knockdown HeLa cells. 18S rRNA served as a loading control. (c) Quantitative real-time PCR measuring the expression levels of *GPI*, *STK19*, *actin*, *CYC1*, and *CCND* in Pf1 knockdown HeLa cells compared to the WT. (d) Chromatin immunoprecipitation analysis of the distribution of HDAC1, H4Ac, and RNAP II within the *RPL13a* gene (r1 to r6) in WT and in Pf1 and Sin3B knockdown HeLa cells. The enrichment was analyzed by real-time PCR. Values represent the ratios of precipitated DNA to input DNA. The enrichment value for H4Ac was additionally normalized to the levels of whole histone H3. The positions of the amplified regions are indicated on the x axis. Error bars represent standard deviations of two independent replicates.

downstream of the TSS, precisely where H3K4me3- and H3K36me3-enriched nucleosomes overlap. The SHMP complex is tethered to these specific loci via Pf1 and Mrg15 association with H3K4me3- and H3K36me3-enriched chromatin, respectively. Finally, our data suggest that the tethering of HDAC1, as part of this complex, to these loci promotes histone deacetylation within the coding region and prevents uncontrolled RNAP II progression at transcribed loci. Therefore, we have identified a new complex that participates in the fine-tuning of transcription in mammalian cells, in a manner that is reminiscent, though distinct, of its yeast homologue complex.

In contrast to the yeast Rpd3S complex, which accumulates toward the 3' region of transcribed regions, the SHMP complex is tethered to a discrete region localized about 1 kb downstream of the TSS on all transcribed regions we have tested in mammals. This particular region is targeted by the SHMP complex despite the presence of the Mrg15 component, which has been reported to bind to H3K36me3-modified histones, enriched throughout the 3' moiety of transcribed regions.



However, we demonstrated that, like Mrg15, the Pf1 component also contributes to the tethering of the SHMP complex to chromatin. This observation is reminiscent of what has been reported for yeast Rco1, which is required for Eaf3 binding to H3K36me3 nucleosomes (21). However, an important distinction between the mode of recruitment of yeast Rco1 and mammalian Pf1 lies in our observation that Pf1 is dispensable for the interaction of Mrg15 with H3K36me3 but instead favors the interaction of the complex with H3K4me3 nucleosomes. Since Pf1 contains PHD fingers and is necessary for the association of the complex with H3K4me3-modified nucleosomes, we first anticipated that Pf1's PHD fingers would be involved in binding directly to such modified histones, as previously reported for other PHD fingers (25, 46). We were, however, unable to detect any reproducible interaction between the recombinant PHD1<sup>Pf1</sup> or PHD2<sup>Pf1</sup> moieties and H3K4me3 peptides (data not shown). It remains possible that PHD1<sup>Pf1</sup> binds H3K4me3 but requires either posttranslational modifications or the surrounding amino acids found in the wild-type Pf1 protein, and both possibilities are presently being explored. Alternatively, Pf1 may interact with a protein that has not yet been identified and that specifically binds H3K4me3-enriched chromatin. Indeed, although it is tempting to speculate that HDAC1, Pf1, Mrg15, and Sin3B suffice to form the mammalian SHMP complex, by homology to the yeast Rpd3S complex, we cannot exclude the existence of additional components yet to be identified.

Our results indicate that in the absence of proper recruitment of the SHMP complex to transcribed loci, histones fail to be deacetylated downstream of the TSS, resulting in increased progression of RNAP II in the coding regions. This result is consistent with the notion that HDAC1 is tethered to transcribed regions through the SHMP complex. Importantly, we and others have previously demonstrated that mammalian Sin3 proteins interact with additional chromatin modifiers. For example, Sin3A interacts with the H3K36me3 histone demethylase KDM2b (Fbl10) (19) and components of the hSWI/SNF remodeling complex (31). Importantly, recent studies have shown that Sin3B associates specifically with the H3K4me3 histone demethylase KDM5A (Rbp2) (31, 39). Together, these observations suggest that, within the SHMP complex, Sin3B could serve as a recruitment platform for different chromatin-modifying activities that converge toward the prevention of uncontrolled chromatin relaxation downstream of the TSS. Surprisingly, recent genome-wide studies documented not only the presence of Sin3B downstream of the TSS, consistent with our results, but also the presence of Sin3A in active genes (39). However, our results strongly suggest that Sin3B plays a specific role at transcribed loci, since (i) we were unable to detect any interaction between Sin3A and Pf1 and (ii) Sin3A was not found to be present in the transcribed regions of the genes we studied. This hypothesis is consistent with our previous reports demonstrating that Sin3A and Sin3B are not redundant in several biological processes (6, 7, 12).

As discussed above, unlike its yeast Rpd3S complex homologue, which acts along the coding region all the way to the 3' end of the gene (4, 16, 21), we found that the SHMP complex is tethered downstream of the TSS, at discrete locations. This distinction may be correlated to the differences in size and organization between yeast and mammalian protein coding

genes; while yeast genes are generally shorter and mainly devoid of introns (15), mammalian genes tend to be larger with multiple introns. In addition, while transcribed regions in mammals exhibit many of the marks that have been reported in yeast, they also contain H3K9me2/3 and HP1 $\gamma$ , both absent from yeast (37). These observations suggest that restoration of a repressive chromatin structure following transcriptional elongation occurs in mammalian cells, but this process may use complexes other than the SHMP complex. A recent study reported the characterization of SetD2, a mammalian homologue of Set2, that specifically trimethylates H3K36 in transcribed regions (10). Small interfering RNA-mediated knockdown of SetD2 resulted in the complete loss of H3K36me3 in the body of transcribed genes, although it did not affect H3K36me1 or -me2. Under these conditions, no aberrant internal initiation of transcription was detected at transcribed genes, leading those authors to conclude that this pathway is not conserved from yeast to mammals. However, Mrg15 was recently shown to bind H3K36me2 with the same affinity as H3K36me3 (45). Therefore, it is possible that Mrg15 is still recruited at transcribed regions despite the absence of SetD2 and thus engages an HDAC-containing complex to prevent internal initiation. In conclusion, all evidence so far points to the evolutionary conservation of the restoration of the repressive chromatin structure pathway at transcribed loci from yeast to mammals. However, upon knockdown of any component of the SHMP complex, no transcripts of aberrant size were detected for multiple loci bound by Sin3B, including RPL13a and GAPDH, as revealed by Northern blot analysis (data not shown). Based on our results, it is unlikely that the Sin3B-containing SHMP complex contributes to the prevention of aberrant internal initiation of transcription. A recent study on mammalian HDACs surprisingly demonstrated that the binding of all tested HDACs (HDAC1, HDAC2, HDAC3, and HDAC6) positively correlates with gene expression, RNAP II binding, and histone acetylation (40). Specifically, HDAC6 has the ability to bind to elongating RNAP II directly and, as a result, is enriched in the coding region of active genes (40). It is therefore possible that in mammals, the prevention of cryptic initiation of transcription in transcribed regions occurs through elongating RNAP II-mediated recruitment of HDAC6 and subsequent histone deacetylation, in a Sin3-independent manner.

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