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The KAP1 Corepressor Functions To Coordinate the Assembly of De Novo HP1-Demarcated Microenvironments of Heterochromatin Required for KRAB Zinc Finger Protein-Mediated Transcriptional Repression▽†

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KAP1/TIF1β is proposed to be a universal corepressor protein for the KRAB zinc finger protein (KRAB-zfp) superfamily of transcriptional repressors. To characterize the role of KAP1 and KAP1-interacting proteins in transcriptional repression, we investigated the regulation of stably integrated reporter transgenes by hormone-responsive KRAB and KAP1 repressor proteins. Here, we demonstrate that depletion of endogenous KAP1 levels by small interfering RNA (siRNA) significantly inhibited KRAB-mediated transcriptional repression of a chromatin template. Similarly, reduction in cellular levels of HP1α/β/γ and SETDB1 by siRNA attenuated KRAB-KAP1 repression. We also found that direct tethering of KAP1 to DNA was sufficient to repress transcription of an integrated transgene. This activity is absolutely dependent upon the interaction of KAP1 with HP1 and on an intact PHD finger and bromodomain of KAP1, suggesting that these domains function cooperatively in transcriptional corepression. The achievement of the repressed state by wild-type KAP1 involves decreased recruitment of RNA polymerase II, reduced levels of histone H3 K9 acetylation and H3K4 methylation, an increase in histone occupancy, enrichment of trimethyl histone H3K9, H3K36, and histone H4K20, and HP1 deposition at proximal regulatory sequences of the transgene. A KAP1 protein containing a mutation of the HP1 binding domain failed to induce any change in the histone modifications associated with DNA sequences of the transgene, implying that HP1-directed nuclear compartmentalization is required for transcriptional repression by the KRAB/KAP1 repression complex. The combination of these data suggests that KAP1 functions to coordinate activities that dynamically regulate changes in histone modifications and deposition of HP1 to establish a de novo microenvironment of heterochromatin, which is required for repression of gene transcription by KRAB-zfps.

Genetic and epigenetic programs that control proper spatial and temporal patterns of gene expression are instrumental for pluripotent stem cells to determine cellular identity and maintain homeostasis of adult metazoans. Though historically viewed as a passive packaging unit, remodeling of chromatin structure has emerged as a key target for programming of gene expression during early embryogenesis and tissue-specific gene transcription. The dynamic regulation of chromatin organization appears to be accomplished in part by at least four families of proteins, including the following: (i) macromolecular protein complexes that utilize energy from ATP hydrolysis to disrupt DNA-protein interactions; (ii) proteins with intrinsic enzymatic activity to posttranslationally modify the core histones; (iii) nonhistone chromosomal proteins; and (iv) histone variants. Increasing experimental evidence indicates that the combinatorial use of histone variants, posttranslational modification of histones (i.e., acetylation, phosphorylation, ubiquitination, and methylation), and nonhistone chromatin-associated proteins that recognize these signals represent an epigenetic marking system responsible for setting and maintaining heritable programs of gene expression (22, 26, 40, 62). However, several key questions in understanding this indexing system include the following: (i) how are histone modifications and variants targeted to gene-specific regulatory elements, (ii) what are the patterns of modifications at transcriptionally silenced loci, (iii) how do patterns of modifications temporally change during active transcriptional silencing of gene expression, (iv) what nonhistone chromosomal proteins interpret this code, and (v) how is recognition of this code mechanistically translated into a change in gene activity?

TFIIIA/C_2H_2-containing zinc finger proteins represent the most abundant family of sequence-specific DNA binding proteins in higher eukaryotes (32). These proteins are characterized by a repeating three-dimensional structural motif of a β-hairpin followed by an α-helix, which is stabilized by the coordination of one zinc ion (31). Concatemers of two or more zinc finger motifs facilitate selective, high-affinity binding to DNA, with each finger module making specific contacts with a 3- to 5-bp subsite in the major groove of double-stranded DNA (47). Nearly one-third of mammalian zinc finger proteins possess the highly conserved Kruppel-associated box (KRAB) motif. Analysis of the human genome sequence revealed 423 independent KRAB-zinc finger protein (KRAB-zfp)-coding loci, yielding alternative transcripts that altogether predict at least 742 structurally distinct proteins (23). Comparative ge-

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nome analyses indicate that this gene family is vertebrate specific and that the repertoire of KRAB-zfps differs significantly between species, suggesting that KRAB-zfps may regulate programs of gene expression that contribute to speciation (16, 29). The KRAB domain, defined by approximately 75 amino acids, is a transferable module that possesses DNA binding-dependent transcriptional repression activity. This activity is common to many KRAB domains of independent zinc finger proteins that have been tested and can be disrupted by mutations at highly conserved amino acids that define the minimal KRAB domain consensus sequence (4, 39, 43, 65, 67). These data emphasize that the transcriptional repression activity associated with the KRAB domain is a common biochemical property of this motif. Moreover, the abundant representation of KRAB-zfps in vertebrates potentially makes KRAB-directed transcriptional regulation one of the most widespread sequence-specific mechanisms to repress gene transcription in higher eukaryotes.

Mechanistically, transcriptional repression by the KRAB domain correlates with its binding to the KAP1 protein (*K*RAB *a*ssociated *p*rotein *1*), also referred to as TIF1β (transcriptional intermediary factor 1β) or KRIP1 (KRAB-interacting protein 1) (18, 28, 35, 42). The role of KAP1 in KRAB domain repression is supported by several pieces of experimental data, including the following: (i) KAP1 binds to multiple KRAB repression domains both in vitro and in vivo; (ii) KRAB domain mutations that abolish repression decrease or eliminate the interaction with KAP1; (iii) exogenous expression of KAP1 enhances KRAB-mediated repression; and (iv) KAP1 directly tethered to DNA is sufficient to repress transcription (2, 18, 28, 35, 42, 44, 52, 56, 64). Despite these observations, it is unclear whether other cellular proteins exist that are necessary and/or sufficient to mediate the repression activity of the KRAB domain.

The primary amino acid sequence of KAP1 reveals the presence of several well-conserved consensus signature motifs, including a RING finger, B-boxes, a coiled-coil region, a PHD finger, and a bromodomain (18, 28, 35, 42). This spatial arrangement of motifs is the prototype for a family of transcriptional regulators that includes TIF1α, TIF1γ, TIF1δ, and Bonus (5, 27, 36, 64). Biochemical analyses of the RING finger, B-boxes, and coiled-coil, collectively referred to as the RBCC/Trim domain, indicate that this tripartite motif is both necessary and sufficient for homo-oligomerization and direct binding to the KRAB repression module. Furthermore, KAP1 is the only member of the TIF1 family that directly binds to the KRAB domain (1, 27, 49–51). KAP1 also displays several biochemical properties that suggest it functions as a molecular scaffold to coordinate activities that regulate chromatin structure, including the following: (i) interaction with Mi-2α, a core component of the multisubunit NuRD histone deacetylase complex (56); (ii) interaction with the histone H3 lysine 9-selective methyltransferase SETDB1 (55); and (iii) direct interaction with the chromoshadow domain of the heterochromatin protein 1 (HP1) family via a core PxVxL motif (HP1BD) in vitro and in vivo (33, 44, 52). The biological significance of the KAP1-HP1 interaction is highlighted by observations in F9 cells, where KAP1 associates with heterochromatin in a PxVxL-dependent manner upon induction of cellular differentiation (12). Furthermore, the KAP1-HP1 interaction is required for differentiation of F9 cells into parietal endoderm-like cells in vitro (11). Moreover, transcriptional repression of a chromatinized reporter gene by a heterologous KRAB repressor protein correlates with localized enrichment of KAP1, SETDB1, and HP1 and methylation of histone H3 lysine 9 at promoter sequences of the transgene (4, 55). Based on these data, we hypothesize that KRAB-zfps require KAP1 and the network of proteins that interact with KAP1 to establish localized microenvironments of heterochromatin at gene-specific loci to repress gene transcription.

Our current model of transcriptional repression by KRAB-zfps is largely based on a network of biochemical interactions between KAP1 and proteins with previously described roles in chromatin metabolism. Previous studies have shown that mutations in the HP1BD/PxVxL motif, PHD finger, and bromodomain of KAP1 that disrupt protein-protein interactions with HP1, Mi-2α, and SETDB1 correlate with attenuated KAP1 repression. These data are consistent with the hypothesis that KRAB-mediated repression is dependent upon KAP1 and the network of proteins that associate with KAP1. However, the interpretation of these data is limited by the fact these experiments were done exclusively in transient-transfection-based reporter assays. Furthermore, many of these conclusions were drawn from the use of minimal peptides in KAP1 that function as autonomous repression domains when tethered directly to DNA. However, these data do not address whether the network of proteins that interact with KAP1 function cooperatively during KRAB-mediated transcriptional repression, especially in the context of a chromatin template. Here, we use hormone-responsive repressor proteins and small interfering RNA (siRNA) approaches to genetically investigate the requirement of KAP1, HP1, and SETDB1 in KRAB-mediated repression of stably integrated reporter transgenes. Furthermore, we have defined molecular changes in specific histone modifications associated with a chromatin template that has been transcriptionally repressed by direct tethering of KAP1 to DNA.

MATERIALS AND METHODS

Plasmids. The p5XGAL4-TK-Luciferase and pM1-KRAB plasmids have been previously described (39, 56).

To construct pSUPERretro-K928, nucleotides 928 to 946 (5′-GCATGAACC CCTTGTGCTG-3′) of MN_005762 were subcloned into the BglII/HindIII sites of pSUPERretro as a short hairpin (10).

To create the FLAG-KAP1 mammalian expression vector, a 1.2-kbp EcoRI/BamHI fragment from pFASTBAC-Flag-KAP1 (51) and a 1.4-kbp BamHI/XbaI fragment from pM2-KAP1 (18) were subcloned into the EcoRI/XbaI restriction sites of pcDNA3 (Invitrogen). The cDNA insert encompassed nucleotides 346 to 2797 of MN_005762, which encodes amino acids 20 to 835 of KAP1 fused to an NH_3-terminal FLAG epitope tag. To create an allele of KAP1 refractory to the short hairpin RNA (shRNA), a double nucleotide substitution at nucleotides 937 (C>A) and 940 (T>A) was introduced into the pC3-FLAG-KAP1 expression vector by QuikChange PCR mutagenesis. These nucleotide substitutions are silent with regard to the coding of amino acids at codons 216 and 217. The incorporation of the corresponding nucleotide substitutions and integrity of the surrounding KAP1 coding sequence were confirmed by DNA sequence analysis. Nucleotide substitutions giving rise to the RV487,488EE, W664A, L720A, and F761A mutations have been previously defined (13, 52, 55, 56). DNA fragments containing these mutations were subcloned into the pC3-FLAG-KAP1 construct, replacing the corresponding wild-type sequence.

The pC3-ERHBD-GAL4 plasmid was created by a series of sequential subcloning steps. First, nucleotides 1023 to 1979 of NM_007956 encoding amino acids 281 to 599 of the murine estrogen receptor hormone binding domain containing the G525R mutation (38) were PCR amplified and subcloned into the

HindIII/BamHI restriction sites of pcDNA3 (Invitrogen). Subsequently, nucleotides encoding the GAL4 DNA binding domain (amino acids 2 to 147) were PCR amplified from pM1 (53) and subcloned into the BamHI/EcoRI restriction sites of pC3-ERHBD, destroying the BamHI site as a result of a BamHI/BglII fusion. The fusion junctions and integrity of PCR-amplified DNA were confirmed by DNA sequence analysis.

The pC3-ERHBD-GAL4-KAP1 plasmid was created by subcloning a ~1.4-kb EcoRI/XbaI fragment from pM2-KAP1(293-835) (18) into the EcoRI/XbaI sites of pC3-ERHBD-GAL4. The pC3-ERHBD-GAL4-KAP1 (RV487,488EE) plasmid was created by subcloning a ~1.4-kb EcoRI/XbaI fragment from pM2-KAP1 (RV487,488EE) (52) into pC3-ERHBD-GAL4. Sequence-confirmed nucleotide changes in the coding region of KAP1 encoding the W664A, L720A, and F761A mutations (13, 55, 56) were first subcloned from pM1-KAP1(618-835) into pM2-KAP1(293-835). Subsequently, each mutation was subcloned from pM2-KAP1(293-835) into the EcoRI/XbaI sites of pC3-ERHBD-GAL4 as described above for the wild-type coding sequence. The pC3-ERHBD-GAL4-KRAB and pC3-ERHBD-GAL4-KRAB (DV) plasmids were created by subcloning an EcoRI/XbaI restriction fragment from pM1-KRAB and pM1-KRAB (DV) (39), respectively, into the EcoRI/XbaI restriction sites of pC3-ERHBD-GAL4.

pQE32-HP1α (nucleotides 70 to 642 of NM_012117, encoding amino acids 1 to 191) and pQE32-HP1β (nucleotides 283 to 840 of NM_006807, encoding amino acids 1 to 185) bacterial expression plasmids have been previously described (33, 34). The HP1γ bacterial expression vector (nucleotides 152 to 703 of NM_016587, encoding amino acids 21 to 173) was created by subcloning an XmaI/XhoI fragment from pC3-FLAG-HP1γ (52) into pQE32 (QIAGEN). Nucleotides encoding the GAL4 DNA binding domain (amino acids 2 to 147) were PCR amplified from pM1 (53) and subcloned into the BamHI/HindIII sites of pQE30 (QIAGEN). Proteins were expressed in *Escherichia coli* and purified as previously described (33, 52). Purified proteins were used to generate custom polyclonal antiserum (Rockland Immunochemicals).

Transient-transfection reporter assays. Cells (5×10^4) were plated in 17-mm tissue culture dishes 24 h prior to transfection. Cells were cotransfected with the indicated plasmid constructs and 500 ng of pC3-β-gal reporter plasmid using Fugene 6 reagent (Roche) at a ratio of 1.5 μl of Fugene per 1 μg of plasmid DNA. Forty-eight hours posttransfection, cells were harvested in 1× reporter lysis buffer, and whole-cell lysates were used to determine luciferase activity (Promega). Raw luciferase values were normalized to β-galactosidase activity. Fold repression was calculated as the ratio of normalized luciferase activity of cells transfected in the absence of an effector plasmid to that of the cells transfected with an effector plasmid.

Generation of cell lines with a stable reduction in endogenous KAP1. HEK293 cells were transfected with pSUPERretro-K928. Twenty-four hours posttransfection cells were grown in growth medium (Dulbecco's modified Eagle's medium plus 10% fetal bovine serum) supplemented with 10 μg/ml puromycin. Individual antibiotic-resistant colonies of cells were expanded and maintained in growth medium containing 10 μg/ml puromycin. The absolute level of KAP1 in antibiotic-resistant cells was determined by Western blotting with two independent antibodies to nonoverlapping antigens in KAP1 (56).

Generation of cell lines with stable integration of the 5XGAL4-TK-luciferase transgene. HEK293 cells were cotransfected with p5XGAL4-TK-luciferase and pBabe-Puro at a molar ratio of 10:1. Twenty-four hours posttransfection, cells were grown in growth medium (Dulbecco's modified Eagle's medium plus 10% fetal bovine serum) supplemented with 1 μg/ml puromycin. Individual colonies of cells were expanded and maintained in growth medium containing 1 μg/ml puromycin. Five micrograms of genomic DNA isolated from established clones was digested with HindIII and subjected to Southern blot analysis to verify stable incorporation of the luciferase plasmid (57). Basal expression of the chromatinized reporter was determined by measurement of luciferase activity in whole-cell extracts. Raw luciferase values were normalized to the total protein concentration. Wild-type or mutant versions of pC3-ERHBD-GAL4-KRAB and pC3-ERHBD-GAL4-KAP1, respectively, were transfected into 5XGAL4-TK-LUC cells to generate double stable cell clones that expressed a hormone-responsive repressor and luciferase. Twenty-four hours posttransfection, cells were grown in growth medium containing 1 μg/ml puromycin and 500 μg/ml of G418. Approximately 50 well-isolated colonies of cells for each repressor plasmid transfected were expanded and maintained in growth medium containing 1 μg/ml puromycin and 500 μg/ml of G418. Doubly antibiotic-resistant cells were screened for 4-hydroxytamoxifen (4-OHT; Sigma)-dependent repression of luciferase activity in whole-cell extracts.

Luciferase assays. Cells were plated in triplicate into 17-mm wells and grown in medium containing either 0.1% ethanol or 500 nM 4-OHT for the indicated times. The cells were harvested with 1× reporter lysis buffer (Promega), and lysates were used to measure luciferase activities. Raw luciferase values were normalized to total protein concentrations. Fold repression was calculated as the ratio of normalized luciferase activity in ethanol-treated cells to normalized luciferase activity in 4-OHT-treated samples.

siRNA transfection. Cells (4×10^5) were plated into 35-mm wells and transiently transfected with double-stranded RNA (dsRNA) oligonucleotides against KAP1 (M-005046; K928 [5′-GCATGAACCCCTTGTGCTG-3′], K1 [5′-GACCAAACCTGTGCTTATGTT-3′], K2 [5′-GATGATCCCTACTCAAGTGTT-3′], K3 [5′-GCGATCTGGTTATGTGCAATT-3′], and K4 [5′-AGAATTATTTCATGCGTGATT-3′]; Dharmacon SMART pool), HP1α (5′-AAGGAGCACAATACTTGGGAA-3′), HP1β (M-009716; Dharmacon SMART pool), HP1γ (M-010033; Dharmacon SMART pool), and SETDB1 (M-020070; Dharmacon SMART pool). Two hundred picomoles of each oligonucleotide was diluted into 250 μl of OPTIMEM (Invitrogen). For transfections designed to simultaneously knock down expression of HP1α, HP1β, and HP1γ, 100 picomoles of each oligonucleotide was diluted in 250 μl of OPTIMEM. One microliter of Lipofectamine 2000 reagent (Invitrogen) per 50 picomoles of siRNA was diluted in 250 μl of OPTIMEM. Diluted Lipofectamine 2000 was added to diluted siRNA and allowed to incubate for 20 min at room temperature before being added to the cells growing in 1.5 ml of standard growth medium minus antibiotics. A second transfection was done 48 h after the first transfection. Twenty-four hours following the second transfection, cells were trypsinized and plated (7×10^4 per 17-mm well in triplicate) in growth medium containing either 0.1% ethanol or 500 nM 4-OHT for 48 h.

Western blot analysis. Whole-cell lysates were prepared in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate [SDS], 10% glycerol) supplemented with 20 mM NaF, 0.1 M phenylmethylsulfonyl fluoride, 10 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotonin, 10 μg/ml pepstatin, and 1 mM benzamidine. Equal amounts of protein (25 μg) were resolved by SDS-polyacrylamide gel electrophoresis and blotted to polyvinylidene difluoride (Millipore) (52). Antigen-antibody complexes were visualized by enhanced chemiluminescence and exposure to X-ray film. Expression levels of specific proteins (i.e., KAP1, HP1, SETDB1, etc.) were determined from densitometric traces of X-ray films and normalized to the expression levels of a loading control (i.e., β-actin or Rbap48).

ChIP. Cells were plated into 100-mm dishes and grown in medium containing either 0.1% ethanol or 500 nM 4-OHT for the indicated times. Cells were fixed with 1% formaldehyde for 10 min at 37°C. Excess formaldehyde was quenched by adding a 1/10 volume of 1.25 M glycine for 5 min at room temperature. Approximately 2×10^6 cell equivalents were lysed in 100 μl of SDS-lysis buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 1% SDS, 0.1 M phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotonin, 10 μg/ml pepstatin, 1 mM benzamidine). Lysed cells were sonicated using a Branson 450 sonicator with a 3-mm two-step tapered microtip at power setting 2 and 70% duty for 12 pulses/cycle and nine cycles (~5-W output for 8 to 10 seconds). Clarified, sonicated chromatin was diluted 20-fold in chromatin immunoprecipitation (ChIP) dilution buffer (16.7 mM Tris, pH 8.0, 1.2 mM EDTA, 167 mM NaCl, 1.1% Triton X-100, 0.01% SDS), bringing the final concentration of SDS to 0.5%. Antibodies used to immunoprecipitate chromatin were RNA polymerase II (MMS-126R; Covance), histone H3 (ab1791; Abcam), acetyl-H3 (06-599; Upstate Biotechnology), histone H3-AcK9 (ab4441; Abcam), histone H3-AcK14 (ab2381; Abcam), acetyl-H4 (06-866; Upstate), histone H4 AcK16 (ab1762; Abcam), histone H3-2XmeK4 (07-030; Upstate), histone H3-3XmeK4 (ab8580; Abcam), histone H3-2XmeK9 (07-441; Upstate), histone H3-3XmeK9 (07-442; Upstate), histone H3-3XmeK27 (07-449; Upstate), histone H3-3XmeK36 (ab9050; Abcam), histone H4-3X-meK20 (07-463; Upstate), antigen-purified custom polyclonal GAL4 (DNA binding domain), HP1α, HP1β, HP1γ, and SETDB1 (55) immunoglobulin G. Antigen-DNA complexes were eluted in 200 μl of elution buffer (50 mM $NaHCO_3$, pH 9.0, 1% SDS), cross-links were reversed for 5 h at 65°C, and the DNA was purified by using spin columns (MoBio Laboratories). A 1/10 volume of purified DNA was amplified under the following PCR conditions: 1 mM $MgCl_2$, 1 μM primer, 200 μM deoxynucleoside triphosphate, and 0.25 U *Taq* DNA polymerase. DNA was denatured for 4 min at 94°C, followed by 28 cycles of 15 seconds at 94°C, 15 seconds at 55°C, and 30 seconds at 72°C. Primer sequences used to amplify immunoprecipitated DNA were as follows: (i) GAL4(DBS), 5′-CACACAGGAAACAGCTATGAC-3′ (sense) and 5′-GAATTCGCCAATGACAAGAC-3′ (antisense); (ii) HSVTK promoter, 5′-GGATCCGACTAGATCTGACTTC-3′ (sense) and 5′-CCAGGAACCAGGGCGTATCTC-3′ (antisense); (iii) LUC3′, 5′-TACTGGGACGAAGACGAACAC-3′ (sense) and 5′-TCGTCCACAAACACAACTCC-3′ (antisense); (iv) poly(A), 5′-CACACAGGCATAGAGTGTCTG-3′ (sense) and 5′-GATACATTGATGAGTTTGGAC-3′ (antisense). PCR-amplified products were run on a 2% agarose gel and visualized by ethidium bromide staining. The fluorescence was captured by an eight-bit digital camera, and signal intensities were quantitated using GeneTools

software from Syngene (Frederick, MD). Signals from specific immunoprecipitations were normalized to signals from input DNA (0.0625%). Enrichment was calculated as the ratio of normalized signal of amplified DNA from chromatin immunoprecipitated from 4-OHT-treated cells to normalized signal of amplified DNA from chromatin immunoprecipitated from ethanol-treated cells.

RESULTS

The KAP1 corepressor is required for KRAB-mediated repression. Although KRAB-mediated repression of transcription correlates with KAP1 binding, the requirement for KAP1 has not been demonstrated. To address this question, we have stably suppressed the expression of cellular KAP1 by 80 to 90% in HEK293 cells by constitutively expressing an shRNA directed against the coding sequence of the KAP1 mRNA (Fig. 1A; see also Fig. S1a in the supplemental material). Stable depletion of KAP1 appeared to have little effect on cellular morphology, viability, or the steady-state levels of proteins previously described to interact with KAP1 (data not shown) (see Fig. S1b in the supplemental material). To test the requirement of KAP1 in KRAB-mediated transcriptional repression, we used these cells in a transient-transfection reporter assay with a 5XGAL4-TK-luciferase reporter plasmid and a plasmid that expresses a GAL4-KRAB repressor protein (Fig. 1B). In parental HEK293 cells, we observed a dose-dependent relationship between the amount of plasmid, expressing GAL4-KRAB, transfected and the absolute level of transcriptional repression. In contrast, KRAB repression was significantly reduced in two independently isolated KAP1 knockdown cells (Fig. 1C). This observation is consistent with the hypothesis that KAP1 is an essential cellular factor for KRAB-mediated transcriptional repression.

Previous site-directed mutagenesis studies have identified key residues that are required for protein-protein interactions between KAP1 and HP1, Mi-2α, and SETDB1 (33, 44, 52, 55, 56). These mutations also reduce the repression activity of a heterologous KAP1 protein (amino acids 293 to 835) when tethered to DNA in transient-transfection based reporter assays (18, 56) (see Fig. S2 in the supplemental material). To test the effect of these mutations on the corepressor activity of full-length KAP1, we exogenously expressed alleles of KAP1 in knockdown cells that are refractory to the shRNA and possess deleterious amino acid substitutions in the HP1BD, PHD finger, and bromodomain (Fig. 2A). As illustrated in Fig. 2B, expression of wild-type KAP1 complemented the repression defect in the KAP1 knockdown cells. This observation suggests that the repression defect observed in the stable KAP1 knockdown cells is unlikely to be the result of an off-target effect of the shRNA. Although expressed at near-equal levels or higher, exogenous expression of a KAP1 protein unable to interact with HP1 (RV487,488EE) was incapable of restoring wild-type levels of repression (Fig. 2B and C). We also observed that exogenous expression of this mutant protein in parental cells dominantly inhibited GAL4-KRAB repression in a dose-dependent manner (data not shown). This observation implies that the interaction between KAP1 and HP1 is essential for KAP1 corepressor activity. Expression of proteins with mutations in either the PHD finger or bromodomain partially complemented the repression defect of the stable KAP1 knockdown cells. The combination of these observations suggests that repression of gene transcription by KRAB-zfps depends on the network of proteins that directly interact with KAP1, especially HP1.

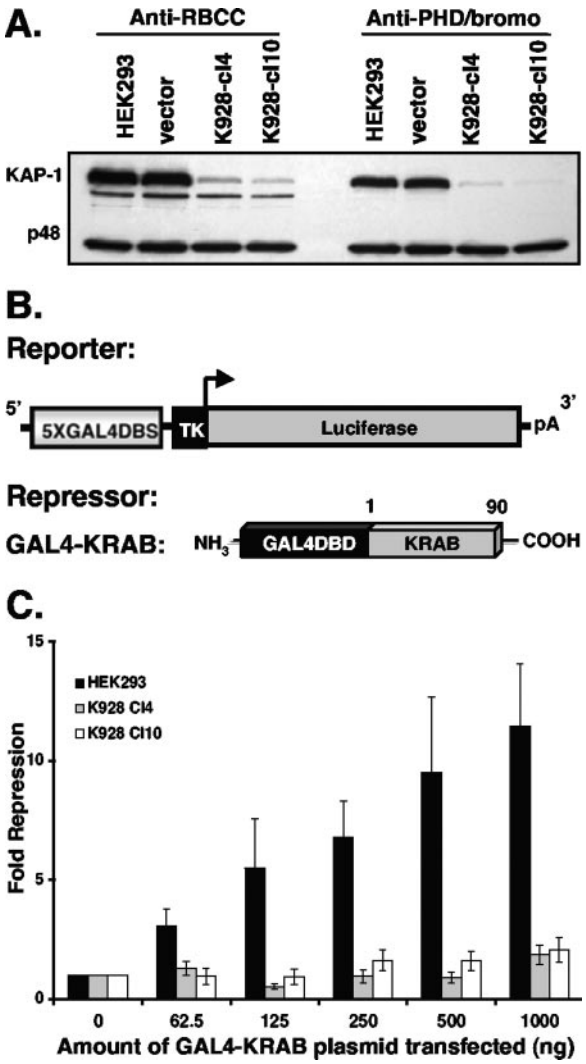

FIG. 1. KAP1 is required for KRAB-mediated repression. (A) Western blot analysis of endogenous KAP1 expression in two independent stable knockdown cell lines, using antibodies that recognize either the N terminus (anti-RBCC) or C terminus (anti-PHD/bromo) of KAP1. Detection of Rbap48 (p48) was used as a loading control. (B) Schematic illustration of the 5XGAL4-TK-luciferase reporter and GAL-KRAB repressor protein. (C) Stable KAP1 knockdown cells (cl4 and cl10) were transiently transfected with the p5XGAL4-TK-luciferase reporter and the indicated amounts of plasmid that expresses the GAL4-KRAB repressor protein. Luciferase activity was measured 48 h posttransfection and normalized for transfection efficiency. Repression (*n*-fold) represents the ratio of normalized luciferase activity in the absence of any effector plasmid to the activity measured in the presence of the indicated amount of GAL4-KRAB plasmid transfected. The data represent the averages of two independent experiments done in triplicate. Error bars represent the standard deviations of the means.

Establishment of GAL4-TK-luciferase cell lines. Our data indicate that KAP1 is an essential factor required for transcriptional repression by the KRAB-zfp superfamily. Therefore, it was important to investigate structure-function relationships of

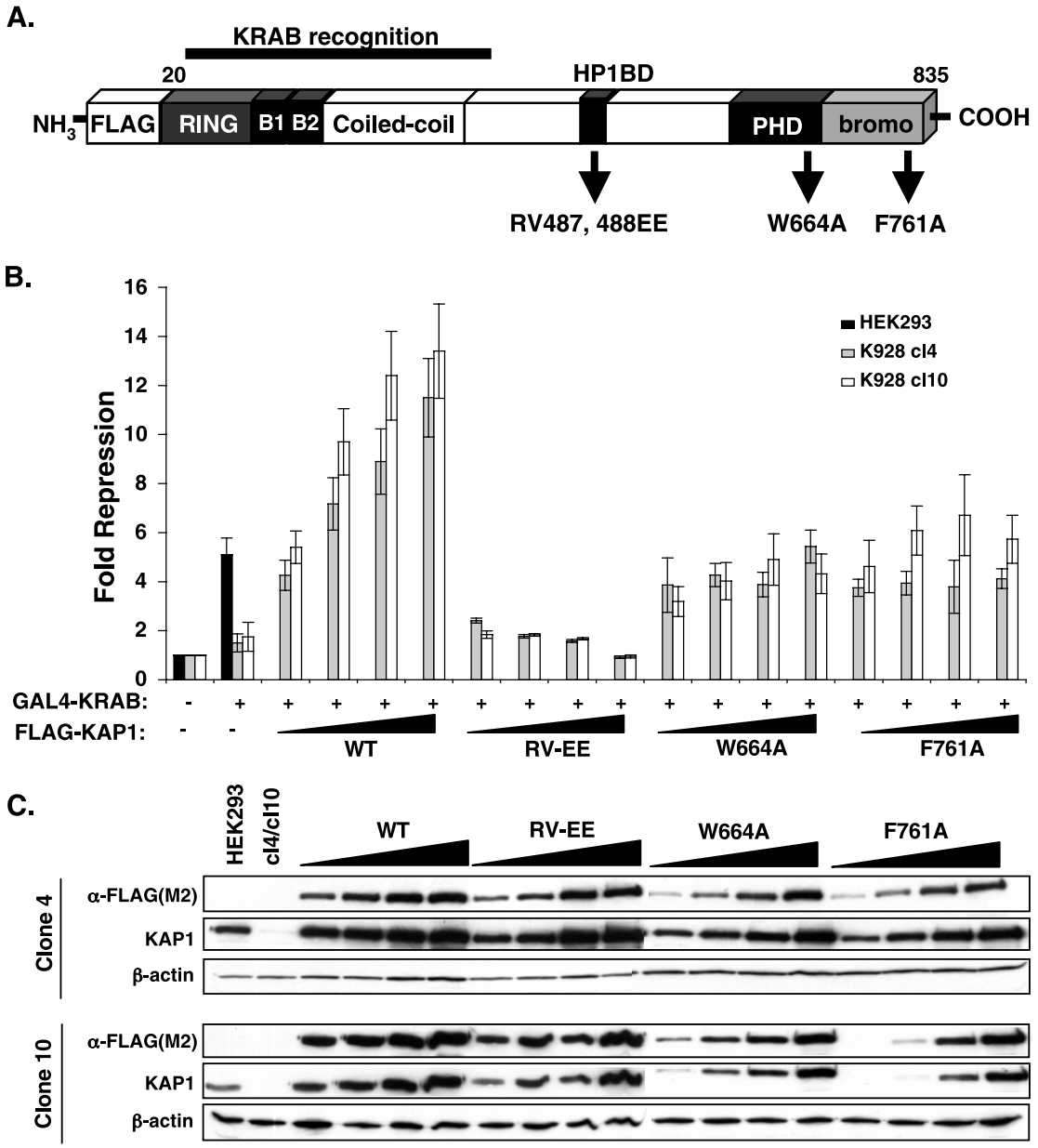

FIG. 2. The corepressor activity of KAP1 depends on its interaction with HP1 and a functional PHD finger and bromodomain. (A) Schematic illustration of KAP1's domain structure and location of synthetically introduced mutations. (B) Stable KAP1 knockdown cells were transiently transfected with the p5XGAL4-TK-luciferase reporter, 100 ng of pM1-KRAB, and increasing amounts of a plasmid that expresses FLAG-tagged KAP1 (wild type [WT], RV487, 488EE, W664A, and F761A). Luciferase activity was measured 48 h posttransfection and normalized for transfection efficiency. Repression was calculated as described for Fig. 1. Data are representative of two independent experiments done in triplicate. Error bars represent the standard deviations of the means. The apparent absence of error bars indicates a standard deviation too small to be physically illustrated. (C) Western blot analysis of transfected HEK293 cells, confirming stable exogenous expression of the FLAG-KAP1 proteins (using anti-FLAG and anti-RBCC antibodies). β-Actin represents a loading control.

the HP1BD, PHD finger, and bromodomain of KAP1 in transcriptional repression of a chromatin template. In the absence of well-characterized endogenous genes regulated by KRAB-zfps, we developed a modification of our previous strategy to conditionally regulate transcription of a chromatinized reporter transgene (4, 55). As schematically represented in Fig. 3A, we employed a sequential transfection strategy to establish cells that possess a stably integrated GAL4-TK-luciferase transgene and a hormone-responsive repressor protein. The advantage of this approach is that we can directly compare the consequences of mutations in KAP1 on regulation of gene transcription at an isogenic locus.

We initially established cell lines possessing a stably integrated copy of the 5XGAL4-TK-luciferase reporter (Fig. 1B). Basal expression of this transgene is driven by a minimal herpes simplex virus thymidine kinase promoter, which can be regulated by heterologous proteins that bind GAL4 DNA binding sites positioned immediately 5′ to the TK promoter

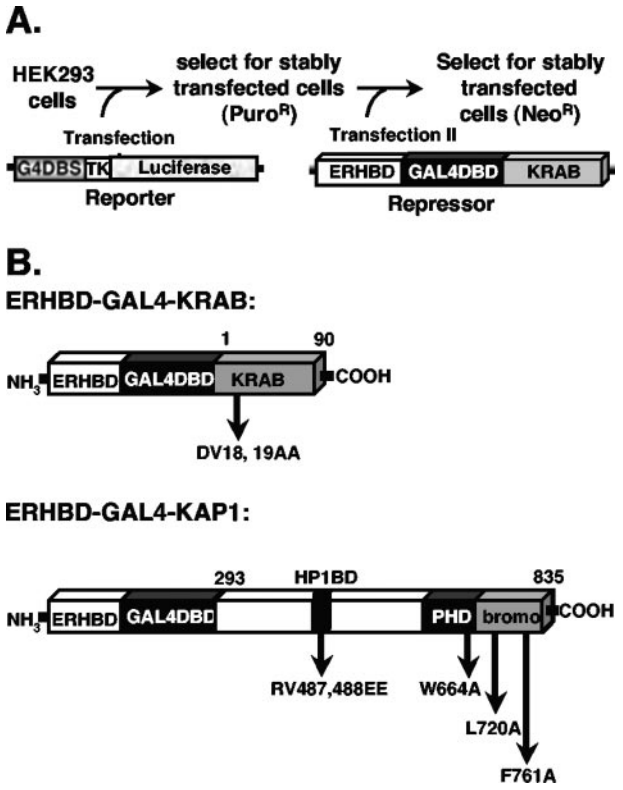

FIG. 3. Establishment of cell lines with a hormone-regulatable reporter transgene. (A) Strategy to generate cell lines that possess a stably integrated 5XGAL4-TK-luciferase reporter and constitutive expression of a hormone-responsive repressor protein. (B) Schematic illustration of heterologous hormone-responsive repressor proteins. The KRAB domain (Kox1 amino acids 1 to 90) or amino acids 293 to 835 of KAP1 (wild type, RV487, 488EE, W664A, L720A, and F761A) were fused in frame to the C terminus of the ERHBD-GAL4 DNA binding domain fusion.

sequences. DNA for the p5XGAL4-TK-luciferase plasmid and a second plasmid conferring puromycin resistance were cotransfected into HEK293 cells. We chose this two-plasmid transfection approach so that we could select for cells whose luciferase transcription was not influenced by the promoter activity driving puromycin resistance during the selection process. Approximately 100 puromycin-resistant cell colonies were isolated and expanded. Southern blot analysis of genomic DNA isolated from a representative selection of clones indicated that the copy number of the transgene ranged from 1 to 10 copies, depending on the cell line, and integrated at multiple independent loci (see Fig. S3b in the supplemental material). The normalized basal luciferase activities of the individual clones varied from 5 to 200 light units/optical density unit of protein (see Fig. S3c in the supplemental material). The variability of basal luciferase expression in each cell clone could be a direct reflection of the transgene copy number incorporated into the host cell genome or its site of integration. Regardless, the detection of stable luciferase activity in these clones is indicative of the transgene integrating in a transcriptionally permissive euchromatic environment.

Hormone-regulated repressor proteins. In order to conditionally regulate the transcription of the chromatinized luciferase transgene, we engineered our GAL4-KAP1 repressor protein to be hormone regulated by fusing a tamoxifen-sensitive derivative of the estrogen receptor hormone binding domain (ERHBD) to the N terminus of the GAL4 DNA binding domain (Fig. 3B). The ERHBD is 1,000-fold less responsive to serum estrogens and contains no intrinsic transcriptional activation potential (38). Unlike other conditional expression systems that are transcriptionally controlled, our chimeric repressor proteins are constitutively expressed. The transcriptional regulatory activity of these proteins is posttranslationally controlled by the addition of 4-OHT to the tissue culture medium. Hormone-regulated GAL4-KAP1 fusion proteins were created for the wild-type KAP1 sequence (amino acids 293 to 835) and mutations in the HP1BD (RV487,488EE), PHD finger (W664A), and bromodomain (L720A, F761A). Each of these mutations disrupts the tertiary structure of these modular domains, which significantly affects the ability of KAP1 to interact with HP1, Mi-2α, and SETDB1, respectively, and attenuates KAP1-mediated transcriptional repression (Fig. 3B). Thus, this set of KAP1 mutant repressor proteins enabled us to comprehensively investigate the functional role of these different domains in KAP1-mediated regulation of transcription and chromatin structure. As a control, an ERHBD-GAL4-KRAB protein was engineered which contained the 90-amino-acid KRAB domain of Kox1. This minimal domain is sufficient to bind KAP1 and is a very potent, DNA binding-dependent repressor in vivo (3, 4, 55). In a transient-transfection reporter assay experiment, we confirmed that the wild-type ERHBD-GAL4-KRAB and ERHBD-GAL4-KAP1 proteins functioned as both hormone-dependent and DNA binding-dependent transcriptional repressors (data not shown). Most importantly, repression was tightly regulated by 4-OHT, as defined by a less-than-twofold change in basal luciferase activity in the absence of hormone.

Individual subclones of stable GAL4-TK-Luc cells were transfected with plasmid DNA encoding either the ERHBD-GAL4-KRAB or ERHBD-GAL4-KAP1 proteins (Fig. 3A). For each repressor protein introduced, we isolated between 5 to 10 independent clones that demonstrated 4-OHT-dependent repression of the chromatinized reporter. Repression of the transgene's expression by the wild-type ERHBD-GAL4-KRAB and ERHBD-GAL4-KAP1 repressor proteins was dependent on the concentration of 4-OHT, with maximal effects being reached between 125 and 250 nM (see Fig. S4a in the supplemental material). Reduced luciferase activity also correlated with a quantitative reduction in steady-state levels of the transgene's mRNA (see Fig. S4b). Chromatin immunoprecipitation experiments with anti-GAL4 immunoglobulin G demonstrated that repression of the transgene was tightly associated with 4-OHT-induced DNA binding of the repressor proteins to the GAL4 DNA binding sites and the HSVTK promoter sequences (data not shown) (see Fig. 6, below).

Hormone-dependent repression of chromatin templates by ERHBD-GAL4-KRAB is dependent upon KAP1. To study temporal characteristics of transcriptional repression by the KRAB-KAP1 complex, we incubated cells expressing ERHBD-GAL4-KRAB with growth medium containing either 0.1% ethanol or 500 nM 4-OHT for various amounts of time. Steady-state repression of the chromatinized transgene was approached between 72 and 96 h of continuous 4-OHT treat-

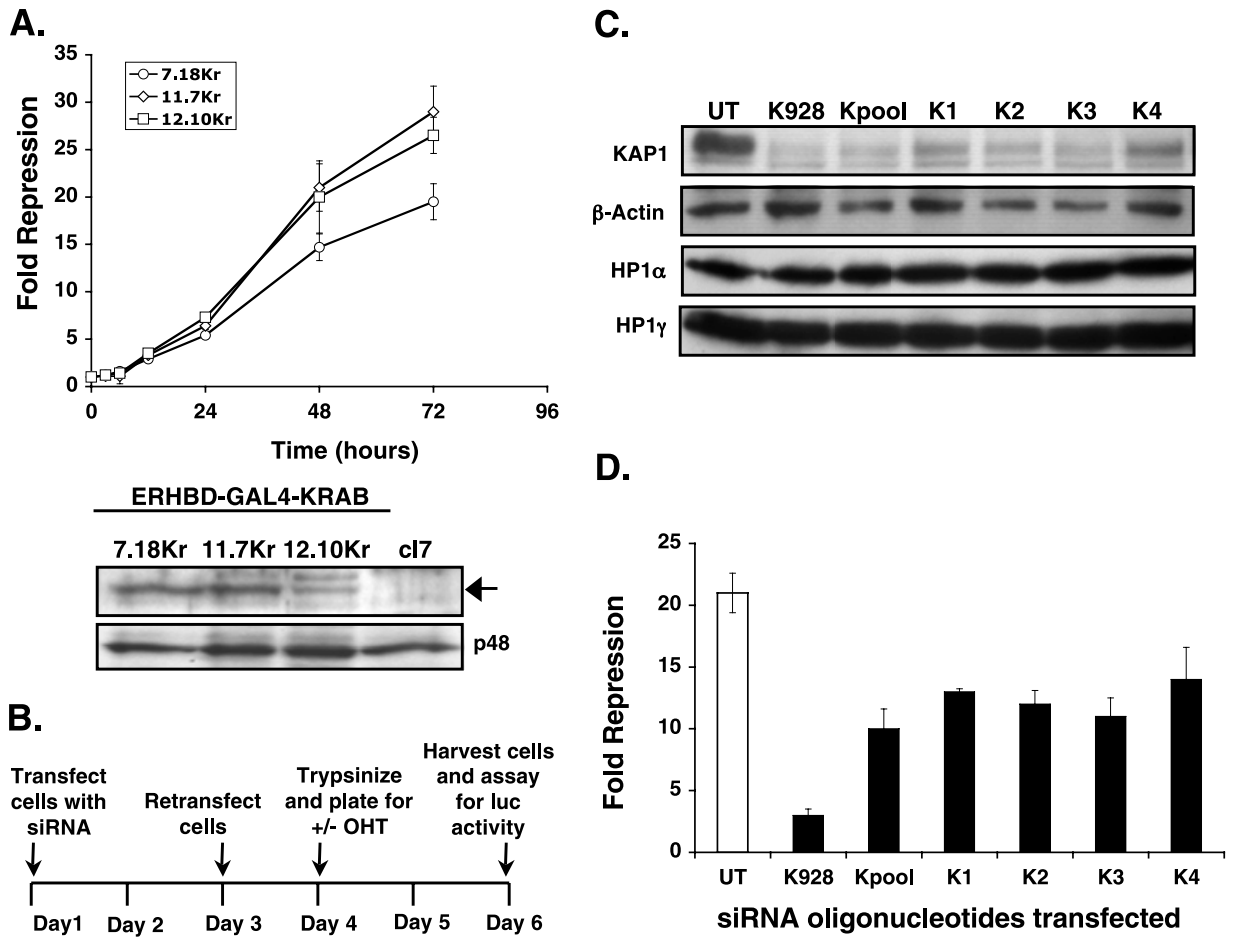

FIG. 4. Hormone-dependent repression of the chromatin template by ERHBD-GAL4-KRAB requires KAP1. (A, upper panel) Kinetics of hormone-dependent repression by ERHBD-GAL4-KRAB. Cells were grown in medium containing either 0.1% ethanol or 500 nM 4-OHT for the indicated times. Repression (*n*-fold) was calculated as the ratio of normalized luciferase activity in the absence of the hormone to normalized luciferase activity in the presence of the hormone. The data represent the averages of three independent experiments done in triplicate, and the error bars represent the standard deviations of the means. (Lower panel) Expression of ERHBD-GAL4-KRAB (arrow) in the indicated cell clones was detected using an antibody against the GAL4 DNA binding domain. Detection of Rbap48 (p48) was used as a loading control. (B) Overview of the experimental scheme. 12.10Kr cells were subjected to two rounds of transfection with 100 nM of independent dsRNA oligonucleotides designed to reduce expression of KAP1 prior to treatment with either 0.1% ethanol or 500 nM 4-OHT for 48 h. (C) Western blot analysis of whole-cell extracts prepared on day 6 from transfected cells with the indicated siRNA. (D) Transient depletion of KAP1 by independent siRNA molecules targeted to different regions of the KAP1 mRNA results in attenuation of hormone-dependent KRAB-mediated repression. Repression was calculated as described for panel A. Data are representative of two independent experiments done in triplicate. The error bars represent the standard deviations of the means. UT, untransfected.

ment (Fig. 4A). Cells expressing a mutant KRAB domain (DV18,19AA) which lacked the ability to bind KAP1 failed to repress the chromatinized transgene at any time point (data not shown). Furthermore, hormone-dependent repression of the reporter transgene was not due to enhanced expression of the repressor protein in cells treated with 4-OHT (data not shown). To investigate the role of KAP1 in KRAB-mediated repression of chromatin templates, we developed an experimental strategy to transiently deplete KAP1 by siRNA transfection (Fig. 4B). The rationale for this scheme was to ensure sufficient knockdown in steady-state levels of KAP1 prior to treating with 4-OHT. Similar to the transfection-based reporter assays illustrated in Fig. 2, transient depletion of KAP1 by siRNA significantly crippled hormone-dependent KRAB-mediated repression of the chromatinized reporter (Fig. 4C and D; see also Fig. S5 in the supplemental material). In general, the extent to which KRAB-mediated repression was inhibited correlated with the efficiency of KAP1 depletion by the siRNA transfection. Furthermore, transient depletion of the highly related TIF1α and TIF1γ proteins by siRNA transfection had no effect on hormone-dependent KRAB-mediated transcriptional repression of a chromatin template (see Fig. S5 and S6 in the supplemental material).

Direct tethering of KAP1 to a chromatin template is sufficient to repress transcription. Our data indicate that KAP1 is an essential cellular factor for KRAB-mediated transcriptional repression. To investigate whether directly tethering KAP1 to DNA was sufficient to repress transcription of a chromatin template, we incubated cells expressing ERHBD-GAL4-KAP1 with growth medium containing either 0.1% ethanol or 500 nM 4-OHT for various amounts of time. In contrast to the ERHBD-GAL4-KRAB repressor protein, direct tethering of

KAP1 to DNA demonstrated lower levels of absolute repression but more rapid kinetics of transcriptional repression, with steady-state repression reached within 48 to 72 h following 4-OHT treatment (Fig. 5A). Furthermore, we observed discrete differences in the absolute level of repression and subtle variations in the kinetic patterns of repression for the ERHBD-GAL4-KAP1 repressor protein in the different clones analyzed. We attribute these variations to differences in the expression levels of the repressor proteins and/or the transgene's integration site. Regardless, the combination of these data validate this model system as a tool to further investigate the mechanism by which KAP1 coordinates changes in histone modifications (i.e., histone deacetylation, methylation, etc.) and deposition of HP1 proteins to alter chromatin structure and repress transcription of a highly transcribed gene.

The HP1 interaction with KAP1 is required for repression of chromatinized reporter transgenes. An advantage of our experimental system is that we can analyze the consequences of biochemically well-defined amino acid substitutions in KAP1 on the transcriptional repression of a chromatinized reporter transgene, positioned at an isogenic locus. To investigate the requirements of the HP1BD, the PHD finger, and bromodomain of KAP1 and their associated activities in the transcriptional repression of a chromatin template, clone 12 cells (see Fig. S3 in the supplemental material) were stably transfected with plasmid DNA encoding the ERHBD-GAL4-KAP1 repressor containing the RV487,488EE, W664A, L720A, or F761A mutation. We isolated between 5 and 10 independent antibiotic-resistant cell clones that expressed either the wild-type or each mutant KAP1 repressor protein (Fig. 5C). Two representative cell clones for each mutant protein were grown in growth medium containing either 0.1% ethanol or 500 nM 4-OHT for 96 h in order to characterize functional consequences associated with these specific mutations on KAP1-mediated transcriptional repression. As illustrated in Fig. 5D, two independent cell clones expressing wild-type ERHBD-GAL4-KAP1 displayed a steady state of 10- to 14-fold repression. In contrast, the cell clones expressing mutant forms of KAP1 demonstrated significantly attenuated levels of transcriptional repression, despite the expression levels of these mutant proteins being equal to or greater than the wild-type protein (Fig. 5C). Temporal analysis of hormone-dependent transcriptional repression in these cell clones indicated that the difference in absolute repression between the wild-type repressor protein and the various mutant proteins was not the result of delayed kinetics (data not shown). Furthermore, decreased repression activity appears to be intrinsic to the mutant KAP1 repressor proteins, as Western blot analyses revealed little variation in HP1α, HP1β, HP1γ, Mi-2α, and SETDB1 expression levels between the different clones (see Fig. S7 in the supplemental material). Moreover, we observed similar results in three additional cell clones that express the wild-type and each mutant repressor protein (data not shown). Overall, these data demonstrate a fundamental requirement for these domains and their associated activities in KAP1-mediated transcriptional repression of a chromosomally integrated target.

Hormone-dependent repression by KAP1 correlates with reduced recruitment of RNA polymerase II and dynamic changes in histone tail modifications. To determine molecular events that correlate with 4-OHT-induced transcriptional repression of the chromatinized transgene by ERHBD-GAL4-KAP1, we did chromatin immunoprecipitation experiments. To define spatial relationships between histone modifications and specific DNA sequences within the transgene, we analyzed four loci along the transgene (Fig. 6A). Following a 96-h incubation with 4-OHT, we observed a hormone-dependent enrichment (four- to sixfold) of the ERHBD-GAL4-KAP1 protein at sequences that overlapped with the GAL4 DNA binding sites, HSVTK promoter, and the transcription start site (Fig. 6b; see also Fig. S8 in the supplemental material). Binding of the repressor protein was coincident with a fourfold reduction in hypophosphorylated RNA polymerase II occupancy at proximal regulatory sequences. Analysis of total histone H3 revealed a twofold hormone-dependent increase in histone H3 occupancy throughout the reporter transgene, coupled with a concomitant decrease in acetylated H3 K9/K14. Analysis of site-specific histone H3 methylation (i.e., K4, K9, K27, and K36) indicated that both dimethyl and trimethyl H3K4 were reduced by ~2-fold at promoter sequences following 4-OHT treatment. Moreover, we observed enrichment of dimethyl histone H3K9 (2-fold) and trimethyl histone H3K9 (2.5- to 6-fold), H3K27 (2.5- to 6-fold), H3K36 (2- to 4-fold), and histone H4K20 (3-fold) (Fig. 6B). When we analyzed sequences ~1.5 kbp and ~2.5 kbp distal to the transcription start site in the 3′ coding region of the luciferase mRNA and polyadenylation signal sequence, respectively, we observed a progressive reduction in trimethyl H3K4, H3K36, and histone H4K20 associated with a transcriptionally repressed transgene. In contrast to proximal promoter sequences, the extent of hormone-dependent changes in di- and trimethyl H3K9 and trimethyl H3K27 levels was less dramatic in nucleosomes positioned in this region of the reporter transgene. Immunoprecipitations with antisera against HP1α, HP1β, and HP1γ revealed a twofold hormone-dependent enrichment of HP1α and HP1β at promoter sequences of the transgene (Fig. 6B; see also Fig. S8 in the supplemental material). Similar results were observed with additional, independent antibodies raised against the HP1 proteins (data not shown). We also observed increased binding of the histone H3 lysine 9-selective methyltransferase SETDB1 to promoter sequences following treatment with 4-OHT, which was coincident with the elevated levels of trimethyl H3K9 we detected in this region. These data indicate that direct tethering of the KAP1 corepressor protein to a chromatinized reporter transgene is sufficient to coordinate dynamic changes in histone modifications that support the recruitment and deposition of HP1 proteins to form a localized heterochromatin-like environment that blocks the recruitment of RNA polymerase II.

Our data indicate that disruption of the interaction between KAP1 and HP1 cripples the corepressor activity of KAP1. Thus, to begin to understand at a molecular level the consequences of this mutation on 4-OHT-induced changes in the chromatin structure of the transgene, we did chromatin immunoprecipitation experiments in cells that express ERHBD-GAL4-KAP1 (RV487,488EE). As illustrated in Fig. 7, we observed a hormone-dependent increase in the amount of GAL4-KAP1 repressor protein bound to promoter sequences of the transgene. Despite the recruitment of the mutant repressor protein to the transgene's promoter, we did not observe any decrease in hypophosphorylated RNA polymerase recruitment to promoter

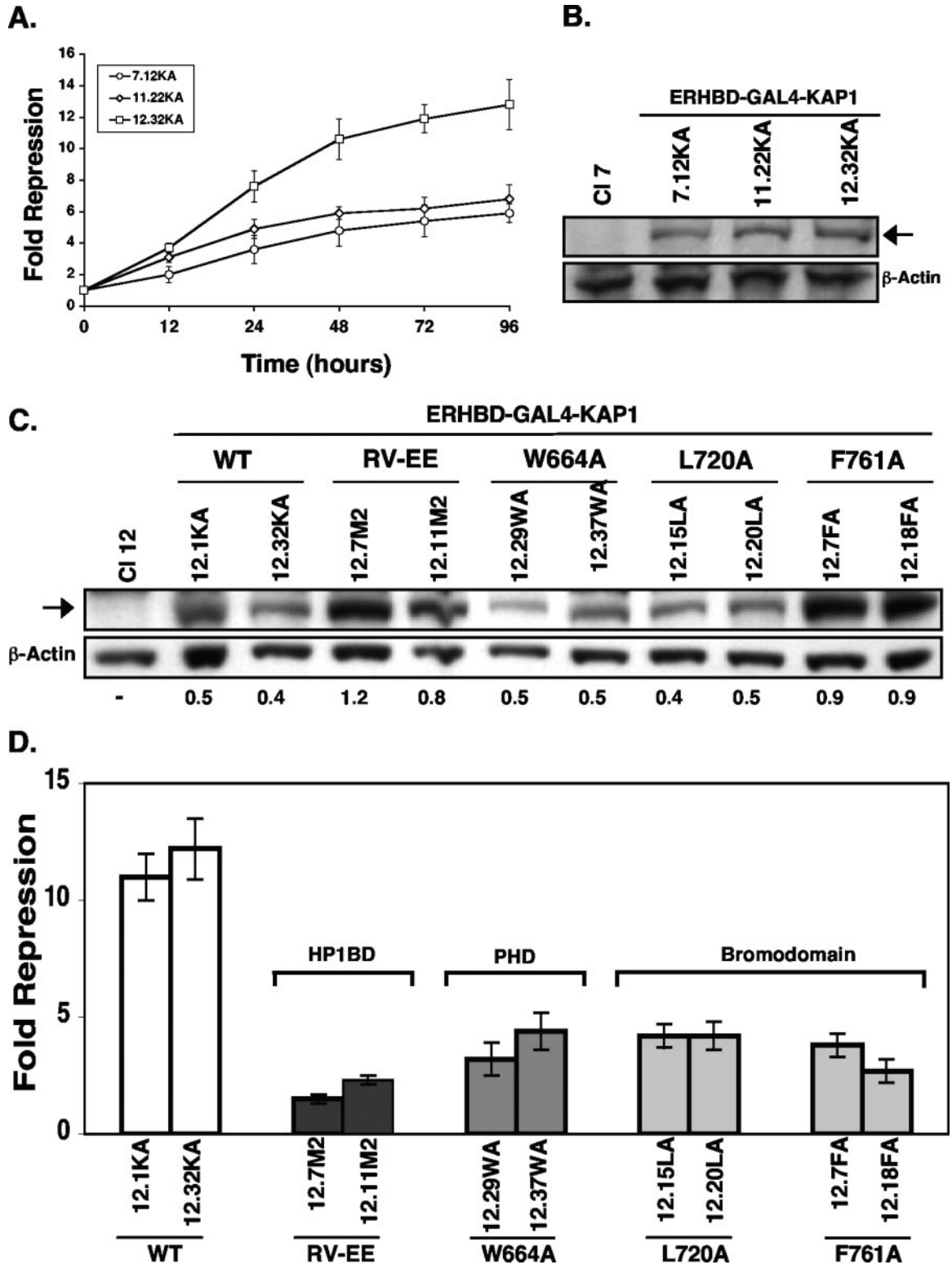

FIG. 5. Hormone-dependent repression of chromatin templates by ERHBD-GAL4-KAP1. (A) Kinetics of hormone-dependent transcriptional repression by ERHBD-GAL4-KAP1 in the indicated cell clones. Cells were grown in medium containing either 0.1% ethanol or 500 nM 4-OHT for the indicated times. Repression was calculated as described for Fig. 4A. The data represent the means of two independent experiments done in triplicate, and the error bars represent the standard deviations of the means. (B) Expression of ERHBD-GAL4-KAP1 (arrow) in the indicated cell clones was detected using an antibody against the GAL4 DNA binding domain. β-Actin expression was used as a loading control. (C) Western blot analysis of stable cell lines expressing the indicated ERHBD-GAL4-KAP1 proteins (wild type [WT], RV487, 488EE, W664A, L720A, and F761A). Numbers at the bottom represent the expression level (arbitrary units) of each ERHBD-GAL4-KAP1 protein, normalized to β-actin expression. (D) The indicated cell lines were grown in medium containing either 0.1% ethanol or 500 nM 4-OHT for 96 h. Repression was calculated as described in the legend for Fig. 4. The data represent the averages of three independent experiments done in triplicate, and the error bars represent the standard deviations of the means.

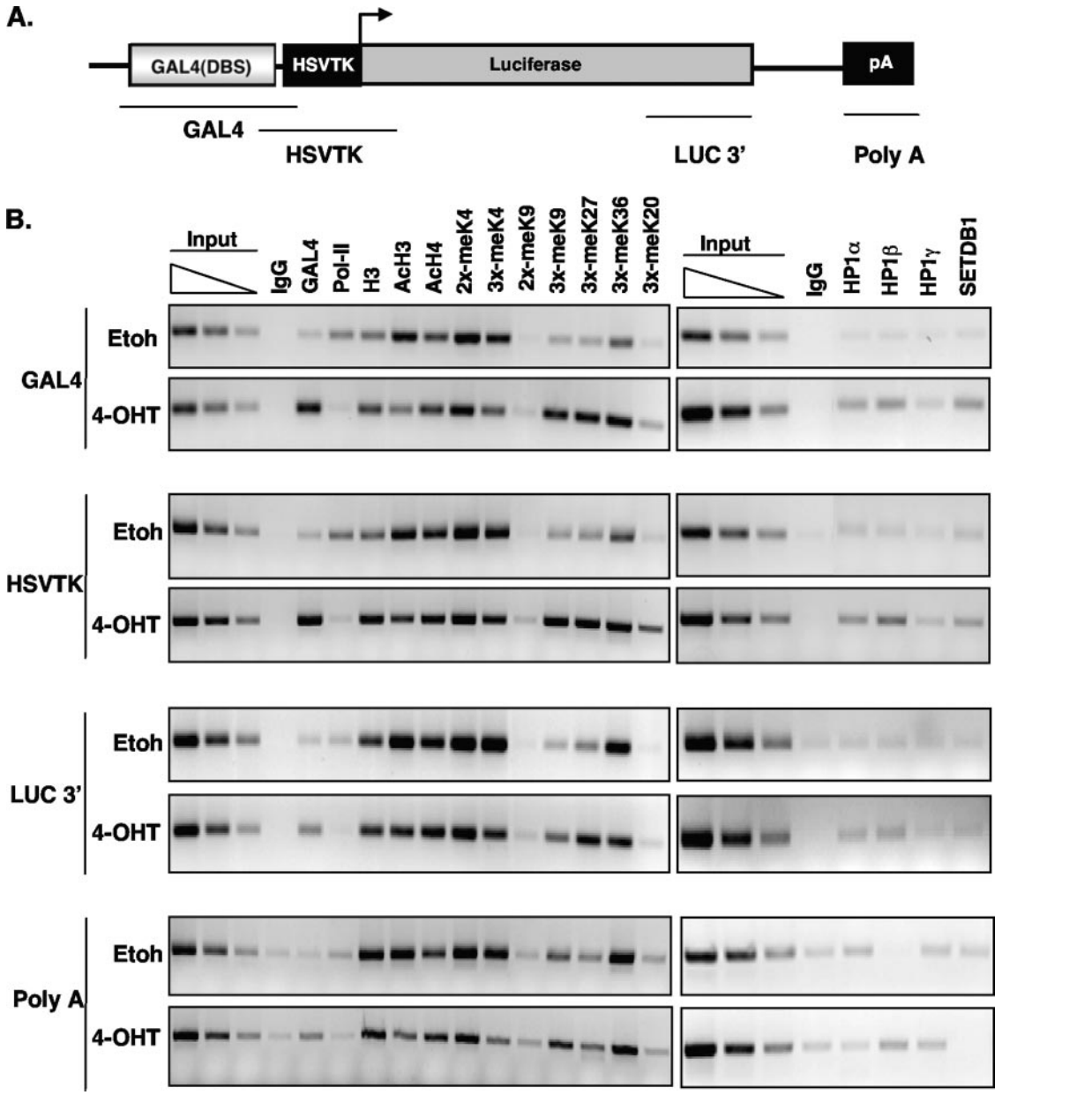

FIG. 6. Chromatin immunoprecipitation analysis of a luciferase transgene repressed by ERHBD-GAL4-KAP1. (A) Schematic illustration of the 5XGAL4-TK-luciferase transgene. Bold lines represent four regions of the transgene amplified by PCR in DNA recovered from immunoprecipitations. (B) Formaldehyde-cross-linked chromatin from 12.32KA cells grown in medium containing either 0.1% ethanol or 500 nM 4-OHT for 96 h was immunoprecipitated with antibodies against the indicated antigens. The immunoprecipitated DNA was PCR amplified at the indicated loci to detect hormone-dependent changes in hypophosphorylated RNA polymerase II recruitment, histone H3 occupancy, and histone modifications (left panel) and changes in HP1 and SETDB1 occupancy (right panel). Input DNA represents 0.25, 0.125, and 0.0625% of the total amount of DNA immunoprecipitated, respectively.

sequences. Similarly, we observed very little 4-OHT-dependent change in any of the histone modifications we examined. Most striking was the absence of hormone-induced enrichment of trimethyl H3K9, H3K27, H3K36, or histone H4K20 at promoter sequences. Furthermore, we did not observe hormone-dependent binding of either SETDB1 or any of the HP1 isoforms to promoter sequences, a result that is consistent with the lack of hormone-dependent enrichment of trimethyl H3K9 (Fig. 7B). ChIP analyses in cells expressing either a PHD finger mutant (W664A) or bromodomain mutant (F761A) ERHBD-GAL4-KAP1 protein yielded nearly identical results as the HP1BD mutant protein (see Fig. S9 in the supplemental material). In summation, our ChIP data indicate that the binding of HP1 to KAP1 is necessary to induce changes in patterns of histone modifications that correlate with KAP1-dependent repression of transcription. Moreover, these data are consistent with a role for KAP1 in de novo assembly of highly localized microenvironments of HP1-demarcated heterochromatin.

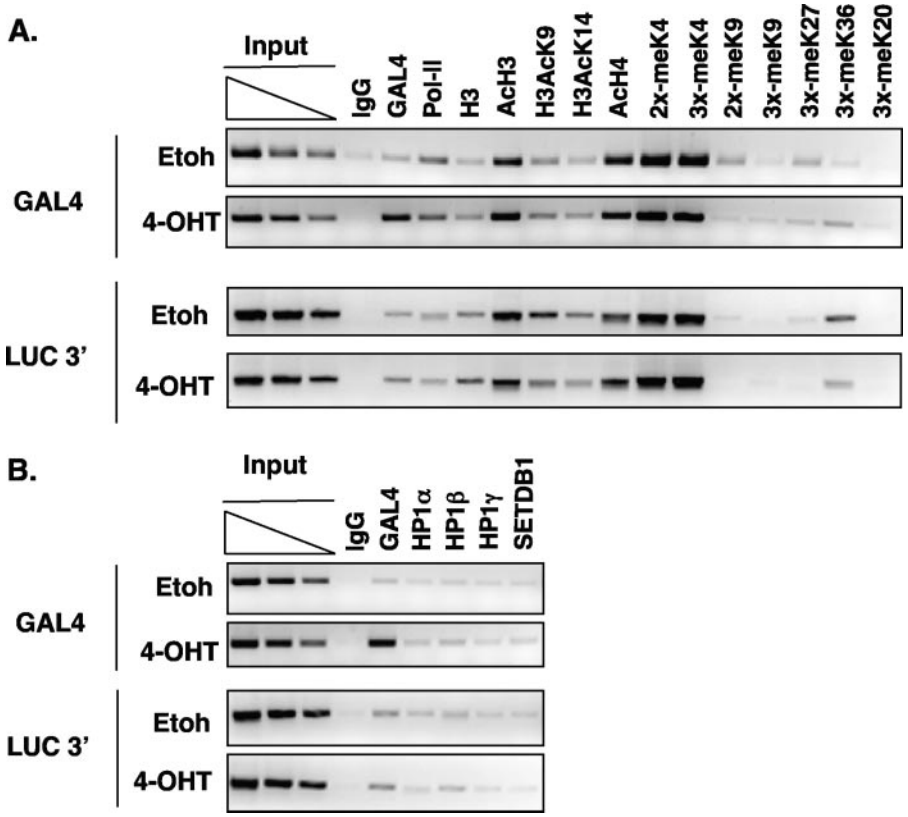

FIG. 7. Disruption of the KAP1-HP1 interaction fails to induce hormone-dependent changes in RNA polymerase II recruitment, histone occupancy, and histone modifications (A) or HP1/SETDB1 recruitment to promoter sequences of the luciferase reporter (B) following treatment with 4-OHT. Formaldehyde-cross-linked chromatin from 12.11M2 cells grown in medium containing either 0.1% ethanol or 500 nM 4-OHT for 96 h was immunoprecipitated with antibodies against the indicated antigens. Promoter and 3′ luciferase coding sequences recovered from the immunoprecipitations were PCR amplified. Input DNA represents 0.25, 0.125, and 0.0625% of the total amount of DNA immunoprecipitated.

HP1 and SETDB1 are required for KRAB-mediated repression of chromatin templates. Our data demonstrate that KRAB repression is dependent upon KAP1 and the network of proteins that interact with the HP1BD, PHD finger, and bromodomain of KAP1. To test the role of known KAP1-interacting proteins in hormone-dependent KRAB repression of a chromatinized transgene, we transiently depleted KAP1, HP1α, HP1β, HP1γ, and SETDB1 using a siRNA approach (Fig. 4B). Western blot analysis of protein extracts from cells transfected with siRNAs targeting the mRNAs of HP1α, HP1β, HP1γ, and SETDB1 indicated that the expression of these proteins was depleted by ≥75% (Fig. 8A; see also Fig. S10 in the supplemental material). Interestingly, we observed a slight reduction in the expression of HP1α in KAP1 knockdown cells, too. In contrast to the reduction of cellular levels of KAP1, depletion of each HP1 isoform individually resulted in little effect on KRAB repression, suggesting that the HP1 proteins are redundant in terms of function with KAP1 (Fig. 8B). However, simultaneous depletion of all three HP1 isoforms resulted in a greater-than-50% loss of KRAB repressor activity. We observed a similar effect on hormone-dependent KRAB-mediated repression in cells where SETDB1 was transiently depleted. Collectively, these genetic data support our biochemical data and further suggest that the HP1 proteins and SETDB1 have a fundamental role in site-specific regulation of chromatin structure and transcriptional repression by the KRAB-zfp–KAP1 repressor-corepressor complex.

DISCUSSION

The KAP1 protein fulfills several important criteria that define it as a corepressor protein for the KRAB-zfp superfamily of transcriptional repressors. However, the abundance of endogenous KAP1 has hindered the ability to investigate its dependence in KRAB-mediated repression. Here we have demonstrated that KRAB-mediated repression of both transiently transfected and chromatinized reporter transgenes was attenuated in cells where the endogenous level of KAP1 was reduced between 50 and 90%. The use of multiple independent siRNAs against KAP1 inhibited KRAB repression to varying extents which correlated with the expression level of the KAP1 protein, suggesting that a critical threshold level of KAP1 is required for KRAB repression. Furthermore, reexpression of the wild-type KAP1 protein in stable knockdown cells complemented the defect in KRAB repression. These data strongly argue against the repression defect arising completely from an off-target effect of the siRNAs. In addition, transient depletion of TIF1α and TIF1γ did not affect KRAB-mediated repression. This observation is consistent with in vitro biochemical experiments demonstrating selective interac-

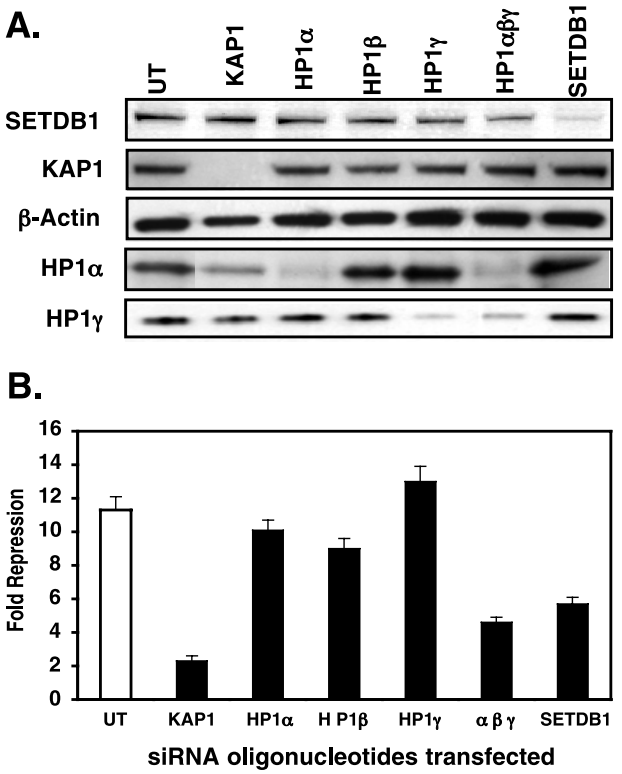

FIG. 8. Hormone-dependent KRAB repression of a chromatin template requires KAP1, HP1, and SETDB1. 12.10Kr cells were subjected to two rounds of transfection with dsRNA oligonucleotides to transiently reduce levels of the indicated proteins, as described in the legend for Fig. 4B. For the triple knockdown of HP1α, -β, and -γ, cells were transfected with 50 nM siRNA to each target. (A) Western blot analysis of whole-cell extracts prepared on day 6 of cells transfected with the indicated siRNA. UT, untransfected. (B) Transient depletion of KAP1, HP1, and SETDB1 attenuates hormone-dependent KRAB repression of a chromatin template. The data represent the averages of two independent experiments done in triplicate. Error bars represent the standard deviations of the means.

tion between the KRAB repression module and KAP1/TIF1β (1, 27, 50, 51). Although we cannot rule out that depletion of KAP1 from cells does not directly or indirectly affect the levels of known and unknown cellular proteins that cooperate with KAP1 to optimally repress transcription, the combination of these data is consistent with the conclusion that KAP1 is an essential cellular factor necessary to repress transcription by KRAB-zfps.

To further study the role of KAP1 and KAP1-interacting proteins in mediating transcriptional repression of a chromatin template, we investigated regulation of a stably integrated GAL4-responsive TK-luciferase transgene by hormone-responsive GAL4-KRAB and GAL4-KAP1 repressor proteins, respectively. In contrast to previous studies that have utilized a similar experimental strategy (4), we first created a series of cell lines that stably express luciferase from a randomly integrated transgene. Subsequently, we transfected these cells with plasmids to stably express either wild-type or mutant repressor proteins. This particular approach enabled us to study the effects of site-directed mutations in KAP1 on its function as a transcriptional repressor within the context of an isogenic, chromosomal locus.

Although direct tethering of KAP1 to a chromatin template is sufficient to rapidly repress transcription, the absolute level of steady-state repression is significantly less compared to tethering a heterologous KRAB repressor protein. Thus, the collection of our data indicates that KAP1 is necessary but may not be sufficient for KRAB repression. We speculate that the reduced efficiency of the heterologous KAP1 repressor protein may be a consequence of the fact that endogenous KAP1 is a trimer in solution (50) and that this native oligomerization state is not maintained by the ERHBD-GAL4-KAP1 protein. Furthermore, it is possible that in addition to facilitating oligomerization and the direct interaction between KAP1 and the KRAB repression module, the RBCC/TRIM domain may bind to additional cellular factors that are required for optimal levels of KAP1-mediated corepression of transcription. Future studies will be needed to determine whether the RBCC/TRIM domain of KAP1 contributes to transcriptional repression beyond simple recognition of the KRAB domain. Alternatively, our data suggest that KRAB-mediated repression results from the additive nature of a very rapid KAP1-dependent mechanism and a slower KAP1-independent mechanism. While our data set does not completely eliminate this possibility, time course experiments in 12.10Kr and 7.18Kr cells in which KAP1 had been either transiently or stably depleted by RNA inhibition-based approaches failed to demonstrate any level of repression following growth in medium containing 4-OHT for 144 h (S. P. Sripathy and D. C. Schultz, unpublished data). Thus, if KRAB-mediated repression were indeed the additive result of a fast KAP1-dependent mechanism and a slow KAP1-independent component, then our data would support a hypothesis that the initiation of the slow component would be dependent upon the presence of KAP1.

Previous studies have defined several KAP1 polypeptides that have the ability to autonomously repress transcription when directly tethered to DNA via a heterologous DNA binding domain. However, the importance of these repression domains in the context of the full-length KAP1 protein, and also their role in regulating transcription of a chromatin template, has not been studied. Data from transient-transfection reporter assays suggest that the repression mechanisms of the PHD finger/bromodomain and the HP1BD may be additive. Alternatively, these domains may work independently of one another and the different functions of these domains may be invoked depending on the nature of the target or the cell type. Our data demonstrate an obligate role for the interaction between KAP1 and HP1 in KRAB-KAP1 repression. In contrast, mutations in the PHD finger and bromodomain, respectively, display quite different results depending on the context of the assay. In transient-transfection reporter assays, mutations in either the PHD finger or bromodomain mildly impair KAP1-dependent repression relative to the wild-type protein but do not ablate its function like the HP1BD mutation. However, our data demonstrate an essential role for these domains in KAP1-mediated transcriptional repression of chromatin templates. In fact, mutations in these domains appear to be epistatic with the HP1BD mutation in KAP1 repression. These observations are not entirely surprising, given that these motifs are almost exclusively found in proteins that have a role in regulating chro-

matin structure and function (7, 25) and have been shown to bind specific posttranslational modifications of the histone proteins (17, 24, 37, 46, 48, 59, 68). Therefore, one might predict that mutations in these domains may have a more pronounced effect on the transcriptional regulation of a chromatin template. Further insights into the functions of these domains in KAP1-directed transcriptional repression will be dependent upon defining the specificity of the potential interactions these domains have with epitopes on histones, nucleosomes, or higher-order chromatin structure and the identification of native target genes regulated by KRAB-zfps.

Our understanding of transcriptionally silent chromatin assembly has been largely limited to studies of cytologically defined heterochromatin in *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and mammalian X-chromosome inactivation (6, 14, 21, 60). Thus, how heterochromatin domains are formed and how they function to repress transcription in euchromatic loci remain important questions. An advantage of our experimental system is that we can induce transcriptional silencing of a well-defined, highly transcribed transgene embedded in a chromatin environment. Therefore, our system has great utility to address fundamental questions regarding targeted gene silencing in time and space. Our ChIP data indicate a reduced steady-state level of hypophosphorylated RNA polymerase II at promoter sequences of a repressed transgene, suggesting that recruitment of RNA polymerase II has been altered. In *S. cerevisiae*, the formation of heterochromatin does not exclude the binding of preinitiation complex components to transcriptionally silenced genes but rather appears to attenuate productive initiation and/or elongation of transcription by RNA polymerase II (58). The disparity between these two observations may represent fundamental differences in heterochromatin assembly in budding yeast and higher eukaryotes. In this regard, *S. cerevisiae* lacks methylation of histone H3K9 and an HP1 orthologue. Alternatively, these differences may be attributed to unique characteristics of the genomic loci targeted for silencing. Thus, further insights into the impact of heterochromatin on RNA polymerase II activity will require the identification and characterization of endogenous targets that become transcriptionally silenced in association with formation of localized heterochromatin environments.

The increase in histone H3 occupancy throughout the transgene under repressed conditions may represent an indirect measurement of increased nucleosome ordering. We have previously shown that repression of a chromatinized reporter transgene by a KRAB repressor protein reduces accessibility of DNA sequences to restriction endonucleases in situ (4). In *D. melanogaster*, HP1 has been shown to induce long-range ordering of nucleosomes associated with transgenes embedded within heterochromatin environments (61). Although our ChIP data indicate a bias in HP1 deposition at sequences surrounding the promoter of the transgene, we did detect hormone-dependent increases in the levels of HP1 within the coding sequences of the transgene. These data could be indicative of HP1 spreading, ultimately leading to increased ordering of nucleosomes throughout the transcription unit. Interestingly, recent data indicate that methylation of histone H1K26 can be recognized by the chromodomain of HP1 (15, 19, 30). Histone H1 is instrumental in the organization of oligonucleosomes into higher-order structures and, therefore, it would be intriguing to investigate the potential role of KAP1 in the recruitment, methylation, and codeposition of methylated histone H1 with HP1. Thus, one possibility is that the KRAB-KAP1 repression complex directs the assembly of a highly organized chromatin environment that sterically interferes with the binding of transcriptional activator proteins and the ultimate recruitment/engagement of RNA polymerase II.

While many studies have investigated the correlation between a specific histone modification and a particular cytological domain or transcriptional state of a gene, few studies have looked into the temporal and spatial patterns of multiple modifications during gene silencing. In our study we looked at the spatial distribution of general histone occupancy, histone acetylation, and site-specific histone methylation. Induction of transcriptional silencing by direct tethering of the KAP1 corepressor to DNA is characterized by increased histone occupancy and a concomitant decrease in histone H3 acetylation, H3K4 methylation, an increase in trimethylation of H3K9, H3K27, H3K36, and H4K20, and enrichment of the HP1 proteins at proximal regulatory elements of the transgene. The enrichment of the H3K9 trimethyl epitope, HP1, and SETDB1 at promoter sequences is consistent with our previous data (4, 55). Furthermore, hormone-dependent KRAB repression is attenuated in cells where SETDB1 has been transiently depleted. The preference for trimethylated H3K9 is consistent with the observation that the SETDB1/mAM enzyme complex possesses processivity to trimethylate substrates (66). Despite recent data linking H3K9 trimethylation and HP1γ localization to coding regions of actively transcribed genes (63), we have not quantitatively detected high levels of these antigens in the coding region of our actively transcribed transgene. The enrichment of trimethyl histone H4K20 is not entirely surprising, as this epitope cytologically localizes to constitutive heterochromatin domains in a histone H3K9 methylation-dependent manner (54). This dependency may possibly explain the absence of a 4-OHT-dependent increase at transgene sequences in cell lines that express the mutant ERHBD-GAL-KAP1 proteins. Moreover, this observation may suggest that the formation of a highly localized domain of heterochromatin mimics the structure of constitutive heterochromatin domains. The enrichment of trimethylated H3K27 is an intriguing observation; however, the patterns of H3K27 methylation consistently mirror the H3K9 methylation patterns, suggesting that this result may be due to cross-reactivity of this antibody with methylated H3K9 or H1K26. Preliminary experiments that transiently depleted EZH2 and EHMT1/G9a, two enzymes that methylate H3K27 in vivo, did not alter hormone-dependent KRAB repression (Sripathy and Schultz, unpublished data). Thus, the relevance of this observation relative to KAP1 repression cannot be fully defined by the current study.

In addition to enhanced methylation of H3K9, H3K27, and H4K20, we observed an increase in histone H3K36 methylation associated with the DNA sequence in the proximal regulatory elements of the transgene. Methylation of histone H3K4, -K36, and -K79 is commonly associated with transcriptional competence (20). Indeed, we did observe a 4-OHT-dependent decrease in H3K4 methylation throughout the transgene and H3K36 methylation associated with DNA sequences in the downstream transcriptional unit, as would be expected for a repressed transcript. However, the precise func-

tion of increased H3K36 methylation at promoter sequences in transcriptional repression is unclear at this time. Interestingly, our data suggest that H3K4 and H3K9 methylation may coexist within the same regions of a transcriptionally silenced transgene. This result may be explained by the fact that SETDB1 can methylate substrates that possess methylation on K4 (55). To determine whether these modifications coexist in the same nucleosome or even on the same histone, reimmunoprecipitation experiments will need to be done in the future. Regardless, these data are in contrast with locus-wide data from the *Schizosaccharomyces pombe* mating type locus, which demonstrate an inverse correlation between these two modifications (45). These data indicate that H3K4 methylation does not need to be completely removed in order for the transcription of a gene to be repressed and, therefore, our data may represent a fundamental difference between constitutive and localized heterochromatin domains. Interestingly, removal of 4-OHT from the growth medium reactivates luciferase gene expression with kinetics that are nearly identical to the time (48 to 72 h) it takes the ERHBD-GAL4-KAP1 protein to reach steady-state repression of the transgene (Sripathy and Schultz, unpublished data). The presence of histone H3K4 and H3K36 methylation may explain the rapid kinetics of the transgene's transcriptional reactivation following withdrawal of 4-OHT. Although these data are the first to define the repertoire of histone modification patterns associated with a transcriptional unit repressed by KAP1 future studies are needed to examine the temporal changes in the patterns of histone modifications as a gene transitions from a transcribed state to a transcriptionally repressed state.

Another unique advantage of our experimental system is that we can evaluate the consequences of well-defined mutations in the various domains of KAP1 on molecular changes in chromatin structure of a target gene. Cells that express the HP1 binding mutant KAP1 repressor protein fail to repress transcription of the integrated target. Consistent with this result, the simultaneous reduction of HP1α/β/γ reduced the efficiency of KRAB-mediated repression. Interestingly, cells that only express a KAP1 protein possessing a mutation in the HP1 binding domain fail to undergo endodermal differentiation in vitro (11). The combination of these data suggests that the HP1-binding-deficient allele of KAP1 in these cells fails to repress transcription of endogenous KRAB-zfp target genes required for the cell to differentiate. The major question that remains is how HP1 mechanistically influences the transcriptional state of a KRAB-zfp target gene. At a molecular level, our data are consistent with a hypothesis that KAP1 and HP1 direct the assembly of a localized microenvironment of heterochromatin at gene-specific loci. In our experiments, the HP1-binding-deficient KAP1 mutant protein not only failed to recruit HP1 to the target locus but also failed to induce methylation of H3K9. Furthermore, the magnitude of other changes in histone modifications appeared to be less severe when compared to the wild-type repressor protein. These data suggest that HP1 has additional functions in KAP1-mediated transcriptional repression beyond simple recognition of methylated H3K9 or H1K26. The binding of HP1 to KAP1 may lead to a change in structural conformation of the corepressor required for the functions of the PHD finger and bromodomain. Alternatively, the binding of HP1 may trigger the translocation of target genes from eu- to heterochromatin in order to silence gene expression, including the coordination of activities that modulate changes in histone modifications. This latter mechanism has been proposed for the transcription factor Ikaros, which regulates the expression of genes involved in T-cell activation (8, 9). The potential role for HP1-directed nuclear compartmentalization in KRAB-KAP1 regulation of gene expression is underscored by several pieces of experimental data. First, KAP1 that is unable to interact with HP1 fails to associate with cytologically defined heterochromatin following stimulation of cellular differentiation in vitro (12). Second, transcriptional repression of an integrated transgene by a hormone-responsive KRAB repressor protein correlated with an increased frequency of association with cytologically defined heterochromatin (4). Finally, the KRAB-zfps KRAZ1 and KRAZ2 colocalize with KAP1 and HP1 proteins within 4′,6′-diamidino-2-phenylindole-stained heterochromatin in fibroblasts (41). Future experiments are needed to identify genes that are direct targets of KRAB-KAP1 transcriptional regulation and how the KAP1 interaction with HP1 regulates the transcription of these genes during cellular differentiation, organismal development, and possibly human disease.

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REFERENCES

1. **Abrink, M., J. A. Ortiz, C. Mark, C. Sanchez, C. Looman, L. Hellman, P. Chambon, and R. Losson.** 2001. Conserved interaction between distinct Kruppel-associated box domains and the transcriptional intermediary factor 1 beta. Proc. Natl. Acad. Sci. USA **98:**1422–1426.
2. **Agata, Y., E. Matsuda, and A. Shimizu.** 1999. Two novel Kruppel-associated box-containing zinc-finger proteins, KRAZ1 and KRAZ2, repress transcription through functional interaction with the corepressor KAP-1 (TIF1β/KRIP-1). J. Biol. Chem. **274:**16412–16422.
3. **Ayyanathan, K., W. J. Fredericks, C. Berking, M. Herlyn, C. Balakrishnan, E. Gunther, and F. J. Rauscher III.** 2000. Hormone-dependent tumor regression in vivo by an inducible transcriptional repressor directed at the PAX3-FKHR oncogene. Cancer Res. **60:**5803–5814.
4. **Ayyanathan, K., M. S. Lechner, P. Bell, G. G. Maul, D. C. Schultz, Y. Yamada, K. Tanaka, K. Torigoe, and F. J. Rauscher III.** 2003. Regulated recruitment of HP1 to a euchromatic gene induces mitotically heritable, epigenetic gene silencing: a mammalian cell culture model of gene variegation. Genes Dev. **17:**1855–1869.
5. **Beckstead, R., J. A. Ortiz, C. Sanchez, S. N. Prokopenko, P. Chambon, R. Losson, and H. J. Bellen.** 2001. Bonus, a Drosophila homolog of TIF1 proteins, interacts with nuclear receptors and can inhibit βFTZ-F1-dependent transcription. Mol. Cell **7:**753–765.
6. **Bernstein, E., and C. D. Allis.** 2005. RNA meets chromatin. Genes Dev. **19:**1635–1655.
7. **Bienz, M.** 2006. The PHD finger, a nuclear protein-interaction domain. Trends Biochem. Sci. **31:**35–40.
8. **Brown, K. E., J. Baxter, D. Graf, M. Merkenschlager, and A. G. Fisher.** 1999. Dynamic repositioning of genes in the nucleus of lymphocytes preparing for cell division. Mol. Cell **3:**207–217.
9. **Brown, K. E., S. S. Guest, S. T. Smale, K. Hahm, M. Merkenschlager, and A. G. Fisher.** 1997. Association of transcriptionally silent genes with Ikaros complexes at centromeric heterochromatin. Cell **91:**845–854.
10. **Brummelkamp, T. R., R. Bernards, and R. Agami.** 2002. Stable suppression of tumorigenicity by virus-mediated RNA interference. Cancer Cell **2:**243–247.
11. **Cammas, F., M. Herzog, T. Lerouge, P. Chambon, and R. Losson.** 2004. Association of the transcriptional corepressor TIF1β with heterochromatin protein 1 (HP1): an essential role for progression through differentiation. Genes Dev. **18:**2147–2160.

12. **Cammas, F., M. Oulad-Abdelghani, J. L. Vonesch, Y. Huss-Garcia, P. Chambon, and R. Losson.** 2002. Cell differentiation induces TIF1β association with centromeric heterochromatin via an HP1 interaction. J. Cell Sci. **115:**3439–3448.
13. **Capili, A. D., D. C. Schultz, I. F. Rauscher, and K. L. Borden.** 2001. Solution structure of the PHD domain from the KAP-1 corepressor: structural determinants for PHD, RING and LIM zinc-binding domains. EMBO J. **20:** 165–177.
14. **Chang, S. C., T. Tucker, N. P. Thorogood, and C. J. Brown.** 2006. Mechanisms of X-chromosome inactivation. Front. Biosci. **11:**852–866.
15. **Daujat, S., U. Zeissler, T. Waldmann, N. Happel, and R. Schneider.** 2005. HP1 binds specifically to Lys26-methylated histone H1.4, whereas simultaneous Ser27 phosphorylation blocks HP1 binding. J. Biol. Chem. **280:**38090–38095.
16. **Dehal, P., P. Predki, A. S. Olsen, A. Kobayashi, P. Folta, S. Lucas, M. Land, A. Terry, C. L. Ecale Zhou, S. Rash, Q. Zhang, L. Gordon, J. Kim, C. Elkin, M. J. Pollard, P. Richardson, D. Rokhsar, E. Uberbacher, T. Hawkins, E. Branscomb, and L. Stubbs.** 2001. Human chromosome 19 and related regions in mouse: conservative and lineage-specific evolution. Science **293:**104–111.
17. **Dhalluin, C., J. E. Carlson, L. Zeng, C. He, A. K. Aggarwal, and M. M. Zhou.** 1999. Structure and ligand of a histone acetyltransferase bromodomain. Nature **399:**491–496.
18. **Friedman, J. R., W. J. Fredericks, D. E. Jensen, D. W. Speicher, X. P. Huang, E. G. Neilson, and F. J. Rauscher III.** 1996. KAP-1, a novel corepressor for the highly conserved KRAB repression domain. Genes Dev. **10:**2067–2078.
19. **Garcia, B. A., S. A. Busby, C. M. Barber, J. Shabanowitz, C. D. Allis, and D. F. Hunt.** 2004. Characterization of phosphorylation sites on histone H1 isoforms by tandem mass spectrometry. J. Proteome Res. **3:**1219–1227.
20. **Gerber, M., and A. Shilatifard.** 2003. Transcriptional elongation by RNA polymerase II and histone methylation. J. Biol. Chem. **278:**26303–26306.
21. **Grewal, S. I., and J. C. Rice.** 2004. Regulation of heterochromatin by histone methylation and small RNAs. Curr. Opin. Cell Biol. **16:**230–238.
22. **Hake, S., A. Xiao, and C. Allis.** 2004. Linking the epigentic 'language' of covalent histone modifications to cancer. Br. J. Cancer **90:**761–769.
23. **Huntley, S., D. M. Baggott, A. T. Hamilton, M. Tran-Gyamfi, S. Yang, J. Kim, L. Gordon, E. Branscomb, and L. Stubbs.** 2006. A comprehensive catalog of human KRAB-associated zinc finger genes: insights into the evolutionary history of a large family of transcriptional repressors. Genome Res. **16:**669–677.
24. **Jacobson, R. H., A. G. Ladurner, D. S. King, and R. Tjian.** 2000. Structure and function of a human TAFII250 double bromodomain module. Science **288:**1422–1425.
25. **Jeanmougin, F., J. M. Wurtz, B. Le Douarin, P. Chambon, and R. Losson.** 1997. The bromodomain revisited. Trends Biochem. Sci. **22:**151–153.
26. **Jenuwein, T., and C. Allis.** 2001. Translating the histone code. Science **293:**1074–1080.
27. **Khetchoumian, K., M. Teletin, M. Mark, T. Lerouge, M. Cervino, M. Oulad-Abdelghani, P. Chambon, and R. Losson.** 2004. TIF1δ, a novel HP1-interacting member of the transcriptional intermediary factor 1 (TIF1) family expressed by elongating spermatids. J. Biol. Chem. **279:**48329–48341.
28. **Kim, S. S., Y. M. Chen, E. O'Leary, R. Witzgall, M. Vidal, and J. V. Bonventre.** 1996. A novel member of the RING finger family, KRIP-1, associates with the KRAB-A transcriptional repressor domain of zinc finger proteins. Proc. Natl. Acad. Sci. USA **93:**15299–15304.
29. **Krebs, C. J., L. K. Larkins, S. M. Khan, and D. M. Robins.** 2005. Expansion and diversification of KRAB zinc-finger genes within a cluster including Regulator of sex-limitation 1 and 2. Genomics **85:**752–761.
30. **Kuzmichev, A., T. Jenuwein, P. Tempst, and D. Reinberg.** 2004. Different EZH2-containing complexes target methylation of histone H1 or nucleosomal histone H3. Mol. Cell **14:**183–193.
31. **Laity, J. H., B. M. Lee, and P. E. Wright.** 2001. Zinc finger proteins: new insights into structural and functional diversity. Curr. Opin. Struct. Biol. **11:**39–46.
32. **Lander, E. S., L. M. Linton, B. Birren, C. Nusbaum, M. C. Zody, J. Baldwin, K. Devon, K. Dewar, M. Doyle, W. FitzHugh, R. Funke, D. Gage, K. Harris, A. Heaford, J. Howland, L. Kann, J. Lehoczky, R. LeVine, P. McEwan, K. McKernan, J. Meldrim, J. P. Mesirov, C. Miranda, W. Morris, J. Naylor, C. Raymond, M. Rosetti, R. Santos, A. Sheridan, C. Sougnez, N. Stange-Thomann, N. Stojanovic, A. Subramanian, D. Wyman, J. Rogers, J. Sulston, R. Ainscough, S. Beck, D. Bentley, J. Burton, C. Clee, N. Carter, A. Coulson, R. Deadman, P. Deloukas, A. Dunham, I. Dunham, R. Durbin, L. French, D. Grafham, S. Gregory, T. Hubbard, S. Humphray, A. Hunt, M. Jones, C. Lloyd, A. McMurray, L. Matthews, S. Mercer, S. Milne, J. C. Mullikin, A. Mungall, R. Plumb, M. Ross, R. Shownkeen, S. Sims, R. H. Waterston, R. K. Wilson, L. W. Hillier, J. D. McPherson, M. A. Marra, E. R. Mardis, L. A. Fulton, A. T. Chinwalla, K. H. Pepin, W. R. Gish, S. L. Chissoe, M. C. Wendl, K. D. Delehaunty, T. L. Miner, A. Delehaunty, J. B. Kramer, L. L. Cook, R. S. Fulton, D. L. Johnson, P. J. Minx, S. W. Clifton, T. Hawkins, E. Branscomb, P. Predki, P. Richardson, S. Wenning, T. Slezak, N. Doggett, J. F. Cheng, A. Olsen, S. Lucas, C. Elkin, E. Uberbacher, M. Frazier, et al.** 2001. Initial sequencing and analysis of the human genome. Nature **409:**860–921.
33. **Lechner, M. S., G. E. Begg, D. W. Speicher, and F. J. Rauscher III.** 2000. Molecular determinants for targeting heterochromatin protein 1-mediated gene silencing: direct chromoshadow domain-KAP-1 corepressor interaction is essential. Mol. Cell. Biol. **20:**6449–6465.
34. **Lechner, M. S., D. C. Schultz, D. Negorev, G. G. Maul, and F. J. Rauscher III.** 2005. The mammalian heterochromatin protein 1 binds diverse nuclear proteins through a common motif that targets the chromoshadow domain. Biochem. Biophys. Res. Commun. **331:**929–937.
35. **Le Douarin, B., A. L. Nielsen, J. M. Garnier, H. Ichinose, F. Jeanmougin, R. Losson, and P. Chambon.** 1996. A possible involvement of TIF1 alpha and TIF1 beta in the epigenetic control of transcription by nuclear receptors. EMBO J. **15:**6701–6715.
36. **Le Douarin, B., A. L. Nielsen, J. You, P. Chambon, and R. Losson.** 1997. TIF1 alpha: a chromatin-specific mediator for the ligand-dependent activation function AF-2 of nuclear receptors? Biochem. Soc. Trans. **25:**605–612.
37. **Li, H., S. Ilin, W. Wang, E. M. Duncan, J. Wysocka, C. D. Allis, and D. J. Patel.** 2006. Molecular basis for site-specific read-out of histone H3K4me3 by the BPTF PHD finger of NURF. Nature **442:**91–95.
38. **Littlewood, T. D., D. C. Hancock, P. S. Danielian, M. G. Parker, and G. I. Evan.** 1995. A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. Nucleic Acids Res. **23:**1686–1690.
39. **Margolin, J. F., J. R. Friedman, W. K. Meyer, H. Vissing, H. J. Thiesen, and F. J. Rauscher III.** 1994. Kruppel-associated boxes are potent transcriptional repression domains. Proc. Natl. Acad. Sci. USA **91:**4509–4513.
40. **Martin, C., and Y. Zhang.** 2005. The diverse functions of histone lysine methylation. Nat. Rev. Mol. Cell. Biol. **6:**838–849.
41. **Matsuda, E., Y. Agata, M. Sugai, T. Katakai, H. Gonda, and A. Shimizu.** 2001. Targeting of Kruppel-associated box-containing zinc finger proteins to centromeric heterochromatin. Implication for the gene silencing mechanisms. J. Biol. Chem. **276:**14222–14229.
42. **Moosmann, P., O. Georgiev, B. Le Douarin, J. P. Bourquin, and W. Schaffner.** 1996. Transcriptional repression by RING finger protein TIF1 beta that interacts with the KRAB repressor domain of KOX1. Nucleic Acids Res. **24:**4859–4867.
43. **Moosmann, P., O. Georgiev, H. J. Thiesen, M. Hagmann, and W. Schaffner.** 1997. Silencing of RNA polymerases II and III-dependent transcription by the KRAB protein domain of KOX1, a Kruppel-type zinc finger factor. Biol. Chem. **378:**669–677.
44. **Nielsen, A. L., J. A. Ortiz, J. You, M. Oulad-Abdelghani, R. Khechumian, A. Gansmuller, P. Chambon, and R. Losson.** 1999. Interaction with members of the heterochromatin protein 1 (HP1) family and histone deacetylation are differentially involved in transcriptional silencing by members of the TIF1 family. EMBO J. **18:**6385–6395.
45. **Noma, K., C. D. Allis, and S. I. Grewal.** 2001. Transitions in distinct histone H3 methylation patterns at the heterochromatin domain boundaries. Science **293:**1150–1155.
46. **Owen, D. J., P. Ornaghi, J. C. Yang, N. Lowe, P. R. Evans, P. Ballario, D. Neuhaus, P. Filetici, and A. A. Travers.** 2000. The structural basis for the recognition of acetylated histone H4 by the bromodomain of histone acetyltransferase gcn5p. EMBO J. **19:**6141–6149.
47. **Pavletich, N. P., and C. O. Pabo.** 1991. Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 Å. Science **252:**809–817.
48. **Pena, P. V., F. Davrazou, X. Shi, K. L. Walter, V. V. Verkhusha, O. Gozani, R. Zhao, and T. G. Kutateladze.** 2006. Molecular mechanism of histone H3K4me3 recognition by plant homeodomain of ING2. Nature **442:**100–103.
49. **Peng, H., G. E. Begg, S. L. Harper, J. R. Friedman, D. W. Speicher, and F. J. Rauscher III.** 2000. Biochemical analysis of the Kruppel-associated box (KRAB) transcriptional repression domain. J. Biol. Chem. **275:**18000–18010.
50. **Peng, H., G. E. Begg, D. C. Schultz, J. R. Friedman, D. E. Jensen, D. W. Speicher, and F. J. Rauscher III.** 2000. Reconstitution of the KRAB-KAP-1 repressor complex: a model system for defining the molecular anatomy of RING-B box-coiled-coil domain-mediated protein-protein interactions. J. Mol. Biol. **295:**1139–1162.
51. **Peng, H., I. Feldman, and F. J. Rauscher III.** 2002. Hetero-oligomerization among the TIF family of RBCC/TRIM domain-containing nuclear cofactors: a potential mechanism for regulating the switch between coactivation and corepression. J. Mol. Biol. **320:**629–644.
52. **Ryan, R. F., D. C. Schultz, K. Ayyanathan, P. B. Singh, J. R. Friedman, W. J. Fredericks, and F. J. Rauscher III.** 1999. KAP-1 corepressor protein interacts and colocalizes with heterochromatic and euchromatic HP1 proteins: a potential role for Kruppel-associated box-zinc finger proteins in heterochromatin-mediated gene silencing. Mol. Cell. Biol. **19:**4366–4378.
53. **Sadowski, I., B. Bell, P. Broad, and M. Hollis.** 1992. GAL4 fusion vectors for expression in yeast or mammalian cells. Gene **118:**137–141.
54. **Schotta, G., M. Lachner, K. Sarma, A. Ebert, R. Sengupta, G. Reuter, D. Reinberg, and T. Jenuwein.** 2004. A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin. Genes Dev. **18:**1251–1262.
55. **Schultz, D. C., K. Ayyanathan, D. Negorev, G. G. Maul, and F. J. Rauscher**

III. 2002. SETDB1: a novel KAP-1-associated histone H3, lysine 9-specific methyltransferase that contributes to HP1-mediated silencing of euchromatic genes by KRAB zinc-finger proteins. Genes Dev. **16:**919–932.
56. **Schultz, D. C., J. R. Friedman, and F. J. Rauscher III.** 2001. Targeting histone deacetylase complexes via KRAB-zinc finger proteins: the PHD and bromodomains of KAP-1 form a cooperative unit that recruits a novel isoform of the Mi-2alpha subunit of NuRD. Genes Dev. **15:**428–443.
57. **Schultz, D. C., L. Vanderveer, K. H. Buetow, M. P. Boente, R. F. Ozols, T. C. Hamilton, and A. K. Godwin.** 1995. Characterization of chromosome 9 in human ovarian neoplasia identifies frequent genetic imbalance on 9q and rare alterations involving 9p, including CDKN2. Cancer Res **55:**2150–2157.
58. **Sekinger, E. A., and D. S. Gross.** 2001. Silenced chromatin is permissive to activator binding and PIC recruitment. Cell **105:**403–414.
59. **Shi, X., T. Hong, K. L. Walter, M. Ewalt, E. Michishita, T. Hung, D. Carney, P. Pena, F. Lan, M. R. Kaadige, N. Lacoste, C. Cayrou, F. Davrazou, A. Saha, B. R. Cairns, D. E. Ayer, T. G. Kutateladze, Y. Shi, J. Cote, K. F. Chua, and O. Gozani.** 2006. ING2 PHD domain links histone H3 lysine 4 methylation to active gene repression. Nature **442:**96–99.
60. **Smale, S. T.** 2003. The establishment and maintenance of lymphocyte identity through gene silencing. Nat. Immunol. **4:**607–615.
61. **Sun, F. L., M. H. Cuaycong, and S. C. Elgin.** 2001. Long-range nucleosome ordering is associated with gene silencing in *Drosophila melanogaster* pericentric heterochromatin. Mol. Cell. Biol. **21:**2867–2879.
62. **Turner, B. M.** 2003. Memorable transcription. Nat. Cell Biol. **5:**390–393.
63. **Vakoc, C., S. Mandat, B. Olenchock, and G. Blobel.** 2005. Histone H3 lysine 9 methylation and HP1gamma are associated with transcription elongation through mammalian chromatin. Mol. Cell **19:**381–391.
64. **Venturini, L., J. You, M. Stadler, R. Galien, V. Lallemand, M. H. Koken, M. G. Mattei, A. Ganser, P. Chambon, R. Losson, and H. de The.** 1999. TIF1γ, a novel member of the transcriptional intermediary factor 1 family. Oncogene **18:**1209–1217.
65. **Vissing, H., W. K. Meyer, L. Aagaard, N. Tommerup, and H. J. Thiesen.** 1995. Repression of transcriptional activity by heterologous KRAB domains present in zinc finger proteins. FEBS Lett. **369:**153–157.
66. **Wang, H., W. An, R. Cao, L. Xia, H. Erdjument-Bromage, B. Chatton, P. Tempst, R. G. Roeder, and Y. Zhang.** 2003. mAM facilitates conversion by ESET of dimethyl to trimethyl lysine 9 of histone H3 to cause transcriptional repression. Mol. Cell **12:**475–487.
67. **Witzgall, R., E. O'Leary, A. Leaf, D. Onaldi, and J. V. Bonventre.** 1994. The Kruppel-associated box-A (KRAB-A) domain of zinc finger proteins mediates transcriptional repression. Proc. Natl. Acad. Sci. USA **91:**4514–4518.
68. **Wysocka, J., T. Swigut, H. Xiao, T. A. Milne, S. Y. Kwon, J. Landry, M. Kauer, A. J. Tackett, B. T. Chait, P. Badenhorst, C. Wu, and C. D. Allis.** 2006. A PHD finger of NURF couples histone H3 lysine 4 trimethylation with chromatin remodelling. Nature **442:**86–90.