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RbAp48 Belongs to the Histone Deacetylase Complex That Associates with the Retinoblastoma Protein*

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The retinoblastoma susceptibility gene product, the Rb protein, is a key regulator of mammalian cell proliferation. One of the major targets of Rb is the S phase inducing E2F transcription factor. Once bound to E2F, Rb represses the expression of E2F-regulated genes. Transcriptional repression by Rb is believed to be crucial for the proper control of cell growth. Recently, we and others showed that Rb represses transcription through the recruitment of a histone deacetylase. Interestingly, we show here that the Rb-associated histone deacetylase complex could deacetylate polynucleosomal substrates, indicating that other proteins could be present within this complex. The Rb-associated protein RbAp48 belongs to many histone deacetylase complexes. We show here that the histone deacetylase HDAC1 is able to mediate the formation of a ternary complex containing Rb and RbAp48. Moreover, less deacetylase activity was found associated with Rb in cell extracts depleted for RbAp48 containing complexes, demonstrating that Rb, histone deacetylase, and RbAp48 are physically associated in live cells. Taken together, these data indicate that RbAp48 is a component of the histone deacetylase complex recruited by Rb. Finally, we found that E2F1 and RbAp48 are physically associated in the presence of Rb and HDAC1, suggesting that RbAp48 could be involved in transcriptional repression of E2F-responsive genes.

The retinoblastoma susceptibility gene is frequently altered in human tumors (1). It encodes a protein that plays a key role in regulating cell proliferation (1). The Rb protein shares a domain with two other proteins, the p107 and p130 proteins, and they are collectively referred to as pocket proteins (for recent reviews see Refs. 2 and 3). These pocket proteins are critical cellular targets of viral transforming proteins such as the adenoviral E1A protein (4). Viral transforming proteins

induce S phase entry at least in part through the inhibition of pocket proteins function, indicating that pocket proteins are negative regulators of cell proliferation. Indeed, the presence of active Rb prevents cell entry into S phase (5). During progression in G₁, Rb is phosphorylated by cyclin-dependent kinases, first cyclin D/cdk4 or 6 and then cyclin E/cdk2 (6). This phosphorylation results in Rb inactivation, thus allowing cells to enter S phase.

Many potential targets of Rb for its antiproliferative effects have been proposed (7). However, it is clear at present that one of the major targets of Rb during G₁ is the E2F transcription factor (8, 9). E2F is involved in the activation at the G₁/S boundary of a subset of genes whose products are required for S phase progression, such as DNA polymerase α , dihydrofolate reductase, or Cdc6 (10–12). The E2F transcription factor is composed of heterodimers between one E2F protein and one DP protein (for recent reviews see Refs. 13 and 14). There are six E2F proteins characterized so far, named E2F1 to E2F6 (15, 16). The various E2Fs contain a conserved dimerization and DNA-binding domain. Furthermore, apart from E2F6, they also share a C terminus domain responsible for transcriptional activation and pocket protein binding. Each E2F protein can heterodimerize with one of the two DP (DP1 and DP2) proteins known at the moment. The respective role of these complexes in the regulation of specific genes remains unclear.

During G₁, pocket proteins interact directly with the E2F activation domain and repress its activity (17, 18). Rb is able to repress E2F1-, E2F2-, E2F3-, and in some cases E2F4-containing complexes, whereas p107 and p130 repress E2F4 and E2F5 (13, 14). Inactivation of pocket proteins either by phosphorylation, by viral transforming proteins, or, in the case of Rb, by mutations such as those found in tumors leads to the accumulation of free E2F, able to activate transcription.

Once recruited to E2F-regulated promoters, pocket proteins act as transcriptional repressors (19). Transcriptional repression by pocket proteins is thought to be very important for their activity, because many E2F sites within promoters, such as *b-myb* or cyclin E promoters, have mainly a negative effect on transcription (20, 21). Furthermore, inactivation of pocket proteins relieves their transcriptional repressor functions (19, 22). Finally, various experiments suggest that a basal unrepresed level of transcription of E2F-regulated genes is sufficient to induce cell transformation (23–26).

Many mechanisms have been proposed for transcriptional repression by Rb. It is clear that Rb can inhibit the transactivating capacities of E2F1, for example by preventing TBP recruitment by E2F1 (27, 28). Recent data indicate that repression of basal transcription by Rb, p107, and p130 involves the recruitment of a histone deacetylase complex (29–35). This histone deacetylase could be HDAC1, because Rb interacts

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directly with HDAC1 through a classical Rb-binding motif used by many viral or cellular proteins, the so-called "LXCXE motif" (31).

Histone deacetylases or histone acetyltransferases regulate the acetylation of core histones N-terminal tails. Acetylation is generally thought to alter nucleosomes structure, thus allowing transcription (36, 37). Therefore, histone acetyltransferases are mostly transcriptional co-activators, whereas histone deacetylases are often associated with transcriptional repressors (38). Histone modifying enzymes are targeted at specific promoters through their association with specific transcription factors. Unliganded nuclear receptors recruit a histone deacetylase complex through their corepressor N-Cor, which interacts directly with Sin3, a histone deacetylase-associated protein (39, 40). This complex also contains the highly related RbAp48 and RbAp46 proteins. RbAp48 and RbAp46 are components of many chromatin-remodelling complexes (41–43). They are generally thought to target histone modifying complexes to nucleosomes, because they can interact directly with histone H4 (44–46).

Interestingly, these two proteins have been cloned as Rb-associated molecules (47, 48). Binding of RbAp48 correlates with the ability of Rb to block cell proliferation (47, 49). Furthermore, the *Caenorhabditis elegans* homologues of both Rb and RbAp48 were recently cloned as two proteins that belong to the same Ras-inhibitory pathway (50). Although these studies clearly show that RbAp48 is functionally linked to Rb, little is known about the molecular mechanisms involved.

In an attempt to identify proteins that could be important for transcriptional repression by Rb, we decided to further characterize the Rb-associated histone deacetylase complex. We show here that the Rb-associated histone deacetylase complex could deacetylate nucleosomal substrates. Because purified HDAC1 cannot function on polynucleosomes (51), this result indicates that other proteins besides HDAC1 are likely to be present. Indeed, our data indicate that RbAp48 is a component of the histone deacetylase complex recruited by Rb. We also show that in the presence of Rb and HDAC1, RbAp48 was able to associate with E2F1. Taken together, these results suggest that RbAp48 could be involved in the transcriptional repression of E2F-responsive genes that occurs throughout most of G₁.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—Jurkat cells were maintained in RPMI 1640 medium with glutamax and SAOS2 cells in Dulbecco's modified Eagle's medium high glucose with glutamax, each of them supplemented with 10% fetal calf serum and antibiotics. SAOS2 cells were transfected at a density of 3×10^6 cells/10-cm dish by calcium phosphate coprecipitation. The amount of cytomegalovirus promoter in the transfection was kept constant using empty vectors.

Vectors—pCMV HA-RbAp48¹ was constructed by inserting the RbAp48 coding sequences in frame in the empty vector pCMV 2N3T (a kind gift from Dr. T. Kouzarides). pGEM-RbAp48 is a kind gift from Dr. E. Y. Lee. pCMV E2F1, pCMV Rb 379–928, pCMV HA-HDAC1, pGEX 2T Rb 379–928, and pGEX 2T Rb 379–928 706 C-F (Rb Mut) were described previously (31, 32).

Immunoprecipitations—Total cell extracts were prepared from transiently transfected SAOS2 cells. Cells were resuspended in 0.5 ml of lysis buffer (50 mM Tris, pH 8, 0.4% Nonidet P-40, 300 mM NaCl, 10 mM MgCl₂, supplemented with protease inhibitors (complete, Roche Diagnostics), and phosphatase inhibitors 10 mM NaF, 0.1 mM NaVO₄, 10 mM β-glycerophosphate, 10 μM okadaic acid) and incubated for 15 min on ice. Samples were cleared by centrifugation, and supernatants were collected and diluted with 0.5 ml of dilution buffer (50 mM Tris, pH 8, 0.4% Nonidet P-40). Extracts were precleared with 20 μl of packed protein A beads (Sigma) for 1 h at 4 °C under gentle rotation. Immunoprecipitation was then performed by adding 1 μg of antibody and 20

μl of packed protein A/protein G beads for 1 h at 4 °C under gentle rotation. Beads were then washed three times with washing buffer (50 mM Tris, pH 8, 150 mM NaCl, 5 mM MgCl₂, 0.4% Nonidet P-40), and bound proteins were subjected to Western blot analysis. Western blotting was performed by standard procedures and revealed by Lumi-Light Plus Substrate (Roche Molecular Biochemicals). For immunoprecipitation of endogenous Rb, 500 μl of Jurkat nuclear extracts (prepared according to Ref. 52) were incubated with 5 μg of the anti-Rb antibody (C15G, Santa Cruz) or a control antibody (anti-Flag M2, Kodak) and 20 μl of protein A/protein G beads overnight at 4 °C in buffer D (52).

Purification of GST Fusion Proteins—Expression in *Escherichia coli* of GST, GST-Rb, and GST-Rb706C-F were performed by standard procedures. Cells were then disrupted by sonication at 4 °C in 20 ml of TBS (50 mM Tris, pH 8, 150 mM NaCl) supplemented with 1% Triton and protease inhibitors (complete, Roche Diagnostic). The soluble fraction was collected by centrifugation and added to 300 μl of preswollen glutathione agarose beads (Sigma). After 1 h of incubation at 4 °C, beads were washed extensively with TBS-1% Triton and TBS. Purification and recovery of the recombinant proteins was monitored by SDS-polyacrylamide gel electrophoresis and Coomassie staining.

GST Pull-down Experiments—Beads (corresponding to about 1 μg of fusion proteins) were washed once with washing buffer and preincubated when necessary for 30 min at 4 °C with 1, 3, 10, or 30 μg of either the HDAC1 peptide (SDKRIACEEEFSDSEE), the LXCXE containing SV40 T-antigen peptide (NEENLFCSEEMPSSDD), or an irrelevant peptide (YVEPTKEYDVALLKLD). Beads were then incubated for 1 h at 4 °C with 25 μl of Jurkat nuclear extracts then washed three times with washing buffer. The presence of RbAp48 was assayed by Western blot analysis. For GST pull-downs followed by deacetylase assays, the reaction was scaled up eight times.

Immunodepletion of RbAp48—Binding of the anti-RbAp48 antibody (11G10, Genetex) or control antibody (anti-Flag M2) to protein A/protein G beads (Sigma) was performed overnight in phosphate-buffered saline. Jurkat nuclear extracts (200 μl for GST pull-down experiments or 500 μl for immunoprecipitation of endogenous proteins) were incubated 4 h or overnight with the bead-bound antibodies (20 or 33 μg of antibodies, respectively). This depletion step was performed three times. An aliquot of supernatants from each step was kept to monitor the immunodepletion efficiency by Western blot. Supernatants from the third step were then used for GST pull-down experiments or immunoprecipitations.

Chromatin Labeling and Purification—For histones labeling, Jurkat cells (1.3×10^9) were incubated for 1 h at 37 °C in 20 ml of phosphate-buffered saline supplemented with ³H-labeled acetic acid (25 mCi), trichostatin A (100 ng/ml), and cycloheximide (100 μg/ml). Permeabilized nuclei were treated with micrococcal nuclease, and polynucleosomes were purified using a sucrose gradient by standard methods. Free histones were purified by hydroxyapatite chromatography.

Deacetylase Assays—Beads from immunoprecipitation of endogenous Rb or GST pull-down experiments were washed twice with TBS and then assayed for deacetylase activity for 2 h at 37 °C using 20,000 cpm of the indicated substrate (purified histones or a peptide as described (53)). Assays with polynucleosomes were performed in 50 mM Tris, pH 8.0, NaCl 20 mM. Assays were performed in duplicate. Trichostatin A (100 ng/ml) was used to check the specificity of the assay.

RESULTS

The Rb-associated Histone Deacetylase Complex Can Deacetylate Polynucleosomal Substrates—Transcriptional repression by Rb is mediated, at least in part, through a histone deacetylase (29, 30, 31). Chromatin immunoprecipitation experiments suggest that the Rb-associated histone deacetylase complex could deacetylate nucleosomal substrates (30). However, these experiments were performed using transiently transfected templates, which are thought not to be correctly arranged in chromatin. To clarify this point, we prepared labeled chromatin from Jurkat cells, and we purified polynucleosomes (Fig. 1A, fraction 1). We then tested whether the Rb-associated deacetylase complex could deacetylate histones in polynucleosomes. Incubation of Jurkat nuclear extracts with GST-Rb beads led to the retention on beads of significant polynucleosomes deacetylase activity (Fig. 1B). This retention was specific, because no activity could be seen using control GST beads or beads harboring an inactive point mutant of Rb (RbMut, Fig. 1B). Similar results were obtained using fraction

¹ The abbreviations used are: HA, hemagglutinin; GST, glutathione S-transferase.

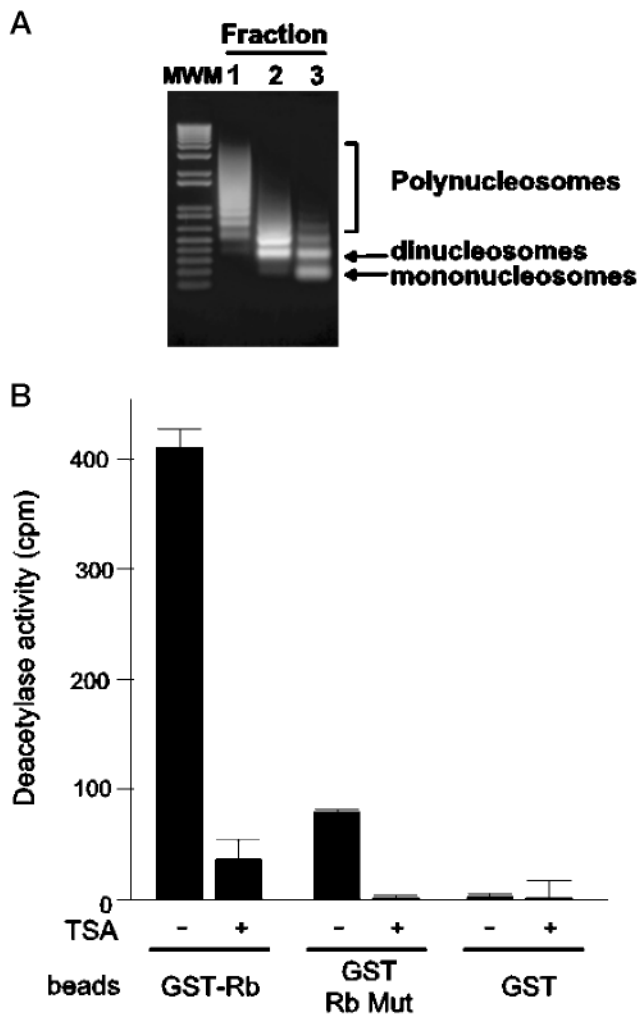


FIG. 1. The Rb-associated histone deacetylase complex deacetylates polynucleosomal substrates. A, nucleosomes were prepared from [3 H]acetic acid-labeled Jurkat cells using a sucrose gradient and pooled in three fractions. The DNA was extracted and analyzed by agarose gel electrophoresis followed by ethidium bromide staining. In the lane labeled *MWM*, 5 μ l of the 1 kilobase plus DNA ladder (Life Technologies, Inc.) were loaded. B, GST-Rb, GST-Rb Mut, or GST beads (corresponding to approximately 2 μ g of fusion proteins) were incubated with Jurkat nuclear extracts (200 μ l). After extensive washing, histone deacetylase activity associated with beads was measured using polynucleosomal substrates (fraction 1 from A) in the presence (+) or absence (-) of a histone deacetylase inhibitor (Trichostatin A (TSA), 100 ng/ml).

3 (mainly mononucleosomes or dinucleosomes) as a substrate (data not shown). This result indicates that the Rb-associated histone deacetylase complex could deacetylate histones in nucleosomes. Note, however, that the activity was about 10-fold lower than with purified histones as a substrate (data not shown).

The Rb-associated Histone Deacetylase Complex Contains a HDAC1-related Protein and Accessory Factors—HDAC1 has been proposed to contact Rb through a direct interaction. Purified HDAC1 is not able to deacetylate polynucleosomal substrates (51). One explanation of Fig. 1 results could be that Rb recruits a multimolecular complex containing proteins allowing accessibility of HDAC1 to polynucleosomes, such as the Mi-2 containing complex (54, 55). Alternatively, the Rb associated histone deacetylase activity could rely on another histone deacetylase that would be active on polynucleosomes. We thus tested by peptide competition experiments whether the Rb-associated histone deacetylase activity relies mainly on

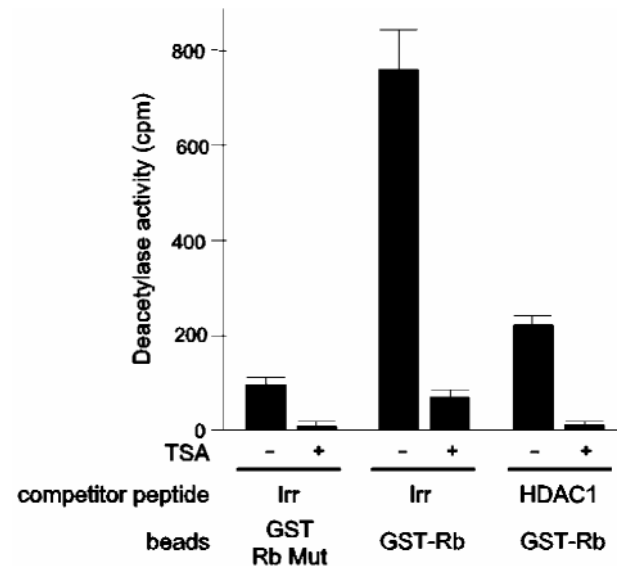


FIG. 2. The Rb-associated histone deacetylase complex contains HDAC1 or a HDAC1-related protein. GST-Rb or GST-Rb Mut beads (corresponding to approximately 0.5 μ g of fusion proteins) were preincubated with 10 μ g of the HDAC1-derived peptide or the irrelevant (*Irr*) peptide as indicated, prior to the incubation with Jurkat nuclear extracts (200 μ l). After extensive washing, histone deacetylase activity associated with beads was measured using labeled purified histones in the presence (+) or absence (-) of a histone deacetylase inhibitor (Trichostatin A (TSA), 100 ng/ml).

HDAC1. HDAC1 is thought to interact directly with Rb through an LXCXE-like motif, a known Rb-binding motif by which a number of viral or cellular proteins interact directly with Rb. Incubation of GST-Rb beads with nuclear extracts resulted in the specific retention of significant histone deacetylase activity assayed using purified histones (Fig. 2). Preincubation of Rb beads with a peptide corresponding to the Rb-binding site of HDAC1 (Fig. 2, *HDAC1*) led to a strong decrease of the Rb/histone deacetylase interaction, whereas addition of an irrelevant peptide had no effect (Fig. 2, *Irr*). In the presence of the HDAC1 peptide, the activity was indeed nearly down to background levels (Fig. 2, *GST-Rb Mut*). The residual activity could be due to residual binding of HDAC1 in the presence of the HDAC1-derived peptide (31). Although we cannot rule out the possibility that other histone deacetylases interact indirectly with Rb through an LXCXE-containing protein, these data suggest that most, if not all, of the Rb-associated histone deacetylase activity is due to a complex containing HDAC1 or a HDAC1-related protein (for example HDAC2, which is the only other known deacetylase related to HDAC1 in this region). Taken together, results from Figs. 1 and 2 suggest that other proteins besides the histone deacetylase are likely to be present within the Rb-associated histone deacetylase complex.

HDAC1 Can Act as a Bridge between Rb and RbAp48—Members of the Rb-associated histone deacetylase complex are likely to play an important role in cell cycle control. In an attempt to better define this complex, we first studied the Rb-associated RbAp48 protein. Indeed, RbAp48 is often found within histone deacetylase complexes (41), and its binding to Rb appears to be functionally important (47, 49, 50). Moreover, RbAp48 can interact directly with histone H4 (44–46), and so might possibly be involved in targeting HDAC1 to nucleosomes.

We reasoned that RbAp48 could be targeted to Rb through the histone deacetylase HDAC1, which is thought to interact directly with Rb (31). Co-immunoprecipitations of extracts from transfected cells were used to determine the parameters of the Rb/RbAp48 interaction. Rb negative cells (SAOS2 cells) were transiently transfected with a vector expressing RbAp48

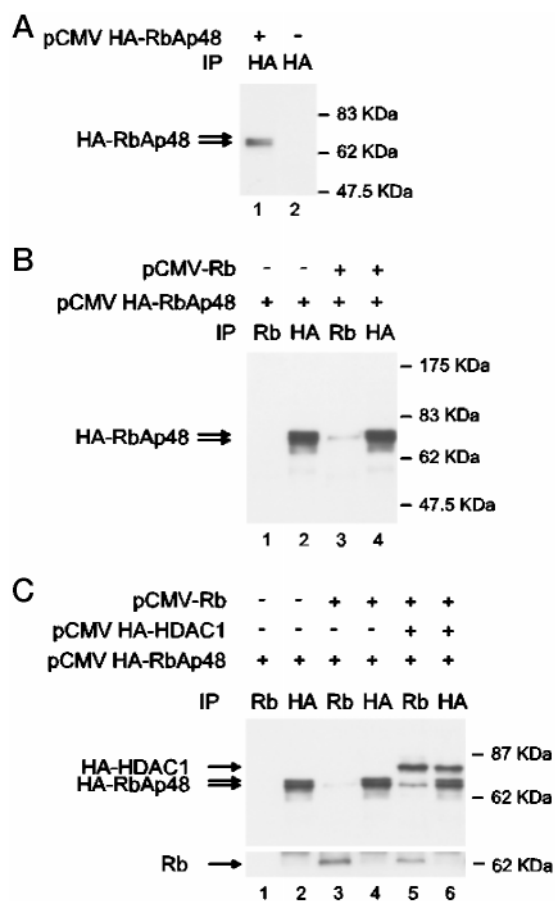


FIG. 3. HDAC1 mediates the Rb/RbAp48 interaction in transfected cells. *A*, SAOS2 cells were transiently transfected with 10 μ g of pCMV HA-RbAp48 expression vector as indicated. Whole cell extracts from transfected cells were immunoprecipitated (IP) using the anti-HA 12CA5 antibody (Roche Diagnostic). Immunoprecipitates were assayed for the presence of transfected RbAp48 by Western blot using the 12CA5 antibody. *B*, SAOS2 cells were transfected with 10 μ g of pCMV-Rb and pCMV HA-RbAp48 expression vectors as indicated. Whole cell extracts were immunoprecipitated using an anti-Rb antibody (C15G, Santa Cruz) (lanes 1 and 3) or the anti-HA antibody (lanes 2 and 4). Immunoprecipitates were then tested for the presence of HA-RbAp48 by Western blot. *C*, SAOS2 cells were transfected with pCMV Rb, pCMV HA-RbAp48, and pCMV HA-HDAC1 expression vectors as indicated. Whole cell extracts were immunoprecipitated using the anti-Rb antibody (lanes 1, 3, and 5) or the anti-HA antibody (lanes 2, 4, and 6). Immunoprecipitates were then tested by Western blot for the presence of HA-RbAp48 and HA-HDAC1 (top panel) or Rb (bottom panel).

tagged with a HA epitope. Transfected RbAp48 protein was detected by Western blot as a doublet (Fig. 3A). These two bands represent two different phosphorylation states of transfected RbAp48.² Immunoprecipitation of Rb from extracts transfected with Rb and RbAp48 expression vectors resulted in the co-immunoprecipitation of the hypophosphorylated form of RbAp48 (Fig. 3B, lane 3). This co-immunoprecipitation was specific, because in the absence of transfected Rb, no RbAp48 was immunoprecipitated with the anti-Rb antibody (lane 1), although RbAp48 was produced at a similar level (compare lanes 2 and 4).

Immunoprecipitation of Rb from cells transfected with Rb, HDAC1, and RbAp48 expression vectors resulted as expected in the co-immunoprecipitation of HDAC1 and RbAp48 (Fig. 3C, lane 5, top panel). Significantly more RbAp48 (about 4.5-fold more in the experiment shown) was found associated with Rb

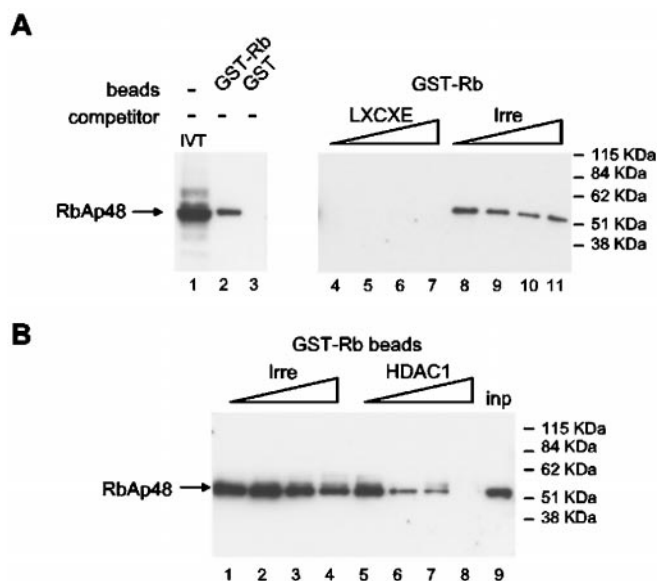


FIG. 4. The *in vitro* interaction between recombinant Rb and cellular RbAp48 is mediated through HDAC1. *A*, nuclear extracts (25 μ l) from Jurkat cells were incubated with GST Rb or control GST beads, as indicated (lanes 2 and 3). In the right panel, GST-Rb beads were preincubated with 1, 3, 10, or 30 μ g of the LXCXE-containing T antigen peptide (lanes 4–7) or 1, 3, 10, or 30 μ g of an unrelated peptide (lanes 8–11). After extensive washing, the presence of RbAp48 on the beads was tested by Western blot using the anti-RbAp48 antibody RB-BP (Transduction Laboratories). In lane 1, *in vitro* translated (IVT) RbAp48 was loaded. *B*, nuclear extracts (25 μ l) from Jurkat cells were incubated with GST Rb beads preincubated with 1, 3, 10, or 30 μ g of the HDAC1-derived peptide (lanes 5–8) or 1, 3, 10, 30 μ g of the irrelevant (Irre) peptide (lanes 1–4). After extensive washing, the presence of RbAp48 on the beads was tested by Western blot using the anti-RbAp48 antibody RB-BP. In lane 9, 1 μ l of Jurkat nuclear extracts was directly loaded.

in the presence of the HDAC1 expression vector than in its absence (compare lanes 3 and 5, top panel), although the levels of transfected RbAp48 (compare lanes 4 and 6, top panel) or of Rb (compare lanes 3 and 5, bottom panel) were similar in all samples. This result suggests that the histone deacetylase HDAC1 can mediate the interaction between Rb and RbAp48 in live cells, leading to the formation of a ternary complex containing Rb, HDAC1, and RbAp48.

Endogenous RbAp48 Is Recruited on Rb Beads by an LXCXE-containing Protein—RbAp48 has been cloned as a cellular protein which is specifically retained on a Rb affinity column, and the possibility of direct contacts between Rb and RbAp48 has been shown, at least *in vitro* (47). In contrast, our results (Fig. 3) suggest that the retention of cellular RbAp48 on Rb beads could reflect the binding to Rb of an HDAC1-containing complex.

Because HDAC1 interacts with Rb through a LXCXE motif, we tested whether a peptide containing this motif, derived from the T antigen, could block the recruitment of RbAp48 by Rb-coated beads (Fig. 4A). As expected, and as previously shown (47), RbAp48 was retained on GST-Rb beads (lane 2) and not on control GST beads (lane 3). Preincubation of GST-Rb beads with various doses of the T antigen peptide resulted in the complete inhibition of cellular RbAp48 recruitment (lanes 4–7), even at the lower dose tested (30 μ g/ml). This competition was specific, because a similar treatment with an unrelated peptide had no significant effect (lanes 8–11). This result indicates that the recruitment of endogenous RbAp48 by Rb involves an LXCXE motif. As already noted (47, 48), RbAp48 does not contain any sequence related to a known LXCXE-like motif, suggesting that its binding to Rb involves another cellular protein.

² E. Nicolas and D. Trouche, unpublished results.

To test whether this protein could be HDAC1, we performed competition experiments using the peptide derived from the HDAC1 site of interaction with Rb (Fig. 4B). Preincubation of GST-Rb beads with increasing doses of this peptide led to a dose-dependent decrease in RbAp48 recruitment (lanes 5–8), whereas a similar treatment with an unrelated peptide had no significant effect (lanes 1–4), as already shown in Fig. 4A. Note that the competition with the peptide derived from HDAC1 was less efficient than with the T antigen peptide (Fig. 4, compare A and B), in agreement with our previous results (31). This probably reflects the fact that to interfere with normal Rb functions, viral transforming proteins bind to Rb more strongly than cellular proteins. Nevertheless, it indicates that HDAC1, or a related protein, mediates the recruitment of cellular RbAp48 to Rb beads.

The Histone Deacetylase Complex Recruited *In Vitro* by Recombinant Rb Contains RbAp48—Recombinant Rb has been shown to recruit histone deacetylase activity from cell extracts (29, 31). The formation of the Rb-HDAC1-RbAp48 ternary complex that we demonstrated above (Figs. 3 and 4) could account for this activity. To test this possibility, we assessed the ability of Rb to recruit histone deacetylase activity from cells extracts immunodepleted for RbAp48-containing complexes.

As previously shown, incubation of GST-Rb-coated beads with Jurkat nuclear extracts resulted in the retention of significant histone deacetylase activity, which was sensitive to the presence of inhibitors (Fig. 5A). This retention was specific, because less deacetylase activity, if any, could be detected using GST-coated beads.

Extracts were incubated with an anti-RbAp48 antibody bound to protein A beads, resulting in lower levels of RbAp48 (Fig. 5B). Depletion was already evident after one step (compare lanes 1 and 2) and increased with three steps (lane 3), whereas a similar treatment with an unrelated antibody (lanes 5 and 6) had only a marginal effect, if any. These immunodepleted extracts (shown in lane 3) or control extracts (shown in lane 6) were then used for GST pull-down experiments using recombinant Rb as in Fig. 5A (Fig. 5C). The ability of GST-Rb-coated beads to recruit histone deacetylase activity (measured using a histone H4-derived peptide) from RbAp48 immunodepleted extracts was significantly lower (by 60%) than from control extracts (Fig. 5C). Thus, the immunodepletion of RbAp48-containing complexes decreased the amount of histone deacetylase activity recruited by Rb. Similar results were obtained using purified histones as a substrate (data not shown).

The significant activity that was found to associate with Rb using immunodepleted extracts could be due to residual RbAp48 (Fig. 5B, lane 3) or to the RbAp48-related protein RbAp46, which was not recognized by the antibody used for the immunodepletion (48) and could be detected by our Western blotting antibody after three rounds of depletion (Fig. 5B). Nevertheless, this result indicates that RbAp48 is a component of the histone deacetylase complex recruited by Rb from cell extracts.

Endogenous RbAp48, Rb, and Histone Deacetylase Are Physically Associated—In live cells, Rb is found complexed with RbAp48 (47), suggesting that RbAp48 could also be present among the histone deacetylase complex associated with endogenous Rb (29, 31). To test this hypothesis, we performed as above an immunodepletion of the RbAp48-containing complexes. (Fig. 6). Immunoprecipitation of Rb from control extracts resulted as expected in the co-immunoprecipitation of a significant histone deacetylase activity (Fig. 6, upper panel). This co-immunoprecipitation was specific, because no deacetylase activity was found in control immunoprecipitates. Immunodepletion of RbAp48 led to a decrease (to 40%) of the Rb-

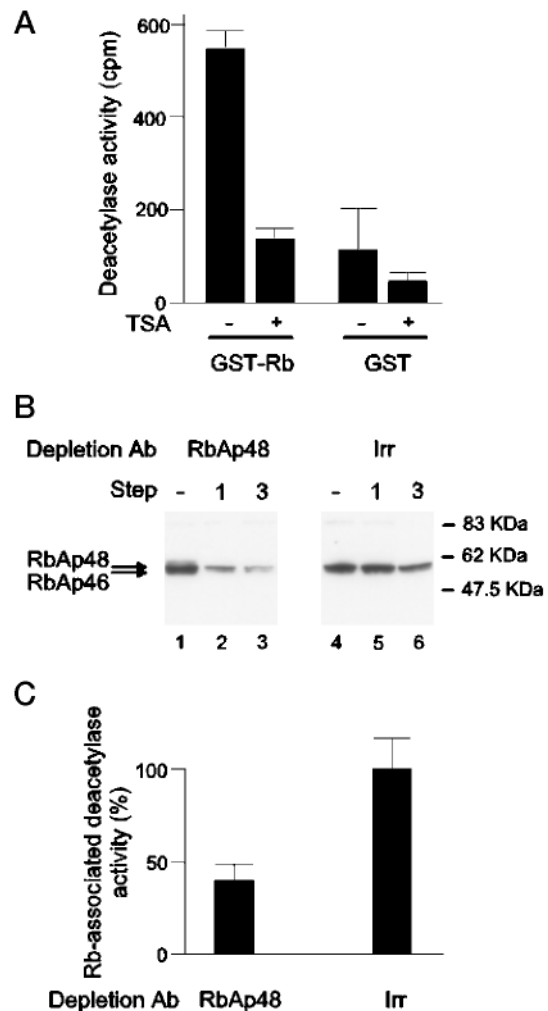


FIG. 5. RbAp48 is a component of the Rb-associated histone deacetylase complex. *A*, GST-Rb or GST beads were incubated with Jurkat nuclear extracts (200 μ l). After extensive washing, histone deacetylase activity associated with beads was measured in the presence (+) or absence (-) of a histone deacetylase inhibitor (Trichostatin A (TSA), 100 ng/ml). *B*, Jurkat nuclear extracts were incubated once (lanes 2 and 5) or three times (lanes 3 and 6) with beads covered with an anti-RbAp48 antibody (11G10, Genetex) (RbAp48) or an irrelevant antibody (anti-Flag M2 antibody, Kodak) (Irr). Supernatants were then subjected to Western blot analysis with the anti-RbAp48 antibody RB-BP. In lanes 1 and 4, 5 μ l of Jurkat nuclear extracts were directly loaded onto the gel. The extracts from lanes 3 and 6 were used for the experiment shown in *C*. *C*, GST-Rb beads were tested as in *A* for their ability to recruit an histone deacetylase activity (measured using a labeled histone H4-derived peptide) from extracts depleted three times with the anti-RbAp48 antibody (left) or control antibody (right).

associated histone deacetylase activity. This result indicates that a large proportion (at least 60%) of the endogenous Rb-histone deacetylase complexes contains RbAp48. Again, the residual Rb-associated histone deacetylase activity could depend on RbAp46-containing complexes or could be due to residual RbAp48-containing complexes, because both proteins could still be detected in the immunodepleted extracts used for the experiment (Fig. 6, lower panel). Whatever the explanation, this result indicates that endogenous Rb, RbAp48 and a histone deacetylase, likely HDAC1, are physically associated in live cells.

E2F1 Can Recruit a Complex Formed of Rb, HDAC1, and RbAp48—Rb has been shown to mediate a physical interaction between E2F1 and HDAC1 (29, 31), resulting in the recruitment via E2F1 of a complex that mediates transcriptional repression (29, 31). Direct contacts between Rb and RbAp48

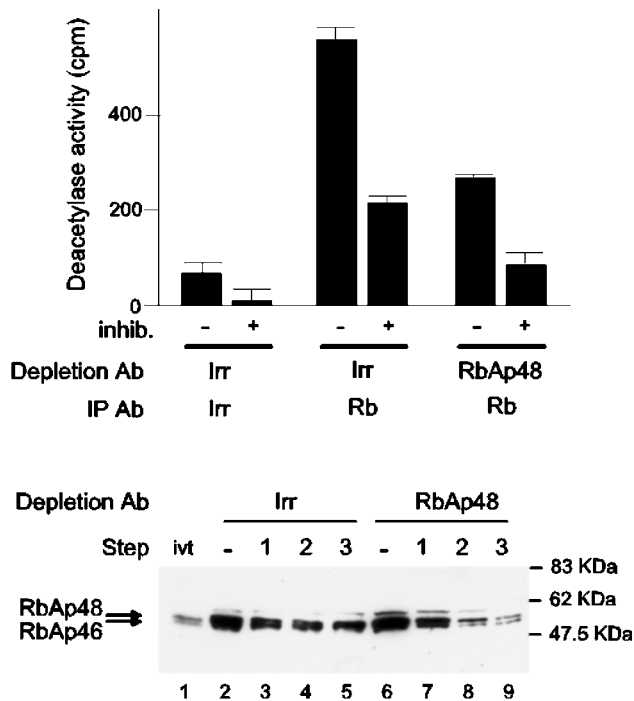


FIG. 6. Endogenous Rb, RbAp48, and histone deacetylase are physically associated. Jurkat Nuclear extracts (500 μ l) were subjected to three steps of immunodepletion with either an anti-RbAp48 antibody (11G10, Genetex) or an irrelevant (*Irr*) antibody (anti-Flag M2 antibody, Kodak) as in Fig. 4. Immunodepletion efficiency was monitored by Western blot analysis using the anti-RbAp48 antibody RBBP (*bottom panel*). Immunodepleted extracts were subjected to immunoprecipitation (*IP*) with either an anti-Rb antibody (C15-G, Santa Cruz) or an irrelevant antibody (anti-Flag M2 antibody, Kodak), as indicated. Immunoprecipitates were then assayed for histone deacetylase activity (measured using a labeled histone H4-derived peptide). *ivt*, *in vitro* translated.

have been documented (47, 48) that could interfere with E2F1 binding to Rb. To test the possibility of the formation on the E2F1 transcription factor of a multimolecular complex containing Rb, HDAC1 and RbAp48, we performed co-immunoprecipitation experiments using extracts from transfected cells (Fig. 7A). In the presence of both Rb and HDAC1 expression vectors, immunoprecipitation of E2F1 led to the co-immunoprecipitation of HDAC1 (*left panel*, lane 2). In addition, the hypophosphorylated RbAp48 was also detected in the immunoprecipitates. This co-immunoprecipitation was specific, because in the absence of exogenous E2F1, no RbAp48 could be detected in the E2F1 immunoprecipitate (*left panel*, lane 4). This result indicates that E2F1 and RbAp48 are able to associate in transfected cells.

In the absence of exogenous Rb or HDAC1, binding of RbAp48 to E2F1 was strongly reduced (*left panel*, lanes 6 and 8). This decrease was not due to a difference in the expression of transfected RbAp48 (*left panel*, compare lanes 1, 5, and 7) or E2F1 (*right panel*). Thus, it indicates that the presence of Rb and HDAC1 allows the formation of a multimolecular complex containing E2F1, Rb, HDAC1, and RbAp48 (Fig. 7B). Note that at higher exposure, a weak band corresponding to the hypophosphorylated RbAp48 could be detected in the absence of exogenous HDAC1 (*lane 8*), likely reflecting the presence of endogenous HDAC1 and thus confirming results from Fig. 3.

DISCUSSION

In this paper, we present evidence that RbAp48 belongs to the histone deacetylase complex recruited by Rb, because: (i) the interaction between RbAp48 and Rb requires a motif ho-

mologous to the Rb binding motif of HDAC1; (ii) HDAC1 can mediate the formation of a multimolecular complex containing Rb and RbAp48; and (iii) immunodepletion of RbAp48 decreases the Rb-associated histone deacetylase activity.

To our knowledge, RbAp48 is the first identified component of this complex apart from the histone deacetylase. Recently, it has been proposed that the Rb-associated histone deacetylase complex could also contain the product of the *c-ski* proto-oncogene (56). However, although this study clearly showed that c-Ski interacts with Rb, there was no direct demonstration that it is a component of the Rb-associated histone deacetylase complex. The presence of Sin3 has also been documented (56), but this result is in disagreement with previous studies (30), and more experiments are required to clarify this point. Finally, although a report points out that the BRCA1 protein could be part of this complex (57), we were unable to detect any Rb/BRCA1 interaction using conditions in which Rb recruits histone deacetylase activity from cell extracts (data not shown).

HDAC1 and HDAC2 have been proposed as the Rb-associated histone deacetylases (29, 30, 31, 58). Our results (Fig. 2) suggest indeed that HDAC1 or a related protein (for example, HDAC2) mediates the formation of the complex. HDAC3 or histone deacetylases from the other class (HDAC4, HDAC5, and HDAC6) do not interact with HDAC1 in live cells (59), suggesting that they are not present within the Rb-associated complex. However, the possibility exists that the Rb-associated histone deacetylase activity involves a still unknown HDAC1-related histone deacetylase or that other known histone deacetylases associate with Rb in other cell types or through indirect interactions with a LXCXE-containing protein. Indeed, it has recently been shown that RBP1 can recruit HDAC3 to Rb through an LXCXE motif (60).

RbAp48 has been previously shown to directly contact Rb, at least *in vitro* (47, 48). However, our results (Fig. 4) clearly show that cellular RbAp48 interacts with Rb, at least in part, through another protein that could be HDAC1 (Figs. 3 and 4). We cannot exclude that direct contacts between Rb and RbAp48 also exist in live cells. However, our results suggest that these direct contacts are not involved in the interaction between Rb and HDAC1, because the presence of exogenous RbAp48 did not affect the amount of HDAC1 that was co-immunoprecipitated with Rb (data not shown). This result suggests that the decrease in the histone deacetylase activity associated with Rb in RbAp48-depleted cell extracts (Fig. 5) is likely to be due to the depletion in RbAp48-containing histone deacetylase complexes. Consistent with this hypothesis, immunoprecipitation of RbAp48 with the antibody used in the depletion experiment resulted in the co-immunoprecipitation of a high level of histone deacetylase activity (data not shown). Furthermore, the total histone deacetylase activity present in the extracts decreased during RbAp48 immunodepletion (data not shown), indicating that extracts have indeed been depleted in histone deacetylase complexes.

Recent data indicate that the Rb-associated histone deacetylase complex is involved in transcriptional repression of E2F responsive genes (29, 30, 31, 32, 33–35). Our results (Fig. 7) indicate that the presence of Rb and HDAC1 allows the formation of a multimolecular complex containing RbAp48 and E2F1. It is thus possible that RbAp48 belongs to the Rb-associated transcriptional repressor complex that forms on E2F sites during G₁ (Fig. 7B). Overexpression of RbAp48 did not have any effect on E2F1 repression by Rb (data not shown), likely reflecting that endogenous RbAp48 is present in excess. Indeed, previous results suggest that HDAC1 is the limiting factor in SAOS2 cells for E2F1 repression by Rb (29, 31). The role of RbAp48 in transcriptional repression by Rb is likely to be

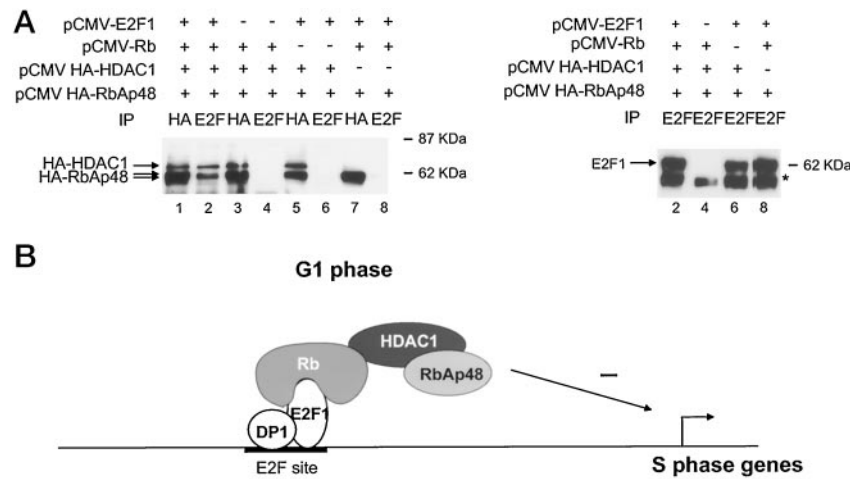


FIG. 7. RbAp48 is recruited to E2F1 in the presence of Rb and HDAC1. A, SAOS2 cells were transfected with pCMV E2F1, pCMV Rb, pCMV HA-RbAp48, and pCMV HA-HDAC1 expression vectors as indicated. Whole cell extracts were then immunoprecipitated (IP) using an anti-E2F1 antibody (KH95, Santa Cruz) (lanes 2, 4, 6, and 8) or the anti-HA antibody (lanes 1, 3, 5, and 7). Immunoprecipitates were then tested by Western blot using the anti-HA antibody (left panel) or the anti-E2F1 antibody (right panel). The asterisk indicates a band corresponding to the heavy chain of the anti-E2F1 antibody that was used for the immunoprecipitations. B, model of the transcriptional repressor complex recruited by Rb to repress transcription of E2F-regulated genes during the G₁ phase of the cell cycle.

important; the *C. elegans* homologues of both Rb and RbAp48 were recently cloned as two functionally related genes that antagonize a *C. elegans* Ras pathway (50). Regulating the binding of RbAp48 to the Rb-associated histone deacetylase complex could thus be a mean of modulating transcriptional repression by Rb.

What could be the role of RbAp48 in the Rb-associated histone deacetylase complex? RbAp48 is often seen as a molecular platform, allowing the formation of various different multimolecular complexes. Interestingly, histones modifying complexes are generally thought to be functionally linked to another class of chromatin remodelling factors, the ATP-dependent chromatin remodelling complexes of the SWI/SNF family. It has recently been shown that the auto-antigen Mi2, which possesses an ATP-dependent chromatin remodelling activity, is a component of some histone deacetylase complexes, providing a physical relationship between these two families of chromatin-modifying enzymes (55, 61). It is generally thought that the presence of nucleosome remodelling activity allows the histone deacetylase to be active on nucleosomal substrates (54, 55). RbAp48 is found not only in histone deacetylase complexes but also in ATP-dependent remodelling complexes (42). It could thus play a role in the sequential formation of these complexes. Consistent with this hypothesis is the fact that the Rb-associated histone deacetylase complex could deacetylate polynucleosomal substrates (Fig. 1). Such a role for RbAp48 would require some ways of regulating its association with the different complexes. It also raises the question of the involvement of ATP-dependent chromatin remodelling complexes in transcriptional repression by Rb.

Interestingly, RbAp48 has also been proposed to target histone-modifying enzymes to nucleosomes, because it can interact with histone H4 (44, 45). RbAp48 contacts directly the helix 1 of the histone fold, a region that is unlikely to be accessible in chromatin (45). ATP-dependent chromatin remodelling complexes could be involved in the unmasking of the RbAp48 interacting domain within nucleosomes. Consistent with this hypothesis, overexpression of Hbrm, the catalytic subunit of an ATP-dependent chromatin remodelling complex was found to increase the efficiency of E2F1 repression by Rb (62).

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REFERENCES

- Sellers, W. R., and Kaelin, W. G., Jr. (1997) *J. Clin. Oncol.* **15**, 3301–3312
- Grana, X., Garriga, J., and Mayol, X. (1998) *Oncogene* **17**, 3365–3383
- Mulligan, G., and Jacks, T. (1998) *Trends Genet.* **14**, 223–229
- Nevins, J. R. (1995) *Curr. Top. Microbiol. Immunol.* **199**, 25–32
- Qin, X. Q., Chittenden, T., Livingston, D. M., and Kaelin, W. G., Jr. (1992) *Genes Dev.* **6**, 953–964
- Planassilva, M. D., and Weinberg, R. A. (1997) *Curr. Opin. Cell Biol.* **9**, 768–772
- Herwig, S., and Strauss, M. (1997) *Eur. J. Biochem.* **246**, 581–601
- Qin, X. Q., Livingston, D. M., Ewen, M., Sellers, W. R., Arany, Z., and Kaelin, W. G., Jr. (1995) *Mol. Cell. Biol.* **15**, 742–755
- Tsai, K. Y., Hu, Y., Macleod, K. F., Crowley, D., Yamasaki, L., and Jacks, T. (1998) *Mol. Cell* **2**, 293–304
- Yan, Z., DeGregori, J., Shohet, R., Leone, G., Stillman, B., Nevins, J. R., and Williams, R. S. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 3603–3608
- Hateboer, G., Wobst, A., Petersen, B. O., Le Cam, L., Vigo, E., Sardet, C., and Helin, K. (1998) *Mol. Cell. Biol.* **18**, 6679–6697
- DeGregori, J., Kowalik, T., and Nevins, J. R. (1995) *Mol. Cell. Biol.* **15**, 4215–4224
- Johnson, D. G., and Schneider-Broussard, R. (1998) *Front. Biosci.* **3**, 447–448
- Nevins, J. R. (1998) *Cell Growth Differ.* **9**, 585–593
- Trimarchi, J. M., Fairchild, B., Verona, K., Moberg, K., Andon, N., and Lees, J. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 2850–2855
- Morkel, M., Wenkel, J., Bannister, A. J., Kouzarides, T., and Hagemeier, C. (1997) *Nature* **390**, 567–568
- Helin, K., Harlow, E., and Fattaey, A. (1993) *Mol. Cell. Biol.* **13**, 6501–6508
- Flemington, E. K., Speck, S. H., and Kaelin, W. G., Jr. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 6914–6918
- Chow, K. N., Starostik, P., and Dean, D. C. (1996) *Mol. Cell. Biol.* **16**, 7173–7181
- Lam, E. W., and Watson, R. J. (1993) *EMBO J.* **12**, 2705–2713
- Le Cam, L., Polanowska, J., Fabbriozzi, E., Olivier, M., Philips, A., Ng Eaton, E., Classon, M., Geng, Y., and Sardet, C. (1999) *EMBO J.* **18**, 1878–1890
- Adnane, J., Shao, Z., and Robbins, P. D. (1995) *J. Biol. Chem.* **270**, 8837–8843
- Krek, W., Xu, G., and Livingston, D. M. (1995) *Cell* **83**, 1149–1158
- Field, S. J., Tsai, F. Y., Kuo, F., Zubiaga, A. M., Kaelin, W. G., Jr., Livingston, D. M., Orkin, S. H., and Greenberg, M. E. (1996) *Cell* **85**, 549–561
- Yamasaki, L., Jacks, T., Bronson, R., Goillot, E., Harlow, E., and Dyson, N. J. (1996) *Cell* **85**, 537–548
- Trouche, D. (1997) *Pathol. Biol.* **45**, 5–8
- Weintraub, S. J., Chow, K. N., Luo, R. X., Zhang, S. H., He, S., and Dean, D. C. (1995) *Nature* **375**, 812–815
- Pearson, A., and Greenblatt, J. (1997) *Oncogene* **15**, 2643–2658
- Brehm, A., Miska, E. A., McCance, D. J., Reid, J. L., Bannister, A. J., and Kouzarides, T. (1998) *Nature* **391**, 597–601
- Luo, R. X., Postigo, A. A., and Dean, D. C. (1998) *Cell* **92**, 463–473
- Magnaghi-Jaulin, L., Groisman, R., Naguibneva, I., Robin, P., Lorain, S., Le Villain, J. P., Troalen, F., Trouche, D., and Harel-Bellan, A. (1998) *Nature* **391**, 601–605
- Ferreira, R., Magnaghi-Jaulin, L., Robin, P., Harel-Bellan, A., and Trouche, D. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 10493–10498

33. Iavarone, A., and Massague, J. (1999) *Mol. Cell Biol.* **19**, 916–922
34. Stiegler, P., De Luca, A., Bagella, L., and Giordano, A. (1998) *Cancer Res.* **58**, 5049–5052
35. De Luca, P., Majello, B., and Lania, L. (1998) *J. Cell. Biochem.* **70**, 281–287
36. Workman, J. L., and Kingston, R. E. (1998) *Annu. Rev. Biochem.* **67**, 545–579
37. Wade, P. A., Pruss, D., and Wolffe, A. P. (1997) *Trends Biochem. Sci.* **22**, 128–132
38. Kuo, M. H., and Allis, C. D. (1998) *Bioessays* **20**, 615–626
39. Torchia, J., Glass, C., and Rosenfeld, M. G. (1998) *Curr. Opin. Cell Biol.* **10**, 373–383
40. Heinzl, T., Lavinsky, R. M., Mullen, T. M., Soderstrom, M., Laherty, C. D., Torchia, J., Yang, W. M., Brard, G., Ngo, S. D., Davie, J. R., Seto, E., Eisenman, R. N., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1997) *Nature* **387**, 43–48
41. Tyler, J. K., Bulger, M., Kamakaka, R. T., Kobayashi, R., and Kadonaga, J. T. (1996) *Mol. Cell Biol.* **16**, 6149–6159
42. Gdula, D. A., Sandaltzopoulos, R., Tsukiyama, T., Ossipow, V., and Wu, C. (1998) *Genes Dev.* **12**, 3206–3216
43. Bulger, M., Ito, T., Kamakaka, R. T., and Kadonaga, J. T. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 11726–11730
44. Verreault, A., Kaufman, P. D., Kobayashi, R., and Stillman, B. (1996) *Cell* **87**, 95–104
45. Verreault, A., Kaufman, P. D., Kobayashi, R., and Stillman, B. (1998) *Curr. Biol.* **8**, 96–108
46. Parthun, M. R., Widom, J., and Gottschling, D. E. (1996) *Cell* **87**, 85–94
47. Qian, Y. W., Wang, Y. C., Hollingsworth, R. E., Jr., Jones, D., Ling, N., and Lee, E. Y. (1993) *Nature* **364**, 648–652
48. Qian, Y.-W., and Lee, E. Y.-H. P. (1995) *J. Biol. Chem.* **270**, 25507–25513
49. Huang, S., Lee, W. H., and Lee, E. Y. (1991) *Nature* **350**, 160–162
50. Lu, X., and Horvitz, H. R. (1998) *Cell* **95**, 981–991
51. Hassig, C. A., Tong, J. K., Fleischer, T. C., Owa, T., Grable, P. G., Ayer, D. E., and Schreiber, S. L. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 3519–3524
52. Dignam, J. D., Lebowitz, R. M., and Roeder, R. G. (1983) *Nucleic Acids Res.* **11**, 1474–1486
53. Taunton, J., Hassig, C. A., and Schreiber, S. L. (1996) *Science* **272**, 408–411
54. Tong, J. K., Hassig, C. A., Schnitzler, G. R., Kingston, R. E., and Schreiber, S. L. (1998) *Nature* **395**, 9179–21
55. Zhang, Y., LeRoy, G., Seelig, H. P., Lane, W. S., and Reinberg, D. (1998) *Cell* **95**, 279–289
56. Tokitou, F., Nomura, T., Khan, M. M., Kaul, S. C., Wadhwa, R., Yasukawa, T., Kohno, I., and Ishii, S. (1999) *J. Biol. Chem.* **274**, 4485–4488
57. Yarden, R. I., and Brody, L. C. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 4983–4988
58. Brehm, A., Nielsen, S. J., Miska, E. A., McCance, D. J., Reid, J. L., Bannister, A. J., and Kouzarides, T. (1999) *EMBO J.* **18**, 2449–2458
59. Grozinger, C. M., Hassig, C. A., and Schreiber, S. L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 4868–4873
60. Lai, A., Lee, J. M., Yang, W. M., DeCaprio, J. A., Kaelin, W. G., Jr., Seto, E., and Branton, P. E. (1999) *Mol. Cell Biol.* **19**, 6632–6641
61. Wade, P. A., Jones, P. L., Vermaak, D., and Wolffe, A. P. (1998) *Curr Biol* **8**, 843–846
62. Trouche, D., Le Chalony, C., Muchardt, C., Yaniv, M., and Kouzarides, T. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 11268–11273