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FAST TRACK

Retinoblastoma Protein Tethered to Promoter DNA Represses TBP-Mediated Transcription

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Abstract The retinoblastoma (RB) tumour suppressor protein negatively regulates cell proliferation by modulating transcription of growth-regulatory genes. Recruitment of Rb to promoters, by association with E2F complex or by fusion with heterologous DNA-binding domains, demonstrated that Rb represses directly transcription. Recent studies also suggest that the RB protein is able to repress gene transcription mediated by the RNA polymerase I and III. Since the TATA-binding protein (TBP) is an important component for transcription mediated by all three RNA polymerases, we have analysed the functional interaction between Rb and TBP in vivo in the context of RNA pol II-driven transcription. We demonstrated that in mammalian cells Rb tethered to promoter represses TBP-mediated activation in vivo, and Rb-mediated repression is reversed in the presence of the inhibition of histone deacetylase activity by trichostatin A (TSA). J. Cell. Biochem. 70:281–287, 1998. © 1998 Wiley-Liss, Inc.

Key words: retinoblastoma protein; TATA-binding protein; repressor; TSA

It is widely recognised that the retinoblastoma tumour suppressor protein Rb is an important cell cycle regulatory protein. The current concept is that Rb suppresses cell growth by preventing the transcription of growth-regulatory genes [Weinberg, 1995]. Convergent genetic and biochemical data suggest that Rb operates through modification of gene expression, achieved by functional interaction with transcription factors such as E2F-1 by the hypophosphorylated form of Rb that is present in early and mid-G1 phase [Bartek et al., 1996; Sanchez et al., 1996]. Phosphorylation of Rb via cyclin-dependent kinases in mid-to late G1 phase results in the release of Rb-bound E2F-1 and other transcription factors, which then activate the transcription of genes associated with proliferative signals [Weintraub and Dean, 1992; Hiebert et al., 1992; Helin et al., 1993].

Many studies have shown that recruitment of Rb to promoters via heterologous DNA binding domains represses transcription directly in a phosphorylation-sensitive manner [Weintraub et al., 1995; Adnane et al., 1995; Bremmer et al., 1995; Chow et al., 1996]. Recent reports suggest that, apart from regulating transcription of some S phase specific Pol II-dependent genes, Rb can also repress the expression of genes transcribed by the other two nuclear RNA polymerases, Pol I and Pol III [Cavanaugh, et al., 1995; White et al., 1996]. These findings indicated that Rb is the only presently known repressor of all three classes of RNA polymerases.

It is conceivable that Rb-mediated repression of different RNA polymerases may involve a putative factor shared in all three of them. Thus, Rb-mediated regulation may be the result of a functional interaction between Rb and a general transcription factor required for the activity of each polymerase. Such factor may be the TATA-binding protein (TBP), that is an essential component for transcription of all three RNA polymerases [Hernandez, 1993].

In this work we investigated the functional interaction between Rb and the TATA-binding protein (TBP) in vivo in the context of RNA pol II-driven transcription. Our data indicate that in mammalian cells Rb tethered to promoter DNA via heterologous DNA-binding domain represses TBP-mediated activation in vivo. Rb-

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mediated repression appears to be dependent upon an intact pocket domain, and it can be abrogated by phosphorylation. Finally, we found that inhibition of histone deacetylase activity by trichostatin A (TSA) relieves Rb repression, suggesting that Rb-mediated represson of basal transcription involves modification of chromatin structure.

MATERIALS AND METHODS

Reporter Plasmids

The CAT reporters G5E1b, G1E1b, T7G5-TATA have been previously described [De Luca et al., 1996]. The T7G1-TATA has been obtained by substitution of the five GAL4 binding sequences of the T7G5-TATA with an oligonucleotide containing a single GAL4 DNA-binding. To construct G1-TGGA, the TATA box of G1E1b (XbaI/KpnI fragment) was substituted with a double stranded oligonucleotide bearing the mutated TGGA box. The G1–150-E1b was constructed by inserting three copies of the pGEM3 polylinker HindIII-EcoR1 51bp fragment inserted into the XbaI site located between the GAL4 site and the TATA-box of G1E1b.

Effector Plasmids

The CMV-hTBP, expressing the human full-length TBP cDNA, was kindly provided by Dr A. Hoffman. The GAL4-hTBP was constructed by PCR amplification of the complete hTBP coding region and inserted into the SmaI site of pSG424. The GAL4-hTBP Δ1–106 was constructed by inserting the HincII fragment from CMV-hTBP, encoding the aa 106–339 of the human TBP, in frame with the GAL4 DNA binding domain of the pSG424 SmaI digested. The expression vectors encoding for the cyclin B1 and E were kindly provided by G. Piaggio. The GAL4-RB (1–928) and the CMV-RB vectors were provided by K. Helin. The expression plasmids encoding the DNA-binding region of the tetracycline repressor (TetR) fused to the KRAB domain [Pengue and Lania, 1996] or to various regions of the Rb cDNA: TetR-RB(379–928), TetR-RB(379–792), TetR-RBΔ21, and TetR-RBΔ22 have been described [Sellers et al., 1995] and kindly provided by W. Kaelin.

Transfection and CAT Assay

C33A cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum. Transfection were performed by calcium phosphate precipitation as previous described [De Luca et al., 1996; Majello et al., 1997]. TSA (Sigma) was added (100 nM) at 10 h after transfection, and cells were harvested 30 h later. For normalization of transfection efficiencies a β-Gal expression plasmid (pSV-β-Gal) was included in the cotransfections. CAT assays were performed as described [De Luca et al., 1996; Majello et al., 1997], and results are presented as mean ± s.d. of a least three duplicated independent transfection experiments.

RESULTS

RB Protein Represses TBP-Mediated Transcription

To analyse the role of Rb on basal transcription the human cervical carcinoma cell line C33A was transfected with a reporter plasmid containing only the adenovirus E1b-TATA sequence (G5E1b). However, the level of expression of this reporter was very low, consequently the role of Rb in regulating basal transcription could not be unambiguously determined. Moreover, enforced expression of exogenous hTBP (CMV-hTBP) did not lead to a significant increase of the G5E1b CAT activity (data not shown). Consequently, given the very modest level under which the hTBP activation was observed, we were uncertain as to its significance.

To investigate the consequences of Rb expression on basal transcription in the absence of any activator, we designed an experimental strategy in which a minimal promoter was effectively activated by hTBP in the absence of any enhancer. Studies in yeast and more recently in mammalian cells, have demonstrated that the binding of TBP to a promoter in vivo is rate-limiting for many TATA-containing promoters, as artificially tethering TBP to a promoter template overcomes the requirement for an activator to generate elevated levels of transcription [Chatterjee and Struhl, 1995; Klages and Strubin, 1995; Xiao et al., 1997]. We constructed an expression plasmid in which the GAL4 DNA-binding domain (amino acids 1–147) was fused to the full-length hTBP (GAL4-hTBP), and we found that tethering hTBP to a promoter via GAL4-DNA binding sites results in a strong (30–50-fold) transcriptional activation of promoter bearing a single (G1E1b) or multiple (G5E1b) GAL4 binding sites located upstream to the E1b TATA box in the absence of any activator (Fig. 1A). However, hTBP has been described to possess a "fortuitous" activation

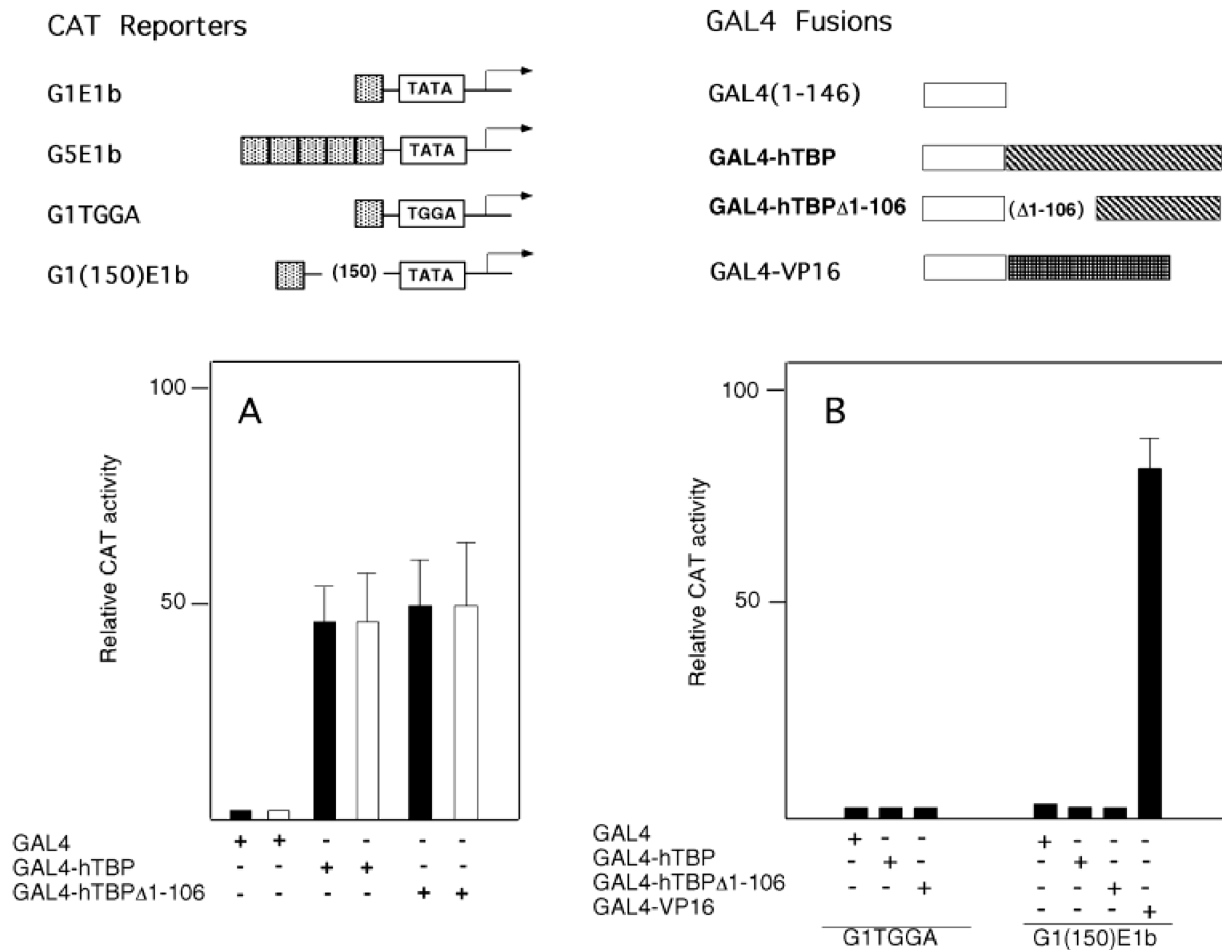

Fig. 1. Effects of Rb on the basal transcription. On top is reported a schematic representation of CAT reporters and GAL4 effectors. **A**: G1E1b (empty bars) and G5E1b (filled bars) reporter plasmids (5 μg) were transfected into C33A cells with the indicated GAL4 expression plasmids (5 μg), as indicated. **B**: The G1-TGGA and G1(150) E1b CAT reporters (5 μg) were transfected into C33A cells with the indicated GAL4 expression plasmids (5 μg), as indicated. The β-Gal expression plasmid (1 μg) was included in the cotransfections for normalization. The bars represent the result of at least three duplicated experiments. Standard deviations are shown by vertical bars.

domain located at the amino terminus [Seipel et al., 1993]. Hence, we could not exclude that GAL4-hTBP was functioning as a conventional activator such as Sp1 in recruiting endogenous hTBP to the TATA box.

To exclude that GAL4-hTBP may act as a conventional activator several control experiments were performed. First, we deleted the activation domain of hTBP which resides in the N-terminal 93 amino acids to create a GAL4-hTBP Δ1–106 expression vector. We found that GAL4-hTBP Δ1–106 yielded a similar extend of activation compared to the GAL4-hTBP, thus deletion of the "fortuitous" activation hTBP domain had no effect in the ability of GAL4-hTBP to activate transcription. Second, when the tested on a promoter bearing a mutated E1b TATA element (TGGAA), both GAL4-hTBP and GAL4-hTBP D1–106 fusions failed to activate transcription (Fig. 1B). Moreover, enforced expression of GAL4-hTBP did not activate a reporter lacking the GAL4 sites (data not shown). Hence, GAL4-hTBP requires both the presence of a GAL4 binding site and a functional TATA element. Finally, both GAL4-hTBP and GAL4-hTBP Δ1–106 were inactive when tested on a reporter with a single GAL4 site located 150 bp upstream the TATA box, whereas GAL4 hybrid containing the VP16 activation domain functioned efficiently (Fig. 1B). Thus, unlike a conventional activator, stimulation by GAL4-hTBP is strongly influenced by the promoter context.

The consequence of Rb co-expression on GAL4-hTBP mediated activation was moni-

tored by co-transfections using as reporter T7G1-TATA plasmid which contains the CAT gene under the control of the E1b TATA box with a single GAL4 DNA-binding site and seven 7 tetO sequences. To evaluate Rb-mediated repression the CMV-RB and the TetR-RB effector plasmids were used [De Luca et al., 1996]. In the latter plasmid the Rb coding region (amino acids 379–928) is connected in frame to the C terminus of the prokaryotic TetR encoded by Tn10 from *Escherichia coli* [Grossen and Bujard, 1992]. Thus, the TetR-RB chimeric protein is able to bind to the tet operator (tetO) sequences. As control we used the TetR-KRAB effector in which the TetR were fused to the KRAB sequences, which have been shown to code for a strong transcriptional repressor domain [Pengue and Lania, 1996]. C33A cells were transfected with the reporter T7G1-TATA in the presence of the GAL4-hTBP. The GAL4-hTBP chimeric protein effectively stimulated transcription when allowed to bind next to the TATA box. However, the GAL4-hTBP dependent transcriptional activation was repressed by the co-expression of TetR-KRAB and by the TetR-RB (379–928) and TetR-RB (379–792) chimeras, the latter construct retaining pocket function (Fig. 2). Conversely, TetR fused to the pocket mutant RB-(379–928Δ21) or RB-(379–928Δ22) did not repress GAL4-hTBP activation.

Electrophoretic mobility retardation analysis ensured that comparable amount of different TetR-RB fusion proteins were made in each transfection (data not shown). Finally, coexpression of CMV-RB, lacking the TetR DNA-binding domain did not cause repression. Thus, RB-binding to the promoter sequences appears to be a prerequisite for repression. Such requirement also proves that TBP-repression is not an indirect effect of the ability of Rb to perturb cell growth.

Phosphorylation Negatively Regulates Repression of Basal Transcription by Rb

Rb activity is regulated by serine/threonine phosphorylation during the cell cycle [Weinberg, 1995; Bartek et al., 1996]. Given that phosphorylation disrupts Rb's ability to interact with other proteins, it is likely that phosphorylation also disrupts an interaction required for repression of basal transcription. Overexpression of cyclin E resulted in a inhibition of the TetR-RB (379–928) repressor activity. Cyclin B1 had no significant effect (Fig. 3).

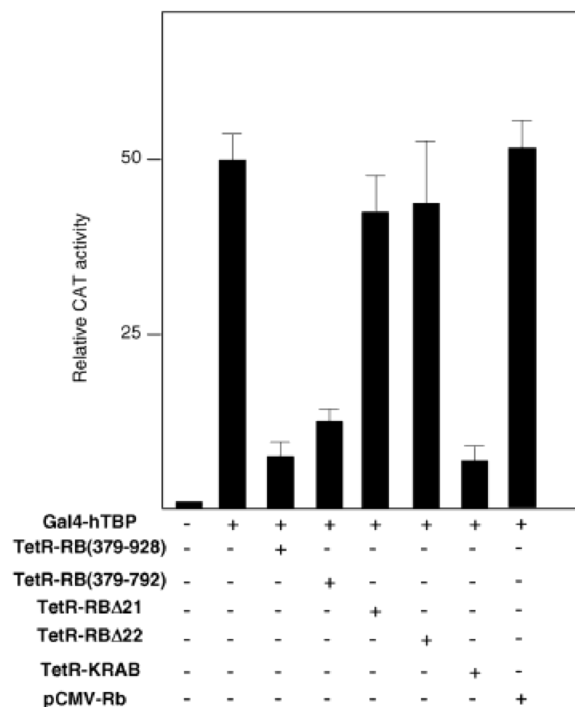

Fig. 2. Rb repression of GAL4-hTBP-mediated transcription. C33A cells were transfected with T7G1-TATA reporter plasmid (5 μg) in the presence of 5 μg of the GAL4-hTBP expression plasmid. The effects of coexpression of the TetR-Rb (379–928), TetR-Rb (379–792) TetR-Rb (379–928)Δ21, TetR-Rb (379–928)Δ22, and TetR-KRAB (1 μg each) are reported. The β-Gal expression plasmid (1 μg) was included in the cotransfections for normalization. The bars represent the results of at four independent duplicated experiments. Standard deviations are shown.

Over-expression of these cyclins did not have any effect on the TetR-RB (379–792) and TetR-KRAB mediated repression. TetR-RB (379–792) chimera, however, lacks the Rb phosphorylation sites required for inhibition of RB growth-suppression function [Bartek et al., 1996] and, perhaps, as a result the repression function is not affected. Moreover, in the absence of TetR-RB effectors, the enforced expression of cyclin E did not affect the GAL4-hTBP-mediated activation. Thus, the abrogation of TetR-RB (379–928) repression of TBP-mediated transcription by cyclin E may be the result of the phosphorylation of TetR-RB (379–928) effector protein.

Trichostatin A Treatment Relieves Rb Repression of GAL4-hTBP

A number of recent studes have shown that several transcriptional repressor are associate with histone deacetylases [Wolffe and Pruss,

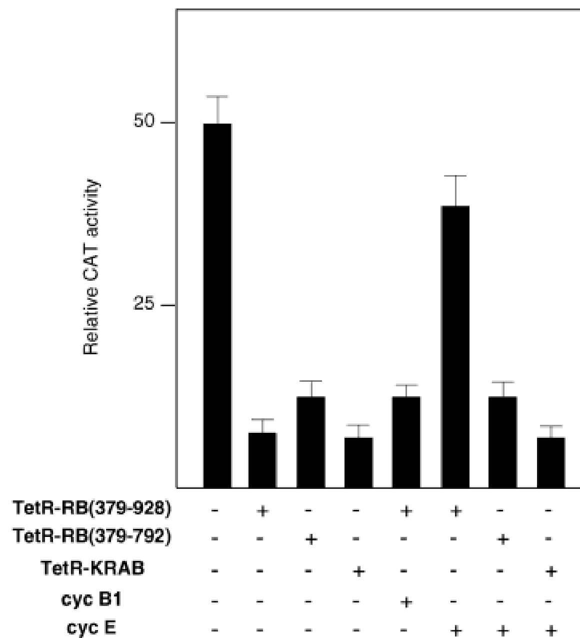

Fig. 3. cycE overexpression specifically abrogates Rb-mediated repression of GAL4-hTBP. C33A cells were transfected with T7G1-TATA and GAL4-hTBP using the conditions described in Figure 2 legend. The effects of the coexpression of the indicated plasmids (5 μg each) on the GAL4-hTBP activation are reported. The results are presented as described in Figure 2.

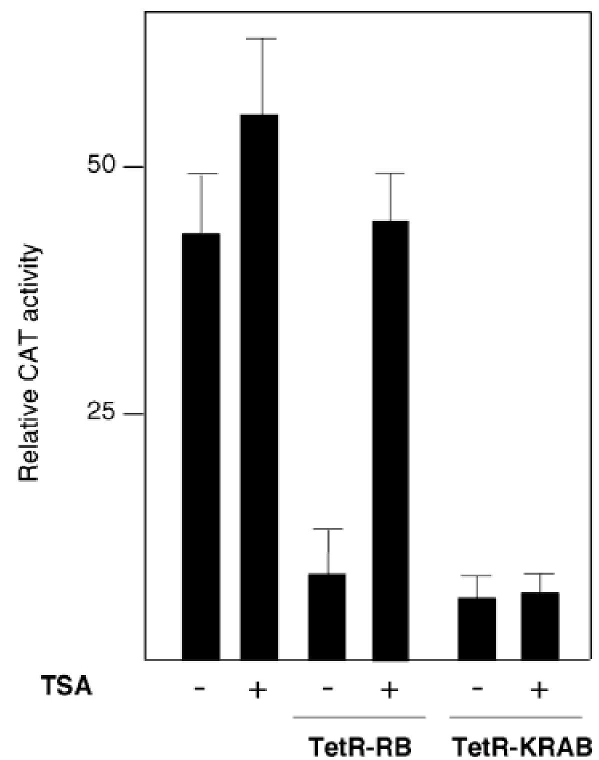

Fig. 4. TSA treatment relieves RB-mediated repression of GAL4-hTBP. C33A cells were transfected with T7G1-TATA and GAL4-hTBP as described in Figure 2 legend. TetR-Rb (379–928), and TetR-KRAB repressors were included in the transfections as indicated. Transfected cells were treated with TSA (100 nM) as indicated. The §-Gal expression plasmid was included in the cotransfections for normalization. The bars represent the results of three independent duplicated experiments. Standard deviations are shown.

1996; Pazin and Kadonaga, 1997], and the use of the drug Trichostatin A, which irreversibly inhibits histone deacetylases [Taunton et al., 1996] has been instrumental to determine the involvement of histone deacetylase activity in transcriptional repression. We sought to determine whether Rb-mediated repression of basal transcription requires histone deacetylase activity. The inhibitor of histone deacetylase, TSA, was used to monitor the involvement of these activity in basal repression by Rb by using transient transfections in C33A cells. As reported in Figure 4, the presence of TSA drastically reduced the inhibiting effect of Rb. To demonstrate the specificty of the abrogation of Rb repression by TSA, we carried out transient tranfections using the KRAB repressor domain. Both Rb and KRAB domain efficiently blocked GAL4-hTBP transcription, but, in contrast to Rb, KRAB-mediated repression was unaffected by TSA treatment.

DISCUSSION

Taken together, our data demonstrated that the Rb protein represses TBP-mediated transcription when directly bound to a promoter in the absence of any activator and we showed that Rb-mediated repression requires the presence of an intact pocket domain, it is reversed by phosphorylation and it is sensitive to the histone deacetylase inhibitor TSA.

To effectively study basal transcription we exploited the observation that the binding of TBP to a promoter in vivo is rate-limiting in vivo, as artificially tethering TBP to a promoter overcomes the requirement for an activator to generate elevated levels of transcription [Chatterjee and Struhl, 1995; Klages and Strubin, 1995; Xiao et al., 1997]. This strategy have then allowed us to analyse the role of Rb in the regulation of basal transcription in the absence of any activator. Transfection experiments have clearly demonstrated that Rb directly inhibits TBP-mediated transcription of a simple TATA-containing promoter. Rb repression was reversed by co-expression of cyclin E, suggesting that phosphorylation disrupts the interaction required for repression of basal transcription.

The ability to repress TBP-mediated transcription and the widespread inhibition of different activators [Weintraub et al., 1995] indicate that the Rb inhibitory domain is likely to act by contacting a common downstream target of different activators within the basal transcription complex bound to the TATA-box.

At present the molecular basis of such interaction are not fully undertood. However, the results reported in Figure 4 clearly demonstrated that Rb-mediated repression is sensitive to the presence of the histone deacetylase inhibitor TSA. The ability of TSA-treatment to reverse the Rb-mediated repression of basal transcription suggest that Rb may repress at least in part by interacting with proteins that remodel chromatin. A large number of studies have provided molecular evidence that chromatin structure is directly involved the regulation of gene transcription [Wolffe and Pruss, 1996; Pazin and Kadonaga, 1997]. The acetylation of histones increases the accessibility of nucleosomal DNA to transcription factors, relieving transcriptional repression. On the other hand, the histone deacetylases such as the mammalian HDAC1, are thought to deacetylate histones and thereby promote formation of nucleosomes [Wolffe and Pruss, 1996; Pazin and Kadonaga, 1997]. Formation of nucleosomes has been shown to inhibit transcription, presumably by blocking access of transcription factors to the promoter [Grunstein, 1997]. It is pertinent to note that our results have been obtained using transiently transfected plasmids reporters, which are not fully assembled into a complete chromatin structure. Consequently, the TSA-mediated relieve of Rb repression may be due to the inhibition of a deacetylation of nonhistone proteins, such as components of the basal transcription machinery.

While this manuscript was in preparation we learned that three groups independently reported that Rb represses transcription by recruiting the histone deacetylase HDAC1 [Luo et al., 1998; Brehm et al., 1998; Magnaghi-Jaulin et al., 1998].

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