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Advances in Brief

The COOH-terminal Region of pRb2/p130 Binds to Histone Deacetylase 1 (HDAC1), Enhancing Transcriptional Repression of the E2F-dependent Cyclin A Promoter[1]

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Abstract

The tumor suppressor retinoblastoma protein family members pRb, p107, and pRb2/p130 are potent negative transcriptional regulators. The best understood target is the transcription factor E2F, which activates cell cycle-dependent transcription of genes controlling and promoting the cell division cycle (*e.g.*, cyclin A). pRb2/p130 is known to be important in implementing cell cycle exit into G_0 due to serum deprivation or various differentiation programs. Several recent studies demonstrated the effect histone acetylases and histone deacetylases (HDACs) have on fine-tuning transcriptional regulation of eucaryotic cells. In this study, we demonstrate that pRb2/p130 binds to HDAC1. This interaction increases the ability of pRb2/p130 to inhibit transcription of the E2F-dependent cyclin A promoter *in vivo*. We also identify the COOH-terminal 35aa as being necessary for stable interaction between HDAC1 and pRb2/p130.

Introduction

Proteins of the retinoblastoma family are principal factors in regulating cell cycle events and act by repressing transcription via binding to various transcription factors. pRb has been shown to regulate gene expression of all three RNA polymerases and to be of central importance at a phase in G_1 known as the "restriction point." pRb2/p130, first identified in our laboratory, shares functional and structural features with pRb, even though it is well established that the time and order of pRb2/p130's action within the cell cycle differs markedly from pRb (1). The principal targets of the retinoblastoma protein family are the E2F transcription factors, which consist of at least seven genes, *E2F1-5* and *DP1* and *DP2*. Individual E2F-DP heterodimers form stable complexes with DNA and are regulated transcriptionally by individual association with either member of the retinoblastoma protein family (2). Although pRb binds to E2F-1-3 in the G_1 phase of the cell cycle, pRb2/p130 mainly is expressed and active in arrested or differentiated cells and binds to E2F-4 (3). E2F-4 activity also is regulated via its subcellular localization. Recent studies show that E2F-4 is primarily nuclear only in G_0 and early G_1 cells, matching pRb2/p130 activity (4, 5). Ectopic expression of pRb2/p130 in tumor cells causes growth arrest, and it was shown that this is accomplished in part by the increased nuclear localization of E2F-4 (2, 5–7). Many identified cell cycle control genes (*e.g.*, *cyclin A*, *cyclin E*, and *cdc2*) and phase-promoting genes (*e.g.*, *DHFR*, *TK*, *TS*, and *DNA polymerase-α*) are known to be regulated by E2F sites within their promoters (3). E2F-4/pRb2/p130 complexes are the main sources of E2F site-dependent transcriptional repression in serum-deprived and differentiated cells and distinguish a G_0 state from a G_1 cell state (8). The E2F-directed regulation of the cyclin A promoter has been studied extensively (9–11). Cell cycle arrest induced by different methods has been shown to cause E2F-dependent transcriptional repression. However, it is not conclusively clear which member of the retinoblastoma protein family is responsible for this action (11–13). Histone acetylation has long been known to be an indicator of active chromatin (14, 15). New insight into transcriptional regulation comes from the identification of histone acetylase activities of p300/CBP and P/CAF, components of multimeric transcriptional activation complexes of diverse promoters (16, 17). Identification of HDACs[3] as sources of transcriptional repression in various multimeric complexes, such as ligand-free hormone receptors, is another piece of the transcriptional regulation puzzle (18).

The recent identification of HDACs as functional binding partners of pRb via a IXCXE motif has linked E2F-dependent transcriptional repression to nucleosome regulation and has shed a new light on the mode of pRb's transcriptional repression potential (15, 19, 20). We set out to investigate whether pRb2/p130 uses histone deacetylation for proper regulation of E2F-regulated genes in the G_0 state of cells, where pRb2/p130 is the main source of E2F regulation. We show that HDAC1 is a binding partner for pRb2/p130 and enhances pRb2/p130's ability to repress transcription of the cyclin A promoter. In addition to the pocket region, we identify the COOH-terminal region of pRb2/p130 as important for HDAC1 binding. We also show that the last 35aa are critical for an *in vitro* binding.

Materials and Methods

Cell Culture and Transient Transfection. NIH-3T3 cells were kept in DMEM supplemented with 10% FCS, penicillin (60 μg/ml), and streptomycin (100 μg/ml) in a 5% CO_2 atmosphere. For serum arrest, cells were grown to 70% confluence and then incubated in DMEM plus 0.2% FCS for 48 h. Cells then were incubated with or without 100 nM TSA (WAKO) for 6 h. Transient transfection experiments were performed according to the CaPhosphate protocol. Cells were seeded at a 50% confluence, incubated with the precipitate for 16 h, washed once in DMEM, and harvested 24 h afterward. Extracts and luciferase assays were performed according to the provided Promega protocol;

Construct Preparation. We used a procaryotic expression vector pGEX-2T (Stratagene) and PCR to generate chimeric GST-pRb2/p130 COOH terminal fusion proteins. We used a eucaryotic expression vector pCDNA3 (Promega) and PCR to generate the pRb2/p130 wt and COOH-terminal deletion mutants. The primers used to generate the above constructs derived from the COOH-terminal region of pRb2/p130. The *myc*-Tag HDAC1 is a generous gift of C. Seiser. The cyclin A promoter fragment-lucifease construct, hpGL2-cycA promoter, spanned the region −89/+11 relative to the start site.

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[3] The abbreviations used are: HDAC, histone deacetylase; GST, glutathione *S*-transferase; ECL, enhanced chemiluminescence; TSA, trichostatin A.

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GST-Fusion Protein Preparation. XL1-Blue or DH5a bacteria carrying the pGEX 2T constructs were grown to midlog phase and then induced to express the fusion-proteins by the addition of 0.25 mM isopropyl-1-thio-b-D-galactopyranoside to the media. The cultures were then incubated at room temperature for 4 h. Bacteria then were pelleted and resuspended in NENT buffer [20 mM Tris/CL (pH 8), 100 mM NaCl, 1 mM EDTA, 0.5% Nondient P-40, 1 mM DTT, and 1 mM PMSF)]. Cell suspensions were sonicated and pelleted. The fusion proteins were extracted from the supernatant by binding to glutathione-agarose (Pharmacia) o.n. at 4°C. The agarose subsequently was pelleted and washed three times in NENT buffer.

***In Vitro* Translation.** One microgram of the indicated plasmids was translated with T7 RNA polymerase according to the provided Promega translation kit.

Immunoprecipitation. Immunoprecipitations were performed as described previously (21). Briefly, equal amounts of whole cell extracts were immunoprecipitated with the polyclonal antibodies, as indicated, and subsequently incubated with A-Sepharose. Recovered proteins were analyzed in immunoblots.

Immunoblotting. NIH3T3 cell pellets were resuspended in 200 μl of lysis buffer (50 mM Tris/Cl (pH 7.4), 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0,1 mM Na3VO4, 0.1% TritonX-100, and protease inhibitors). Proteins (30–50 μg) were separated on polyacrylamide gels and transferred to polyvinylidene difluoride membranes in CAPS buffer [10 mM CAPS and 20% methanol (pH 11)]. Membranes were blocked in 5% nonfat milk in TBS-T buffer [2 mM Tris/Cl (pH 7.6), 13.7 mM NaCl, and 0.1% Tween 20) and then washed in TBS-T. Primary antibodies were incubated in TBS-T/3% milk; secondary antibodies coupled with horseradish peroxidase (Amersham Corp.) were incubated in TBS-T. The presence of the secondary antibody was detected with an ECL system (NEN Life Sciences).

Results

We investigated the effect of histone deacetylation on the transcriptional repression of the *cyclin A* gene in G_0 state cells, which is thought to be mediated by E2F-4/pRb2/p130 complexes. TSA has been identified as a potent inhibitor of HDACs, and we first analyzed the effects of this drug on the cyclin A promoter in serum-deprived NIH3T3 cells. In immunoblots, we detected the expected increase in pRb2/p130 protein in extracts prepared from arrested *versus* logarithmical cell populations, confirming a G_0 state of these cells (data not shown). We also confirmed by Electro Mobility Shift Assay that E2F/pRb2/p130 is the primary complex binding to the cyclin A promoter E2F site in quiescent cells treated with or without TSA (data not shown).

Incubation of serum-deprived cells with TSA for 6 h led to an increase in cyclin A protein detected in immunoblots, most likely reflecting an up-regulation of the cyclin A promoter, possibly due to a release of pRb2/p130-mediated repression (Fig. 1). These data led us to investigate whether pRb2/p130 and HDAC are present in the same

Fig. 1. Immunoblot analysis for cyclin A protein expression in NIH 3T3 cells. Whole cell extracts (30 μg) from logarithmically growing and serum-deprived cell populations (in the absence or presence of 100 nM TSA for 6 h) were resolved in a 10% SDS polyacrylamide gel. Membrane was probed with an affinity purified polyclonal rabbit anticyclin A serum. Immunoreactive proteins were visualized using the ECL detection system.

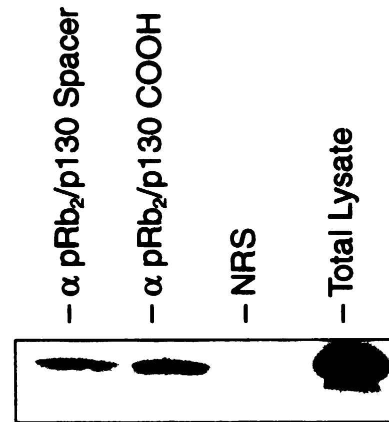

Fig. 2. pRb2/p130 interacts with HDAC1. Extracts of NIH3T3 cells transiently cotransfected with 10 μg of pRb2/p130 and 10 μg of *myc*-HDAC1 were incubated with normal rabbit serum (*NRS*) and polyclonal rabbit anti-Spacer pRb2/p130 and anti-COOH-20 pRb2/p130 antibodies. *myc*-HDAC1 was detected in immunoblots using anti-mouse *myc*-antiserum and the ECL detection system.

complexes *in vivo* and *in vitro*, helping to explain the above observed effect on cyclin A expression. We transiently transfected NIH3T3 cells with *myc*-tagged HDAC1 and pRb2/p130 and performed coimmunoprecipitations. We applied polyclonal pRb2/p130 antisera raised in our laboratory against the spacer region and the COOH-terminal 20aa to avoid potential steric hindrance effects. In this setting, we were able to detect an *in vivo* interaction between *myc*-tagged HDAC1 and pRb2/p130. No *myc*-tagged HDAC1 precipitated with the control serum (Fig. 2).

As a consequence of this interaction, we also determined the regions important for the HDAC1 and pRb2/p130 interaction. We performed coprecipitations of *in vitro*-translated ^{35}S-labeled HDAC1 with GST-fusion proteins expressing the NH2-terminus, the A-domain, the spacer region, the B-domain, and the COOH-terminal region of pRb2/p130 or with GST alone. We observe a strong interaction between HDAC1 and the COOH-terminus of pRb2/p130. Weak interactions are detected with the A- and B-domains and there are no significant interactions with the NH2-terminus and the spacer domain (Fig. 3*A*). We also subdivided the COOH-terminal region in order to more carefully define the region of interaction (Fig. 3*B*). Deletion of the terminal 35aa abolishes the interaction with HDAC1 (Fig. 3*A*).

The interaction between pRb and HDAC1 was defined to be dependent on the pocket domain and, to some extent, on the COOH-terminus (15, 19, 20). The pocket region alone has weak interaction with HDAC1 (18). We verify that the pRb pocket domain plus the COOH-terminus forms a stable *in vitro* complex with the ^{35}S-labeled HDAC1 (Fig. 3*C*). In contrast to the COOH-terminus of pRb2/p130, we find that the COOH-terminus of pRb (Rb-792-928) alone only weakly binds to the HDAC1 protein. This interaction, however, is also strictly dependent on the very COOH-terminal residues as the shorter pRb construct (Rb-768-833) does not bind the deacetylase (Fig. 3*C*). We next expressed the pocket domain of pRb2/p130 as a GST-fusion protein and compared it with the binding capacities of the pRb2/p130 COOH-terminus. The pRb2/p130 pocket domain alone, or its separated regions, only weakly bind to HDAC (Fig. 3*D*), similar to what has been shown for pRB (19, 20). We find that equal amounts of the COOH-terminus of pRb2/p130 coprecipitate with several times the amount of ^{35}S-labeled HDAC1 as the pocket domain (Fig. 3*D*).

We also examined the effect of deleting the COOH-terminal 35aa on pRb2/p130's ability to repress transcription in dependence of HDAC1. We performed transient cotransfection experiments using either pRb2/p130 wt or the 35aa deletion (Fig. 4*A*), together with a

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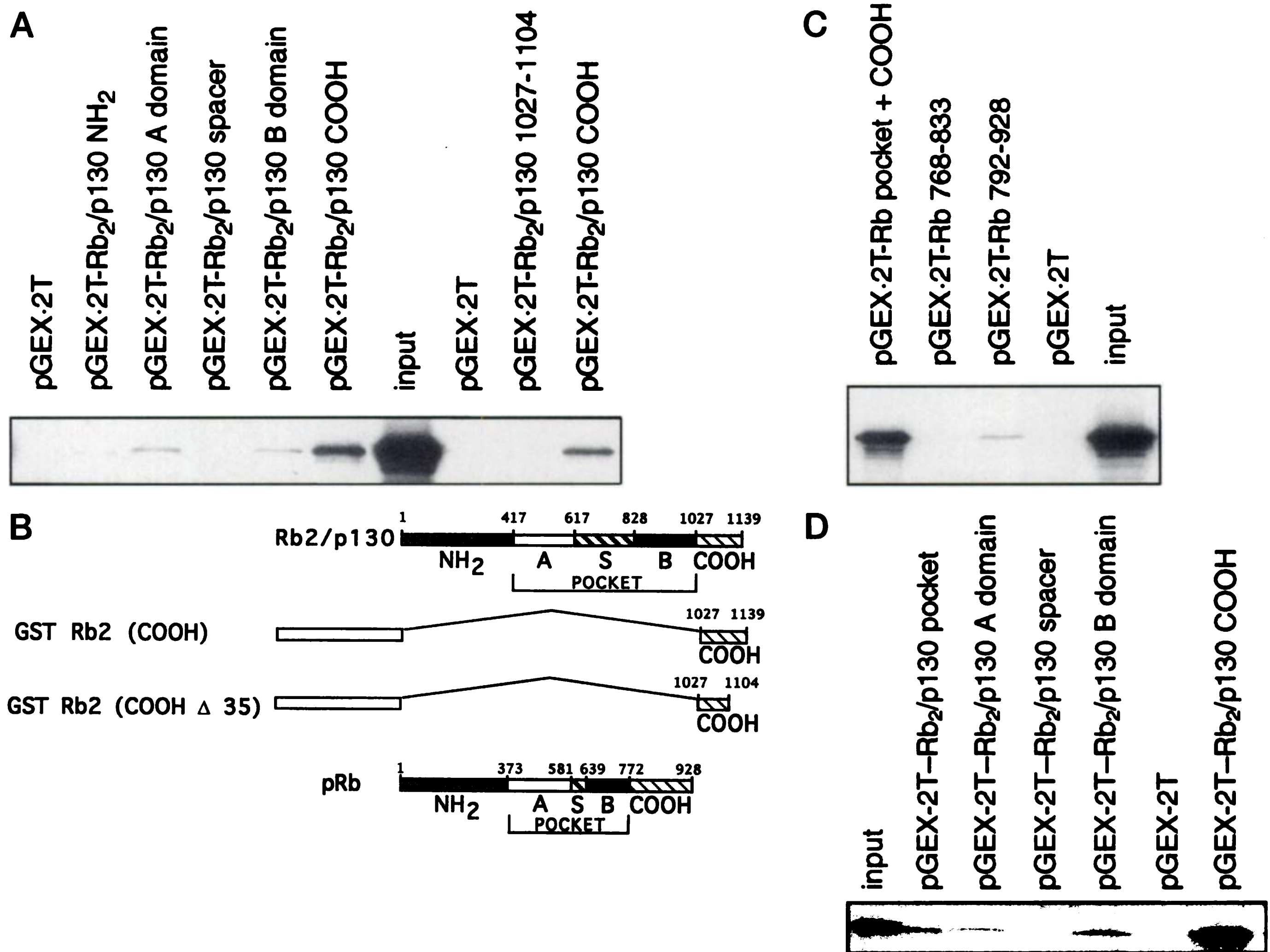

Fig. 3. *In vitro* analysis of the pRb2/p130 interaction with HDAC1. *A*, equal amounts of GST and the indicated GST-fusion proteins of pRb2/p130 were incubated with ^{35}S-labeled HDAC1 in GST-pull down experiments. Recovered proteins were resolved on a 10% SDS polyacrylamide gel. The signal was detected with enhanced autoradiography. *B*, schematic representation of the pRb2/p130, the COOH-terminal GST-fusion constructs used, and pRb. *C*, equal amounts of GST and the indicated GST-fusion proteins of pRb were incubated with ^{35}S-labeled HDAC1 in GST-pull down experiments and further analyzed as in *B*. *D*, equal amounts of GST and the indicated GST-fusion proteins of pRb2/p130 were incubated with ^{35}S-labeled HDAC1 in GST-pull down experiments and analyzed as in *B*. Only 1 of 10 of the input was loaded onto the gel.

−89/+11 fragment of the human cyclin A promoter containing the E2F site upstream of the luciferase gene. pRb2/p130 wt reduces the expression from the cyclin A promoter, whereas deletion of the last 35aa diminishes the amount of transcriptional repression (Fig. 4*B*). To demonstrate that the reduced repression activity of pRb2/p130 Δ35 was due to its inability to interact with HDAC1, we included HDAC1 into the above cotransfections. These experiments show that HDAC1 enhances the transcriptional repressive activity of the pRb2/p130-wt *in vivo*, but does not significantly add to the ability of pRb2/p130-Δ35 to repress transcription of the cyclin A promoter (Fig. 4*B*).

Discussion

In the present study we set out to analyze whether pRb2/p130, an important source of transcriptional down-regulation of numerous cell cycle-control and DNA synthesis-promoting genes in quiescent cells is linked to histone deacetylation. In one present model, E2F regulation during the cell division cycle and G_0 state is ascribed to one or two of the three members of the retinoblastoma gene family (1). Cyclin A has been identified as promoting the cell cycle in G_1 and G_2 phases of the cell cycle. The analysis of the cyclin A promoter has revealed an E2F binding site to be necessary for properly timed expression of this gene in mouse and human cells (9, 10, 13).

According to recent data, pRb2/p130 actively represses E2F transcription in complexes with E2F-4 in differentiated and arrested cells. pRb also is active in differentiation and arrest, but it is the main E2F regulator during early G_1 and is an integrator of external growth signals at the "restriction point" (1, 2). If differentiation and prolonged serum arrest require a distinct set of genes expressed, one might assume that such transcriptional repression may include histone deacetylation to generate inactive chromatin (14, 18). pRb recently has been shown to form complexes with HDACs via its pocket domain, and *in vitro* can include the E2F-1 transcription factor (15, 19, 20).

We demonstrate that the E2F-directed cyclin A promoter is sensitive to TSA treatment in its repressed form in quiescent cells and, based on our data and published work, we suggest that this is due to inactivation of E2F/pRb2/p130-mediated transcriptional repression. Our coimmunoprecipitation experiments reveal a stable interaction between HDAC1 and pRb2/p130 *in vivo*. We identify the COOH-terminus, and specifically the last 35aa of pRb2/p130, as important for

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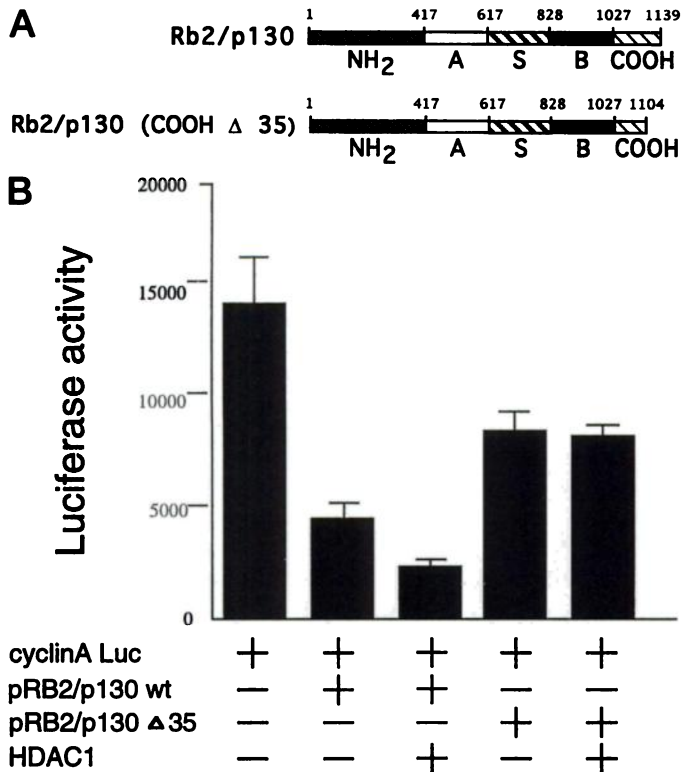

Fig. 4. Influence of the pRb2/p130 COOH-terminal 35aa and HDAC1 on the transcriptional regulative potential of pRb2/p130. *A*, schematic representation of pRb2/p130 wt and the COOH-terminal 35aa deletion. *B*, NIH3T3 cells were transiently transfected with 1 μg of cyclin A-luciferase reporter vector and, where indicated, with 1 μg of pCDNA3 pRb2/p130 or 1 μg of pCDNA3 pRb2/p130Δ35, together with or without 3 μg of pCIneo HDAC1. Data were normalized to β-galactosidase activity.

HDAC1 complex formation *in vitro*. Transient transfection experiments with the human cyclin A promoter −89/+11 revealed that HDAC1 enhances transcriptional repression by pRb2/p130 and that this *in vivo* biological function is dependent on the last 35aa of pRb2/p130. Deletion of this 35aa led to a diminished ability of pRb2/p130 to repress transcription in transient assays, which is, to our knowledge, the first demonstration that this region is functionally involved in pRb2/p130-mediated regulation.

The COOH-termini of pRb and pRb2/p130 have less homology than do the pocket domains, which suggests the COOH-terminus of pRb2/p130 may have unique properties among the retinoblastoma family in recruiting factors and regulating transcription. This is supported by our data showing that the COOH-terminus of pRb2/p130 binds much stronger to HDAC1 than does the same region in pRb. Our laboratory already has identified pRb2/p130 as being unique in its growth-repressive ability among the retinoblastoma family members and in its ability to inhibit CDK2 kinase activity (6, 21). These new findings strengthen the role of pRb2/p130 as being a key regulatory element in cell cycle regulation with unique properties and activities. Our data also shed new light on pRb/HDAC1 complexes. The first reports about pRb/HDAC interactions concentrate on the importance of the pocket domain of pRB and the IxCxE motif in HDAC1 to form a stable complex (19, 20). We show that the COOH-terminus of pRb also, albeit weakly, binds to the HDAC and that the very terminus of this region is essential for a stable *in vitro* complex formation. According to our published data, the above model of the pRb pocket domain as being sufficient for HDAC binding must be modified. We propose that both members of the retinoblastoma family, pRb and pRb2/p130, need a functional pocket as well as the COOH-terminus to form a stable complex with HDAC1 *in vitro* as well as *in vivo*. This also implies that even short COOH-terminal deletions in pRb and pRb2/p130 will have strong impacts on growth regulation, as it will negatively influence the recruitment of histone deacetylation to promoters. Future studies will be needed to carefully assess the functional role of histone deacetylation on the level of retinoblastoma family member-dependent gene regulation. Additional work is in progress to characterize the COOH-terminal region of pRb2/p130 and its role in cell cycle control.

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References

1. Stiegler, P., and Giordano, A. Big brothers are watching: the retinoblastoma family and growth control. *In:* A. Marcieira-Coelho (ed.), Progress in Molecular and Subcellular Biology, Vol. 20, pp. 25–42. Berlin and Heidelberg, Germany: Springer Verlag, 1998.
2. Beijersbergen, R. L., and Bernards, R. Cell cycle regulation by the retinoblastoma family of growth inhibitory proteins. Biochim. Biophys. Acta, *1287:* 103–120, 1996.
3. Slansky, J. E., and Farnham, P. J. Introduction of the E2F family: protein structure and gene regulation. Curr. Top. Microbiol. Immunol., *208:* 1–30.
4. Müller, H., Moroni, M. C., Vigo, E., Petersen, B. O., Bartek, J., and Helin, K. Induction of S-phase entry by E2F transcription factors depends on their nuclear localisation. Mol. Cell. Biol., *17:* 5508–5520, 1997.
5. Verona, R., Moberg, K., Estes, S., Starz, M., Vernon, J. P., and Lees, J. A. E2F activity is regulated by cell cycle-dependent changes in subcellular localization. Mol. Cell. Biol., *17:* 7268–7282, 1997.
6. Claudio, P. P., Howard, C. M., Baldi, A., De Luca, A., Fu, Y., Condorelli, G., Sun, Y., Colburn, N., Calabretta, B., and Giordano, A. p130/pRb2 has growth suppressive properties similar yet distinctive from those of the retinoblastoma family members pRb and p107. Cancer Res., *54:* 5555–5560, 1994.
7. Lindeman, G. J., Gaubatz, S., Livingston, D. M., and Ginsberg, D. The subcellular localisation of E2F-4 is cell cycle dependent. Proc. Natl. Acad. Sci. USA, *94:* 5095–5100, 1997.
8. Smith, E. J., Leone, G., DeGeorgi, J., Jakoi, L., and Nevins, J. R. The accumulation of an E2F-p130 transcriptional repressor distinguishes a G_0 state from a G_1 cell state. Mol. Cell. Biol., *16:* 6965–6976, 1996.
9. Fan, J., and Bertino, J. R. Functional roles of E2F in cell cycle regulation. Oncogene, *14:* 1191–1200, 1998.
10. Schulze, A., Zerfass, K., Spitkovsky, D., Middendorp, S., Berges, J., Helin, K., Jansen-Dürr, P., and Henglein, B. Cell cycle regulation of the cyclin A gene promoter is mediated by a variant E2F site. Proc. Natl. Acad. Sci. USA, *92:* 11264–11268, 1995.
11. Zerfass-Thome, K., Schulze, A., Zwerschke, W., Vogt, B., Helin, K., Bartek, J., Henglein, B., and Jansen-Dürr, P. p27kip1 blocks cyclin E-dependent transactivation of cyclin A gene expression. Mol. Cell. Biol., *17:* 407–415, 1997.
12. Schulze, A., Mannhardt, B., Zerfass-Thome, K., Zwerschke, W., and Jansen-Dürr, P. Anchorage-independent transcription of the cyclin A gene induced by the E7 oncoprotein of human papillomavirus type 16. J. Virol., *72:* 2323–2334, 1998.
13. Philips, A., Huet, X., Plet, A., Le Cam, L., Vie, A., and Blanchard, J. M. The retinoblastoma protein is essential for cyclin A repression in quiescent cells. Oncogene, *16:* 1373–1381, 1998.
14. Grunstein, M. Histone acetylation in chromatin structure and transcription. Nature (Lond.), *389:* 349–352, 1997.
15. Luo, R. X., Postigo, A. A., and Dean, D. C. Rb interacts with histone deacetylase to repress transcription. Cell, *92:* 463–473, 1998.
16. Yang, X. J., Ogryzko, V. V., Nishikawa, J., Howard, B. H., and Nakatani, Y. A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. Nature (Lond.), *382:* 319–324, 1996.
17. Ogryzko, V. V., Schilz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. The transcriptional coactivators p300 and CBP are histone acetylases. Cell, *87:* 953–959, 1996.
18. Pazin, M. J., and Kadonaga, J. T. What's up and down with histone deacetylation and transcription? Cell, *89:* 325–328.
19. Brehm, A., Miska, E. A., McCance, D. J., Reid, J. L., Bannister, A. J., and Kouzarides, T. Retinoblastoma protein recruits histone deacetylase to repress transcription. Nature (Lond.), *391:* 597–601, 1998.
20. Magnaghi-Jaulin, L., Groisman, R., Naguibneva, I., Robin, P., Lorain, S., LeVillain, J. P., Troalen, F., Trouche, D., and Harel-Bellan, A. Retinoblastoma protein represses transcription by recruiting a histone deacetylase. Nature (Lond.), *391:* 601–604, 1998.
21. De Luca, A., MacLachlan, T. K., Bagella, L., Dean, C., Howard, C. M., Claudio, P. P., Baldi, A., Khalili, K., and Giordano, A. A unique domain of pRb2/p130 acts as an inhibitor of CDK2 kinase activity. J. Biol. Chem., *272:* 20971–20974, 1997.

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