

except that the reverse transcriptase was replaced by four units of *Thermus aquaticus* DNA polymerase (Promega). Amplification cycles for the first round of the PCR were 96 °C for 4 min, 94 °C for 50 s, 55 °C for 30 s, and 72 °C for 1 min for 32 cycles, followed by a final extension at 72 °C for 10 min. The first-round PCR products (4 µl) were used in a second-round PCR to amplify specific HIV-1 regions with primer pairs P63 and P56 (residues 3757–3734, 5'-TGT CCA CCA TGC TTC CCA TGT TTC-3') for the *pol* region, PV32 and PV33 (residues 7053–7079, 5'-TCA CAG ACA ATG CTA AAA CCA TAA TAG-3') for the V3 region, PE2 and P31 (residues 7704–7736, 5'-TAG GAG TAG CAC CCA CCA AGG CAA AGA GAA GAG-3') for the gp120–gp41-junction region, or SP15 and PE6 (residues 8822–8796, 5'-ACT ACT TTT TGA CCA CTT GCC ACC CAT-3') for the C terminus of gp41. Amplification conditions were 94 °C for 2 min, 94 °C for 30 s, 55 °C for 25 s, and 72 °C for 1 min for 35 cycles, followed by a final extension at 72 °C for 10 min. All PCR reactions were carried out in the Perkin–Elmer model 9600 thermocycler. PCR products were purified with the QIAEX II gel extraction kit (QIAGEN) and then inserted into the PCR3.1 vector (Invitrogen) before DNA sequencing in an automated sequencer (ABI PRISM 377).

Phylogenetic analyses. The neighbour-joining tree was generated using PHYLIP 3.5 (J. Felsenstein). We used the 111 sequences from the *env* alignment (in the Los Alamos Database) that spanned all three regions of interest, a total of 468 bases in the alignment after gap-stripping. By using all available taxa, we could determine that the phylogenetic behaviour of the ZR59 sequence was unique, and that no sequences from the 1980s or 1990s gave a similar result. The distance matrix for the neighbour-joining tree was created using the F84 option of the program DNADIST with the likelihood estimates of the relative rates of site mutations described below (when a simpler Kimura two-parameter model was used to calculate the distance matrix, the ZR59 sequence preferentially branched off from the B clade in the neighbour-joining tree). We selected a representative subset of the 111 sequences to represent the major-group viruses; we tried to include sequences with known years of sampling. These sequences and the ZR59 sequence were subjected to tree-building methods that are computationally intensive, namely maximum likelihood and weighted parsimony. Maximum-likelihood analyses were done using several programs. FastDNAm1²⁹ was used for the bootstrap analysis³⁰ and the initial maximum-likelihood tree. PHYLIP 3.5 was used for likelihood-ratio tests to compare the trees. We used PHYLIP 3.6 (J. Felsenstein) to reconstruct the most probable sequence at the ancestral nodes. All likelihood trees incorporated an estimate of rate variation between sites, an important element of accurate tree reconstruction, using the DNArates program (G. Olsen, personal communication) which optimizes the relative rate of substitution at each position in an alignment using maximum likelihood. A maximum-likelihood tree incorporating a REV model with an optimized gamma distribution using PAML (version 1.1, Z. Yang) was also generated²²; this tree confirmed results obtained with fastDNAm1. Statistical comparisons of the maximal-likelihood trees were made using the method of Kishino and Hasegawa²¹. When using weighted parsimony, we estimated substitution rates between the four bases from a parsimony tree generated by PAUP (D. Swofford), using the MacClade program; we then used the inverse of the observed substitution frequency to weight the character changes, by PAUP, in a subsequent parsimony tree. The accuracy of the branching pattern can be improved markedly by this approach, but the branch lengths do not reflect true distances.

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Retinoblastoma protein recruits histone deacetylase to repress transcription

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The retinoblastoma protein (Rb) silences specific genes that are active in the S phase of the cell cycle and which are regulated by E2F transcription factors¹. Rb binds to the activation domain of E2F and then actively represses the promoter by a mechanism that is poorly understood^{2,3}. Here we show that Rb associates with a histone deacetylase, HDAC1, through the Rb 'pocket' domain. Association with the deacetylase is reduced by naturally occurring mutations in the pocket and by binding of the human papilloma virus oncoprotein E7. We find that Rb can recruit histone deacetylase to E2F and that Rb cooperates with HDAC1 to repress the E2F-regulated promoter of the gene encoding the cell-cycle protein cyclin E. Inhibition of histone deacetylase activity by trichostatin A (TSA) inhibits Rb-mediated repression of a chromosomally integrated E2F-regulated promoter. Our results indicate that histone deacetylases are important for regulating the cell cycle and that active transcriptional repression by Rb may involve the modification of chromatin structure.

Histone deacetylase activity has been ascribed to two mammalian proteins, HDAC1 and HDAC2 (refs 4, 5). These deacetylases exist in

a complex with other proteins and are implicated in transcriptional repression resulting from nucleosome remodelling⁶⁻¹². Given that a known Rb-binding protein, Rbp48 (refs 13, 14), is found in the deacetylase complex, we investigated whether the Rb repressor is associated with histone deacetylase activity. Figure 1a shows that a fusion protein of Rb with glutathione-S-transferase (GST) can purify histone deacetylase activity from nuclear extracts derived from HeLa cells, whereas several GST fusions to other nuclear proteins fail to bind significant amounts of deacetylase. Association with deacetylase activity is also seen in two other cell types (U2OS and lymphocytes transformed by Epstein-Barr virus (EBV)) and the activity is sensitive to nanomolar concentrations of the histone deacetylase inhibitor TSA (data not shown). Furthermore, Rb-specific antibodies precipitate deacetylase activity from extracts of mammalian cells (Fig. 1b). These results indicate that Rb is associated with deacetylase activity *in vivo*.

The tumour-suppressor functions of Rb reside in the 'pocket' domain. We find that the integrity of the pocket is crucial for binding deacetylase (Fig. 1c). A tumour-derived point mutation (C706F) or deletion (Δ ex21) abolishes binding of deacetylase, as do two carboxy-terminal truncations that impinge on or delete the pocket domain B.

Viral oncoproteins can stimulate E2F-regulated promoters by sequestering Rb¹. Binding of these viral proteins to the pocket domain is mediated by an LXCXE sequence motif. Given that histone deacetylase associates with the pocket, we investigated whether binding of the human papilloma virus (HPV) E7 oncoprotein could displace the deacetylase. Figure 1d shows that association of Rb with deacetylase is abrogated in the presence of E7. An E7 protein with a mutated, non-functional LXCXE motif (C24) fails to displace deacetylase efficiently compared with a mutation elsewhere in the protein (Δ 5-10). In addition, a peptide containing the Rb-binding LXCXE motif sequence of E7 is sufficient to displace

deacetylase activity from Rb. In contrast, a control peptide (haemagglutinin, HA) has no effect on deacetylase binding. These results suggest that E7 can effectively compete with histone deacetylase for occupancy of the Rb pocket. Our finding that GST-Rb can associate with deacetylase in nuclear extracts of HPV-transformed HeLa cells indicates that there is not enough E7 in these extracts to disrupt the interaction of histone deacetylase with excess GST-Rb.

Figure 2a shows that GST-Rb can complex with HDAC1 translated *in vitro* (lane 3). GST-Rb can also associate with HDAC1 and HDAC2 present in nuclear extract, as revealed by western blotting using anti-HDAC1/2 antibody (Fig. 2b). When Rb and epitope-tagged HDAC1 (HDAC1-Flag) are coexpressed by transient transfection of mammalian cells, an interaction is detected by western blot analysis following precipitation with either anti-Flag (Fig. 2c) or anti-Rb antibody (Fig. 2d). The interaction between Rb and HDAC1 can also be demonstrated in untransfected cells (Fig. 2e). In this experiment, an immunoprecipitate obtained with Rb-specific antibody was shown to contain HDAC1 (Fig. 2e, lane 3), whereas an immunoprecipitate obtained with an irrelevant antibody (anti-HA) did not contain HDAC1 (Fig. 2e, lane 2). Thus Rb associates with the HDAC1 deacetylase *in vivo*, consistent with the ability of Rb to complex with deacetylase activity in nuclear extracts (Fig. 1).

The integrity of the Rb pocket is important for interaction with HDAC1. Binding of *in vitro* translated HDAC1 to GST-Rb is compromised by the tumour-derived mutations C706F and Δ ex21 and by other pocket deletions (Fig. 2f). These Rb pocket mutants have also lost the ability to purify deacetylase activity from nuclear extracts (Fig. 1c). Consistent with HDAC1 binding to the pocket of Rb is the observation that the LXCXE peptide can displace HDAC1 from Rb (data not shown).

If Rb is able to recruit histone deacetylase to E2F, then E2F should be complexed with HDAC1 in the presence of Rb. Figure 3a shows that the activation domain of E2F can interact with *in vitro*

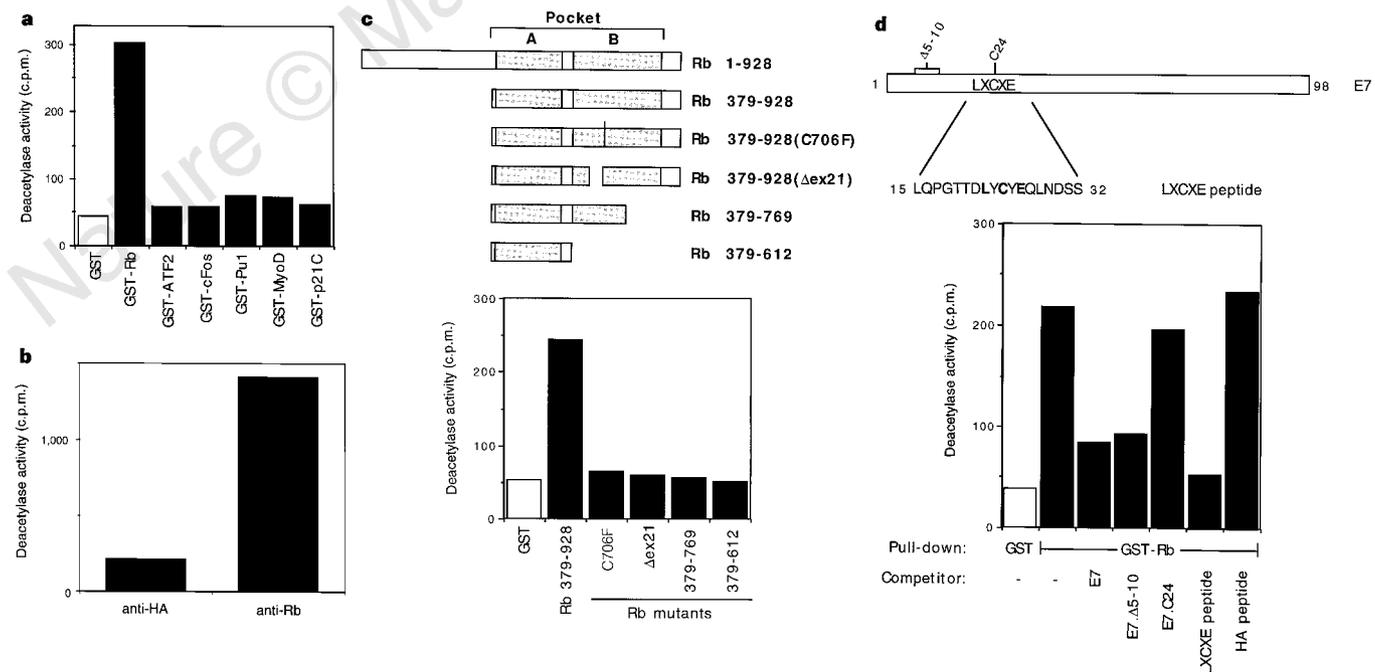


Figure 1 Rb associates with histone deacetylase activity *in vitro* and *in vivo*. **a**, GST-Rb fusion protein binds histone deacetylase activity from nuclear extract. Histone deacetylase activity is given as c.p.m. of ³H-acetate released from an acetylated histone H4 peptide⁴. **b**, Histone deacetylase activity coimmunoprecipitates with Rb. Jurkat nuclear extract was immunoprecipitated with 12C5A (anti-HA) or G3-245 (anti-Rb) antibody, as indicated. **c**, Binding of deacetylase activity is abrogated by pocket domain mutations. Top: representation of mutants. Pocket

subdomains A and B are represented as grey boxes. Bottom: binding of deacetylase activity by GST-Rb379-928 and pocket-domain mutants. **d**, HPV16 E7 competes Rb-associated deacetylase activity. LXCXE: Rb-binding motif. Deacetylase activity was bound in the presence and absence of purified recombinant E7 proteins (E7, E7 Δ 5-10, E7C24) or competitor peptides (HA, LXCXE; 20 μ g ml⁻¹), as indicated.

translated HDAC1 (lane 4). Several observations indicate that this interaction is indirectly mediated by the binding of Rb to E2F. First, mutating the Rb-binding site¹⁵ in E2F (E2FΔRb; Fig. 3a, lane 6) abolishes the interaction with HDAC1, whereas mutating the Mdm2-binding site¹⁶ (E2FΔMDM2; Fig. 3a, lane 5) has no effect. Second, addition of recombinant histidine-tagged Rb to the pull-down reaction (see Methods) increases the interaction of GST–E2F with HDAC1 considerably (Fig. 3b: compare lanes 1 and 2), whereas addition of a control protein, XRCC4, has no effect. These experiments suggest that the interaction between E2F and HDAC1 in reticulocyte lysate is indirectly mediated by the limiting amount of Rb present in the lysate. Coimmunoprecipitation shows that E2F can complex with HDAC1 in transfected cells (data not shown).

To confirm that HDAC1 recruited by Rb to E2F still retained its biological activity, we tested whether an E2F–Rb complex could associate with histone deacetylase. Figure 3c shows that a GST–E2F column does not purify deacetylase activity from nuclear extract (lane 2) unless it is first saturated with recombinant Rb (lane 5). Binding of deacetylase activity is dependent on Rb because GST–E2F bearing a mutated Rb-binding site (GST–E2FΔRb) fails to bind deacetylase activity in this assay (Fig. 3c, lane 6). These results indicate that Rb can recruit active HDAC1 enzyme to the E2F activation domain.

The finding that Rb can recruit HDAC1 to E2F raises the possibility that HDAC1 might function as a co-repressor of E2F. We tested this using the cyclin E promoter, an E2F-regulated target^{17,18}, and found that expression of HDAC1 moderately repressed cyclin E promoter activity (Fig. 4a). In the presence of coexpressed Rb, HDAC1 represses more efficiently and in a cooperative manner.

Given that Rb can tether HDAC1 to E2F, we investigated whether

Rb uses deacetylase activity to mediate repression of E2F. If so, then Rb-mediated repression should be sensitive to the histone deacetylase inhibitor TSA. We therefore used a cell line containing an integrated chloramphenicol acetyltransferase (CAT) gene reporter linked to Gal4-binding sites (3T3/208) (ref. 19). This reporter should be arranged as a complete chromatin structure. Figure 4b shows that the activity of this gene is stimulated by expression of a Gal4-E2F fusion (lane 2), and that coexpression of Rb results in promoter silencing (lanes 3 and 4), but that Rb-mediated repression is compromised in the presence of TSA (Fig. 4b: compare lanes 3 and 4 to lanes 7 and 8). The abrogation of Rb repression by TSA is also observed with non-integrated reporters under conditions in which a Gal4-dependent reporter is introduced by transient transfection (data not shown). These results confirm that Rb uses deacetylase activity to repress E2F.

Our results suggest a new mechanism for repression of E2F-regulated genes by Rb: Rb silences these genes by recruiting a repressive histone deacetylase to E2F (Fig. 4c). Once bound to the promoter, the deacetylase may convert the surrounding chromatin from a transcriptionally active (hyperacetylated) to a transcriptionally repressed (hypoacetylated) state. The demonstration that transfected DNA templates, which may not adopt a complete chromatin structure, are still responsive to HDAC1 repression and TSA-mediated stimulation^{5,9–11} (Fig. 4b, and data not shown) raises the possibility that deacetylases may target non-histone proteins involved in transcriptional regulation. This view is supported by the fact that the histone acetyl transferase CBP/p300 (refs 20, 21) regulates by acetylation the function of non-histone transcription factors²².

Rb binds to HDAC1 through its pocket domain using sequences that are distinct from the E2F-binding site. We have no evidence that

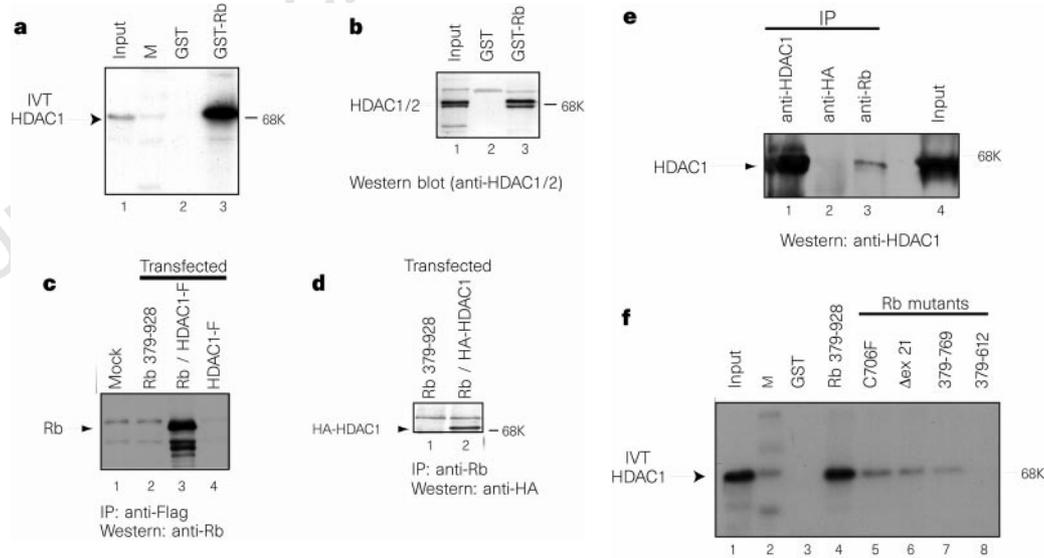


Figure 2 Rb interacts with histone deacetylases *in vitro* and *in vivo*. **a**, A GST–Rb fusion protein interacts with *in vitro*-translated ³⁵S-methionine-labelled HDAC1 in a GST pull-down assay. Lane 1: ³⁵S-HDAC1 input (2.5%). M: relative molecular mass marker. **b**, GST–Rb affinity-purifies histone deacetylases HDAC1 and HDAC2 from HeLa nuclear extract. Histone deacetylases were visualized by western blot analysis using the 1121 antibody, which recognizes both HDAC1 and HDAC2 (ref. 4). Lane 1: HeLa nuclear extract input (5%). **c**, Rb coimmunoprecipitates with Flag-tagged HDAC1 from extracts of transfected U2OS cells. Cells were transfected with pCMV-Rb379–928 and pcDNA3-HDAC1-F as indicated. Whole-cell extracts were precipitated with M2 (anti-Flag) antibody and the presence of Rb379–928 in immunoprecipitates was visualized by western blot analysis using XZ55 (anti-Rb) antibody. **d**, HA-tagged HDAC1 coimmunoprecipitates with Rb from extracts of transfected U2OS cells. Cells were transfected with pCMV-

Rb379–928 and pCMV-HA-HDAC1 as indicated. Whole-cell extracts were precipitated with XZ55 (anti-Rb) antibody and the presence of HA-HDAC1 in the immunoprecipitates was visualized by western blot analysis using 12C5A antibody. **e**, HDAC1 coimmunoprecipitates with Rb from Jurkat-cell extracts. Whole-cell extracts of untransfected Jurkat cells were precipitated with 1123 (anti-HDAC1; lane 1) (ref. 19), 12C5A (anti-HA; lane 2) and G3-245 (anti-Rb; lane 3) antibody, respectively. The presence of HDAC1 in immunoprecipitates was visualized by western blot analysis using 1121 (anti-HDAC1) antibody. Lane 4: Jurkat whole-cell extract input (1%). **f**, Mutations of the pocket domain of Rb compromise the interaction with HDAC1 *in vitro*. Binding of GST–Rb fusion proteins to *in vitro*-translated HDAC1 was assessed in a GST pull-down assay as indicated. Lane 1: ³⁵S-HDAC1 input (10%).

the interaction between Rb and HDAC1 is direct. The fact that Rb and HDAC1 do not interact in a yeast two-hybrid assay (data not shown) argues that the interaction in mammalian cells may be mediated by a component of the Sin3 complex—for example, by Rbp48 or by Sin3 itself^{6–12}.

The recruitment of histone deacetylase activity may explain why Rb has an active repression capacity when tethered to the promoter by a heterologous DNA-binding domain³. The ability to deliver deacetylase activity may allow Rb to silence any promoter to which it is recruited. Thus, the repression of pol I (ref. 23) and pol III genes^{24,25} by Rb may also involve the recruitment of deacetylase. Taken together, our results suggest that histone deacetylases are important in cell-cycle control. S-phase-specific genes, as well as other genes repressed by Rb, may be regulated, at least in part, at the chromatin level. □

Methods

Histone deacetylase assay. Histone deacetylase was assayed as described⁴.
Affinity purification and immunoprecipitation of histone deacetylase activity from cell extracts. Equivalent amounts of GST fusion proteins prebound to glutathione beads were added to 30 μl HeLa nuclear extract (Computer Cell Culture Centre, Belgium) in 250 μl IPH buffer²⁰ and incubated at 4 °C for 45 min. Beads were washed 2–3 times with IPH buffer and assayed for histone deacetylase activity. For peptide and E7 competition experiments, GST fusion proteins were preincubated with competitors for 5 min at room temperature before adding to the extract. Immunoprecipitations were carried out using standard procedures¹⁶. Antibody complexes were collected on protein A/G Sepharose beads, washed 3–4 times with IPH buffer and assayed for histone deacetylase activity.
Immunoprecipitations and western blot analysis. Standard procedures were used for coimmunoprecipitation and western blotting¹⁶. For coimmuno-

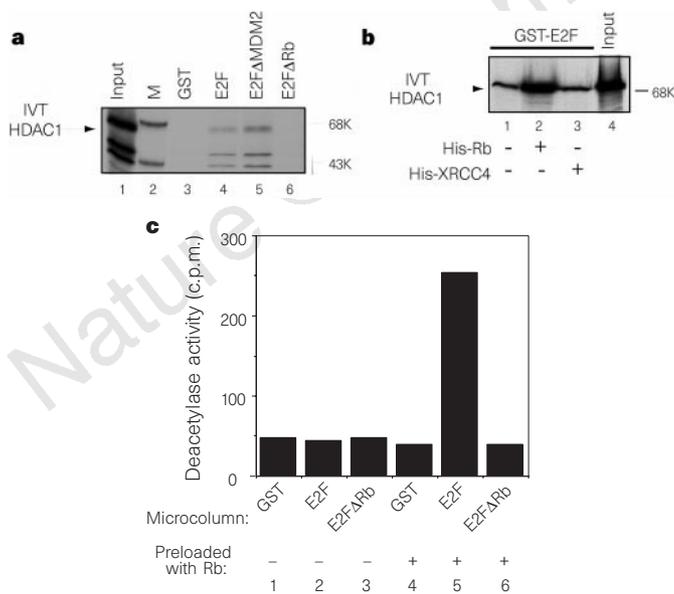


Figure 3 Rb recruits HDAC1 to E2F. **a**, Interaction of E2F and HDAC1 is dependent on the Rb-binding site of E2F. Binding of GST-E2F380–437 and GST-E2F mutants to *in vitro*-translated HDAC1 was assessed in a GST pull-down assay as indicated. Lane 1: ³⁵S-HDAC1 input (10%). E2F: GST-E2F380–437. E2FΔM2M2 contains a mutated Mdm2-binding site, E2FΔRb a mutated Rb-binding site. **b**, Binding of E2F to HDAC1 *in vitro* is enhanced by addition of recombinant Rb protein. Binding of *in vitro*-translated HDAC1 to GST-E2F380–437 was tested in the presence and absence of recombinant purified histidine-tagged Rb379–928 and XRCC4 protein, respectively, as indicated. Lane 4: ³⁵S-HDAC1 input (10%). **c**, Affinity purification of histone deacetylase activity from nuclear extract by a GST-E2F column requires Rb. GST, GST-E2F380–437 and GST-E2FΔRb microcolumns were preloaded with recombinant His-tagged Rb379–928 protein and used to bind deacetylase activity from nuclear extract.

precipitation of Rb and HDAC1, whole-cell extract from 10⁹ Jurkat cells in 10 ml IPH buffer was used.

Cell culture, transfection, CAT and luciferase assays. Cell lines were maintained in DMEM supplemented with 10% fetal calf serum and grown at 37 °C, 5% CO₂. The 3T3/208 cell line was maintained in the presence of 0.5 mg ml⁻¹ G418. Jurkat cells were maintained in RPMI medium supplemented

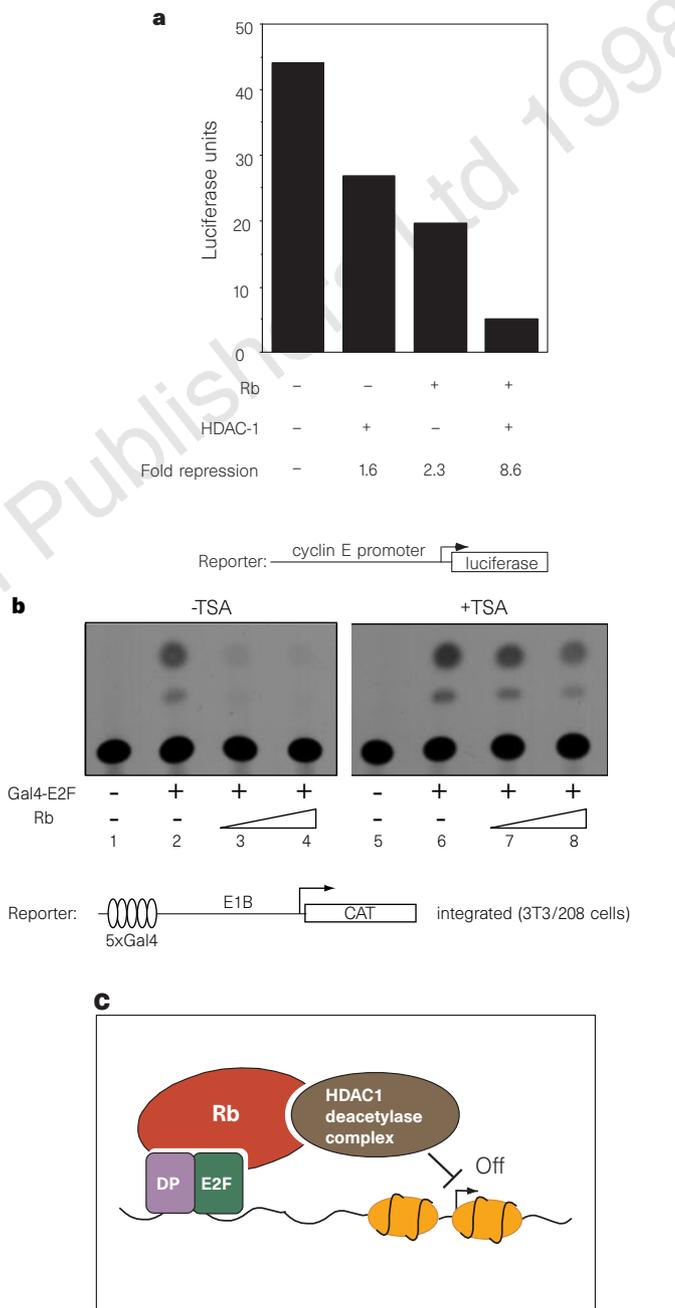


Figure 4 Histone deacetylase is required for full Rb-mediated transcriptional repression *in vivo*. **a**, HDAC1 enhances Rb-mediated repression of E2F *in vivo*. U2OS cells were transfected with pCE(-543/+263)luc reporter (1 μg), pCMV-Rb379–928 (0.25 μg) and pcDNA3-HDAC1-F (0.1 μg) as indicated. **b**, Inhibition of histone deacetylase activity compromises repression of E2F by Rb *in vivo*. 3T3/208 cells containing a chromosomal Gal4-dependent CAT reporter gene (5GE1B-CAT) (ref. 19) were transfected with pHKG-E2F380–437 (10 μg) and increasing amounts of pCMV-Rb379–928 (lanes 3 and 7: 0.3 μg, lanes 4 and 8: 1.0 μg) expression vectors in the absence (left) or presence (right) of 330 nM TSA as indicated. **c**, Repression of E2F by Rb involves deacetylase activity. Rb can bind simultaneously to E2F and HDAC1 and uses deacetylase activity to silence the promoter. This mechanism may account for the ‘active’ repression of Gal4-Rb and the E2F-Rb complex.

with 10% fetal calf serum. Transfections, CAT and luciferase assays were done using standard techniques¹⁵. TSA (Wako BioProducts) was added at 330 nM 24 h before collecting cells.

Recombinant GST fusion and histidine-tagged proteins. Recombinant proteins were expressed in and purified from *E. coli* XA90 as described²⁰. For competition experiments, GST-E7 proteins were cleaved from GST using thrombin.

GST microcolumns, pull-down assays and *in vitro* translation. Microcolumns were prepared by placing a glass bead in a yellow Gilson tip and applying 30–50 μ l GST fusion protein prebound to glutathione beads. GST-E2F380–437 columns were loaded with recombinant Rb protein and washed 4 times with IPH buffer. Beads were then transferred to a fresh reaction tube for affinity purification of histone deacetylase activity. *In vitro* translation reactions and GST pull-down experiments have been described¹⁵. HDAC1 was translated *in vitro* from pING14A-HDAC1. The sequences of the competitor peptides were LQPGTTDLYCYQLNDSS (LXCXE) and YPYDVDPDYA (HA), respectively.

Plasmids. pING14A-HDAC1 was subcloned from pBJ5-HD1-F by PCR (ref. 4). pGEX-Rb, pGEX-E7 and pGEX-E2F expression vectors, pCMV-Rb379–928, pHKG-E2F380–437 and pCycE-luc have all been described^{15–17}.

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Retinoblastoma protein represses transcription by recruiting a histone deacetylase

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The retinoblastoma tumour-suppressor protein Rb¹ inhibits cell proliferation by repressing a subset of genes that are controlled by the E2F family of transcription factors² and which are involved in progression from the G1 to the S phase of the cell cycle. Rb, which is recruited to target promoters by E2F1 (ref. 3), represses

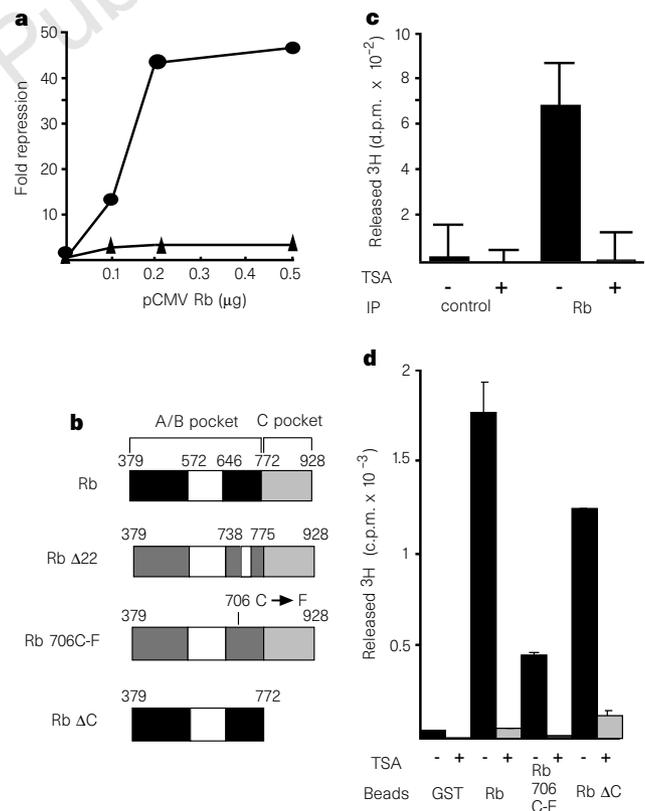


Figure 1 A histone deacetylase activity is associated with Rb. **a**, Trichostatin A (TSA) treatment relieves Rb repression of E2F1. SAOS2 cells were transiently transfected with 1 μ g pE2F-luc reporter vector, 50 ng pCMV E2F1 (ref. 19), and the indicated doses of pCMV Rb. Cells were treated (triangles) or not (circles) with 100 ng ml⁻¹ TSA for 8 h; repression by pCMV Rb was calculated. **b**, Diagram of the Rb mutants used. The Rb A/B pocket is indicated by filled boxes, and the C pocket by grey boxes. pRb Δ 22 lacks exon 22 and Rb706C-F contains a point mutation at codon 706; both mutations result in inactivation of the A/B pocket. pRb Δ C lacks the C pocket. **c**, A histone deacetylase is immunoprecipitated (IP) by anti-Rb antibodies: Jurkat whole-cell extracts were immunoprecipitated with anti-Rb or control (anti-HA) antibodies and immunoprecipitates were assayed for deacetylase activity. **d**, Recombinant Rb binds to a histone deacetylase activity: beads covered with the indicated GST fusion proteins were incubated with nuclear extracts (1 mg) and assayed for histone deacetylase activity.