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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

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Mammalian TIF1α and TIF1β (KAP-1/KRIP-1) are related transcriptional intermediary factors that possess intrinsic silencing activity. TIF1α is believed to be a euchromatic target for liganded nuclear receptors, while TIF1β may serve as a co-repressor for the large family of KRAB domain-containing zinc finger proteins. Here, we report an association of TIF1β with both heterochromatin and euchromatin in interphase nuclei. Co-immunoprecipitation of nuclear extracts shows that endogenous TIF1β, but not TIF1α, is associated with members of the heterochromatin protein 1 (HP1) family. However, *in vitro*, both TIF1α and TIF1β interact with and phosphorylate the HP1 proteins. This interaction involves a conserved amino acid motif, which is critical for the silencing activity of TIF1β but not TIF1α. We further show that trichostatin A, an inhibitor of histone deacetylases, can interfere with both TIF1 and HP1 silencing. The silencing activity of TIF1α appears to result chiefly from histone deacetylation, whereas that of TIF1β may be mediated via both HP1 binding and histone deacetylation.

Keywords: chromatin remodelling/KRAB domain/phosphorylation/protein kinase/transcriptional repression

Introduction

Transcriptional regulation of gene expression in eukaryotes in response to developmental or environmental signals is a complex multi-step process that requires the concerted action of many cellular factors. Central players in this elaborate process are sequence-specific transcription factors that positively and/or negatively control transcription through interactions with transcriptional intermediary factors (TIFs; also designated as co-activators and co-repressors), whose function is to remodel chromatin structure (reviewed in Kornberg and Lorch, 1999; Wolffe and Hayes, 1999), to stimulate or inhibit (pre)initiation complex formation (reviewed in Orphanides *et al*., 1996; Hampsey and Reinberg, 1999 and references therein) or to associate target genes with specialized nuclear compartments that confer transcriptional activation or repression (Brown *et al*., 1997, 1999; Cocktell and Gasser, 1999).

Mammalian TIF1α and TIF1β are members of a family of TIFs that are believed to function at the level of the chromatin template (Le Douarin *et al*., 1996). These proteins display a characteristic domain structure comprising an N-terminal RBCC (RING finger, B boxes, coiled coil) motif, a poorly conserved central region, and a C-terminal region that contains a PHD finger and a bromodomain (see Figure 5A). Whereas RING fingers are present in many proteins with diverse functions (Freemont, 1993; Saurin *et al*., 1996), PHD fingers and bromodomains are restricted to nuclear proteins, including transcriptional cofactors and chromatin-remodelling proteins (Aasland *et al*., 1995; Jeanmougin *et al*., 1997 and references therein). Initially identified in a yeast genetic screen for proteins increasing the transactivation potential of retinoid X receptors (RXRs) (Le Douarin *et al*., 1995a), TIF1α was subsequently found: (i) to interact *in vitro* with members of the nuclear receptor superfamily in the presence of their cognate agonistic ligand (Le Douarin *et al*., 1995a; vom Baur *et al*., 1996); (ii) to be a phosphoprotein that undergoes a ligand-dependent hyperphosphorylation as a consequence of nuclear receptor binding *in vivo* (Fraser *et al*., 1998); (iii) to possess an intrinsic protein kinase activity (Fraser *et al*., 1998); (iv) to be tightly and preferentially associated with highly accessible euchromatic regions (Remboutsika *et al*., 1999); and (v) to be a particularly abundant protein in toti- and pluripotent cells, suggesting that it may represent a marker for numerous euchromatic sites in the genome of these cells (Remboutsika *et al*., 1999). Supporting the notion that TIF1α could play a dual role in transcription, being involved in both activation and repression, TIF1α was also reported to silence transcription when tethered to DNA through fusion to a heterologous DNA binding domain (DBD) and to interact *in vitro* with members of the heterochromatin protein 1 (HP1) family (Le Douarin *et al*., 1996), a subfamily of the chromatin organization modifier (chromo) superfamily (Koonin *et al*., 1995), which is thought to be important for regulating heterochromatin-mediated silencing and chromosome structure. The prototype of this family is *Drosophila* HP1, which is preferentially localized in pericentric heterochromatin (James and Elgin, 1986) and involved in position effect variegation (PEV) (Eissenberg *et al*., 1990), a well-known epigenetic silencing activity exhibited by

euchromatic genes placed within or near heterochromatin (Wakimoto, 1998). In mammals, three HP1 homologues have been described: HP1α, HP1β (MOD1/M31) and HP1γ (MOD2/M32) (Singh *et al*., 1991; Saunders *et al*., 1993). We and others found that HP1α and HP1γ can significantly repress transcription when fused to a heterologous DBD in transiently transfected cells (Le Douarin *et al*., 1996; Lehming *et al*., 1998; Seeler *et al*., 1998).

Using HP1α as bait in a two-hybrid screen (Le Douarin *et al*., 1996), we isolated TIF1β, also referred to as KAP-1 (KRAB-associated protein 1) or KRIP-1 (KRAB-interacting protein 1), by virtue of its ability to interact with the transcriptional silencing KRAB domain of human zinc finger proteins, including KOX1 and Kid-1 (Friedman *et al*., 1996; Kim *et al*., 1996; Moosmann *et al*., 1996). The so-called KRAB domain, which is present in about one-third of the 300–700 human zinc finger proteins of the Krüppel Cys2His2-type (Bellefroid *et al*., 1991), is one of the most widely distributed transcriptional silencing domains identified yet in vertebrates (Margolin *et al*., 1994; Witzgall *et al*., 1994; Vissing *et al*., 1995). When fused to a heterologous DBD, it silences both basal and activated transcription in a dose-dependent manner and over large distances (Pengue *et al*., 1994; Deuschle *et al*., 1995; Moosmann *et al*., 1997). TIF1β interacts with numerous KRAB domains both in yeast and *in vitro*, but not with KRAB mutants that do not repress transcription *in vivo*. An overexpression of TIF1β can enhance KRAB-mediated repression, and TIF1β itself acts as a repressor when tethered to DNA (Friedman *et al*., 1996; Kim *et al*., 1996; Moosmann *et al*., 1996). Thus, TIF1β exhibits properties expected for a transcriptional co-repressor of the large family of KRAB domain-containing zinc finger proteins (KRAB ZFPs).

The molecular mechanisms by which TIF1α and TIF1β may exert their repressing effects on transcription have not yet been elucidated. However, both proteins share structural and functional features that strongly favour chromatin-mediated mechanisms. Supporting this view, our present results provide evidence for a direct role of the HP1 proteins in TIF1β silencing and implicate histone deacetylation in both TIF1α- and TIF1β-mediated silencing.

Results

Immunolocalization of TIF1β and HP1 proteins

The intracellular localization of TIF1β, HP1α, HP1β and HP1γ was investigated in mouse NIH 3T3 cells using indirect immunofluorescence labelling and confocal microscopy. DNA-containing structures were visualized by Hoechst 33258 staining and endogenous proteins were detected using specific monoclonal antibodies (mAbs) and a Cy3-fluorochrome-conjugated secondary antibody (Figure 1). As previously reported (Wreggett *et al*., 1994; Remboutsika *et al*., 1999), HP1α was detected predominantly in heterochromatic regions, revealed by Hoechst staining as spots of brighter fluorescence (Figure 1C and D). These large blocks of condensed heterochromatin were also labelled by HP1β and HP1γ antibodies; however, HP1β and even more so HP1γ were detected in many additional sites dispersed within the nucleus (Figure 1E–H). Note that the present distribution of HP1γ is in contrast to that observed in mouse C1271 interphase cells, in which HP1γ appears to be largely excluded from heterochromatin (Horsley *et al*., 1996).

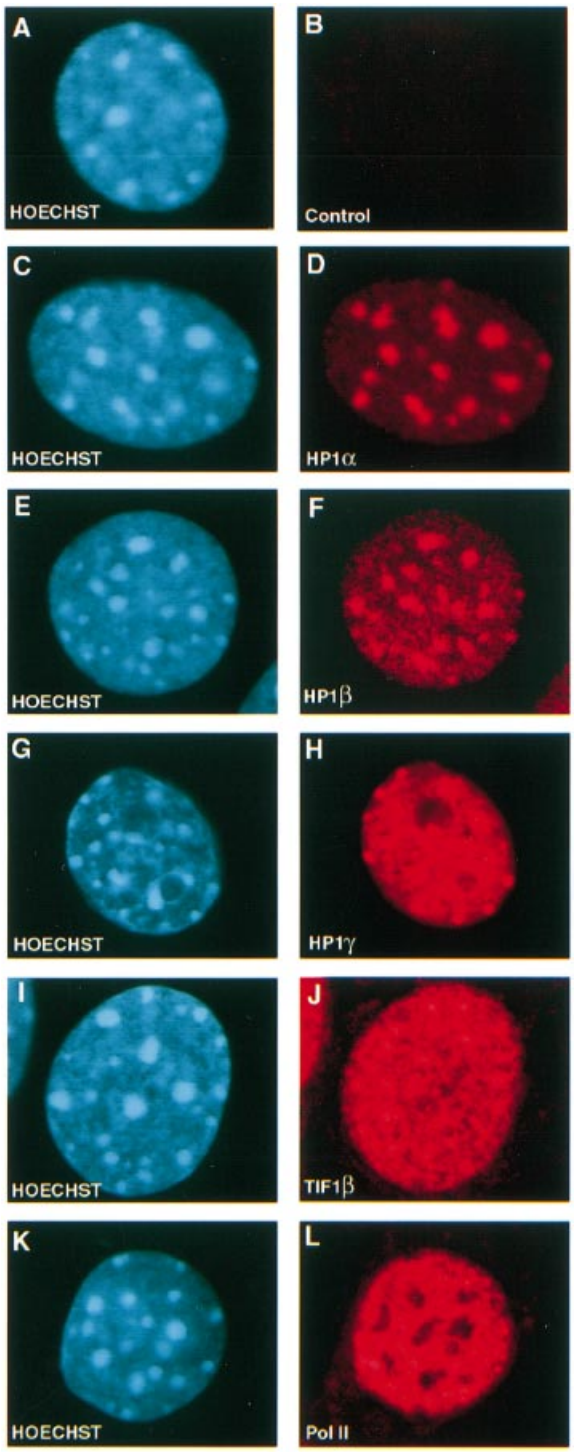

Fig. 1. Distribution of endogenous TIF1β and HP1 proteins within the nuclei of NIH 3T3 cells. Confocal images of single optical sections through the nucleus of representative individual cells are shown. Left panels show the Hoechst DNA staining, and right panels correspond to immunodetection with specific mAbs, as indicated. A mouse IgG fraction was used as a negative control in (B).

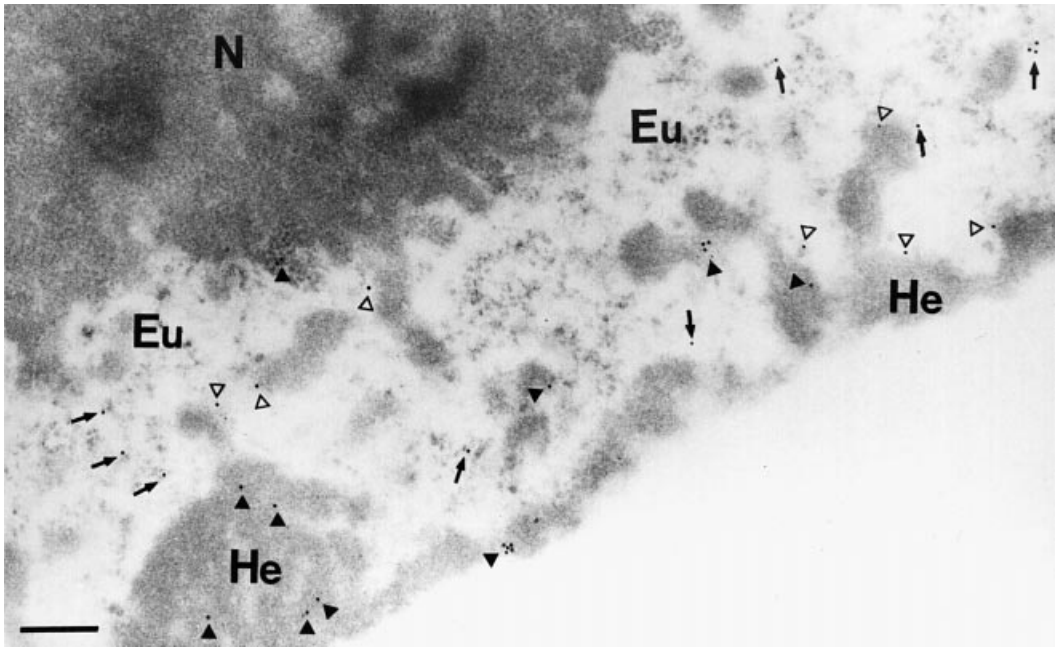

Fig. 2. Ultrastructural analysis of TIF1β localization in P19 EC nuclei by indirect immunogold electron microscopy using a mouse anti-TIF1β mAb and a 10 nM gold-conjugated secondary antibody. Eu, euchromatin; He, heterochromatin; N, nucleolus. Gold particles are seen as black dots. TIF1β was localized in Eu (arrows), He (closed arrowheads), and at the borders between Eu and He (open arrowheads). No significant labelling was obtained when a polyclonal anti-mouse IgG fraction was used (data not shown). Scale bar, 0.25 μm.

TIF1β exhibited a diffuse as well as a punctuate or speckled distribution throughout the nucleoplasm (Figure 1J). In contrast to the largest RPB1 subunit of RNA polymerase II (Pol II; Figure 1K and L), TIF1β was not excluded from the regions of condensed heterochromatin, although the staining with the anti-TIF1β antibody was less intense in the large blocks of heterochromatin than in the surrounding euchromatin (Figure 1I and J). TIF1β was similarly distributed in nuclei of P19 and F9 EC cells (data not shown). In an attempt to localize TIF1β further, immunogold electron microscopy was performed on P19 cell nuclei (Figure 2). TIF1β was found in low-contrast areas of euchromatin (arrows), at the borders between euchromatin and heterochromatin (open arrowheads), and in electron-dense or heterochromatic regions located at the nuclear periphery, around nucleoli or dispersed as clumps in the nucleoplasm (Figure 2, closed arrowheads; and data not shown). In contrast, almost no signal was observed in the nucleoli or in the interchromatin granule clusters (Figure 2; and data not shown). These results, which are in keeping with the immunocytofluorescence data, indicate that TIF1β is present in both euchromatin and heterochromatin.

Association of TIF1α and TIF1β with HP1α, -β and -γ in mammalian cells

The above results and the previous observation that TIF1β can interact with HP1α, -β and -γ in the yeast two-hybrid system (Le Douarin *et al*., 1996; Venturini *et al*., 1999) prompted us to investigate whether these proteins could be physically associated in mammalian cells. When TIF1β was immunoprecipitated from nuclear extracts of P19 EC cells, all three HP1s, HP1α, -β and -γ, were found in the TIF1β immunoprecipitate (Figure 3A, B and C, respectively). This co-immunoprecipitation was specific as none of the HP1 proteins could be detected in control immunoprecipitations with an irrelevant antibody (Figure 3A–C, lane 2). A negative result was also obtained by using specific antibodies against TIF1α (data not shown).

In a reciprocal experiment, P19 EC cell nuclear extracts were immunoprecipitated with either HP1α, -β or -γ antibodies, and the immunoprecipitates were probed for the presence of TIF1β. Co-immunoprecipitation of endogenous TIF1β was clearly detected in each HP1 immunoprecipitate (Figure 2D, lanes 3–5), but not in controls (Figure 3D, lane 2). In contrast, we found no evidence for a co-immunoprecipitation of TIF1α with HP1 antibodies (data not shown), indicating that unlike TIF1β, TIF1α may not be stably associated with HP1 proteins in P19 EC cells.

TIF1α and TIF1β bind directly to HP1α, -β and -γ in vitro

To investigate whether the association of TIF1β with the HP1 proteins in nuclear extracts corresponds to a direct interaction, binding assays were performed *in vitro* using purified recombinant proteins. N-terminally epitope-tagged His-TIF1β was expressed in Sf9 cells using a baculovirus vector and purified by affinity chromatography. Glutathione *S*-transferase (GST)–HP1 fusion proteins expressed in *Escherichia coli* were immobilized on glutathione *S*–Sepharose beads and incubated with purified His-TIF1β in the presence of various salt and detergent concentrations (Figure 4A). His-TIF1β bound to GST–HP1α (lanes 5–7), GST–HP1β (lanes 8–10) and GST–HP1γ (lanes 11–13), but not to GST alone (lanes 2–4). Interactions with all three HP1s were stable between 0.1 and 1 M KCl (upper panel) and between 0.5 and 5% NP-40 (Figure 4A, lower panel). Thus, TIF1β interacts directly and stably with all three HP1s *in vitro*.

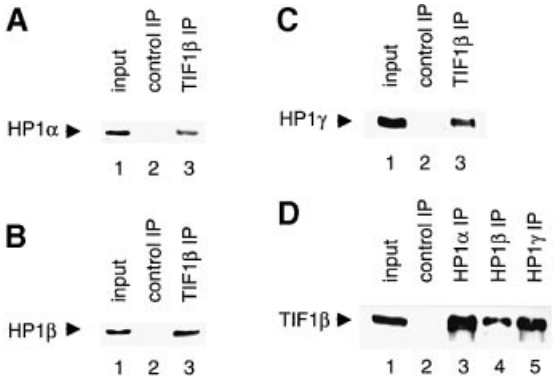

Fig. 3. P19 EC cell endogenous TIF1β is associated with HP1α, HP1β and HP1γ. (**A**–**C**) Detection of HP1α, HP1β and HP1γ in TIF1β immunoprecipitates. Nuclear extracts were analysed by Western blotting either directly (input) or following immunoprecipitation with a TIF1β mAb (TIF1β IP) or with an irrelevant antibody (anti-Flag antibody; control IP). Western blots were probed with either HP1α (A), HP1β (B) or HP1γ (C) mAbs. Arrowheads indicate the position of the protein recognized by each mAb. (**D**) Detection of TIF1β in HP1 immunoprecipitates. Nuclear extracts of P19 cells were used for IP with mAbs against HP1α (lane 3), HP1β (lane 4) or HP1γ (lane 5) or an irrelevant anti-Flag mAb (lane 2). A Western blot of the immunoprecipitates probed with a TIF1β mAb is shown. In all panels, lane 1 (input) corresponds to 1/20 the amount of nuclear extract used for immunoprecipitation.

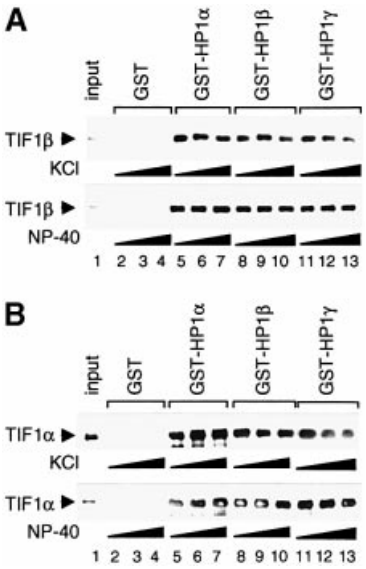

Fig. 4. TIF1α and TIF1β bind to HP1α, HP1β and HP1γ *in vitro*. Purified His-TIF1β (**A**) or His-TIF1α (**B**) was incubated in a batch assay with 'control' GST (lanes 2–4), GST–HP1α (lanes 5–7), GST–HP1β (lanes 8–10) or GST–HP1γ (lanes 11–13) in the presence of various concentrations of salt (0.1, 0.5 and 1 M KCl; upper panels) and detergent (0.5, 2 and 5% NP-40; lower panels). Bound TIF1 was detected by Western blotting. Lane 1 shows one-fifth of the amount of input His-TIF1, the position of which is indicated by an arrowhead.

His-tagged TIF1α interacts with HP1α and HP1β *in vitro* (Le Douarin *et al*., 1996). We show here that it can also bind to HP1γ, whatever the concentration of salt and detergent (Figure 4B). Thus, although no association with HP1 proteins could be detected in mammalian cells, TIF1α interacts in a stable manner with all three HP1s (α, β and γ) *in vitro*.

The HP1-interacting domains of TIF1α and TIF1β lie within a conserved 25-amino-acid region

To identify the HP1-interacting domain of TIF1β, a deletion analysis of TIF1β was performed using the yeast two-hybrid system (Figure 5). Various TIF1β deletion mutants expressed as fusion proteins with the VP16 acidic activation domain (denoted AAD) were tested for interaction with HP1α fused to the DBD of the oestrogen receptor (ERα; amino acid residues 176–282) in a yeast strain containing an integrated URA3 reporter driven by three ERα binding sites (Le Douarin *et al*., 1995b). As indicated by the orotidine 5′-monophosphate decarboxylase (OMPdecase) activity of the URA3 gene product (Figure 5B), this deletion analysis showed that an HP1α-interacting domain is present between residues 468 and 535 of TIF1β. Interestingly, the TIF1β sequence from residues 484–506 is markedly similar to the HP1 box of TIF1α, which is sufficient for interaction with HP1α (Le Douarin *et al*., 1996; Figure 5C). An interaction was effectively observed when DBD–TIF1β(483–507) was co-expressed with either AAD–HP1α, AAD–HP1β or AAD–HP1γ in PL3 (Figure 5D). To demonstrate that TIF1β actually binds HP1 proteins through this HP1 box, an internal deletion from residues 484–493 was introduced into full-length TIF1β. No interaction with DBD–HP1α in yeast (Figure 5B, AAD–TIF1βΔ484–493) and no binding to GST–HP1α *in vitro* could be detected, while binding to the KRAB domain of KOX1 was not affected (Figure 5E, TIF1βΔHP1 box). As expected, an internal deletion of the HP1 box in TIF1α (from residues 678–687) resulted in a similar lack of binding to GST–HP1α, while binding to the liganded oestrogen receptor [GST–ERα(DEF)] was not affected (Figure 5F). Taken together, these data indicate that TIF1α and TIF1β both contain a conserved HP1 box that is necessary and sufficient for specific interactions with the HP1 proteins.

TIF1α and TIF1β are autophosphorylatable protein kinases that also phosphorylate HP1α, -β and -γ

We previously demonstrated that recombinant TIF1α possesses an intrinsic kinase activity catalysing *in vitro* autophosphorylation, as well as phosphorylation of several proteins such as TFIIEα, $TAF_{II}55$ and $TAF_{II}28$ (Fraser *et al*., 1998). We therefore investigated whether TIF1β could also be a protein kinase, and whether both TIF1α and TIF1β may phosphorylate HP1α, -β and -γ to which they bind directly. Recombinant epitope-tagged full-length His-TIF1α and His-TIF1β proteins were expressed in Sf9 cells, purified to homogeneity by affinity chromatography and immunoprecipitation, and subjected to *in vitro* kinase assays (Figure 6). His-TIF1α eluted from immunoprecipitates obtained with three distinct mAbs was phosphorylated (Figure 6A, lanes 1–4), confirming the autokinase activity of TIF1α (Fraser *et al*., 1998). Interestingly, recombinant GST–HP1α (lanes 13–16) and GST–HP1β (lanes 17–20), as well as His-HP1γ (lanes 21–24), but not 'control' GST (lanes 9–12), were phosphorylated efficiently by TIF1α (Figure 6A). Using the same kinase reaction conditions, TIF1α was also tested for its ability to phosphorylate a well established kinase substrate, histone H1, to which TIF1α also binds (our unpublished results). Under similar conditions, neither calf thymus histone H1 (Figure 6A,

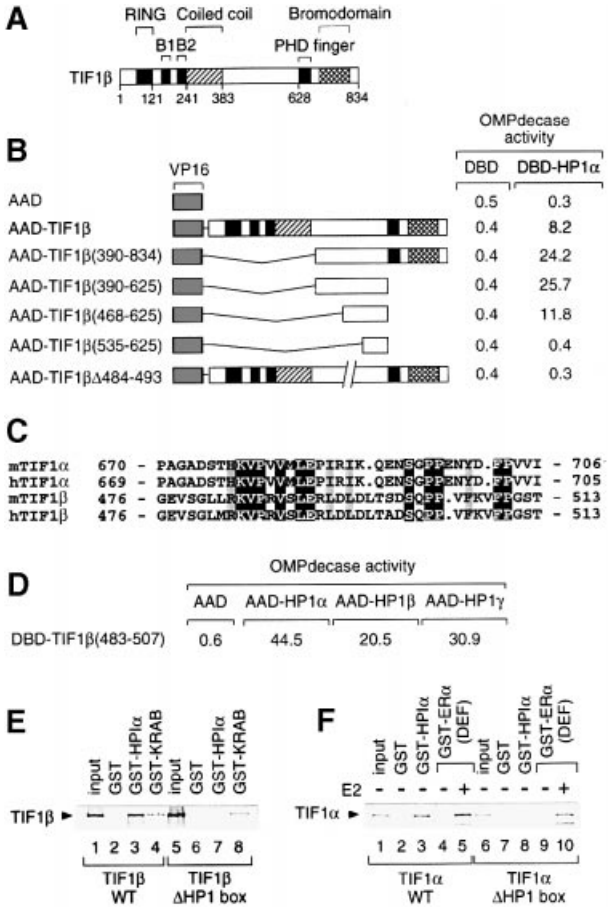

Fig. 5. Mapping of the HP1-interacting domain in mouse TIF1β. (**A**) Domain organization of mouse TIF1β. Numbers refer to amino acid positions (see Le Douarin *et al*., 1996). (**B**) Residues 484–493 of TIF1β are required for interaction with HP1α in yeast. Various deletion mutants of TIF1β were fused to the VP16 AAD and assayed for interaction with the 'unfused ' ERα DBD or a DBD fusion of HP1α in the yeast strain PL3, which contains a URA3 reporter gene driven by three oestrogen response elements (EREs) (Pierrat *et al*., 1992). Transformants were grown in liquid medium containing uracil. OMPdecase activities determined for each cell-free extract are expressed in nmol substrate/min/mg protein. The values (± 20%) are the average of at least three independent transformants. Note that expression of all fusion proteins was confirmed by Western blotting using the antibody 2GV4 against VP16 (data not shown). (**C**) Alignment of the HP1-interacting domain of TIF1α (Le Douarin *et al*., 1996) with TIF1β amino acids 468–535 reveals a highly conserved region, referred to as the HP1 box. Invariant amino acids are highlighted in bold. Shading indicates conserved amino acid residues. Database accession nos: mouse TIF1α (mTIF1α, S78219); human TIF1α (hTIF1α, AF119042); mouse TIF1β (mTIF1β, X99644); human TIF1β (hTIF1β, U95040). (**D**) Residues 483–507 of TIF1β are sufficient for interaction with all three HP1s in yeast. DBD–TIF1β(483–507) was co-expressed with either the 'unfused' VP16 AAD (negative control) or the VP16 AAD fused to HP1α, HP1β or HP1γ in yeast strain PL3. OMPdecase activities are given as in (B). (**E** and **F**) The HP1 box is required for binding to HP1α *in vitro*. In (E), *in vitro* ^{35}S-labelled TIF1β (lanes 1–4) and TIF1βΔHP1box lacking residues 484–493 (lanes 5–9) were incubated in a batch assay with immobilized 'control' GST, GST–HP1α or GST–KRAB containing the KRAB repression domain of KOX1 (residues 1–97). After extensive washing, the bound TIF1β protein was eluted with SDS, resolved on SDS–PAGE and visualized by autoradiography. Lanes 1 and 5 represent one-tenth of the amount of input labelled TIF1β and TIF1βΔHP1box, the positions of which are indicated by an arrowhead. In (F), similar GST 'pull-down' assays were performed with *in vitro* ^{35}S-labelled TIF1α (lanes 1–5) or TIF1αΔHP1box lacking residues 678–687 (lanes 6–10) and 'control' GST or GST–HP1α or GST–ERα(DEF). A 10^{-6} M concentration of oestradiol (E2) was added to the incubations as indicated.

lanes 5–8) nor a recombinant GST–mouse histone H1 fusion (data not shown) were phosphorylated by TIF1α (Figure 6A). Similarly, affinity-purified and immunoprecipitated recombinant His-TIF1β exhibited an autokinase activity (Figure 6B, lane 3) and phosphorylated HP1α (lane 6), HP1β (lane 9) and HP1γ (lane 12), thus indicating that TIF1β, like TIF1α, has an intrinsic protein kinase activity.

To extend the above results obtained with recombinant proteins to more physiological conditions, we also investigated whether endogenous TIF1α and TIF1β exhibited kinase activity when immunopurified from nuclear extracts from P19 EC cells. As shown in Figure 6C and D, autokinase and HP1 kinase activities were specifically co-immunoprecipitated with mAbs against TIF1α and TIF1β, respectively, but not with a control antibody. Thus, endogenous P19 EC cell TIF1α and TIF1β also appear to be associated with HP1 kinase activity.

Involvement of HP1 interaction and histone deacetylation in transcriptional repression by TIF1α and TIF1β

Similar to HP1 proteins (see below and the references in Introduction), TIF1α and TIF1β can repress transcription when fused to a heterologous DBD such as the GAL4 DBD (hereafter designated as GAL4–TIF1 fusion proteins) (Le Douarin *et al*., 1996; Moosmann *et al*., 1996). To investigate whether an interaction with HP1s is required for the silencing activity of TIF1α and TIF1β, GAL4–TIF1 fusion derivatives containing an internal deletion of the HP1 box, GAL4–TIF1αΔHP1box and GAL4–TIF1βΔHP1box, were generated and tested in transiently transfected Cos-1 cells for repression of the chimeric transactivator ER(C)-VP16 using a GAL4 reporter containing two GAL4 DNA binding sites (UAS, 17M2) and an oestrogen response element (ERE) in front of a β-globin (G) promoter–CAT fusion (17M2-ERE-G–CAT; Figure 7A). GAL4–TIF1αΔHP1box repressed the reporter gene activity to the same degree as the wild-type TIF1α fusion, indicating that an intact HP1 box is not required for the silencing activity of TIF1α (Figure 7B). This finding is consistent with our previous conclusion that point mutations in the HP1 box (V681E/V682E; Le Douarin *et al*., 1996) impair the interaction of TIF1α with HP1α in yeast, but not its transcriptional silencing effect in transfected mammalian cells. In contrast, deletion of the HP1 box in TIF1β resulted in a marked decrease in repression (with no effect on the expression level of the GAL4 fusion protein; Figure 7C; and data not shown), suggesting that the silencing effect of TIF1β requires an interaction with the HP1 proteins. However, additional silencing mechanism(s) appear to be involved as a residual level of repression was reproducibly retained by the GAL4–TIF1βΔHP1box mutant (Figure 7C).

Because, in many instances, recruitment of histone deacetylases (HDACs) has been shown to be involved in transcriptional repression (Pazin and Kadonaga, 1997; Ashraf and Ip, 1998; Kuo and Allis, 1998 and references therein), we analysed the effects of the specific inhibitor of HDAC, trichostatin A (TSA; Yoshida *et al*., 1995), on repression by GAL4–TIF1 fusion proteins. Repression by both GAL4–TIF1α and GAL4–TIF1αΔHP1box was almost fully relieved upon treatment with TSA

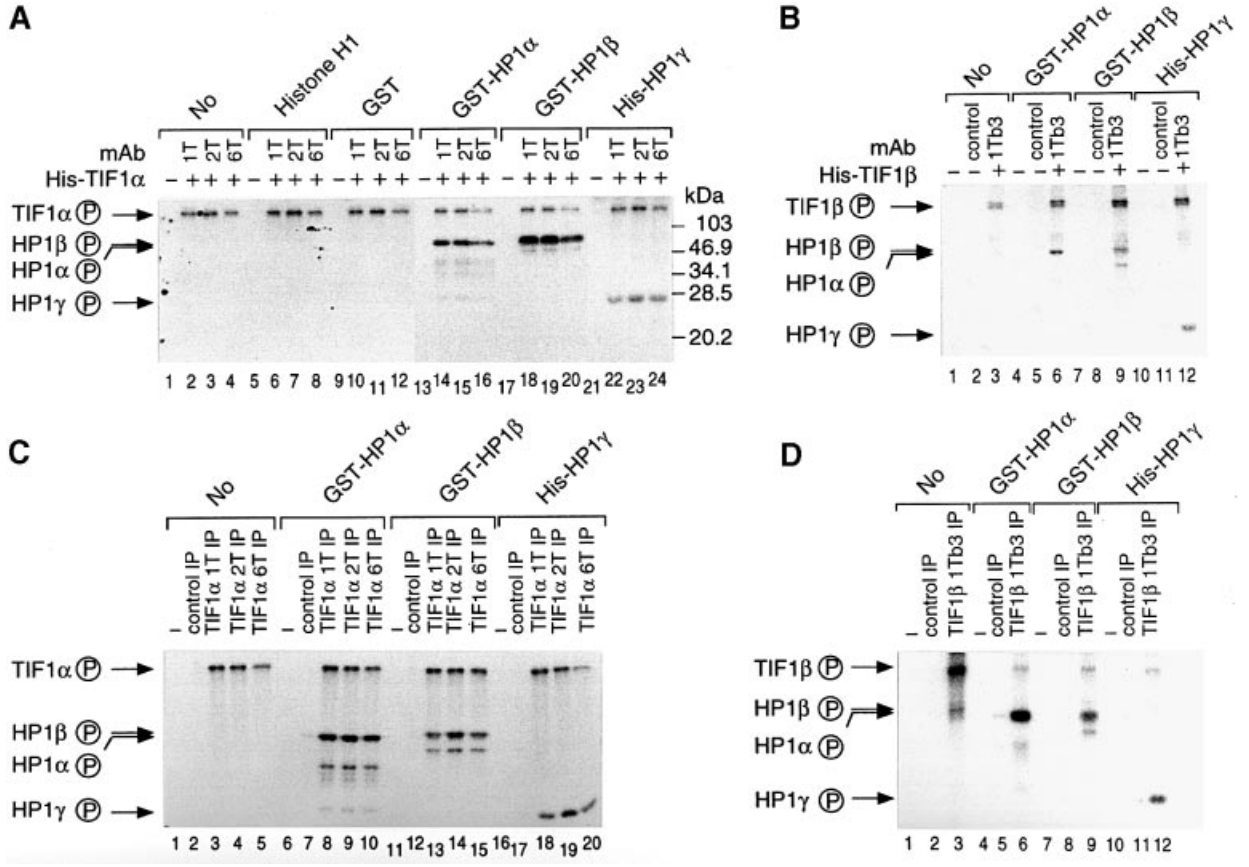

Fig. 6. Association of autokinase and HP1 kinase activity with recombinant and endogenous TIF1α and TIF1β. (**A**) Recombinant His-TIF1α expressed in Sf9 cells and purified by nickel chelate affinity chromatography was immunoprecipitated with three different TIF1α mAbs (mAb1T, mAb2T and mAb6T) as indicated. TIF1α was eluted from the three immunoprecipitates with the respective epitope peptides, and the peptide eluates were individually tested for autokinase activity (lanes 1–4) and transphosphorylation activity using calf thymus histone H1 (lanes 5–8), purified *E.coli*-expressed unfused GST (lanes 9–12), GST–HP1α (lanes 13–16), GST–HP1β (lanes 17–20) and His-HP1γ (lanes 21–24). As a negative control, *in vitro* kinase reactions were performed in the absence of His-TIF1α (buffer alone; lanes 1, 5, 9, 13, 17 and 21). The proteins were resolved by SDS–PAGE and analysed by Coomassie Blue staining (data not shown) and autoradiography. The sizes of protein markers are indicated on the right side of the figure. Arrows mark positions of phosphorylated TIF1α and HP1s. (**B**) An *in vitro* kinase assay was performed as in (A) in the absence (buffer alone; lanes 1, 4, 7 and 10) or presence of recombinant His-TIF1β expressed from Sf9 cells, purified by nickel chelate affinity chromatography and immunoprecipitated with a specific TIF1β antibody (mAb TIF1β 1Tb3; lanes 3, 6, 9 and 12). As an additional control, an Sf9 extract containing His-TIF1β was processed in the presence of an irrelevant anti-Flag mAb (control mAb; lanes 2, 5, 8 and 11). The positions of phosphorylated TIF1β and HP1s are shown. The identity of the upper band as His-TIF1β was confirmed by Western blot analysis (data not shown). (**C**) Co-immunoprecipitation of HP1 kinase activity with endogenous TIF1α. Nuclear extracts from P19 EC cells were immunoprecipitated with antibodies against TIF1α (TIF1α 1T, 2T or 6T IP) or an irrelevant anti-Flag antibody (control IP), and the resulting immunoprecipitates were tested for autokinase activity (lanes 2–5) and kinase activity using recombinant GST–HP1α (lanes 7–10), GST–HP1β (lanes 12–15) and His-HP1γ (lanes 17–20). *In vitro* kinase reactions were also performed in the absence of immunoprecipitates (buffer alone, lanes 1, 6, 11 and 16). (**D**) Co-immunoprecipitation of HP1 kinase activity with endogenous TIF1β. Nuclear extracts from P19 EC cells were immunoprecipitated with a TIF1β antibody (anti-TIF1β 1Tb3 IP) or an irrelevant anti-Flag antibody (control IP), and the resulting immunoprecipitates were tested for kinase activity as in (C).

(Figure 8A), suggesting that histone deacetylation is chiefly involved in TIF1α-mediated repression. In contrast, under similar conditions, the silencing activity of GAL4–TIF1β was only partially inhibited by TSA treatment (Figure 8B). However, an almost complete relief of repression was achieved upon TSA treatment of GAL4–TIF1βΔHP1box-expressing cells (Figure 8B), thus suggesting that repression by TIF1β involves both the binding of HP1 and histone deacetylation.

TSA treatment partially relieves HP1-dependent repression

HP1α (Le Douarin *et al*., 1996; Lehming *et al*., 1998; Seeler *et al*., 1998) and HP1γ (Lehming *et al*., 1998) have been shown to repress transcription when directly tethered to a promoter in mammalian cells. We show here that a GAL4–HP1β fusion protein can also function as a transcriptional repressor in transiently transfected Cos-1 cells (Figure 8C). We reproducibly found that GAL4–HP1α was a more efficient repressor than either GAL4–HP1β or GAL4–HP1γ (Figure 8C); Western blot analysis using an antibody against the GAL4 DBD indicated similar expression levels for all three fusion proteins (data not shown). Interestingly, treatment of the transfected cells with TSA significantly reduced the silencing activity of each GAL4–HP1 (~3- to 4-fold; Figure 8C), indicating that HP1-dependent repression also involves a histone deacetylase activity. Note, however, that repression was not fully relieved by TSA (Figure 8C; and data not shown), suggesting that a deacetylase-independent mechanism is also involved in HP1-mediated silencing.

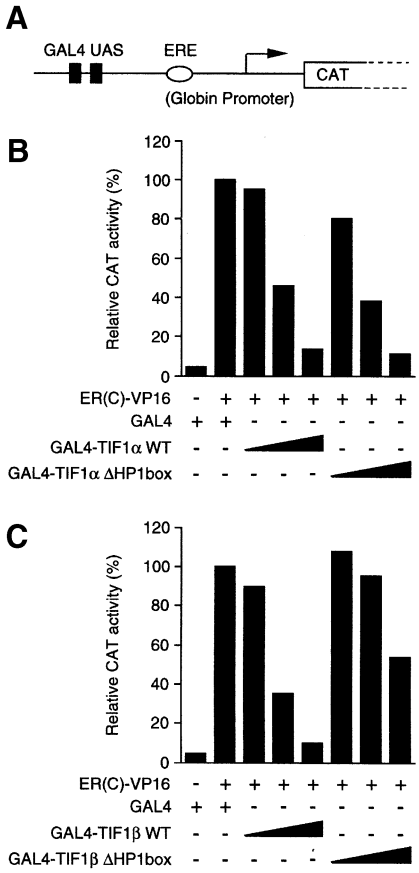

Fig. 7. Effects of HP1 box deletion on the repression activity of TIF1α and TIF1β. (**A**) Schematic representation of the 17M2-ERE-G–CAT reporter gene used. GAL4 UAS motifs are represented by filled squares, the ERE by an open oval and the transcription initiation site by an arrow. (**B**) TIF1α-mediated repression is not affected by deletion of the the HP1 box. Increasing amounts of GAL4–TIF1α wild type (WT) and GAL4–TIF1α lacking the HP1 box (ΔHP1box) expression plasmids (0.2, 2 or 20 ng) were transiently transfected into Cos-1 cells with 1 μg of 17M2-ERE-G–CAT reporter, 1 μg of pCH110 (expressing β-galactosidase) and 100 ng of ER(C)-VP16. CAT activities are expressed relative to that measured in the presence of the unfused GAL4 expression vector (20 ng; taken as 100%). Values (± 10%) represent the averages of two independent triplicated transfections after normalization for the internal control β-galactosidase activity of pCH110. (**C**) HP1 box deletion reduces the repression activity of TIF1β. Cos-1 cells were transiently co-transfected with 1 μg of 17M2-ERE-G–CAT reporter, 1 μg of pCH110, 100 ng of ER(C)-VP16, and 0.2, 2 or 20 ng of GAL4–TIF1βWT and GAL4–TIF1βΔHP1box expression plasmids. CAT activities are expressed as in (B).

Discussion

HP1 proteins are differentially involved in the silencing activity of members of the TIF1 family

In an attempt to characterize the molecular mechanism(s) underlying the silencing activity of TIF1α and TIF1β, we investigated the possibility that they may interact physically and functionally with members of the HP1 gene family, a class of non-histone chromosomal proteins with a well established epigenetic gene silencing function in *Drosophila* (for reviews, see Elgin, 1996; Wallrath, 1998). Using yeast as well as GST pull-down experiments, we have demonstrated that both TIF1α and TIF1β can bind to HP1α, HP1β and HP1γ, and we have identified a conserved amino acid motif within the central region of TIF1α and TIF1β that is both necessary and sufficient for HP1 binding (HP1 box). However, in mouse P19 cells, an association with HP1 proteins was detected for TIF1β only, suggesting that the interaction between HP1s and TIF1α *in vivo* might be transient and/or involve only a small subset of the proteins. We have also demonstrated that TIF1β, but not TIF1α, requires an intact HP1 box for full repression activity, indicating that the formation of a complex with HP1 proteins is instrumental in transcriptional repression by TIF1β. After completion of this manuscript, a study was published by Ryan *et al*. (1999) in which *in vitro* studies confirm that TIF1β (designated KAP-1) is capable of interacting directly with the HP1 proteins. Interestingly, the authors observed a co-localization of TIF1β with HP1β and HP1γ in subnuclear territories of pericentromeric heterochromatin and in numerous punctate euchromatic domains, respectively (Ryan *et al*., 1999), supporting our co-immunoprecipitation data showing that endogenous TIF1β and members of the HP1 family are associated.

Taken together with the observation that, similarly to TIF1β, HP1 proteins function as potent transcriptional repressors when tethered to DNA through fusion to a GAL4 DBD (Le Douarin *et al*., 1996; Lehming *et al*., 1998; Seeler *et al*., 1998; this study), our results raise the possiblity that HP1 proteins may mediate, at least in part, the TIF1β silencing effect, whereas these proteins may not be involved in TIF1α repression. A novel member of the TIF1 gene family, termed TIF1γ, has been identified recently (Venturini *et al*., 1999). TIF1γ resembles TIF1α and TIF1β in that it possesses an intrinsic silencing effect (Venturini *et al*., 1999). However, in contrast to TIF1α and TIF1β, TIF1γ has no HP1 box and does not interact with HP1 proteins in yeast or *in vitro* (Venturini *et al*., 1999; our unpublished results). Thus, TIF1β may represent the only member of the TIF1 gene family to silence transcription through an interaction with HP1 proteins.

Histone deacetylation is involved in the silencing activity of TIF1s and HP1s

We have shown that TSA, a specific and potent inhibitor of HDACs, can interfere with TIF1α- and TIF1β-mediated repression. In the case of TIF1α, the repression was almost fully relieved by HDAC inhibition, suggesting that histone deacetylation is a key determinant in TIF1α-mediated silencing. In the case of TIF1β, HP1-independent repression (as exhibited by the TIF1βΔHP1box mutant) was also almost abrogated by HDAC inhibition, while repression by the wild-type protein was only partially relieved, suggesting that TIF1β repression requires both HP1 binding and histone deacetylation. In the case of TIF1γ, our preliminary results indicate that repression by TIF1γ can also be relieved, at least partially, by treating cells with TSA (J.Ortiz, R.Losson and P.Chambon, unpublished data). Thus, as observed in many cases of transcriptional repression (see Ashraf and Ip, 1998 and references therein), histone deacetylation appears to be involved in repression by all three TIF1 family members. Several transcriptional co-repressors have recently been shown to be components of or to recruit multi-protein complexes that contain HDACs (Kuo and Allis, 1998; Pazin and Kadonaga, 1998 and references therein). Once tethered to target promoters,

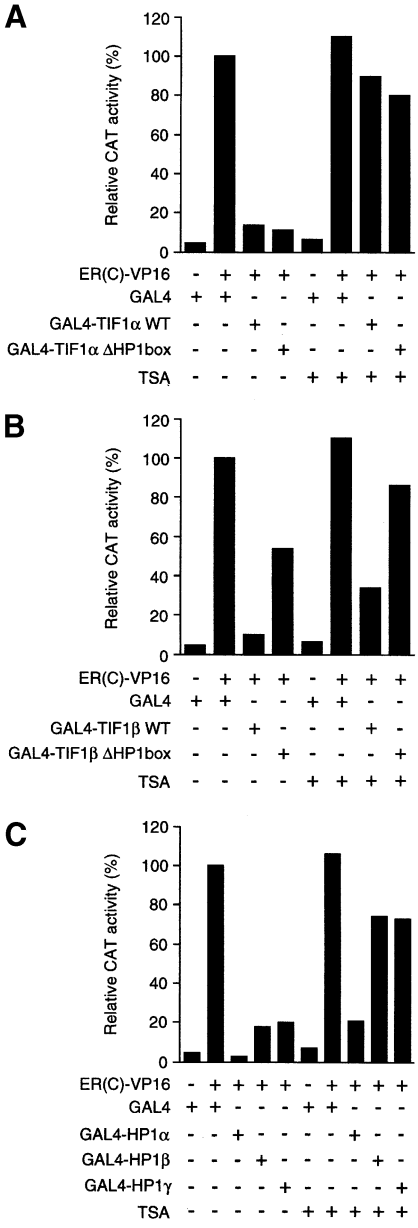

Fig. 8. Relief of TIF1- and HP1-mediated repression of transcription by the histone deacetylase inhibitor TSA. (**A**) TSA treatment relieves the repression activity of TIF1αWT and TIF1αΔHP1box. Cos-1 cells were transiently co-transfected with 1 μg of 17M2-ERE-G–CAT reporter, 1 μg of pCH110, 100 ng of ER(C)-VP16 and 20 ng of the indicated GAL4 expression vectors. For TSA treatment, 300 nM TSA was added 24 h before collecting the cells. CAT activities are expressed as in Figure 7A. (**B**) TSA and HP1 box deletion have additive relieving effects on TIF1β-mediated repression in Cos-1 cells transfected and treated as described in (A). CAT activities are expressed as in Figure 7A. (**C**) TSA treatment partially relieves HP1 repression. Cos-1 cells were transiently co-transfected with 1 μg of 17M2-ERE-G–CAT reporter, 1 μg of pCH110, 100 ng of ER(C)-VP16 and 200 ng of the indicated GAL4 expression vectors. For TSA treatment, 300 nM TSA was added 24 h before collecting the cells. CAT activities are expressed as in Figure 7A. In each panel, values (± 10%) represent the averages of two independent triplicated transfections after normalization for the internal control β-galactosidase activity of pCH110.

these HDACs are thought to interfere with transcription by inducing local repressive chromatin conformations as a consequence of deacetylation of nucleosomal histones. Future experiments will show whether TIF1α, -β and -γ can interact with one of these HDAC-containing complexes (Sin3–HDAC, MI2–HDAC; Zhang *et al*., 1997, 1998).

We have also investigated whether histone deacetylation is involved in HP1-mediated silencing. TSA treatment of transfected cells partially relieved repression by HP1α, -β and -γ. Thus, as observed in budding and fission yeast cells (Braunstein *et al*., 1996; Ekwall *et al*., 1997), a functional relationship between histone deacetylation and heterochromatin-mediated silencing may exist in mammals. At least two non-exclusive possibilities could account for our results. First, HP1α is known to self-associate and to interact with HP1β and HP1γ in yeast as well as *in vitro* (Le Douarin *et al*., 1996; A.L.Nielsen, unpublished data). Thus, by analogy with the known function of *Drosophila* HP1, which, presumably through self-association and the formation of a silencing complex, exerts dose-dependent effects on heterochromatin-mediated gene silencing (for reviews, see Eissenberg *et al*., 1995; Weiler and Wakimoto, 1995; Elgin, 1996), HP1α, -β and -γ could form dimers or higher order multimeric complexes, leading to the establishment of a repressive heterochromatin-like structure. The formation and/or stabilization of this structure may involve specific interactions between HP1 proteins (or HP1-associated proteins) and hypoacetylated histones. Note that interactions have been observed in budding yeast between histone tails and components of heterochromatin (i.e. the silent informator regulator SIR proteins), which, interestingly, are affected by acetylation (Hecht *et al*., 1995, 1996). That HP1 proteins could be involved in the transition from a euchromatic to a heterochromatic state is supported by the observation that HP1β and HP1γ are not exclusively associated with heterochromatin (Horsley *et al*., 1996; this study). A second possibility is suggested by the recent observation that in proliferating B cells Ikaros DNA-binding proteins are capable of recruiting repressed genes (and HDAC complexes) to regions of heterochromatin (Brown *et al*., 1997, 1999; Hahm *et al*., 1998; Klug *et al*., 1998; Kim *et al*., 1999). Similarly, HP1 proteins may recruit the DNA template to which they are bound into heterochromatic nuclear compartments where other HP1s and silencing proteins localize. The structure and/or function of these heterochromatic domains, which, in contrast to euchromatic domains, are rich in hypoacetylated histones (Grunstein, 1998 and references therein), might be affected by histone acetylation. This view is supported by the observation that treating fission yeast cells with TSA is sufficient to induce a hyperacetylated state in centromeric heterochromatin accompanied by a delocalization of the SWI6 protein and a loss of centromeric silencing (Ekwall *et al*., 1997).

We also show here that all three HP1 proteins can be phosphorylated by both endogenous and recombinant TIF1α and TIF1β. Although the biological relevance of these phosphorylations has not yet been established, it is reminiscent of *Drosophila* HP1 phosphorylation, which has been correlated with heterochromatin assembly and/or maintenance (Eissenberg *et al*., 1994). This phosphorylation of HP1 may favour self-association or interaction with other proteins. In the case of TIF1α, which is mostly associated with euchromatic regions readily accessible to micrococcal nuclease digestion (Remboutsika *et al*., 1999), it is tempting to speculate that phosphorylation of HP1s

by TIF1α may favour their incorporation into heterochromatin, thereby preventing their association with euchromatic components. On the other hand, in the case of TIF1β, which requires HP1 binding for full repression activity, our results raise the possibility that phosphorylation of HP1s by TIF1β may increase their effects on gene silencing and/or chromatin condensation at specific loci.

The three members of the TIF1 family are differentially involved in transcriptional repression

Although our present study indicates that the silencing effect of TIF1α and -β is chiefly mediated through chromatin remodelling, it is not clear how these TIF1s could be recruited to chromatin as they have no known DNA binding motif and apparently no sequence-specific DNA binding activity (Remboutsika *et al*., 1999). Therefore, their recruitment to chromatin may result from association(s) with other proteins. In the case of TIF1β, this possibility is supported by a number of co-transfection and biochemical interaction data showing that TIF1β is a putative mediator of transcriptional repression for the large class of KRAB-ZFPs (Friedman *et al*., 1996; Kim *et al*., 1996; Moosmann *et al*., 1996; see Introduction). Although there is currently no known DNA/RNA recognition sequence for any of the KRAB ZFPs identified so far, these proteins possess Krüppel Cys2His2-type zinc fingers in their C-termini, which suggests the existence of a specific nucleic acid binding function (Hollemann *et al*., 1996 and references therein). Moreover, their differential spatial and temporal expression patterns indicate that these proteins may play an important role in regulating expression of specific genes during cell differentiation and development (Bellefroid *et al*., 1993, 1998; Witzgall *et al*., 1993; Mazarakis *et al*., 1996; Poncelet *et al*., 1998). Based on our present findings, we propose a molecular mechanism for the function of these proteins in transcriptional repression, which involves epigenetic effects resulting from association of TIF1β with members of the HP1 family and targeted histone deacetylation. In this model, the KRAB domain of a DNA-bound KRAB ZFP recruits TIF1β to specific loci. TIF1β in turn binds HP1 proteins (and perhaps also directly HDACs) to silence transcription, via the local conversion of a euchromatic region into a heterochromatin-like structure and/or the recruitment of the chromatinic DNA template to heterochromatic compartments. That both mechanisms could operate within the same cell is strongly suggested by our present finding that TIF1β localizes to both euchromatin and highly condensed heterochromatic compartments.

Similar to TIF1β, TIF1α has been reported to interact with the KRAB silencing domain of the human KOX1 protein both in yeast and *in vitro* (Moosmann *et al*., 1996; our unpublished data). However, in contrast to TIF1β, this interaction has not been reproduced with typical KRAB domains of other ZFPs (M.Ebrink, C.Lotta, J.Ortiz, C.Sanchez and R.Losson, unpublished data). TIF1α, but not TIF1β, was previously shown to interact with transcriptionally active nuclear receptors in an agonistic ligand- and activation function AF-2-dependent manner (Le Douarin *et al*., 1995a; vom Baur *et al*., 1996). Thus, TIF1α could be recruited to DNA by agonist-bound nuclear receptors and be involved in ligand-dependent repression through a mechanism that involves histone deacetylation. However, as TIF1α is tightly and preferentially associated with euchromatic sites in toti- and multipotent cells (Remboutsika *et al*., 1999), we favour another model in which TIF1α functions in euchromatin as a protein anchor to which nuclear receptors bind in the presence of their cognate agonistic ligands. As a consequence, TIF1α would become hyperphosphorylated (Fraser *et al*., 1998), and this modification would decrease its silencing activity, thus resulting in an activation of transcription.

To date, no interaction between TIF1γ and DNA binding proteins has been described, but the fact that neither KRAB domains nor nuclear receptors interact with TIF1γ, at least in yeast and *in vitro* (Venturini *et al*., 1999), supports the view that TIF1α, TIF1β and TIF1γ are functionally distinct. Gene knockouts are in progress to determine their respective biological role(s).

Materials and methods

Plasmids

Details on individual plasmid constructs, which were all verified by sequencing, are available upon request. For *in vitro* binding assays, the indicated cDNAs were fused to GST in the pGEX2TK plasmid (Pharmacia; Le Douarin *et al*., 1995a, 1996). For expression of ^{35}S-labelled proteins, the coding sequences of TIF1α and -β were inserted into the pSG5 vector and coupled transcription/translation was performed using T7 RNA polymerase with the TNT lysate system (Promega). The His-TIF1α/β constructs have been cloned into a modified version of the pAcSGHisNT-B baculovirus expression vector (Pharmingen), in which the *Bam*HI–*Xho*I fragment was replaced with the *Bgl*II–*Xho*I fragment of pet15b, thus eliminating the protein kinase A site, but preserving the reading frame and the His_6 tag (Fraser *et al*., 1998). For yeast two-hybrid assays, DBD and AAD fusion proteins were expressed from the yeast multicopy plasmids pBL1 and pASV3, respectively (Le Douarin *et al*., 1995b). These plasmids express inserts under the control of the phosphoglycerate kinase (PGK) promoter. pBL1 contains the HIS3 marker and directs the synthesis of epitope (region F of ERα)-tagged ERα DBD fusion proteins. pASV3 contains the LEU2 marker and a cassette expressing a nuclear localized VP16 AAD, preceding a polylinker and stop codons in all reading frames. For transfection studies in mammalian cells, the GAL4(1–147) chimeras were contructed into pG4MpolyII (Le Douarin *et al*., 1996). The chimeric protein ER(C)-VP16, which encodes amino acids 176–280 of ERα and amino acids 413–490 of VP16, has been described previously as well as the reporter gene 17M2-ERE-G–CAT (Le Douarin *et al*., 1996).

Antibodies

mAbs used include: (i) anti-HP1α mAbs, 2HP1-1H5 for immunocytochemistry and immunoprecipitation and 2HP-2G9 for Western blot analysis (no cross-reactivity with HP1β or HP1γ; Remboutsika *et al*., 1999); (ii) anti-HP1β mAb, 1 Mod-1A9, raised against recombinant *E.coli*-expressed mouse HP1β (no cross-reactivity with HP1α or HP1γ); (iii) anti-HP1γ mAb, 2Mod-1G6, raised against recombinant *E.coli*-expressed mouse HP1γ (no cross-reactivity with HP1α or HP1β); (iv) anti-TIF1α mAbs, 5T-1E8, 1T, 2T and 6T (Le Douarin *et al*., 1995a, 1996; Fraser *et al*., 1998); (v) anti-TIF1β mAb, 1Tb3, raised against TIF1β(123–834); (vi) anti-RPB1 mAb, 1PB-7C2, raised against the heptad repeat CTD-containing peptide of the RPB1 largest subunit of the human RNA polymerase II (Nguyen *et al*., 1996); (vii) anti-GAL4(1–147), 2GV3 (Le Douarin *et al*., 1996); (viii) anti-VP16 mAb, 2GV4 (Le Douarin *et al*., 1996); and (ix) anti-ERα F mAb, F3, raised against the F region of human ERα (Le Douarin *et al*., 1996). Confocal immunomicroscopy and immunoelectron microscopy were performed as described previously (Remboutsika *et al*., 1999).

Nuclear extract preparation, immunoprecipitation and Western blot analysis

P19 EC cells were grown in monolayers in Dulbecco's modified Eagle's medium supplemented with 5% (v/v) normal and 5% delipidated fetal calf serum, and incubated in a 7% CO_2/93% air humidified atmosphere at 37°C. A total of 10^7 cells were harvested, washed twice with ice-cold 10 mM phosphate-buffered saline (PBS) pH 7.2 and suspended in nuclei

isolation buffer [NIB; 15 mM Tris–HCl pH 7.5, 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM dithiothreitol (DTT), 2 mM sodium vanadate, 250 mM sucrose, and protease inhibitor mixture]. An equal volume of NIB buffer containing 0.6% NP-40 was added to the cells, the suspension was gently mixed and incubated on ice for 5 min. Nuclei were pelleted by centrifugation at 2000 *g* for 5 min at 4°C and washed with NIB buffer before final suspension in the same buffer. DNA was estimated by measuring UV absorption at 260 nM (A_{260}) in a 2 M NaCl solution (3 A_{260} units correspond to 100 µg DNA; Bellard *et al*., 1989). After centrifigation, nuclei were suspended in nuclei extraction buffer (NEB; 25 mM Tris–HCl pH 8.0, 250 mM NaCl, 1 mM EDTA, 10% glycerol, 0.2% NP-40, and protease inhibitor mixture). A total of 250 U of DNase I and 5 mM $MgCl_2$ were added to 1 mg DNA equivalent. Samples were digested for 1 h at 4°C. After addition of 10 mM EDTA and centrifugation (at 15 000 *g* for 30 min at 4°C), the resulting nuclear extract (5 mg protein/10^8 initial cells) was incubated with 50 µl of specific antibody coupled to protein G–Sepharose beads for 12 h at 4°C. The immunoprecipitates were washed five times with 800 µl of NEB buffer, and the final pellets were resuspended in 50 µl of Laemmli buffer (Laemmli, 1970); 10 µl were subjected to SDS–PAGE and electrotransferred to nitrocellulose filters. The filters were incubated with specific mAbs followed by a peroxidase-conjugated anti-mouse IgG secondary antibody and developed using an ECL detection kit (Amersham Pharmacia Biotech.).

In vitro binding assays

The assay was performed as described previously (Le Douarin *et al*., 1995a). Briefly, GST or GST–HP1 fusion proteins were expressed in *E.coli* and purified on glutathione *S*–Sepharose (Pharmacia). Purified proteins were quantified by Coomassie staining after SDS–PAGE separation and by Bradford protein assay. Then 12 µg of GST or GST fusion proteins loaded on glutathione *S*–Sepharose beads for 2 h at 4°C in NEB buffer were incubated with 1 µg of baculovirus-expressed His-tagged TIF1β or 10 µl of *in vitro*-translated proteins. Incubation was carried out for 6 h at 4°C with gentle agitation. The beads were washed six times with 750 µl of NEB buffer, resuspended in 25 µl of Laemmli buffer, boiled for 10 min, and proteins analysed by SDS–PAGE. Bound proteins were detected by immunoblotting or autoradiography.

Transactivation assays

Yeast cells grown in yeast extract/peptone/dextrose (YPD) or selective medium were transformed by the lithium acetate procedure (Gietz *et al*., 1995). Yeast PL3 (*Matα ura3-Δ1 his3-Δ200 leu2-Δ1 trp1::3ERE-URA3*; Le Douarin *et al*., 1995b) transformants were grown exponentially for about five generations in selective medium supplemented with uracil. Yeast extracts were prepared and assayed for OMPdecase activity as described previously (Pierrat *et al*., 1992). The transient transfection of mammalian cells and CAT assays were as described previously (Le Douarin *et al*., 1996). TSA (Sigma) was added 24 h after transfection, and cells were harvested 24 h later.

In vitro phosphorylation

Recombinant His-TIF1α and His-TIF1β, expressed in SF9 cells using a baculovirus vector, were purified as described previously (Fraser *et al*., 1998). Immunopurified TIF1α and TIF1β from P19 EC nuclear extracts were prepared as described above. *In vitro* phosphorylation was performed with 50 ng of recombinant purified His-TIF1α/β or 10 ng of immunopurified TIF1α/β in 20 mM Tris–HCl pH 7.9, 15 mM $MgCl_2$, 50 mM NaCl, 10 µM ATP, 1 mM DTT and 0.5 µCi of [γ-^{32}P]ATP with or without substrate (1 µg) as indicated. Reactions were performed at 30°C for 30 min and stopped by addition of Laemmli buffer and boiling. The phosphorylated proteins were subjected to SDS–PAGE, Coomassie Blue staining and autoradiography.

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References

Aasland,R., Gibson,T.J. and Stewart,A.F. (1995) The PHD finger: implication for chromatin-mediated transcriptional regulation. *Trends Biochem. Sci.*, **20**, 56–59.

Ashraf,S.I. and Ip,Y.T. (1998) Transcriptional control: repression by local chromatin modification. *Curr. Biol.*, **8**, 683–686.

Bellard,M., Dretzen,G., Giangrande,A. and Ramain,P. (1989) Nuclease digestion of transcriptionally active chromatin. *Methods Enzymol.*, **170**, 317–347.

Bellefroid,E.J., Poncelet,D.A., Lecocq,P.J., Revelant,O. and Martial,J.A. (1991) The evolutionary conserved Krüppel-associated box domain defines a subfamily of eukaryotic multifingered proteins. *Proc. Natl Acad. Sci. USA*, **88**, 3608–3612.

Bellefroid,E.J. *et al*. (1993) Clustered organization of homologous KRAB zinc-finger genes with enhanced expression in human T lymphoid cells. *EMBO J.*, **12**, 1363–1374.

Bellefroid,E.J. *et al*. (1998) Kzfl—a novel KRAB zinc finger protein encoding gene expressed during rat spermatogenesis. *Biochim. Biophys. Acta*, **1398**, 321–329.

Braunstein,M., Sobel,R.E., Allis,C.D., Turner,B.M. and Broach,J.R. (1996) Efficient transcriptional silencing in *Saccharomyces cerevisiae* requires a heterochromatin histone acetylation pattern. *Mol. Cell. Biol.*, **16**, 4349–4356.

Brown,K.E., Guest,S.S., Smale,S.T., Hahm,K., Merkenschlager,M. and Fisher,A.G. (1997) Association of transcriptionally silent genes with Ikaros complexes at centromeric heterochromatin. *Cell*, **91**, 845–854.

Brown,K.E., Baxter,J., Graf,D., Merkenschlager,M. and Fisher,A.G. (1999) Dynamic repositioning of genes in the nucleus of lymphocytes preparing for cell division. *Cell*, **3**, 207–217.

Cocktell,M. and Gasser,S. (1999) Nuclear compartments and gene regulation. *Curr. Opin. Genet. Dev.*, **9**, 199–205.

Deuschle,U., Meyer,W.K. and Thiesen,H.J. (1995) Tetracycline-reversible silencing of eukaryotic promoters. *Mol. Cell. Biol.*, **15**, 1907–1914.

Eissenberg,J.C., James,T.C., Forster-Hartnett,D.M., Hartnett,T., Ngan,V. and Elgin,S.C.R. (1990) Mutation in a heterochromatin-specific chromosomal protein is associated with suppression of position-effect variegation in *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA*, **87**, 9923–9927.

Eissenberg,J.C., Ge,Y.-W. and Hartnett,T. (1994) Increased phosphorylation of HP1, a heterochromatin-associated protein of *Drosophila*, is correlated with heterochromatin assembly. *J. Biol. Chem.*, **269**, 21315–21321.

Eissenberg,J.C., Elgin,S.C.R. and Paro,R. (1995) Epigenetic regulation in *Drosophila*: a conspiracy of silence. In Elgin,S.C.R. (ed.), *Chromatin Structure and Gene Expression*, Frontiers in Molecular Biology, Oxford University Press, Oxford, UK, pp. 147–171.

Ekwall,K., Olsson,T., Turner,B.M., Cranston,G. and Allshire,R.C. (1997) Transient inhibition of histone deacetylation alters the structural and functional imprint at fission yeast centromeres. *Cell*, **91**, 1021–1032.

Elgin,S.R.C. (1996) Heterochromatin and gene regulation in *Drosophila*. *Curr. Opin. Genet. Dev.*, **6**, 193–202.

Fraser,R.A., Heard,D.J., Adam,S., Lavigne,A.C., Le Douarin,B., Tora,L., Losson,R., Rochette-Egly,C. and Chambon,P. (1998) The putative cofactor TIF1α is a protein kinase that is phosphorylated upon interaction with liganded nuclear receptors. *J. Biol. Chem.*, **273**, 16199–16204.

Freemont,P.S. (1993) The RING finger: a novel protein sequence motif related to the zinc finger. *Ann. NY Acad. Sci.*, **684**, 174–192.

Friedman,J.R., Fredericks,W.J., Jensen,D.E., Speicher,D.W., Huang,X.-P., Neilson,E.G. and Rauscher,F.J.,III (1996) KAP-1, a novel corepressor for the highly conserved KRAB repression domain. *Genes Dev.*, **10**, 2067–2078.

Gietz,R.D., Schiestl,R.H., Willems,A.R. and Woods,R.A. (1995) Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. *Yeast*, **11**, 355–360.

Grunstein,M. (1998) Yeast heterochromatin: regulation by its assembly and inheritance by histones. *Cell*, **93**, 325–328.

Hahm,K. *et al*. (1998) Helios, a T cell-restricted Ikaros family member that quantitatively associates with Ikaros at centromeric heterochromatin. *Genes Dev.*, **12**, 782–796.

Hampsey,M. and Reinberg,D. (1999) RNA polymerase II as a control panel for multiple coactivator complexes. *Curr. Opin. Genet. Dev.*, **9**, 132–139.

Hecht,A., Laroche,T., Strahl-Bolsinger,S., Gasser,S.M. and Grunstein,M. (1995) Histone H3 and H4 N-termini interact with SIR3 and SIR4 proteins: a molecular model for the formation of heterochromatin in yeast. *Cell*, **80**, 583–592.

Hecht,A., Strahl-Bolsinger,S. and Grunstein,M. (1996) Spreading of transcriptional repressor SIR3 from telomeric heterochromatin. *Nature*, **383**, 92–96.

Hollemann,T., Bellefroid,E., Stick,R. and Pieler,T. (1996) Zinc finger proteins in early *Xenopus* development. *Int. J. Dev. Biol.*, **40**, 291–295.

Horsley,D., Hutchings,A., Butcher,G.W. and Singh,P.B. (1996) M32, a murine homologue of *Drosophila* heterochromatin protein 1 (HP1), localises to euchromatin within interphase nuclei and is largely excluded from constitutive heterochromatin. *Cytogenet. Cell Genet.*, **73**, 308–311.

James,T.C. and Elgin,S.C.R. (1986) Identification of a nonhistone chromosomal protein associated with heterochromatin in *Drosophila melanogaster* and its gene. *Mol. Cell. Biol.*, **6**, 3862–3872.

Jeanmougin,F., Wurtz,J.-M., Le Douarin,B., Chambon,P. and Losson,R. (1997) The bromodomain revisited. *Trends Biochem. Sci.*, **22**, 151–153.

Kim,J. *et al*. (1999) Ikaros DNA-binding proteins direct formation of chromatin remodeling complexes in lymphocytes. *Cell*, **10**, 345–355.

Kim,S.-S., Chen,Y.-M., O'Leary,E., Witzgall,R., Vidal,M. and Bonventre,J.V. (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.

Klug,C.A., Morrison,S.J., Masek,M., Hahm,K., Smale,S.T. and Weissman,I.L. (1998) Hematopoietic stem cells and lymphoid progenitors express different Ikaros isoforms and Ikaros is localized to heterochromatin in immature lymphocytes. *Proc. Natl Acad. Sci. USA*, **95**, 657–662.

Koonin,E.V., Zhou,S. and Lucchesi,J.C. (1995) The chromo superfamily: new members, duplication of the chromo domain and possible role in delivering transcription regulators to chromatin. *Nucleic Acids Res.*, **23**, 4229–4233.

Kornberg,R.D. and Lorch,Y. (1999) Chromatin-modifying and remodeling complexes. *Curr. Opin. Genet. Dev.*, **9**, 148–151.

Kuo,M.-H. and Allis,C.D. (1998) Role of histone acetyltransferases and deacetylases in gene regulation. *BioEssays*, **20**, 615–626.

Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Le Douarin,B. *et al*. (1995a) The N-terminal part of TIF1, a putative mediator of the ligand-dependent activation function (AF-2) of nuclear receptors, is fused to B-raf in the oncogenic protein T18. *EMBO J.*, **14**, 2020–2033.

Le Douarin,B., Pierrat,B., vom Baur,E., Chambon,P. and Losson,R. (1995b) A new version of the two-hybrid assay for detection of protein–protein interactions. *Nucleic Acids Res.*, **23**, 876–878.

Le Douarin,B., Lade Nielsen,A., Garnier,J.-M., Ichinose,H., Jeanmougin,F., Losson,R. and Chambon,P. (1996) A possible involvement of TIF1α and TIF1β in the epigenetic control of transcription by nuclear receptors. *EMBO J.*, **15**, 6701–6715.

Lehming,N., Le Saux,A., Schüller,J. and Ptashne,M. (1998) Chromatin components as part of a putative transcriptional repressing complex. *Proc. Natl Acad. Sci. USA*, **95**, 7322–7326.

Margolin,J.F., Friedman,J.R., Meyer,W.K.-H., Vissing,H., Thiesen,H.-J. and Rauscher,F.J.,III (1994) Krüppel-associated boxes are potent transcriptional repression domains. *Proc. Natl Acad. Sci. USA*, **91**, 4509–4513.

Mazarakis,N., Michalovich,D., Karis,A., Grosveld,F. and Galjart,N. (1996) Zfp-37 is a member of the KRAB zinc finger gene family and is expressed in neurons of the developing and adult CNS. *Genomics*, **33**, 247–257.

Moosmann,P., Georgiev,O., Le Douarin,B., Bourquin,J.-P. and Schaffner,W. (1996) Transcriptional repression by RING finger protein TIF1β that interacts with the KRAB repression domain of KOX1. *Nucleic Acids Res.*, **24**, 4859–4867.

Moosmann,P., Georgiev,O., Thiesen,H.-J., Hagmann,M. and Schaffner,W. (1997) Silencing of RNA polymerases II and III-dependent transcription by the KRAB protein domain of KOX1. *Biol. Chem.*, **378**, 669–677.

Nguyen,V.T., Gianonni,F., Dubois,M.F., Seo,S.J., Vigneron,M., Kedinger,C. and Bensaude,O. (1996) *In vivo* degradation of RNA polymerase II largest subunit triggered by α-amanitin. *Nucleic Acids Res.*, **24**, 2924–2929.

Orphanides,G., Lagrange,T. and Reinberg,D. (1996) The general transcription factors of RNA polymerase II. *Genes Dev.*, **10**, 2657–2683.

Pazin,M.J. and Kadonaga,J.T. (1998) What's up and down with histone deacetylation and transcription? *Cell*, **89**, 325–328.

Pengue,G., Calabro,V., Bartoli,P.C., Pagliuca,A. and Lania,L. (1994) Repression of transcriptional activity at a distance by the evolutionary conserved KRAB domain present in a subfamily of zinc finger proteins. *Nucleic Acids Res.*, **22**, 2908–2914.

Pierrat,B., Heery,D.M., Lemoine,Y. and Losson,R. (1992) Functional analysis of the human estrogen receptor by a phenotypic transactivation assay in yeast. *Gene*, **119**, 237–245.

Poncelet,D.A. *et al*. (1998) Functional analysis of ZNF85 KRAB zinc finger protein, a member of the highly homologous ZNF91 family. *DNA Cell Biol.*, **17**, 931–943.

Remboutsika,E., Lutz,Y., Gansmuller,A., Vonesh,J.-L., Losson,R. and Chambon,P. (1999) The putative nuclear receptor mediator TIF1α is tightly associated with euchromatin. *J. Cell Sci.*, **112**, 1671–1683.

Ryan,R.F., Schultz,D.C., Ayyanathan,K., Singh,P.B., Friedman,J.R., Fredericks,W.J. and Rauscher,F.J.,III (1999) KAP-1 corepressor protein interacts and colocalizes with heterochromatic and euchromatic HP1 proteins: a potential role for Krüppel-associated box-zinc finger proteins in heterochromatin-mediated gene silencing. *Mol. Cell. Biol.*, **19**, 4366–4378.

Saunders,W.S. *et al*. (1993) Molecular cloning of a human homologue of *Drosophila* heterochromatin protein HP1 using anti-centromere autoantibodies with anti-chromo specificity. *J. Cell Sci.*, **104**, 573–582.

Saurin,A.J., Borden,K.L.B., Boddy,M.N. and Freemont,P.S. (1996) Does this have a familiar RING? *Trends Biochem. Sci.*, **21**, 208–214.

Seeler,J.S., Marchio,A., Sitterlin,D., Transy,C. and Dejean,A. (1998) Interaction of SP100 with HP1 proteins: a link between the promyelocytic leukemia-associated nuclear bodies and the chromatin compartment. *Proc. Natl Acad. Sci. USA*, **95**, 7316–7321.

Singh,P.B., Miller,J.R., Pearce,J., Kothary,R., Burton,R.D., Paro,R., James,T.C. and Gaunt,S.J. (1991) A sequence motif found in a *Drosophila* heterochromatin protein is conserved in animals and plants. *Nucleic Acids Res.*, **19**, 789–794.

Venturini,L. *et al*. (1999) TIF1γ, a novel member of the transcriptional intermediary factor 1 family. *Oncogene*, **18**, 1209–1217.

Vissing,H., Meyer,W.K., Aagaard,L., Tommerup,N. and Thiesen,H.-J. (1995) Repression of transcriptional activity by heterologous KRAB domains present in zinc finger proteins. *FEBS Lett.*, **369**, 153–157.

vom Baur,E. *et al*. (1996) Differential ligand-dependent interactions between the AF-2 activating domain of nuclear receptors and the putative transcriptional intermediary factors mSUG1 and TIF1. *EMBO J.*, **15**, 110–124.

Wakimoto,B.T. (1998) Beyond the nucleosome: epigenetic aspects of position-effect variegation in *Drosophila*. *Cell*, **93**, 321–324.

Wallrath,L.L. (1998) Unfolding the mysteries of heterochromatin. *Curr. Opin. Genet. Dev.*, **8**, 147–153.

Weiler,K.S. and Wakimoto,B.T. (1995) Heterochromatin and gene expression in *Drosophila*. *Annu. Rev. Genet.*, **29**, 577–605.

Witzgall,R., O'Leary,E., Gessner,R., Ouellette,A.J. and Bonventre,J.V. (1993) Kid-1, a putative renal transcription factor: regulation during ontogeny and in response to ischemia and toxic injury. *Mol. Cell. Biol.*, **13**, 1933–1942.

Witzgall,R., O'Leary,E., Leaf,A., Önaldi,D. and Bonventre,J.V. (1994) The Krüppel-associated box-A (KRAB-A) domain of zinc finger proteins mediates transcriptional repression. *Proc. Natl Acad. Sci. USA*, **91**, 4514–4518.

Wolffe,A.P. and Hayes,J.J. (1999) Chromatin disruption and modification. *Nucleic Acids Res.*, **27**, 711–720.

Wreggett,K.A., Hill,F., James,P.S., Hutchings,A., Butcher,G.W. and Singh,P.B. (1994) A mammalian homologue of *Drosophila* heterochromatin protein 1 (HP1) is a component of constitutive heterochromatin. *Cytogenet. Cell Genet.*, **29**, 577–605.

Yoshida,M., Horinouchi,S. and Beppu,T. (1995) Trichostatin A and trapoxin: novel chemical probes for the role of histone acetylation in chromatin structure and function. *BioEssays*, **17**, 423–430.

Zhang,Y., Iratni,R., Erdjument-Bromage,H., Tempst,P. and Reinberg,D. (1997) Histone deacetylases and SAP18, a novel polypeptide, are components of a human SIN3 complex. *Cell*, **9**, 357–364.

Zhang,Y., LeRoy,G., Seelig,H.-P., Lane,W.S. and Reinberg,D. (1998) The dermatomyositis-specific autoantigen Mi2 is a component of a complex containing histone deacetylase and nucleosome remodeling activities. *Cell*, **95**, 279–289.

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