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HDAC1, HDAC4, and HDAC9 Bind to PC3/Tis21/Btg2 and Are Required for Its Inhibition of Cell Cycle Progression and Cyclin D1 Expression

LAURA MICHELI, GIORGIO D'ANDREA, LUCA LEONARDI, AND FELICE TIRONE*

Institute of Cell Biology and Neurobiology, National Research Council, Fondazione Santa Lucia, Rome, Italy

PC3/Tis21 is a transcriptional cofactor that inhibits proliferation in several cell types, including neural progenitors. Here, we report that PC3/Tis21 associates with HDAC1, HDAC4, and HDAC9 in vivo, in fibroblast cells. Furthermore, when HDAC1, HDAC4, or HDAC9 are silenced in fibroblasts or in a line of cerebellar progenitor cells, the ability of PC3/Tis21 to inhibit proliferation is significantly reduced. Overexpression of HDAC1, HDAC4, or HDAC9 in fibroblasts and in cerebellar precursor cells synergizes with PC3/Tis21 in inhibiting the expression of cyclin D1, a cyclin selectively inhibited by PC3/Tis21. Conversely, the depletion of HDAC1 or HDAC4 (but not HDAC9) in fibroblasts and in cerebellar precursor cells significantly impairs the ability of PC3/Tis21 to inhibit cyclin D1 expression. An analysis of HDAC4 deletion mutants shows that both the amino-terminal moiety and the catalytic domain of HDAC4 associate to PC3/Tis21, but neither alone is sufficient to potentiate the inhibition of cyclin D1 by PC3/Tis21. As a whole, our findings indicate that PC3/Tis21 inhibits cell proliferation in a way dependent on the presence of HDACs, in fibroblasts as well as in neural cells. Considering that several reports have demonstrated that HDACs can act as transcriptional corepressors on the cyclin D1 promoter, our data suggest that the association of PC3/Tis21 to HDACs is functional to recruit them to target genes, such as cyclin D1, for repression of their expression.

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Tis21, also known as PC3 or BTG2 (in mouse, rat, and human, respectively), is an anti-proliferative and pro-differentiative transcriptional cofactor acting in neural and non-neural cells (Bradbury et al., 1991; Guardavaccaro et al., 2000; Prévôt et al., 2000; Canzoniere et al., 2004; Passeri et al., 2006; Tirone et al., 2013). In non-neural cells, such as fibroblast cells (NIH3T3), mouse embryo fibroblasts, granulosa cells of the ovary, or breast and prostate cancer cells, overexpression of Tis21 induces arrest in the G0–G1 phase of the cell cycle (Montagnoli et al., 1996; Rouault et al., 1996; Ficazzola et al., 2001; Kawakubo et al., 2004; Li et al., 2009). In neural progenitor cells of different areas of the brain—such as the cerebellum, the hippocampus, and the subventricular zone—Tis21 induces exit from the proliferative state and differentiation (Canzoniere et al., 2004; Farioli-Vecchioli et al., 2007, 2008, 2009). In fibroblasts and in neural progenitor cells, Tis21 inhibits the G1–S progression through direct repression of the *cyclin D1* promoter activity (Guardavaccaro et al., 2000; Farioli-Vecchioli et al., 2007). Moreover, in neural progenitor cells, *Tis21* activates pro-neural genes through direct repression of the promoter of the Id3 gene, an inhibitor of pro-neural bHLH transcription factors (Canzoniere et al., 2004; Farioli-Vecchioli et al., 2009).

Notably, in neural cells (cerebellar precursors and PC12 cells), the Tis21-mediated inhibition of cyclin D1 expression correlates with recruitment of Tis21 to the cyclin D1 promoter, accompanied by histone deacetylation. Our previous observation that Tis21 binds in vitro to histone deacetylases 1 and 4 (HDAC1 and HDAC4; Farioli-Vecchioli et al., 2007) suggested that the anti-proliferative action of Tis21 could be HDAC-dependent. Moreover, among the genes regulated by Tis21 in cerebellar precursors, a significant number of genes have expression which is modified by the HDAC inhibitor trichostatin. This further suggests a functional implication of HDACs in Tis21 action (Farioli-Vecchioli et al., 2013). Among the genes regulated by Tis21 in the cerebellum, we noticed also HDAC9, whose protein can associate with HDAC1 and HDAC4 (Sparrow et al., 1999; Petrie et al., 2003; Farioli-Vecchioli et al., 2013).

In this study, we further investigated whether the proliferation of fibroblasts and neural cells (immortalized cerebellar precursor cells) is jointly controlled by HDACs and Tis21.

In general, HDACs influence proliferation in a cell type-specific fashion, depending on the physiological environment (see for review Reichert et al., 2012).

Germline deletion of HDAC1 in mice leads to early embryonic lethality before E9.5 due to severe proliferation defects and retardation in development (Lagger et al., 2002). Moreover, HDAC1-deficient embryonic stem cells show reduced proliferation, which correlates with decreased cyclin A and cyclin E-associated kinase activities and elevated levels of the cyclin-dependent kinase inhibitors p21 and p27 (Lagger et al., 2002). Also, HDAC4 appears to promote colon cancer cell proliferation by repressing p21

Laura Micheli and Giorgio D'Andrea contributed equally to this work.

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*Correspondence to: Dr. Felice Tirone, Institute of Cell Biology and Neurobiology, Consiglio Nazionale delle Ricerche, Fondazione S. Lucia, via del Fosso di Fiorano 64, Rome 00143, Italy.
E-mail: felice.tirone@cnr.it

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transcription (Wilson et al., 2008). In parallel with the evidence for a positive role for HDAC1 and HDAC4 in proliferation, there are also opposite data. For instance, deletion of HDAC1 (alone or with HDAC2) in T cells or in neural progenitor cells induces increased proliferation (Montgomery et al., 2009; Grausenburger et al., 2010), also indicating a cell type-specific action. Moreover, recruitment of HDAC1 by Rb is important for its growth-inhibitory effect (Zhang and Dean, 2001), while HDAC4 inhibits the activity of cyclin-dependent kinase-1 and the progression of proliferating HEK293T and HT22 cells through the cell cycle (Majdzadeh et al., 2008).

Here, we report that Tis21 associates in vivo with HDAC1 and HDAC4 as well as with HDAC9. Moreover, the silencing of either HDAC1, HDAC4, or HDAC9 leads to a loss of the cell cycle inhibitory action of Tis21, whereas overexpression of either HDAC1, HDAC4, or HDAC9 potentiates the Tis21-mediated inhibition of cyclin D1. Altogether, our findings indicate that the inhibition of proliferation exerted by Tis21 requires HDAC1, HDAC4, or HDAC9, and suggest that HDAC1, HDAC4, and HDAC9 impact on cell cycle and cyclin D1 expression by associating with Tis21.

Materials and Methods

Expression vectors and plasmids

pcDNA3-HDAC1-Flag and pcDNA3-HDAC4-Flag were kindly provided by T. Kouzarides (Brehm et al., 1998; Miska et al., 2001). pSCT-HDAC1-Flag was generated, as previously described (Farioli-Vecchioli et al., 2007), by excising from the pcDNA3-HDAC1-Flag vector the BamHI-5′ XbaI-3′ fragment containing the HDAC1-Flag cDNA and subcloning in pSCT vector (Guardavaccaro et al., 2000) at the same sites. The pSCT vector is a CMV promoter-driven vector previously described (Guardavaccaro et al., 2000). pSCT-PC3-HA was generated with a two step procedure, first by subcloning in BamHI-5′ HindIII-3′ of pSCT the PC3 (rat) cDNA ORF fragment amplified by PCR and devoid of stop codon; then a double strand fragment containing the 2xHA sequence was cloned in frame in the HindIII-5′ NotI-3′ sites, downstream PC3 cDNA. The pSCT-PC3 vector also contains the PC3 (rat) cDNA ORF (cloned in the XbaI-5′ HindIII-3′ sites), whereas pSCT-βGal contains the βGalactosidase ORF; both were previously generated as described (Guardavaccaro et al., 2000). The pSCT-HDAC9-2xFlag vector was generated by cloning into XbaI-5′ NotI-3′ sites the mouse HDAC9 cDNA sequence, previously synthesized by Genscript (Piscataway, NJ). The pSCT-HDAC4-1-610 2xFlag or the pSCT-HDAC4-611-1084 2xFlag vectors were generated by cloning into BamHI-5′ NotI-3′ sites of pSCT the human HDAC4 cDNA sequence (BglII-5′ NotI-3′) corresponding to aa 1-610 or 611-1084, respectively, and carrying a 2xFlag sequence at the carboxy terminal, previously synthesized by Genscript.

The prCD1-1810 reporter, containing about 1,650 nt of the rat cyclin D1 promoter and 5′ UTR region cloned in the pGL2 Promega vector, was kindly provided by E. Ziff (Yan and Ziff, 1997). For luciferase experiments, we used the constructs pSCT-HDAC1-Flag and pcDNA3-HDAC4-Flag.

The sequence of all these constructs was checked.

Cell lines

The NIH3T3 fibroblast cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 3% fetal calf serum and 7% defined bovine serum (HyClone, Logan, UT), as previously described (Guardavaccaro et al., 2000). C17.2 cells—an immortalized line of cerebellar precursor cells (Ryder et al., 1990)—and HEK 293 (human embryonic kidney) cells were cultured in DMEM containing 10% fetal calf serum. All cell lines were kept in a humidified atmosphere of 5% CO_2 at 37°C.

Transfection of the plasmids was performed by the liposome technique using the Lipofectamine reagent (Life Technologies, Gaithersburg, MD) as per the manufacturer's instructions.

Immunoprecipitation

NIH3T3 cells grown in 90-mm dishes, transfected with pSCT-PC3-HA and pSCT-HDAC1-Flag or pcDNA3-HDAC4-Flag, or pSCT-HDAC9-Flag, were lysed by sonication in buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.4% Nonidet P-40, 10% glycerol, protease inhibitors, 0.1 mM Na_3VO_4, 10 mM 2-glycerophosphate, 5 mM NaF, 5 mM sodium butyrate, 600 nM trichostatin A. Then 1 mg of total protein lysate was immunoprecipitated overnight with either agarose-conjugated mouse monoclonal F-7 anti-HA (Santa Cruz Biotechnology, Santa Cruz, CA; sc-7392AC), or with agarose-conjugated mouse monoclonal anti-Flag antibodies (M2; Sigma–Aldrich, S.Louis, MO; A2220).

In immunoprecipitated lysates, the HDAC1-Flag, HDAC4-Flag, and HDAC9-Flag proteins were revealed by Western blots with an anti-Flag mouse monoclonal (M2; Sigma–Aldrich; F3165); HA-PC3 protein was revealed by Western blot with an anti-HA mouse monoclonal antibody (clone 12CA5; ATCC, Manassas, VA).

Luciferase assays

NIH3T3 fibroblasts were transfected with the prCD1-1810 reporter (i.e., the rat cyclin D1 promoter in the pGL2 Promega vector, see above), with pSCT-PC3 (containing the rat PC3 coding region, Montagnoli et al., 1996), and either the pSCT-HDAC1-Flag or the pcDNA3-HDAC4-Flag expression constructs, by using the Lipofectamine reagent. The pRL-TK control reporter (Renilla luciferase driven by the thymidine kinase promoter) was included in all transfections. Luciferase assays were performed 48 h after transfection using the Dual-Luciferase reporter assay system (Promega, Madison, WI), according to the manufacturer's instructions, as previously described (Micheli et al., 2011). Differences in the luciferase activity of each sample (Li) dependent on the transfection were normalized by measuring in each transfected cell extract the expression levels of Renilla luciferase (Ri). The normalized activity of the reporter gene was thus calculated as Li × Li/Ri. The fold activity was then obtained as ratio of the average normalized reporter activity value to the average normalized reporter activity units of the corresponding control culture (set to unit). The statistical analysis between groups was performed by Student 's *t*-test on normalized reporter activity values.

Design of siRNAs

The 19-nucleotide siRNA sequences specific to mouse HDAC1, HDAC4, and HDAC9 were designed by the on-line Design Tool software (MWG, Ebersberg, Germany). The best candidate sequences were used to synthesize a pair of 64-mer oligonucleotides that were annealed and cloned in the pSUPER.retro.puro siRNA expression vector, according to the manufacturer's instructions (Oligoengine, Inc., Seattle, WA). The HDAC1 siRNA sequence was as follows: 5′-GTGCTGTGAAGCTTAATAA-3′; the HDAC4 siRNA sequence was 5′-AACAGCTTCTGAACCTAAC-3′ (sequence common to mouse and rat HDAC4 mRNAs); the HDAC9 siRNA sequence was 5′-GCAACTGCAGCAAGAGTTA-3′ (sequence common to mouse and rat HDAC9 mRNAs). The control sequence from the luciferase gene was 5′-ACGGATTACCAGGGATTTC-3′ (see also Micheli et al., 2011). The presence of the correct sequence cloned in pSUPER.retro.puro was confirmed by sequencing.

Generation of recombinant viruses and infections

Retroviruses from the pSUPER.retro.puro-shHDAC1, pSUPER.retro.puro-shHDAC4, pSUPER.retro.puro-shHDAC9, and

pSUPER.retro.puro-shLUC constructs were generated as described (Micheli et al., 2011), by transfecting these constructs into the packaging Phoenix helper cells using Lipofectamine (Invitrogen, San Diego, CA). The supernatants were collected after 48 and 72 h and used for infection. NIH3T3 or C17.2 cells were plated (2×10^5 cells) and infected the first time with the viral supernatant after 24 h and then a second time after 48 h. Seventy-two hours after plating, the cells were split and selected with puromycin (2 μg/ml) for 5 days. Infected cells were then reseeded (60-mm dishes; 4×10^5 cells) to analyze HDAC1, HDAC4, or HDAC9 protein expression by Western blot or mRNA by real time PCR; alternatively, infected cells were reseeded (35-mm dishes; 1×10^5 cells) for transfection with the pSCT-PC3 or pSCT-βGal expression vectors and analyzed by immunofluorescence staining for Bromodeoxyuridine (BrdU) incorporation (see below) or cyclin D1 expression. Retroviruses from the pBABE.puro-PC3 or pBABE.puro-empty constructs were also generated by packaging Phoenix helper cells and used to infect NIH3T3 and C17.2 cells (90-mm dishes with 2×10^5 cells) with the procedure described above, except only one infection was performed, followed by selection in puromycin; infected cells were then transfected with pSCT-HDAC1-Flag and pcDNA3-HDAC4-Flag and pSCT-HDAC9-Flag or with empty vectors, and then analyzed for cyclin D1 or HDAC1/4/9 expression (see below).

Immunofluorescence staining and bromodeoxyuridine treatment in NIH3T3 and C17.2 cells

In NIH3T3 or C17.2 cell cultures infected with shRNA targeting HDAC1 or HDAC4 or HDAC9 and transfected with pSCT-PC3 or pSCT-βGal, the BrdU incorporation was determined after an 18 h pulse (50 μg/ml) as previously described (Guardavaccaro et al., 2000). BrdU, PC3, and βGal staining was performed with a rat monoclonal antibody against BrdU (AbD Serotec; MCA2060; 1:300), or with rabbit polyclonal antibodies against PC3 (A3H antibody; 1:50; Guardavaccaro et al., 2000) or against βGal (Millipore [Chemicon, Billerica, MA]; AB1211; 1:100), respectively. Anti-BrdU, anti-PC3, and anti-βGal antibodies were visualized by donkey anti-rat TRITC-conjugated, and by donkey anti-rabbit Alexa 488-conjugated secondary antibodies, respectively (Jackson ImmunoResearch, West Grove, PA). Cyclin D1 expression was detected by immunofluorescence staining using the mouse monoclonal antibody against cyclin D1 (72-13G; Santa Cruz; sc-450; 1:50), and visualized by a donkey anti-mouse TRITC-conjugated antibody.

Immunoblots

Western blot analysis of HDAC1, HDAC4, HDAC9, cyclin D1, or PC3 protein expression in HEK 293, C17.2, or NIH3T3 cells was performed as described (Micheli et al., 2011). Briefly, cells were lysed by sonication in buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.2% Nonidet P-40, with protease inhibitors 1 mM Na_3VO_4, 10 mM 2-glycerophosphate, 10 mM NaF, 5 mM ATP, 5 mM $MgCl_2$. Proteins were then electrophoretically separated by SDS–PAGE and transferred to nitrocellulose filters. Immunoblots were performed hybridizing filters as indicated, either to a rabbit polyclonal antibody against HDAC1 (Santa Cruz Biotechnology; sc-7872; 1:200) or to a rabbit polyclonal antibody against HDAC4 (Abcam, Cambridge, United Kingdom; ab1437; 1:500) or against PC3 (A3H antibody; 1:1,000), or to mouse monoclonal antibodies against Flag (M2; Sigma–Aldrich; F3165; 1:350), HA (clone 12CA5; ATCC; 1:4), or against cyclin D1 (Santa Cruz; sc-450; 1:200).

Detection of the second antibody (goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated antibody; Jackson ImmunoResearch) was performed by chemiluminescent assay.

Results

Binding in vivo of HDAC1/4 to PC3/Tis21

As we had previously observed that HDAC1 and HDAC4 bind PC3/Tis21 in vitro (as we refer hereafter to either the rat or mouse PC3/Tis21/Btg2 sequence; Farioli-Vecchioli et al., 2007), in the first place we sought to ascertain whether the association of HDAC1 and HDAC4 with PC3/Tis21 occurred also in vivo, using

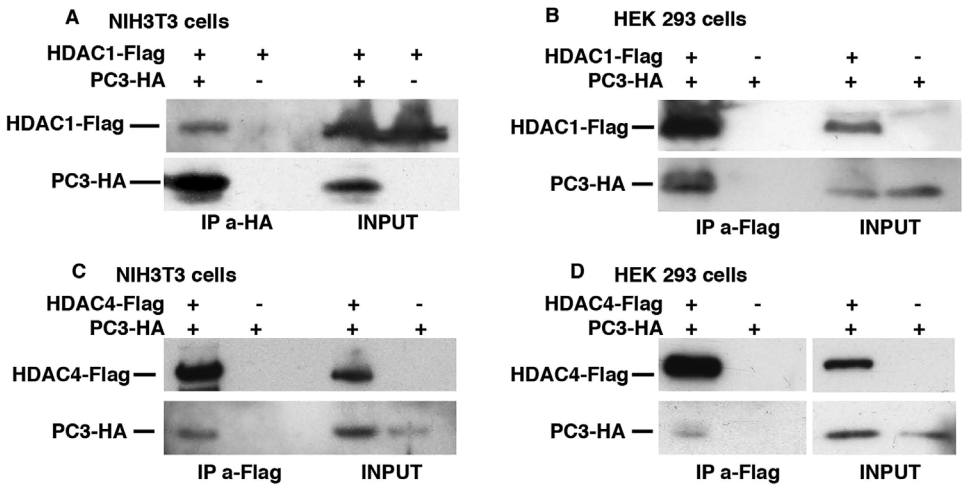

Fig. 1. Coimmunoprecipitation of PC3/Tis21 and HDAC1. (A) NIH3T3 cells (90 mm dish) or (B) HEK 293 cells were transfected (90 mm dish) with pSCT-HDAC1-Flag and with pSCT-PC3-HA or the empty vector (6.0 mg each); 48 h after transfection the cells were lysed and immunoprecipitated with (A) the mouse monoclonal anti-HA antibody or with (B) the mouse monoclonal anti-Flag antibody, covalently bound to Sepharose resin. The anti-HA or the anti-Flag antibodies were used for Western blot analysis of the immunoprecipitated complexes (IP: a-HA lanes, (A); IP: a-Flag lanes, (B)) and of the input cell lysate (1/30 of the immunoprecipitated lysate). (C) NIH3T3 cells (90 mm dish) or (D) HEK 293 cells (90 mm dish) were transfected with pcDNA3-HDAC4-Flag and with pSCT-PC3-HA or the empty vector (6.0 mg each); 48 h after transfection the cells were lysed and immunoprecipitated with the mouse monoclonal anti-HA antibody, covalently bound to Sepharose resin. The anti-HA or the anti-Flag antibodies were used for Western blot analysis of the immunoprecipitated complexes (IP: a-Flag lanes) and of the input cell lysate (1/30 of the immunoprecipitated lysate).

a co-immunoprecipitation assay. NIH3T3 or HEK 293 cells were transfected with pSCT-HDAC1-Flag and with pSCT-PC3-HA or empty vector (Fig. 1A and B). Western blot analysis of either anti-HA or anti-Flag immunoprecipitates in NIH3T3 or HEK 293 cells, respectively, indicated that HDAC1 and PC3/Tis21 proteins did associate in vivo in both cell lines (Fig. 1A and B). An equivalent result was obtained when NIH3T3 or HEK 293 cells were transfected with pcDNA3-HDAC4-Flag and with pSCT-PC3-HA or empty vector (Fig. 1C and D). In fact, Western blot analysis of anti-Flag immunoprecipitates indicated that also HDAC4 and PC3/Tis21 proteins associated in vivo (Fig. 1C and D).

Requirement of HDAC1/4 for the inhibition of cell proliferation by PC3/Tis21

This demonstration of an in vivo binding of HDAC1 and HDAC4 with PC3/Tis21 raised the key question as to whether the functional activity of PC3/Tis21 requires HDAC1 and HDAC4. Thus, we tested whether the inhibition of proliferation exerted by PC3/Tis21 at the G1- to S-phase transition, as determined by bromodeoxyuridine (BrdU) incorporation, was prevented by silencing the expression of HDAC1 and/or HDAC4 through specific short hairpin RNA (shRNA) targeting.

We identified specific (19-nt) HDAC1 and HDAC4 shRNA targeting sequences and cloned them in the pSUPER.retro.puro vector. The corresponding retroviruses were used to infect NIH3T3 cells. Analysis of the selection-resistant fibroblast populations showed that the chosen shRNA sequence was capable of silencing endogenous HDAC1 or HDAC4 protein expression (Fig. 2A), as compared to a control retrovirus expressing an shRNA targeting luciferase (shLUC; Micheli et al., 2011; Farioli-Vecchioli et al., 2012).

Then, we tested the effect of HDAC1 or HDAC4 silencing on the proliferation of NIH3T3 cells. NIH3T3 cells, infected with shRNA to LUC and overexpressing PC3/Tis21 (transfected with pSCT-PC3), showed, as expected, a large decrease of BrdU incorporation relative to controls (transfected with pSCT-βGal) (pSCT-PC3 + shLUC vs. pSCT-βGal + shLUC: Fig. 2C $P < 0.0001$, Fig. 2D $P = 0.001$). Notably, however, overexpression of PC3/Tis21 in NIH3T3 silenced for HDAC1 or HDAC4 only weakly inhibited BrdU incorporation, this being significantly higher than in control shLUC-infected

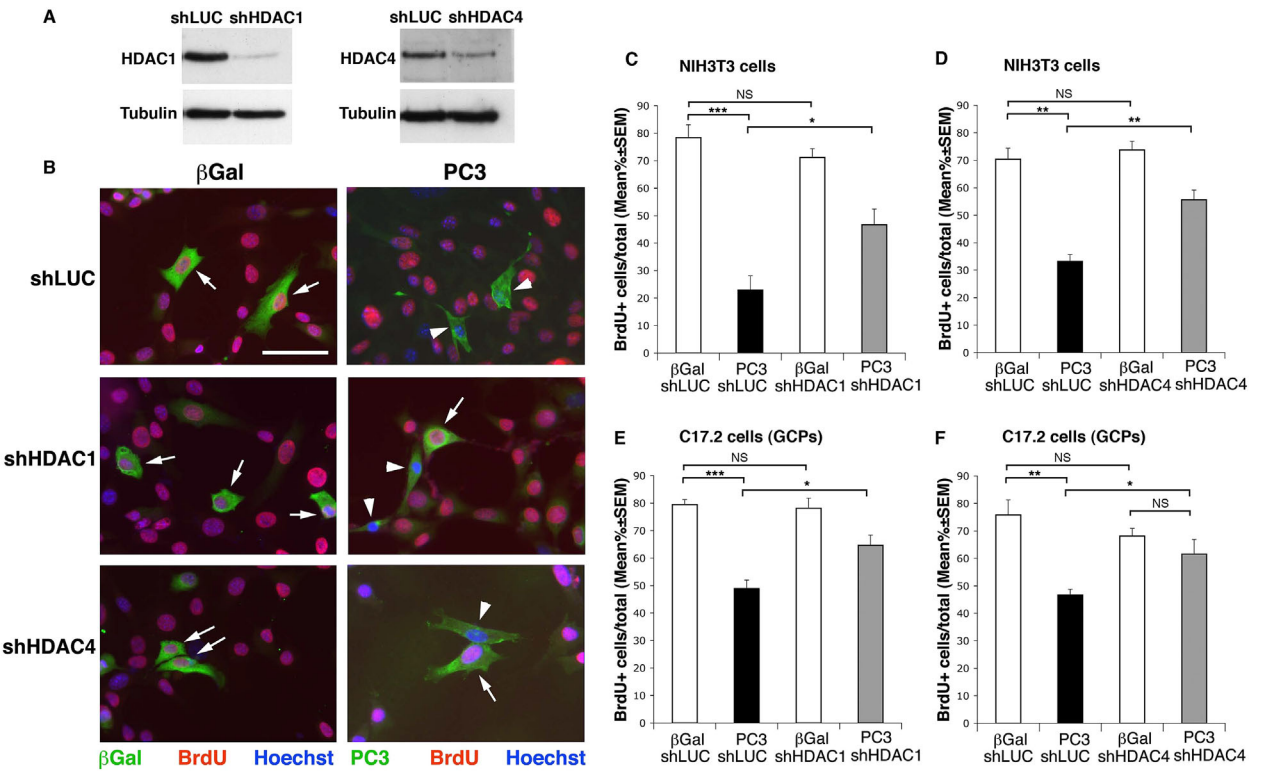

Fig. 2. HDAC1 and HDAC4 are required for the PC3/Tis21-dependent inhibition of proliferation. (A) shRNA-mediated silencing of HDAC1 and HDAC4 expression. The expression of endogenous HDAC1 and HDAC4 proteins was analyzed by Western blot using specific anti-HDAC1/4 antibodies in NIH3T3 fibroblasts, infected with retroviruses generated by the pSUPER.retro.puro vector expressing the HDAC1- or HDAC4-specific shRNA sequences (shHDAC1 or shHDAC4) or the shRNA sequence targeting luciferase (shLUC). After infection, cells were selected for 5 days with puromycin and then analyzed by Western blot. (B) Representative immunofluorescence photomicrographs of BrdU incorporation in NIH3T3 cells transfected with PC3 or βGal and silenced for HDAC1 or HDAC4. NIH3T3 cells, infected with either shHDAC1 or shHDAC4 retroviruses, after selection in puromycin were transfected with the pSCT-PC3 or pSCT-βGal expression vectors (in 35-mm dishes) and analyzed for BrdU incorporation and PC3 and βGal expression by immunofluorescence staining (after a 18 h BrdU pulse). White arrows indicate cells double-positive for BrdU (red) and PC3 or βGal (green), whose nuclei are stained with Hoechst 33258 (blue). Arrowheads indicate cells negative for BrdU (red) and positive for PC3 (green). Size bars 65 μm. (C and D) NIH3T3 cultures transfected with PC3 or βGal and silenced for HDAC1 or HDAC4 and immunostained for PC3, βGal, and BrdU as described in (B) were analyzed for the percentages of $BrdU^+PC3^+$ cells to the total number of $PC3^+$ cells; BrdU-positive cells expressing βGal were calculated as percentages of $BrdU^+$ $βGal^+$ cells to the total number of $βGal^+$ cells. (E) BrdU-positive C17.2 cells infected with shHDAC1 or (F) shHDAC4 retroviruses were selected and transfected with the pSCT-PC3 or pSCT-βGal expression vectors and the percentages of $BrdU^+PC3^+$ or $BrdU^+$ $βGal^+$ cells to the total number of $PC3^+$ or $βGal^+$ cells were measured. (C–F) Mean ± SEM values are from at least three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$, Student's t-test.

cells overexpressing PC3/Tis21 (Fig. 2C, pSCT-PC3 + shHDAC1 vs. pSCT-PC3 + shLUC, 103% increase, $P = 0.011$; Fig. 2D pSCT-PC3 + shHDAC4 vs. pSCT-PC3 + shLUC, 68% increase, $P = 0.003$). Moreover, the silencing of HDAC1 or HDAC4 in NIH3T3 cells in itself did not significantly change the BrdU incorporation ($P = 0.22$ and 0.56, respectively; Fig. 2C and D).

This indicated that the expression of either HDAC1 or HDAC4 is necessary for the full inhibitory activity of PC3/Tis21 on the G1 to S-phase cell cycle transition.

Comparable results were obtained when HDAC1 or HDAC4 were silenced in the continuous cell line of cerebellar granule neuron precursors, C17.2. In fact, PC3/Tis21 strongly inhibited BrdU incorporation, relative to control cells (pSCT-PC3 + shLUC vs. pSCT-βGal + shLUC: Fig. 2E, $P = 0.0002$, Fig. 2F $P = 0.0029$); whereas in C17.2 cells transfected with PC3/Tis21 and silenced for HDAC1 or HDAC4, the levels of BrdU incorporation were significantly higher than in cells transfected with PC3/Tis21 but infected with control shRNA LUC (Fig. 2E, pSCT-PC3 + shHDAC1 vs. pSCT-PC3 + shLUC, 32% increase, $P = 0.024$. Fig. 2F, pSCT-PC3 + shHDAC4 vs. pSCT-PC3 + shLUC, 32% increase, $P = 0.049$; pSCT-PC3 + shHDAC4 vs. pSCT-βGal + shHDAC4, $P = 0.34$).

The inhibition by PC3/Tis21 on cyclin D1 expression is potentiated by HDAC1/4

Given that the inhibitory control of the cell cycle exerted by PC3/Tis21 in normal, non-tumoral cells, occurs mainly, though not exclusively, by down-regulating the expression of cyclin D1 (Guardavaccaro et al., 2000), next, we asked if the requirement of HDAC1 and HDAC4 in the PC3/Tis21-dependent control of cell cycle involved cyclin D1.

Thus, we analyzed the effect of HDAC1 or HDAC4 overexpression on cyclin D1 protein levels in NIH3T3 cells with or without PC3/Tis21. NIH3T3 cells, infected with pBABE-PC3 retrovirus, showed a large decrease of cyclin D1 levels relative to control cultures transfected with empty virus (Fig. 3A,A', pBABE-PC3 vs. pBABE-empty, 48% decrease as judged by densitometry analysis, normalized to tubulin; Fig. 3B, B', pBABE-PC3 vs. pBABE-empty, 46% decrease). On the other hand, the overexpression of HDAC1 or of HDAC4 alone did not appreciably change the expression of cyclin D1 (Fig. 3A,A' and B,B'), consistent with the lack of significant effect of HDAC1 or HDAC4 silencing on cell proliferation (Fig. 2A and B). When, however, NIH3T3 cells infected with pBABE-PC3 retrovirus were transfected with the pSCT-HDAC1-FLAG vector, the levels of cyclin D1 resulted further inhibited, relative to cells infected with pBABE-PC3 retrovirus only (Fig. 3A,A'; pBABE-PC3 vs. pBABE-PC3 + pSCT-HDAC1-FLAG, 52% decrease).

Similarly, when NIH3T3 cells infected with pBABE-PC3 retrovirus were transfected with the pcDNA3-HDAC4-FLAG vector, the levels of cyclin D1 resulted further inhibited (Fig. 3B,B'; pBABE-PC3 vs. pBABE-PC3 + pcDNA3-HDAC4-FLAG, 17% decrease).

Equivalent results were obtained in the neural C17.2 cell line. In fact, infection of C17.2 cells with pBABE-PC3 retrovirus strongly inhibited the expression of cyclin D1 (pBABE-PC3 vs. pBABE-empty, 72% and 52% decrease, Fig. 3C,C' and D,D',

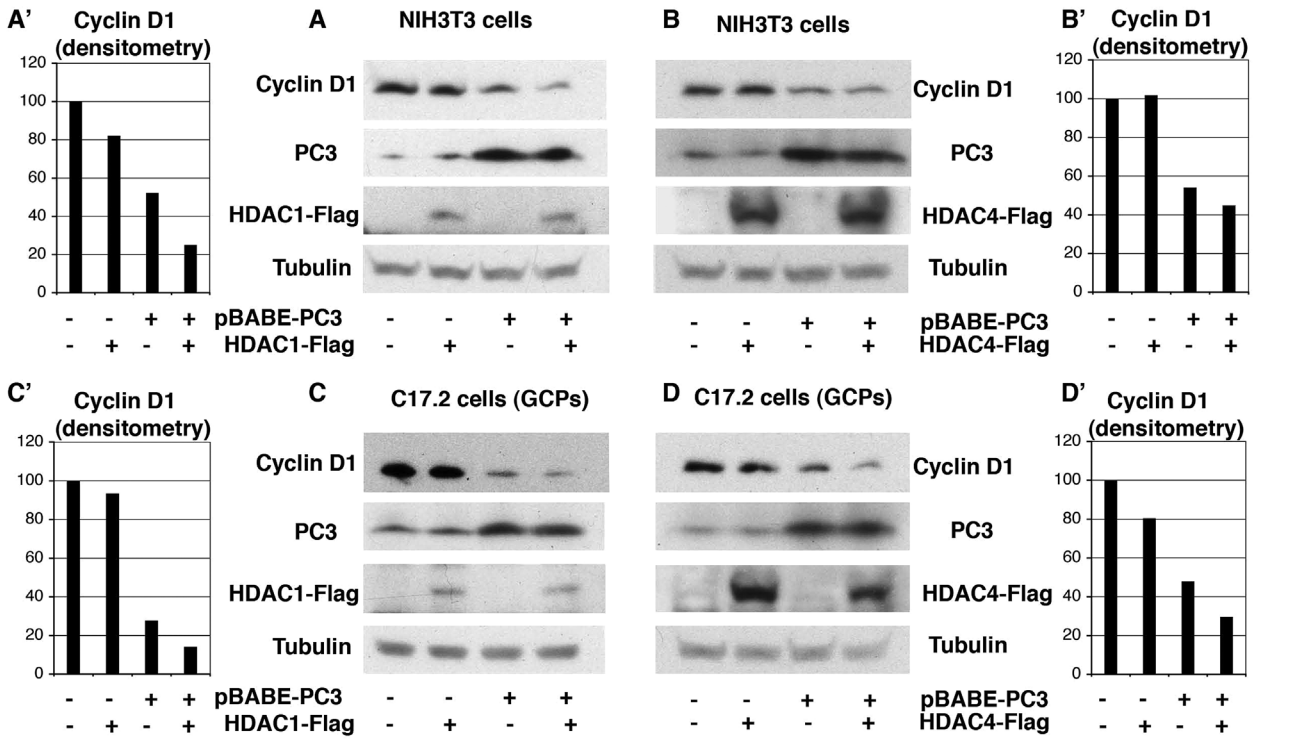

Fig. 3. Overexpression of HDAC1 or HDAC4 potentiates the PC3/Tis21-dependent inhibition of cyclin D1 expression. (A,A' and B,B') Western blot of cyclin D1 protein in NIH3T3 cells or (C,C' and D,D') in C17.2 cells and densitometry analysis. Cells were infected with either pBABE.puro-PC3 or pBABE.puro-empty retroviruses, selected with puromycin for 5 days, then transfected with either pSCT-HDAC1-Flag (A,A' and C,C') or pcDNA3-HDAC4-Flag (B,B' and D,D') or with empty vectors, and after 48 h were analyzed for cyclin D1, PC3, HDAC1-Flag, and HDAC4-Flag expression. Representative results from three independent experiments are shown. In (A',B' and C',D') values are presented as percent decrease of protein expression in cells infected and transfected as indicated, relative to the group of control cells (infected with pBABE-.puro-empty virus and transfected with empty vectors), after normalization to the corresponding values of α-tubulin expression (the control base line is set to 100%).

respectively); moreover, the coexpression of pBABE-PC3 retrovirus with transfected pSCT-HDAC1-FLAG or pcDNA3-HDAC4-FLAG vectors, further inhibited the levels of cyclin D1, relative to cells infected with pBABE-PC3 retrovirus only (Fig. 3C,C' and D,D'; pBABE-PC3 vs pBABE-PC3 + pSCT-HDAC1-FLAG, 49% decrease; pBABE-PC3 vs pBABE-PC3 + pcDNA3-HDAC4-FLAG, 38% decrease).

Thus, the overexpression of HDAC1 or HDAC4 potentiates the ability of PC3/Tis21 to inhibit the expression of cyclin D1. As a whole, these data indicate that the full inhibitory

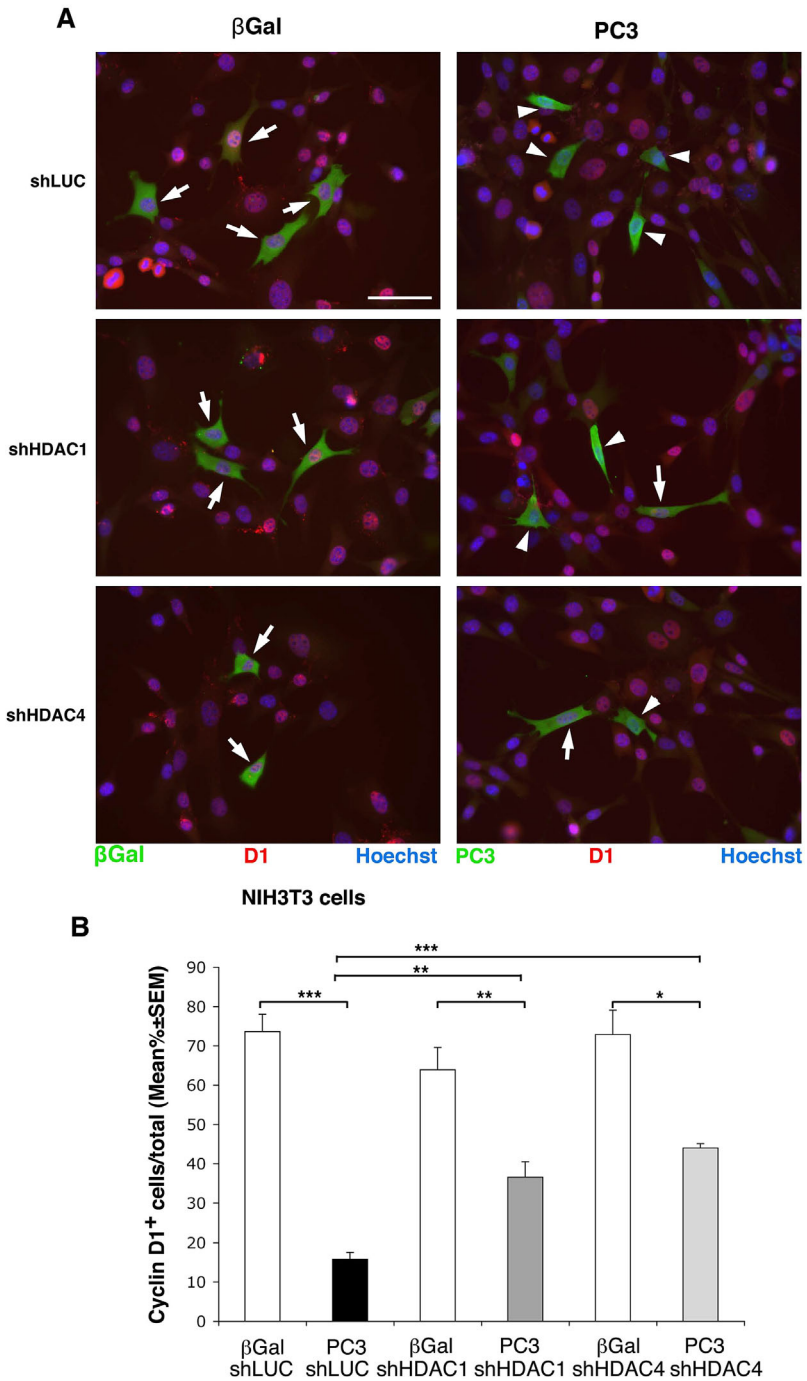

Fig. 4. Silencing of HDAC1 or HDAC4 impairs the PC3/Tis21-dependent inhibition of cyclin D1 expression. (A) Representative immunofluorescence photomicrographs of cyclin D1 nuclear expression in NIH3T3 cells transfected with PC3 or βGal and silenced for HDAC1 or HDAC4. NIH3T3 cells, treated as described below in (B), were analyzed for cyclin D1, PC3, and βGal expression. White arrows: double-positive cells for cyclin D1 (red) and PC3 or βGal (green); arrowheads: cells negative for cyclin D1 (red) and positive for PC3 (green). Nuclei were stained with Hoechst 33258 (blue). Size bars 65 μm. (B) NIH3T3 cells, infected with either shHDAC1, shHDAC4, or shLUC retroviruses, after selection in puromycin were transfected with the pSCT-PC3 or pSCT-βGal expression vectors (in 35-mm dishes) and analyzed for cyclin D1 and PC3 or βGal expression by immunofluorescence staining. The percentages of cyclin D1$^+$PC3$^+$ or cyclin D1$^+$ βGal$^+$ cells were calculated as percentages to the total number of PC3$^+$ cells or βGal$^+$ cells, respectively. Mean ± SEM values are from at least three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$, Student's t-test.

control of PC3/Tis21, either on cell cycle progression or on cyclin D1 expression, requires HDAC1 and HDAC4; this regulation of cyclin D1 appears to be effective also in neural cells.

The inhibition of cyclin D1 expression by PC3/Tis21 requires HDAC1/4

The above data would predict that silencing the expression of HDAC1 or HDAC4 may impair the ability of PC3/Tis21 to inhibit cyclin D1 expression. To test this possibility, we counted by immunofluorescence staining the NIH3T3 cells positive for cyclin D1, after infection with either shLUC, or shHDAC1 or shHDAC4 and overexpressing PC3/Tis21 by transfection of the pSCT-PC3 or control pSCT-βGal vectors (Fig. 4A and B). The inhibition of cyclin D1 expression by PC3/Tis21 was significantly lower in NIH3T3 cells silenced for HDAC1 or HDAC4 than in control NIH3T3 cells infected with shLUC (Fig. 4B, pSCT-PC3 + shHDAC1 vs. pSCT-PC3 + shLUC, 130% increase of cyclin $D1^+$ cells, $P = 0.003$; pSCT-PC3 + shHDAC4 vs. pSCT-PC3 + shLUC, 178% increase of cyclin $D1^+$ cells, $P = 0.00006$). However, PC3/Tis21 was still able to significantly reduce the cyclin $D1^+$ cells in NIH3T3 cultures silenced for HDAC1 or HDAC4, when compared to control cells silenced for HDACs overexpressing βGal (Fig. 4B, pSCT-PC3 + shHDAC1 vs. pSCT-βGal + shHDAC1, $P = 0.007$; pSCT-PC3 + shHDAC4 vs. pSCT-βGal + shHDAC4, $P = 0.018$). Overall, these data confirm that HDAC1 or HDAC4 are required for the PC3/Tis21-dependent inhibition of cyclin D1 expression, but also suggest that neither HDAC1 nor HDAC4 alone is totally responsible for this inhibition.

The inhibition of cyclin D1 promoter by PC3/Tis21 is potentiated by HDAC1/4

To further test the role of HDAC1 and HDAC4 on the control of cyclin D1 expression by PC3/Tis21, we checked whether the down-regulation that PC3/Tis21 exerts on cyclin D1 transcription (Guardavaccaro et al., 2000) is modulated by HDACs. For our experiments, we transfected NIH3T3 fibroblasts with a reporter construct (prCD1-1810) carrying about 1650 nucleotides of the cyclin D1 promoter region, together with expression vectors for HDAC1 (pSCT-HDAC1-Flag) or HDAC4 (pcDNA3-HDAC4-Flag), and increasing concentrations of PC3/Tis21 (pSCT-PC3). We observed that HDAC1 and HDAC4 alone significantly reduced the transcriptional activity of the cyclin D1 promoter (Fig. 5; HDAC1 vs. control, $P = 0.013$; HDAC4 vs. control, $P = 0.0004$), but when HDAC1 or HDAC4 were cotransfected with increasing concentrations of PC3 (0.75 and 1 μg), this inhibition was significantly potentiated (Fig. 5; HDAC1 with 0.75 or 1 μg PC3 vs. HDAC1, $P = 0.03$ and $P < 0.001$, respectively; HDAC4 with 0.75 or 1 μg PC3 vs. HDAC4, $P = 0.017$ and $P = 0.011$, respectively). This indicated that HDAC1 and HDAC4 synergized with PC3/Tis21 in the negative regulation of cyclin D1 transcriptional activity.

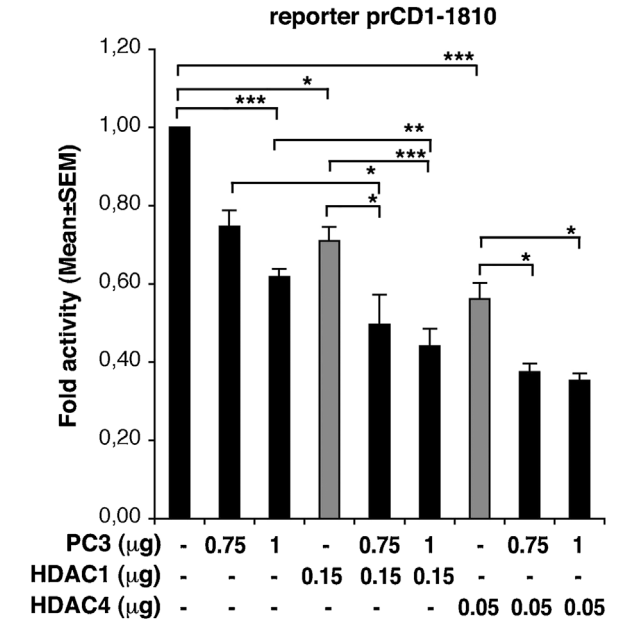

Fig. 5. HDAC1 and HDAC4 synergize with PC3/Tis21 in repressing the transcriptional activity of cyclin D1. NIH3T3 cells were plated in 35-mm culture dishes and cotransfected the next day with the cyclin D1 promoter reporter (prCD1, 0.1 μg), with the pSCT-PC3 expression construct or the empty vector (0.75 or 1 μg), and either HDAC1 (pSCT-HDAC1-Flag, 0.15 μg) or HDAC4 (pcDNA3-HDAC4-Flag, 0.05 μg) expression vectors. The pSCT and/or pcDNA3 empty vectors were included also where necessary to normalize for DNA content. Cells were harvested 48 h after transfection. Luciferase activity from cell extracts is expressed as fold induction relative to the activity of the control prCD1 reporter sample (cotransfected only with empty vectors). HDAC1 and HDAC4 inhibit cyclin D1 promoter activity in synergy with Tis21. Bars represent the average fold induction ± SEM determined from four independent experiments, each performed in duplicate. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (Student's t-test).

PC3/Tis21 binds and requires HDAC9 to inhibit cell proliferation

Recently, a genome-wide analysis performed by us has shown that the ablation of PC3/Tis21 in cerebellar precursors leads to changes in the expression of a number of genes, which are significantly enriched for gene products interacting with HDAC1 or HDAC4 or modified by the HDAC inhibitor trichostatin (Farioli-Vecchioli et al., 2013). Among these genes are HDAC9 and the corepressor Ncor1; as they are part of complexes with HDAC1 or HDAC4 (Sparrow et al., 1999; Petrie et al., 2003), we sought to verify whether PC3/Tis21 was able to bind in vivo also HDAC9, in addition to HDAC4 and HDAC1.

Thus, NIH3T3 and HEK 293 cells were transfected with pSCT-HDAC9-Flag and with pSCT-PC3-HA-or empty vector (Fig. 6A and B). Western blot analysis of anti-Flag immunoprecitates showed that HDAC9 and PC3/Tis21 proteins did associate in vivo (Fig. 6A and B).

Next, we tested whether the inhibition of the proliferation exerted by PC3/Tis21 at the G1- to S-phase transition, required HDAC9 expression. Thus, we assessed if BrdU incorporation was impaired after silencing the expression of HDAC9 by specific shRNA targeting. A specific 19-nt HDAC9 targeting shRNA sequence was cloned into pSUPER.retro.puro vector, and the corresponding retroviruses expressing shHDAC9 resulted capable of silencing endogenous HDAC9 mRNA expression in NIH3T3 cells (Fig. 6C).

As expected, NIH3T3 cells, infected with shLUC and overexpressing PC3/Tis21 by transfection of the pSCT-PC3 vector, showed a great decrease of BrdU incorporation (Fig. 6D and E; pSCT-PC3 + shLUC vs. pSCT-βGal + shLUC, $P < 0.0001$). The silencing of HDAC9 with shHDAC9 in itself did not significantly change the BrdU incorporation ($P = 0.18$ for shLUC + pSCT-βGal vs. shHDAC9 + pSCT-βGal; Fig. 6D and E). Remarkably, however, in PC3-overexpressing

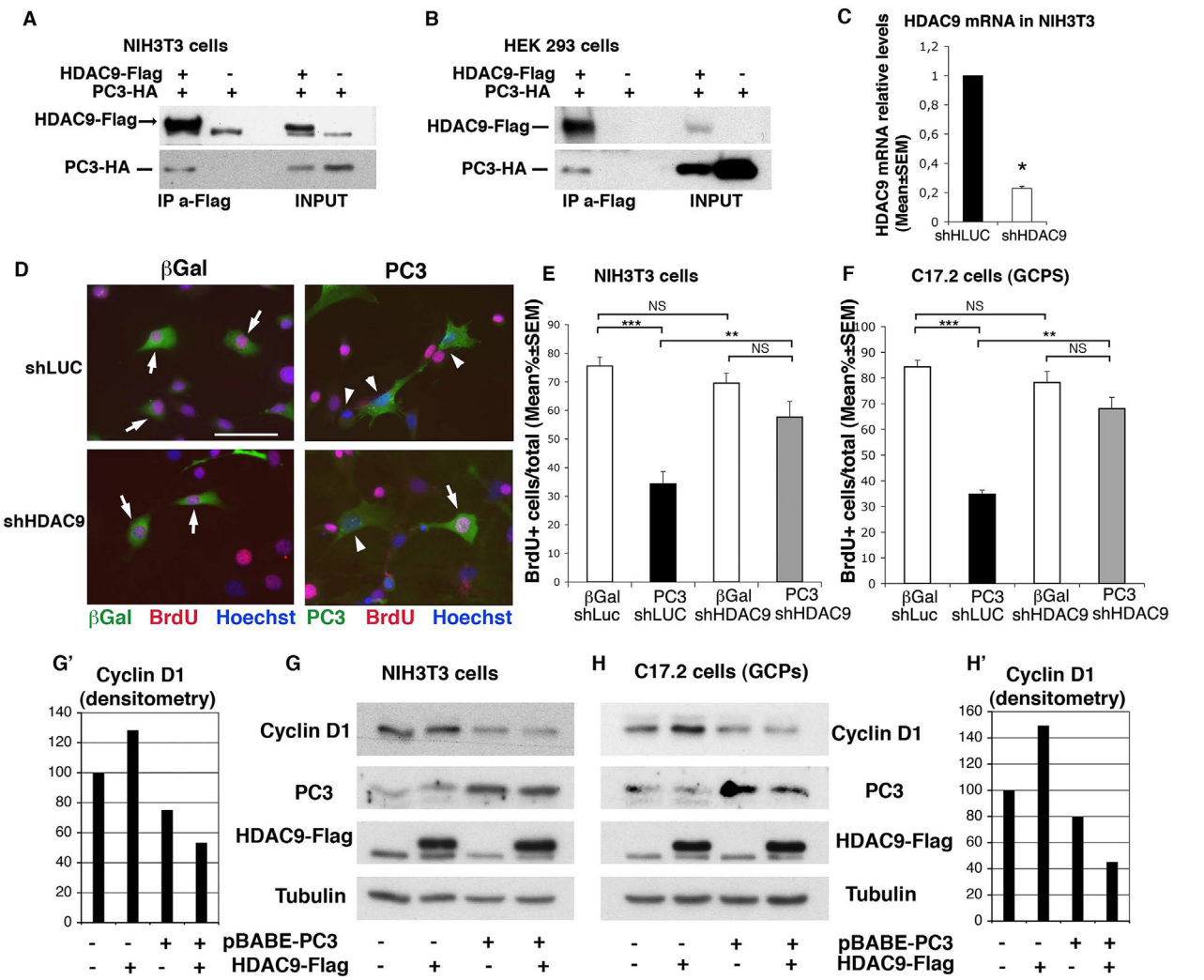

Fig. 6. HDAC9 binds PC3/Tis21 and is required for its inhibitory activity on the cell cycle. (A) NIH3T3 cells (90 mm dish) or (B) HEK 293 cells (90 mm dish) were transfected with pSCT-PC3-HA and with pSCT-HDAC9-Flag or with the empty vector (6.0 μg each); 48 h after transfection the cells were lysed and immunoprecipitated with the mouse monoclonal anti-Flag antibody, covalently bound to Sepharose resin. The anti-Flag or the anti-HA antibodies were used for Western blot analysis of the immunoprecipitated complexes (IP: a-Flag lanes) and of the input cell lysate (1/30 of the immunoprecipitated lysate). (C) shRNA-mediated silencing of HDAC9 expression. The graph shows the levels of HDAC9 mRNA expression in NIH3T3 fibroblasts, infected with retroviruses generated by the pSUPER.retro.puro vector expressing the HDAC9-specific shRNA sequences (shHDAC9) or the control shRNA sequence targeting luciferase (shLUC). After infection, cells were selected for 5 days with puromycin, reseeded, and analyzed. (mean ± SEM fold increases; TBP was used to normalize data; $^{*}P < 0.05$, Student's *t*-test). (D) Representative immunofluorescence photomicrographs of BrdU incorporation in NIH3T3 cells overexpressing PC3/Tis21 or βGal and silenced for HDAC9. NIH3T3 cells were infected with shHDAC9 retrovirus, selected in puromycin, then transfected with the pSCT-PC3 or pSCT-βGal expression vectors and analyzed for BrdU incorporation (after a 18 h BrdU pulse) and PC3 and βGal expression. White arrows: double-positive cells for BrdU (red) and PC3 or βGal (green); arrowheads: cells negative for BrdU (red) and positive for PC3 (green). Size bars 65 μm. (E) NIH3T3 cells or (F) C17.2 cells overexpressing PC3/Tis21 or βGal and silenced for HDAC9 as described in (D) were analyzed for the percentages of $BrdU^{+}PC3^{+}$ or $BrdU^{+}$ $βGal^{+}$ cells to the total number of $PC3^{+}$ or $βGal^{+}$ cells. Mean ± SEM values are from at least three independent experiments. $^{**}P < 0.01$, or $^{***}P < 0.001$, Student's *t*-test. (G,G') Western blot and densitometry analysis of cyclin D1 protein in NIH3T3 cells or (H,H') in C17.2 cells. Cells were infected with either pBABE.puro-PC3 or pBABE.puro-empty retroviruses, selected with puromycin for 5 days, then transfected with either pSCT-HDAC9-Flag or with empty vectors, and after 48 h were analyzed for cyclin D1 or for HDAC9 expression. Representative results from three independent experiments are shown. In (G',H') values are percent change of protein expression in cells infected/transfected as indicated, relative to control cells infected with pBABE-.puro-empty virus and transfected with empty Flag vector (set to 100%), after normalization to the corresponding values of α-tubulin expression.

cultures, the ability of PC3/Tis21 to inhibit BrdU incorporation was significantly reduced by the silencing of HDAC9 (Fig. 6D and E; pSCT-PC3 + shHDAC9 vs. pSCT-PC3 + shLUC, 68% increase of incorporation, $P = 0.005$). Moreover, in NIH3T3 cells silenced for HDAC9 the levels of BrdU incorporation did not significantly differ whether PC3/Tis21 was overexpressed or not (Fig. 6D and E; pSCT-PC3 + shHDAC9 vs. pSCT-βGal + shHDAC9, $P = 0.08$).

Similarly, in PC3-overexpressing C17.2 cultures, the silencing of HDAC9 significantly reduced the ability of PC3/Tis21 to inhibit BrdU incorporation (Fig. 6F; pSCT-PC3 + shHDAC9 vs. pSCT-PC3 + shLUC, 95% increase of incorporation, $P = 0.002$). Moreover, in C17.2 cells silenced for HDAC9, the BrdU incorporation did not differ whether PC3/Tis21 was overexpressed or not (Fig. 6F; pSCT-PC3 + shHDAC9 vs. pSCT-βGal + shHDAC9, $P = 0.17$).

As a whole, this indicated that without the expression of HDAC9 the inhibitory activity of PC3/Tis21 on the G1 to S-phase transition is impaired, thus pointing to a requirement of HDAC9 activity in the antiproliferative effect of PC3/Tis21.

We then analyzed the effect on cyclin D1 protein levels of the overexpression of HDAC9 in NIH3T3 and C17.2 cells with or without PC3/Tis21. As shown above, infection with pBABE-PC3 retrovirus reduced cyclin D1 levels relative to control cultures transfected with empty virus (Fig. 6G,G', pBABE-PC3 vs. pBABE-empty, 25% and 21% decrease in NIH3T3 and C17.2 cells, respectively). Interestingly, the overexpression of HDAC9 alone, unlike that of HDAC1/4, increased the expression of cyclin D1 in both cells types, (Fig. 6G,G' and H, H'), despite the lack of effect of HDAC9 silencing alone on cell proliferation (Fig. 6D and E). However, when NIH3T3 or C17.2 cells infected with pBABE-PC3 retrovirus were transfected with the pSCT-HDAC9-FLAG vector, cyclin D1 levels were further inhibited by 29% and 43%, respectively, relative to cells infected with pBABE-PC3 retrovirus only (Fig. 6G,G' and H,H'; pBABE-PC3 + pSCT-HDAC1-FLAG vs. pBABE-empty, 47% decrease in NIH3T3 and 55% decrease in C17.2 cells).

Thus, the overexpression of HDAC9 potentiates the ability of PC3/Tis21 to inhibit the expression of cyclin D1.

Nevertheless, the silencing of HDAC9 did not affect the ability of PC3/Tis21 to inhibit cyclin D1 expression. In fact, when we counted by immunofluorescence staining the NIH3T3 and C17.2 cells overexpressing PC3/Tis21 that were positive for cyclin D1, the levels of cyclin D1 expression was the same after infection with either shLUC or shHDAC9 (Fig. S1A and B, percentage of cyclin $D1^+$ NIH3T3 cells: pSCT-

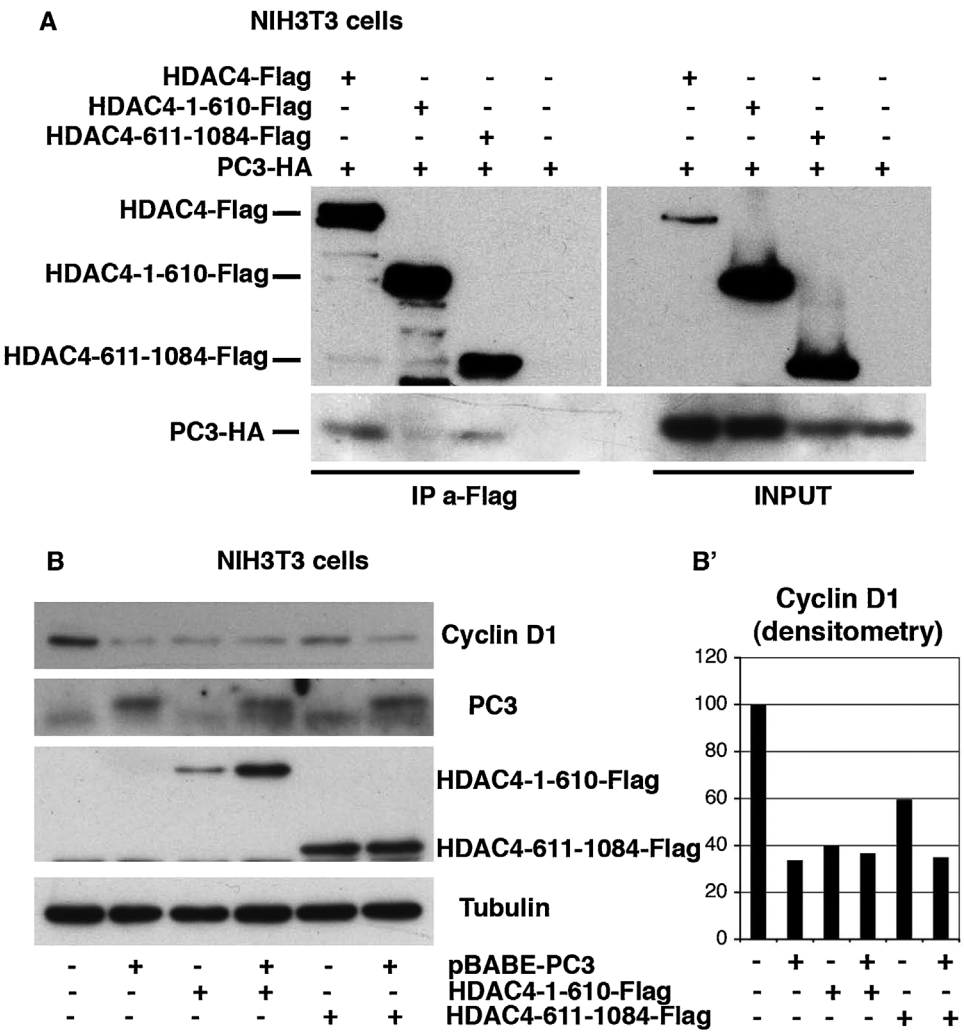

Fig. 7. The amino-terminal moiety and the catalytic domain of HDAC4 both bind PC3/Tis21 but do not potentiate the inhibition of cyclin D1 expression by PC3/Tis21. (A) Co-immunoprecipitation of PC3/Tis21 and HDAC4 deletion mutants. NIH3T3 cells (90 mm dish) were transfected with pcDNA3-HDAC4-Flag or pSCT-HADC4-1-610-Flag, or pSCT-HADC4-611-1084-Flag and with pSCT-PC3-HA or the empty vectors (6.0 μg each); 48 h after transfection the cells were lysed and immunoprecipitated with the mouse monoclonal anti-Flag antibody, covalently bound to Sepharose resin. The anti-HA or the anti-Flag antibodies were used for Western blot analysis of the immunoprecipitated complexes and of the input cell lysate (1/30 of the immunoprecipitated lysate). (B) Western blot of cyclin D1 protein in NIH3T3 cells and (B') densitometry analysis. Cells were infected with either pBABE.puro-PC3 or pBABE.puro-empty retroviruses, selected with puromycin for 5 days, then transfected with either pSCT-HDAC4-1-610-Flag or pSCT-HDAC4-611-1084-Flag or with empty vectors, and after 48 h were analyzed for cyclin D1, PC3, HDAC4 mutant (Flag), and for tubulin expression. (B) A representative result from three independent experiments is shown. (B') Percent decrease of cyclin D1 protein expression in cells infected and transfected as indicated, relative to the group of control cells (infected with pBABE-.puro-empty virus and transfected with empty vectors), after normalization to the corresponding values of α-tubulin expression (the control base line is set to 100%).

PC3 + shHDAC9 vs. pSCT-PC3 + shLUC, $P = 0.62$; Fig. S1C, cyclin D1$^+$ C17.2 cells: pSCT-PC3 + shHDAC9 vs. pSCT-PC3 + shLUC, $P = 0.73$).

This clearly indicates that HDAC9, unlike HDAC1 and HDAC4, is not required for the full inhibitory control of PC3/Tis21 on cyclin D1 expression.

The amino-terminal moiety and the catalytic domain of HDAC4 both bind PC3/Tis21 but do not potentiate the inhibition of cyclin D1 expression by PC3/Tis21.

Since we have observed that HDAC1/4/9 are required for the inhibitory action of PC3/Tis21 on cell proliferation, we further sought to define which HDAC domain was involved. To this aim, we performed a mutant analysis of the HDAC4 protein, as this displays a well separated catalytic domain. We generated two HDAC4 deletion mutants, one spanning the amino-terminal moiety carrying the MEF-binding domain and nuclear localization signal (aa 1-610; pSCT-HADC4-1-610-Flag vector), and a second one carrying the whole catalytic domain and nuclear export signal (aa 611-1084; pSCT-HADC4-611-1084-Flag vector), as described by Bertos et al. (2001).

NIH3T3 cells were transfected with pcDNA3-HDAC4-Flag, or pSCT-HADC4-1-610-Flag or pSCT-HADC4-611-1084-Flag vectors and with pSCT-PC3-HA or empty vectors (Fig. 7A). Western blot analysis of anti-Flag immunoprecipitates indicated that both the N-terminal domain (1-610) and the catalytic domain of HDAC4 (611-1084) were able to associate with the PC3/Tis21 protein, although the latter showed a more efficient binding (Fig. 7A).

Next, we tested whether the HDAC4 amino-terminal moiety and/or the catalytic domain alone were sufficient to potentiate the ability of PC3/Tis21 to inhibit the expression of cyclin D1. Thus, we analyzed cyclin D1 protein levels after overexpression of HDAC4-1-610 and of HDAC4-611-1084 mutants in NIH3T3 cells with or without PC3/Tis21 overexpression (pBABE-PC3 retrovirus). Interestingly, the overexpression of HDAC4-1-610 or HDAC4-611-1084 mutants alone was able to inhibit the expression of cyclin D1 to an extent similar, or less pronounced than PC3/Tis21, respectively (Fig. 7B,B'). When, however, the pSCT-HDAC4-611-1084-Flag vector was transfected in NIH3T3 cells infected with the pBABE-PC3 retrovirus, the levels of cyclin D1 resulted further inhibited to the same level attained with pBABE-PC3 retrovirus alone (Fig. 7B,B'; pBABE-empty vs. pBABE-PC3, 33% decrease, pBABE-empty vs. pBABE-PC3 + pSCT-HDAC4-FLAG-611-1084, 34% decrease). Furthermore, the addition of pBABE-PC3 retrovirus to pSCT-HDAC4-1-610 did not produce any evident change (Fig. 7B,B').

Thus, no synergy was attained with PC3/Tis21 to inhibit cyclin D1 expression, suggesting that the binding of the whole HDAC4 molecule is necessary to synergize with the inhibitory action of PC3/Tis21 on cyclin D1 expression.

Discussion

Our findings indicate that PC3/Tis21 inhibits cell proliferation in a way that is dependent on the function of HDACs. In fact, when HDAC1, HDAC4, or HDAC9 are silenced, the ability of PC3/Tis21 to inhibit proliferation in fibroblasts and in cerebellar precursor cells is significantly reduced. These functional data are compatible with our present observations that HDAC1, HDAC4, and HDAC9 associate in vivo with PC3/Tis21, and that the overexpression of HDAC1/4/9 potentiates—while the silencing of HDAC1/4 reduces—the inhibition exerted by PC3/Tis21 on the expression of cyclin D1, in fibroblasts and in cerebellar precursor cells. Moreover, the inhibitory activity of PC3/Tis21 on the cyclin D1 promoter, where PC3/Tis21 binds, is significantly increased by HDAC1 and HDAC4.

This strongly suggests that the binding of HDACs to PC3/Tis21 is required for the inhibitory activity of PC3/Tis21 on the cyclin D1 promoter, through which PC3/Tis21 preferentially inhibits the cell cycle entry in normal neural and non-neural cells (Guardavaccaro et al., 2000). In fact, we have previously observed that the antiproliferative gene PC3/Tis21 impairs G1–S transition by direct repression of the cyclin D1 promoter, acting as a transcriptional regulator (Guardavaccaro et al., 2000).

This is the first evidence that HDAC9 associates with PC3/Tis21 and is implicated in cell cycle regulation through cyclin D1. However, since we observed that the silencing of HDAC9 does not affect cyclin D1 levels, the activity of HDAC9 might be redundant with that of other HDACs.

D cyclins play a unique role in the regulation of cell proliferation, since these are the only cyclins directly regulated by proliferation factors, that is, the mitogenic signals. Moreover, D cyclins control the "restriction point" responsible for the cell decision to enter or not the cycle; in fact D cyclins, being the first cyclins to phosphorylate pRb, control the activation of E2F and thus control the mechanism by which the cell starts proliferating. The other S-phase cyclins phosphorylate pRB only after D cyclins have inactivated pRb, thus when the cell has already entered the cycle, and therefore do not play a decisional role (Sherr and Roberts, 1995). A misregulation of these controls can be lost during cellular transformation, and cyclin D1 is correspondingly overexpressed in a number of cancers.

Furthermore, the requirement of HDACs for the inhibition by PC3/Tis21 of the cyclin D1 promoter activity is consistent with our previous finding that the recruitment of PC3/Tis21 on cyclin D1 promoter is inversely correlated with the acetylation of histone H4 (Farioli-Vecchioli et al., 2007). This suggests that the HDACs associated with PC3/Tis21 in complexes binding the cyclin D1 promoter can transcriptionally repress its activity. This corepressor activity may be particularly necessary for class II HDACs, such as HDAC4/9, which do not bind directly to DNA but require other coactivators (Martin et al., 2007).

It is known that HDACs exert an important repressive activity interacting, either directly or indirectly, with a variety of DNA-binding transcription factors, including nuclear hormone receptors, nuclear factor kB, myocyte enhancer factor 2, and Sox2 (Nagy et al., 1997; Zhong et al., 2002; Tang and Goldman, 2006; Baltus et al., 2009).

Concerning the role of HDACs in cell cycle regulation, they act in cell-type and environment-specific fashion. In fact, despite the numerous reports describing the use of HDAC inhibitors as anticancer agents with inhibitory activity on the cell cycle (Lakshmaiah et al., 2014), it is known that the HDAC complex is required for the repression of particular promoters responsive to the E2F family of transcription factors that play a critical role in regulation of G1 cyclin genes (Telles and Seto, 2012).

Different reports have demonstrated that HDACs can act as transcriptional corepressors on the cyclin D1 promoter (Klein and Assoian, 2008), that is, the SMAR1 factor targets cyclin D1 promoter and represses its expression interacting with HDAC1, SIN3, and pRb to form a multiprotein repression complex (Rampalli et al., 2005). Similarly, Sox6 represses cyclin D1 promoter activity by interacting with β-catenin and HDAC1, in pancreatic βcells (Iguchi et al., 2007). Interestingly, yin and yang1 (YY-1) represses cyclin D1 by recruiting HDAC1 to a TRE/Oct-1-binding site on the cyclin D1 promoter (Cicatiello et al., 2004) in this way maintaining quiescence in mammary epithelial cells.

As a whole, these observations are consistent with our present results about the inhibitory role of PC3/Tis21 on the

cell cycle by repression of cyclin D1 expression via binding of HDACs.

An involvement of HDACs and chromatin remodeling factors was previously reported for Tob1, another member of the Btg gene family, which was observed to exert its inhibitory activity on the cell cycle by recruiting HDAC1 on the cyclin D1 promoter (Yoshida et al., 2003).

A component of the complexes PC3/Tis21-HDACs may also be the arginine methyltransferase PRMT1, which in fact has been shown to bind PC3/Tis21 (Lin et al., 1996). In this regard, we have previously observed that PRMT1 is recruited to histone complexes on the RARβ promoter, where it primes the demethylation and acetylation of histones, favoring the transcriptional activity of the RARβ promoter (Passeri et al., 2006). Thus, PRMT1 and HDACs may exert opposite activity on the cyclin D1 promoter. Future studies should investigate the role of PRMT1 on the cyclin D1 promoter.

As a comparison between our present observations in the cerebellar precursor cell line C17.2 and the role of PC3/Tis21 in cerebellum in vivo, we have previously observed that the overexpression of PC3/Tis21 in cerebellar precursor cells in a transgenic conditional mouse highly impairs their proliferation, reducing cyclin D1 levels, and also prevents tumorigenesis (Farioli-Vecchioli et al., 2007). This report suggests a functional explanation. Conversely, the ablation of PC3/Tis21 in cerebellar precursor cells in vivo, although highly increasing medulloblastoma frequency, does not affect their proliferation (Farioli-Vecchioli et al., 2012). In this regard, we have recently observed that the role of PC3/Tis21 in the control of cerebellar precursor cells proliferation may be redundant with Btg1, a family-related gene, thus explaining how the inactivation of PC3/Tis21 in vivo does not affect their proliferation (Ceccarelli et al., 2015).

Furthermore, we have previously observed that PC3/Tis21 regulates in proliferating cerebellar precursor cells several genes involved in epigenetic control (Farioli-Vecchioli et al., 2012, 2013). Among these are HDAC9, which also binds HDAC1 or HDAC4 (Petrie et al., 2003), or the histone modification editor Ankrd11, which regulates HDAC3 (Gallagher et al., 2015), or Padi4, which demethylates histones and binds HDAC1 (Denis et al., 2009). Thus, PC3/Tis21 may control the transcription of cyclin D1 and of other genes, by acting as coregulator of complexes including HDACs, PRMT1, and/or several other chromatin modifying enzymes such as a Ankrd11. Our analysis of HDAC4 deletion mutants further indicates that the binding of either the amino-terminal moiety or the catalytic subunit of HDAC4 to PC3/Tis21 is not sufficient to potentiate its inhibition of cyclin D1 expression, suggesting that the full activity of the HDAC molecule is required and that complex interactions may take place.

In this scenario, in neural tissues, it has been observed that deletion of HDAC1 or HDAC2 separately has weak effects on neuronal development (Montgomery et al., 2009), but the deletion of both results in increased proliferation of neuronal precursors at the ventricular zone and reduced number of differentiating neurons (Montgomery et al., 2009). Moreover, in cerebella of HDAC1-2 double knockouts Purkinje cells fail to migrate (Montgomery et al., 2009). Interestingly, the deletion of HDAC4 in vivo leads to a reduced development of brain and cerebellum (Majdzadeh et al., 2008). Such defects of proliferation and migration of neural cells are compatible with the phenotypes observed in cerebella after overexpression or deletion of PC3/Tis21 in cerebellar granule precursor cells (i.e., inhibition of proliferation with increase of differentiation, or impairment of migration of cerebellar precursors, respectively), and are in line with the idea that PC3/Tis21 may vehiculate HDACs to inactivate proliferative target promoter genes such as cyclin D1.

Considering the implication of cyclin D1 in tumorigenesis and since PC3/Tis21 acts as tumor-suppressor gene in several systems (Micheli et al., 2015), the requirement of HDAC1, HDAC4, and HDAC9 for the cell cycle inhibitory activity of PC3/Tis21 may offer new perspectives and targets for cancer therapy.

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