Che-1 affects cell growth by interfering with the recruitment of HDAC1 by Rb

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Summary

DNA tumor virus oncoproteins bind and inactivate Rb by interfering with the Rb/HDAC1 interaction. Che-1 is a recently identified human Rb binding protein that inhibits the Rb growth suppressing function. Here we show that Che-1 contacts the Rb pocket region and competes with HDAC1 for Rb binding site, removing HDAC1 from the Rb/E2F complex in vitro and from the E2F target promoters in vivo. Che-1 overexpression activates DNA synthesis in quiescent NIH-3T3 cells through HDAC1 displacement. Consistently, Che-1-specific RNA interference affects E2F activity and cell proliferation in human fibroblasts but not in the pocket protein-defective 293 cells. These findings indicate the existence of a pathway of Rb regulation supporting Che-1 as the cellular counterpart of DNA tumor virus oncoproteins.

Introduction

The Rb tumor suppressor plays a critical role in the control of cell proliferation and differentiation (Weinberg, 1995). Mutations of the *Rb* gene are present in a variety of human cancers, including retinoblastoma, osteosarcoma, and small-cell lung cancer (Weinberg, 1995; Riley et al., 1994). Rb and the related proteins p130 and p107 control cell cycle progression through their interactions with the E2F family of transcription factors (Dyson, 1998; Harbour and Dean, 2000). E2F is known to activate transcription of a number of genes required for the S phase of the cell cycle, including cyclin A, cyclin E, dihydrofolate reductase (*Dhfr*) and thymidyne kinase (*Tk*) (Dyson, 1998; Nevins et al., 1997). The interaction with E2F1 and the ability of Rb to prevent cell proliferation depends on its functional domains (residues 379–792), termed A and B motifs or the "pocket" region (Weinberg, 1995; De Gregori et al., 1997). The pocket can interact with E2F1 and several oncoproteins, such as SV40 Large T antigen, adenovirus E1A, and papilloma virus E7, that inactivate the pocket proteins by a direct physical association through a conserved Leu-X-Cys-X-Glu (LXCXE) sequence (De Caprio et al., 1988; Hu et al., 1990; Whyte et al., 1988). Inactivation of the E2F1 target gene transcription entails the recruitment of Rb by E2F1 to the promoters of these genes. This Rb recruitment masks the activation domain of E2F1 and prevents its interactions with the general transcription machinery (Hagemeier et al., 1993). Before cells proceed into the S phase of the cell cycle, Rb is phosphorylated at multiple sites by cyclin-dependent kinases (Cdks). This phosphorylation leads to Rb inactivation, release of E2F1, and subsequent transcriptional activation of its target genes (Dyson, 1998). Recently, an additional mechanism of Rb-mediated transcriptional repression of E2F1 target genes has been proposed (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998). Indeed, Rb was shown to recruit class I histone deacetylase proteins (HDAC 1–3) that can repress transcription by removing acetyl groups from histone octamers. This removal facilitates the condensation of nucleosomes into chromatin, which blocks access to transcription factors and leads to gene repression (Kingston and Narlikar, 1999; Woffle and Hayes, 1999; Kornberg and Lorch, 1999; Hassig et al.,

SIGNIFICANCE

The retinoblastoma gene (Rb) was identified over a decade ago as the first tumor suppressor. It plays a fundamental role in cellular regulation and is the target of tumorigenic alterations in many cell types. Although Rb mutations are not frequent, disruption of Rb activity occurs in the majority of human tumors through several mechanisms, including viral oncoprotein binding and deregulated Rb phosphorylation by mutations of cyclins or cyclin-dependent kinase inhibitors. In this paper we propose a novel mechanism of Rb inactivation through its binding with the human Che-1 protein that antagonizes the recruitment of HDAC1 on Rb, supporting Che-1 as the first cellular protein behaving as a viral oncoprotein.

1997). HDAC1 and HDAC2 bind to the pocket region of Rb through an LXCXE-like motif (i.e., IXCXE) (Magnaghi-Jaulin et al., 1998; Ferreira et al., 1998), whereas HDAC3 does not contain this motif, even though it binds Rb (Lai et al., 1999). During the cell cycle, phosphorylation of Rb by Cdks triggers sequential intramolecular interactions that progressively disrupt the Rb/HDAC1 connections by blocking G1 progression (Harbour et al., 1999). Moreover, peptides containing the LXCXE motif present in the viral SV40 large T or E7 oncoproteins displace HDAC1 from Rb (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998; Ferreira et al., 1998), indicating that viral transforming proteins can interrupt the Rb/HDAC1 interaction through Rb binding.

By means of a two-hybrid screening with the subunit 11 of the RNA polymerase II as bait, we have recently cloned the human *Che-1* gene (Fanciulli et al., 2000). The *Che-1* gene product is a nuclear protein involved in transcription control and is highly conserved during evolution (Lindfors et al., 2000; Thomas et al., 2000; Page et al., 1999; Kogel et al., 1999). We can demonstrate that Che-1 can form complexes with Rb by two distinct portions (Fanciulli et al., 2000), one of which is highly homologous to the SV40 Large T region required for Rb inactivation. Furthermore, the binding of Che-1 to Rb interferes with the Rb-mediated inhibition of E2F1 transcriptional activity in transient luciferase reporter assays and with the Rb-mediated inhibition of colony formation in the Rb-negative Saos-2 cells (Fanciulli et al., 2000). In this report, we describe a mechanism by which Che-1 exerts its inhibitory effect on Rb. Che-1 contacts the pocket region and removes HDAC1 from the Rb/E2F1 complex, affecting the activation of E2F-dependent promoters and cell proliferation.

Results

The pocket region of Rb is essential for Che-1/Rb interaction

We had previously shown that Che-1 binds Rb in vitro and in vivo (Fanciulli et al., 2000). Several cellular and viral proteins bind and inactivate Rb within the pocket region (Hu et al., 1990; Huang et al., 1990; Kaelin et al., 1990); thus, we investigated whether this region is also relevant for the association with Che-1. GST-Rb fusion proteins encompassing various domains (Figures 1A and 1C) were employed for in vitro binding experiments with ^{35}S-labeled Che-1 (Figure 1B). The entire wild-type pocket region bound efficiently to Che-1 (Figure 1B), whereas the mutant GST-Rb 379–928 C706F, which does not bind E1A, exhibited a diminished binding to Che-1. Comparable results were obtained with GST-Rb 379–928 Δex21, which lacks exon 21. GST-Rb 1–379 and GST-Rb 792–928, encoding for the N- and the C-terminal domains respectively, did not bind Che-1 at all. These results indicate that Che-1 binds to the Rb pocket region and that the pocket integrity is required for the interaction.

The pocket domains of the Rb family members p107 and p130 are similar to that of Rb. Thus, we analyzed whether these proteins were also able to interact with Che-1. Total cell extracts from NIH-3T3 fibroblasts were used for coimmunoprecipitation analyses. As shown in Figure 1D, Che-1 bound the three members of the pocket proteins family.

Che-1 competes with HDAC1 for Rb interaction through the 305–323 region

To repress transcription, Rb can recruit HDAC1 protein into the E2F complexes (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998). HDAC1 contacts Rb through a degenerate LXCXE motif, and this interaction can be disrupted by Rb phosphorylation or by DNA virus oncogenes, which also possess a LXCXE motif (Luo et al., 1998; Ferreira et al., 1998; Zhang et al., 2000). Though Che-1 does not have a LXCXE motif, it contains an amino acid sequence (aa 305–323) with a strong homology to the region that includes this motif (Figure 2A), suggesting that the interactions of Che-1 and HDAC1 with Rb might be mutually exclusive events. To test this hypothesis, ^{35}S-labeled HDAC1 protein was incubated with bacterial recombinant GST-Rb fusion protein in the absence or presence of increasing amounts of recombinant His-Che-1. HDAC1 was specifically retained on GST-Rb beads, but this interaction was gradually abolished in the presence of recombinant Che-1 (Figure 2B). To confirm this result, human Rb-negative Saos-2 cells were cotransfected with myc-HDAC1 and increasing amounts of myc-Che-1 expression vectors, and cell lysates were incubated with GST-Rb beads. As shown in Figure 2C, Che-1 strongly bound to Rb and decreased the Rb/HDAC1 interaction in a dose-dependent manner. The relative densitometric analyses showed that Che-1 displaces more than 70% of HDAC1 from GST-Rb. Consistent with these results, Che-1 and HDAC1 cotransfection in Saos-2 cells led to a marked decrease in the ability of GST-Rb to retain histone deacetylase activity from cell extracts (Figure 2D). To further characterize the Che-1-mediated displacement of HDAC1 from Rb, competition experiments were performed with synthetic peptides. The Che-1 region 305–323, the sequence homologous to HDAC1 (see Figure 2A), was employed together with a nonspecific control peptide. As shown in Figure 2E, Che-1 305–323 peptide markedly decreased HDAC1 binding in a dose-dependent manner. Indeed, the amount of HDAC1 associated to GST-Rb decreased to background levels, indicating that this peptide had disrupted the majority of Rb/HDAC1 complexes.

The results obtained raised the question of whether Che-1 binds Rb with sufficiently high affinity to justify HDAC1 displacement. Thus, use was made of synthetic peptides corresponding to "LXCXE" motifs of HDAC1, SV40 Large T, and the Che-1 305–323 region to specifically inhibit the Rb/HDAC1 interaction in GST pull-down assays. Consistent with the results of Magnaghi-Jaulin et al. (1998), Large T was a better inhibitor than the homologous HDAC1 sequence (Figure 2F). Significantly, the inhibitory ability of Che-1 peptide was similar to that of Large T (Figure 2F), indicating a higher affinity of Che-1 to Rb than HDAC1. These data indicate that the physical interaction between Che-1 and Rb can interfere with HDAC1 recruitment and suggest that Che-1 can function in the same manner as LXCXE-containing viral oncoproteins.

The relevance of the Che-1 305–323 region in HDAC1 displacement from Rb was further evaluated by employing a Che-1 mutant (ΔChe-1) with this region deleted. As previously shown, Che-1 contacts Rb at two distinct portions (Fanciulli et al., 2000); therefore, as expected, ΔChe-1 expressed in Saos-2 cells still retained the ability to bind GST-Rb. However, it showed a reduced capacity in competing with HDAC1 for Rb binding (Figure 2G). These results were confirmed when GST-Rb-associated histone deacetylase activity from the same cell extracts was analyzed (data not shown). Taken together, these results indicate that Che-1 competes with HDAC1 on the Rb binding site through its 305–323 region.

To evaluate the biological relevance of this finding, ΔChe-1 was engaged in the same assays we previously employed to

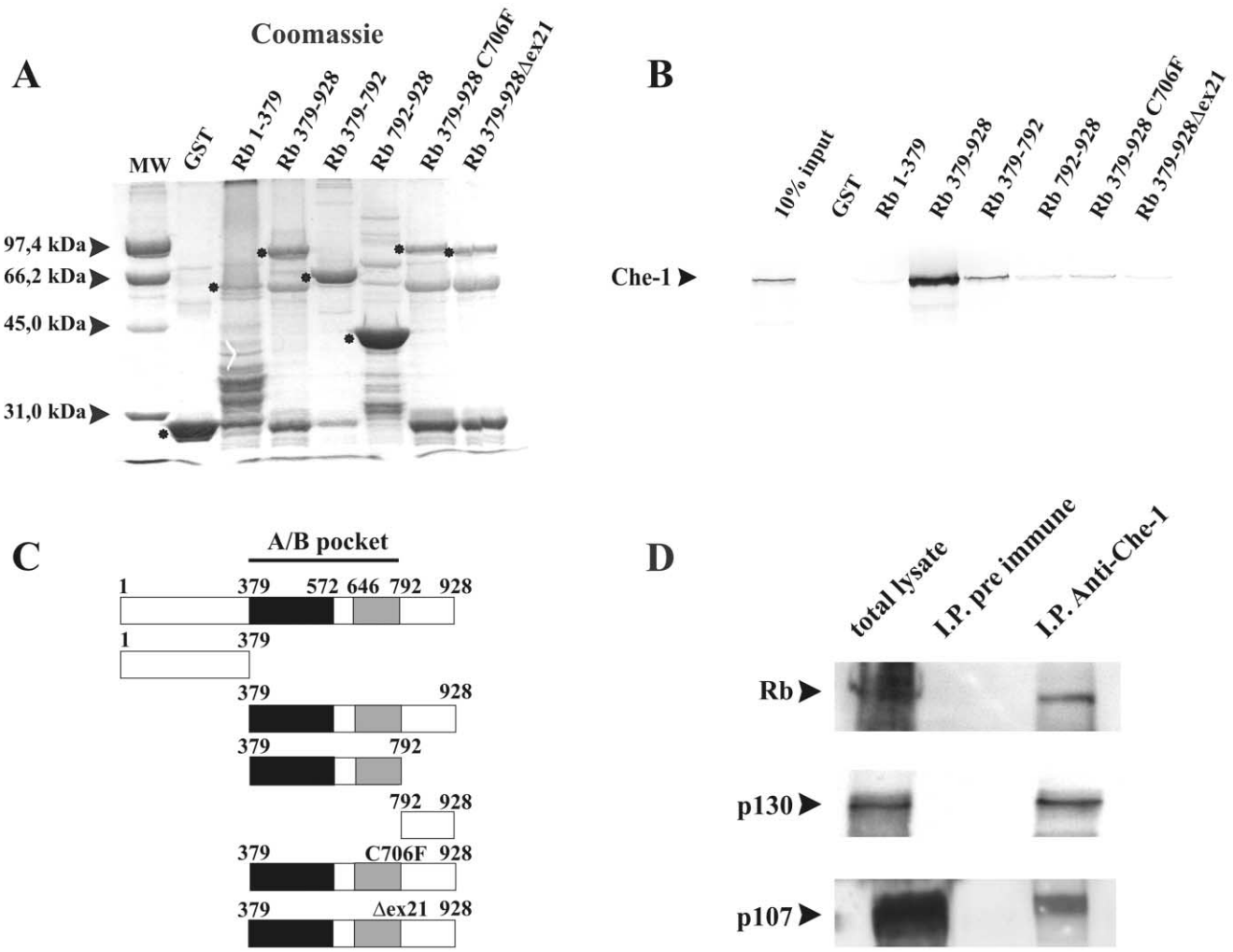


Figure 1. Rb contacts Che-1 through the pocket region

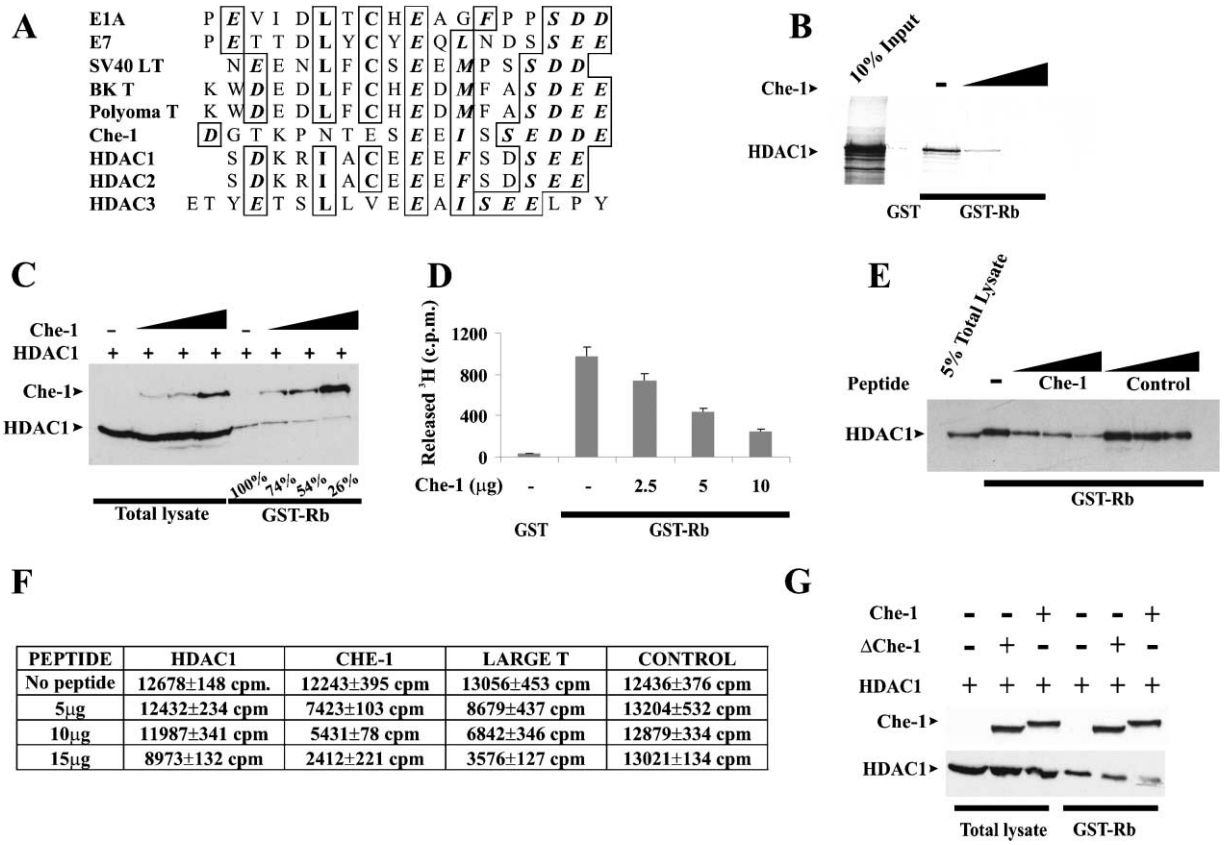
A: Coomassie blue stain of purified GST and GST-Rb proteins (as indicated by *). **B:** Labeled Che-1 was subjected to GST pull-down analysis using GST or GST-Rb fusion proteins beads indicated in **A**. **C:** Schematic representation of GST-Rb fusion proteins used for the analysis. Rb 379–928 C706F represents the mutation C→F at the amino acid 706, whereas Rb 379–928Δex21 represents deletion mutant lacking exon 21. **D:** Che-1 coimmunoprecipitates the three members of the pocket family. Cell lysates from NIH 3T3 fibroblasts were immunoprecipitated with anti-Che-1 polyclonal antibody or preimmune serum and analyzed by Western blot using specific anti-Rb, anti-p130, and p107 polyclonal antibodies as described in Experimental Procedures.

study the Che-1 activity on Rb (Fanciulli et al., 2000). In contrast to wild-type Che-1, ΔChe-1 expression in Saos-2 cells did not counteract the Rb inhibitory effect on E2F1 transacting function on the *Dhfr* promoter in transient luciferase reporter expression assays (Supplemental Figure S1A at http://www.cancercell.org/cgi/content/full/2/5/387/DC1). Moreover, ΔChe-1 did not interfere with the growth suppression activity of Rb in colony forming assays (Supplemental Figures S1A and S1C), suggesting that the Che-1 305–323 region and its activity on HDAC1 recruitment by Rb represents one possible mechanism through which Che-1 inhibits Rb functions.

Che-1 displaces the HDAC1 associated to E2F target gene promoters in vivo

To evaluate whether Che-1 can decrease the level of HDAC1 at the E2F binding sites of E2F-responsive gene promoters in vivo, Saos-2 cells were transfected either with Rb or with Rb and Che-1 (Figure 3A), and chromatin immunoprecipitation (ChIP) assays were performed by immunoprecipitating crosslinked genomic DNA with an anti-HDAC1 specific antibody. Immunoprecipitated DNA was analyzed by PCR utilizing promoter-specific primers that encompass the E2F binding sites (see Experimental Procedures) under conditions of linear amplification (Figure 3B). As shown in Figure 3C, HDAC1 levels were low or undetectable on the E2F target *cyclin A, Dhfr*, and *Tk* gene promoters and increased after Rb overexpression, confirming the HDAC recruitment by this tumor suppressor, but were low or undetectable in cells cotransfected with Rb and Che-1. Either Rb or Rb and Che-1 transfection did not affect the E2F-unresponsive *Gapdh* promoter, used as negative control.

It has been demonstrated that recruitment of HDAC1 by Rb to gene promoters decreases their level of histone acetylation (Luo et al., 1998). Thus, we evaluated whether Che-1 mediated displacement of HDAC1 was associated with the presence of



PEPTIDE	HDAC1	CHE-1	LARGE T	CONTROL
No peptide	12678±148 cpm.	12243±395 cpm	13056±453 cpm	12436±376 cpm
5μg	12432±234 cpm	7423±103 cpm	8679±437 cpm	13204±532 cpm
10μg	11987±341 cpm	5431±78 cpm	6842±346 cpm	12879±334 cpm
15μg	8973±132 cpm	2412±221 cpm	3576±127 cpm	13021±134 cpm

Figure 2. Che-1 competes with HDAC1 for Rb binding

A: Sequence alignment of Che-1 and LXCXE-containing proteins. Amino acid sequences spanning the LXCXE motif of 5 viral proteins and HDAC1–3. Conserved LXCXE motifs are in bold and boxed. Conserved acid residues N-terminal to the LXCXE motif, and conserved hydrophobic and acid residues C-terminal to the motif, are in bold, italic, and boxed. **B:** Labeled HDAC1 was subjected to GST pull-down analysis using GST or GST-Rb beads with or without (−) 5 or 10 μg of recombinant histidine-tagged Che-1 (amount indicated by the height of the black triangle). **C:** In vitro binding analysis of GST-Rb fusion protein with lysates from Saos-2 cells transiently transfected with myc-tagged HDAC1 (5 μg) or with HDAC1 and 1, 2, or 5 μg of myc-tagged Che-1 (amount indicated by the height of the black triangle). The membrane was analyzed by Western blot using specific monoclonal antibody anti-myc. Numbers show percentages of HDAC1 binding to GST-Rb deduced by densitometric analysis of the blot. **D:** Che-1 reduces histone deacetylase activity associated with Rb. Column diagram shows deacetylase activity recovered by performing experiments as described in **C**. Data are presented as the mean ± SD from three independent experiments performed in duplicate. **E:** Lysates from Saos-2 cells transiently transfected with myc-tagged HDAC1 were subjected to a pull-down analysis using GST-Rb beads. Before GST pull-down analysis, beads were incubated with or without (−) 5, 10, or 15 μg of Che-1 or control peptide (amount indicated by the height of the blank triangle). **F:** Inhibition of Rb/HDAC1 interaction by an excess of "Rb-interacting motifs": labeled HADC1 (5×10^5 cpm) was subjected to pull-down analysis using GST-Rb beads in the absence or in the presence of increasing amounts (5, 10, 15 μg) of the indicated synthetic peptides described in the Experimental Procedures. The radioactivity associated with pellet of each sample was evaluated by counting on an open channel, and the value of the pellet of each sample was corrected by subtracting the count of a background sample (GST alone). Data are presented as the mean ± SD from three independent experiments performed in duplicate. **G:** ΔChe-1 does not displace HDAC1 from Rb. In vitro binding analysis of GST-Rb fusion protein with lysates from Saos-2 cells transiently transfected with myc-tagged HDAC1 alone, HDAC1 and myc-tagged Che-1, or HDAC1 and myc-tagged ΔChe-1 lacking region 305–323. The membrane was analyzed by Western blot using specific monoclonal antibody anti-myc.

acetylated histones on in vivo promoters. ChIP was performed by immunoprecipitating the DNA with an anti-acetylated histone H3 antibody. Control vector-transfected Saos-2 cells exhibited high levels of acetylated histone H3 in association with the E2F binding sites of the analyzed promoters (Figure 3D). Overexpression of Rb decreased acetylated histone H3 association with the same promoters, but not with the *Gapdh* unrelated promoter. In contrast, coexpression of Rb and Che-1 completely abolished the effects of Rb (Figure 3D). These results are consistent with the hypothesis that Che-1 efficiently competes with HDAC1 for Rb binding.

Finally, to evaluate whether the results observed by performing transcriptional analysis (Supplemental Figures S2A–S2C at http://www.cancercell.org/cgi/content/full/2/5/387/DC1) and ChIP correspond to a real regulation of the expression of these genes in vivo, a semiquantitative reverse transcriptase-PCR was carried out. The expression levels of *Dhfr*, *Tk*, and *Cyclin A* mRNA were measured in Saos-2 cells upon overexpression of Rb or Rb and Che-1. Consistent with the previous analysis, these genes were specifically downregulated following Rb overexpression, but not upon coexpression of Rb and Che-1 (Figure 3E).

Exogenous Che-1-inducible expression affects E2F activity and cell proliferation

The experiments described above were carried out by using protein overexpression in transient assays. To test whether

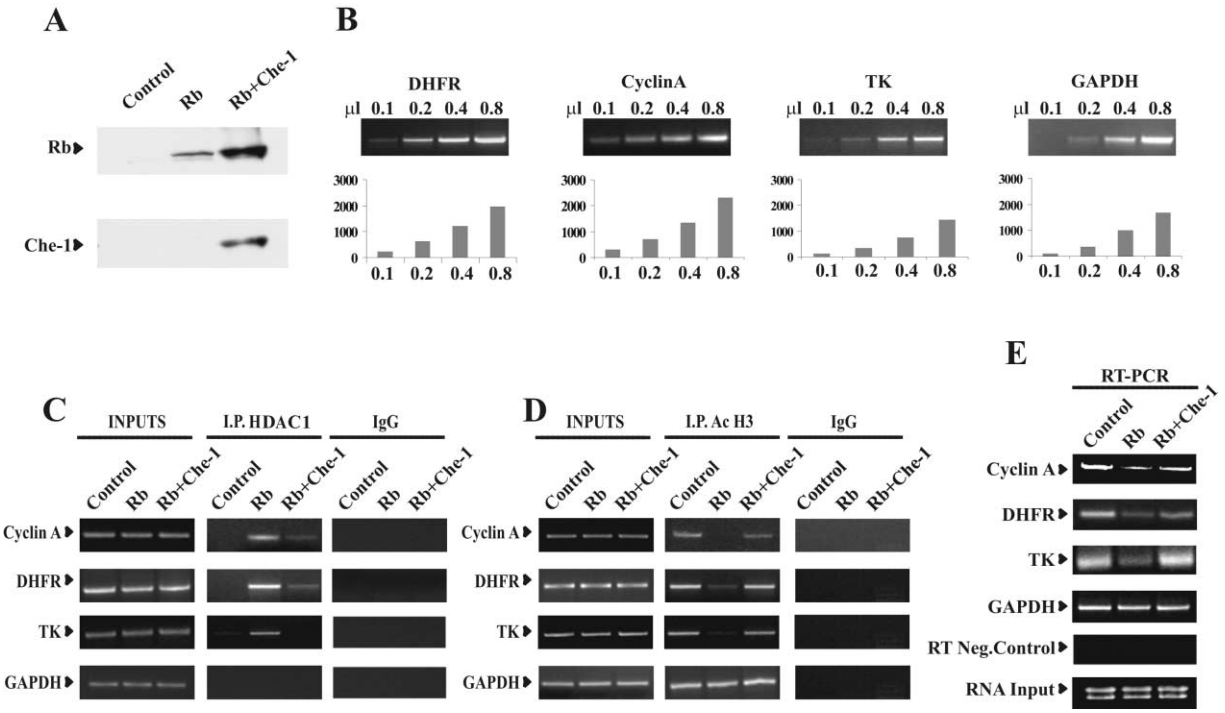


Figure 3. Che-1 displaces the HDAC1 from E2F target gene promoters in vivo

A: Lysates from Saos-2 cells transiently transfected with empty vector, pCMV Rb or pCMV Rb, and myc-Che-1, respectively, were subjected to Western blot analysis with anti-Rb and anti-myc antibodies to verify protein expression. **B**, **C**, and **D:** Lysates from Saos-2 cells transfected as described in **A** were subjected to chromatin immunoprecipitation using specific polyclonal antibodies anti-HDAC1 (**C**) or anti-acetylated histone H3 (**D**). **B:** Increasing amounts of input samples (0.1, 0.2, 0.4, 0.8 μl) were used as template in PCR amplifications performed using primers specific for the different promoters including E2F binding sites. "DHFR" indicates dihydrofolate reductase, "TK" indicates thymidine kinase, and "GAPDH" indicates glyceraldehyde-3-phosphate dehydrogenase. Immunoprecipitates from each sample were analyzed by PCR, and a sample representing linear amplification (0.2–0.4 μl) of the total input chromatin (Input) was included in the PCRs as a control. Additional control included a precipitation performed with no specific IgGs. **E:** RNA from Saos-2 cells transiently transfected as described in **A** was isolated. Equal amounts of RNA (RNA input) were analyzed by RT-PCR (25 cycles) for the expression of cyclin A, *Dhfr*, *Tk*, and *Gapdh*. The RT control lanes represent RT-PCR in the absence of reverse transcription.

physiological levels of Che-1 can affect HDAC1/Rb binding on E2F-dependent promoters, we generated NIH-3T3 cells that conditionally express human Che-1 in a tetracycline-regulated way. Human and murine Che-1 are highly homologous, but the human gene possesses an extra exon coding for aa 146–177 that results in a 3 kDa larger protein (Thomas et al., 2000). As shown in Figure 4A, a 24 hr treatment with the tetracycline analog doxycycline (Dox) induced an expression of the exogenous human Che-1 at levels comparable to the endogenous murine Che-1. Interestingly, exogenous Che-1 downregulated the level of the endogenous protein, suggesting the existence of a feedback mechanism for the tight regulation of Che-1. The expression of exogenous Che-1 resulted in an increase of Che-1 levels at E2F-dependent promoters with a parallel decrease of HDAC1 levels as measured by ChIP (Figure 4B), thus confirming the ability of Che-1 to displace HDAC1 from Rb/E2F1 complex. Consistently, Che-1 induction affected the regulation of E2F-dependent transcription of *Dhfr*, *Tk*, and *cyclin E* promoters (Figure 4C). These findings, underscoring an involvement of Che-1 in the regulation of E2F-dependent transcription, prompted us to analyze the growth characteristics of induced and uninduced cells. Figure 4D shows that NIH-3T3 cells expressing the human Che-1 protein exhibited an increase rate of proliferation. Furthermore, Dox treatment after serum starvation accelerated the cell cycle reentry, as evaluated by simultaneous flow cytometric analyses for DNA synthesis (BrdUrd incorporation) and DNA content (propidium iodide staining) (Figure 4E). These results were confirmed by immunoblots for the levels of cyclins A and D1 (Figure 4F). Taken together, these results strongly indicate that Che-1 is implicated in the regulation of E2F activity and entry into S phase of the cell cycle.

Che-1 association with E2F target gene promoters is cell cycle-regulated

We have shown that Che-1 is able to displace HDAC1 from Rb, and this phenomenon can account, at least in part, for the effects of Che-1 on Rb inhibition of colony formation in tumor cells. Next, we evaluated whether and when this displacement takes place within the cells on the endogenous proteins. To this aim, NIH-3T3 cells were synchronized by 60 hr serum starvation, harvested at 0, 8, 12, and 16 hr after serum stimulation, and analyzed for DNA content to confirm cell cycle reentry (Figure 5A). Western blot analysis showed that Che-1 expression is regulated during cell proliferation. Indeed, the levels of Che-1 increased at the 12 hr time point (Figure 5B), corresponding to the G_1/S transition (Figure 5A). Consistent with these results, coimmunoprecipitation of Che-1 with anti-Rb antibody demonstrated increased amounts of Che-1 associated to Rb during

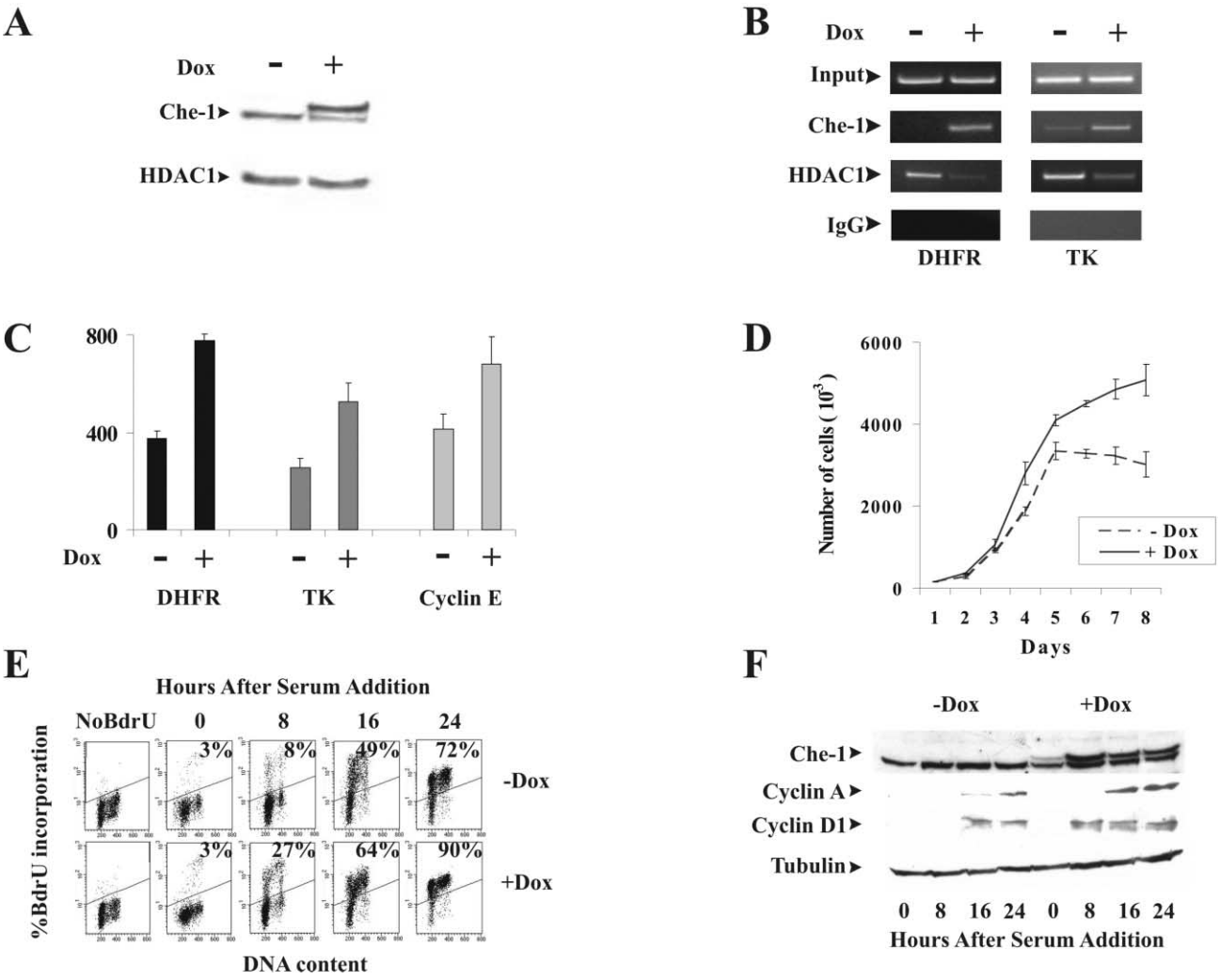


Figure 4. Exogenous Che-1-inducible expression affects E2F activity and cell proliferation and accelerates S phase entry

A: Lysates from NIH 3T3 cells stably transfected with inducible human Che-1, uninduced (−Dox), and induced (+Dox) with the tetracycline analog doxycycline (Dox), respectively, were subjected to Western blot analysis with anti-Che-1 and anti-HDAC1 antibodies to verify protein expression. **B:** Uninduced and induced NIH 3T3 cells were subjected to chromatin immunoprecipitation using specific polyclonal antibodies anti-HDAC1 or anti-Che-1. Immunoprecipitates from each sample were analyzed by PCR using primers specific for the different promoters. As a control, a sample representing linear amplification (see Figure 5D) of the total input chromatin (Input) was included in the PCRs. Additional control included a precipitation lacking specific antibodies (IgG). **C:** Che-1-inducible NIH 3T3 cells were transiently transfected with 1 μg of the indicated luciferase reporters and treated (+Dox) or not (−Dox) with doxycycline for 48 hr. Data are presented as the mean ± SD from three independent experiments performed in duplicate. **D:** Proliferation curves of −Dox and +Dox NIH 3T3 cells. The curves represent the average of three experiments with standard deviations (error bars). **E:** Simultaneous flow cytometric analyses for DNA synthesis (BrdU incorporation) and DNA content (propidium iodide staining) of −Dox and +Dox NIH 3T3 cells were performed at the indicated times following the end of starvation. Dox was added at the end of starvation. Percentages represent BrdU-incorporating cells. **F:** Immunoblot analysis of protein lysates prepared after the readdition of serum to starved cells. Blots contain total protein lysates from −Dox and +Dox NIH 3T3 cells stimulated with serum for indicated times.

this period (Figure 5B). Since Che-1 promoter characterization provided evidence that Che-1 transcription does not change during the cell cycle (our unpublished data), it is possible that posttranscriptional modification of Che-1 can regulate its accumulation. Che-1 amino-acidic sequence showed several putative phosphorylation sites for protein kinases CKII, PKA, and PKC (Page et al., 1999); we therefore investigated whether Che-1 is phosphorylated during the cell cycle. Measurement of ^{32}P incorporation at different times after serum stimulation showed that Che-1 is hyperphosphorylated during G_1/S transition (Figure 5C), thus suggesting that phosphorylation can mediate Che-1 stability.

Next, we analyzed the presence of Che-1 on E2F target gene promoters during cell cycle progression. As shown in Figure 5E, ChIP experiments performed under conditions of linear amplification (Figure 5D) revealed that the amount of Che-1 physically associated with the E2F binding sites of *Dhfr, cyclin A*, and *Tk* promoters drastically increased during cell cycle progression. A reciprocal result was observed by evaluating the amount of HDAC1 on the same promoters. Indeed, as reported by Ferreira et al. (2001a), HDAC1 expression does not change during the cell cycle, but HDAC1 was stably bound to E2F binding sites during G_0 and early G_1 and gradually released during G_1/S transition (Figure 5E).

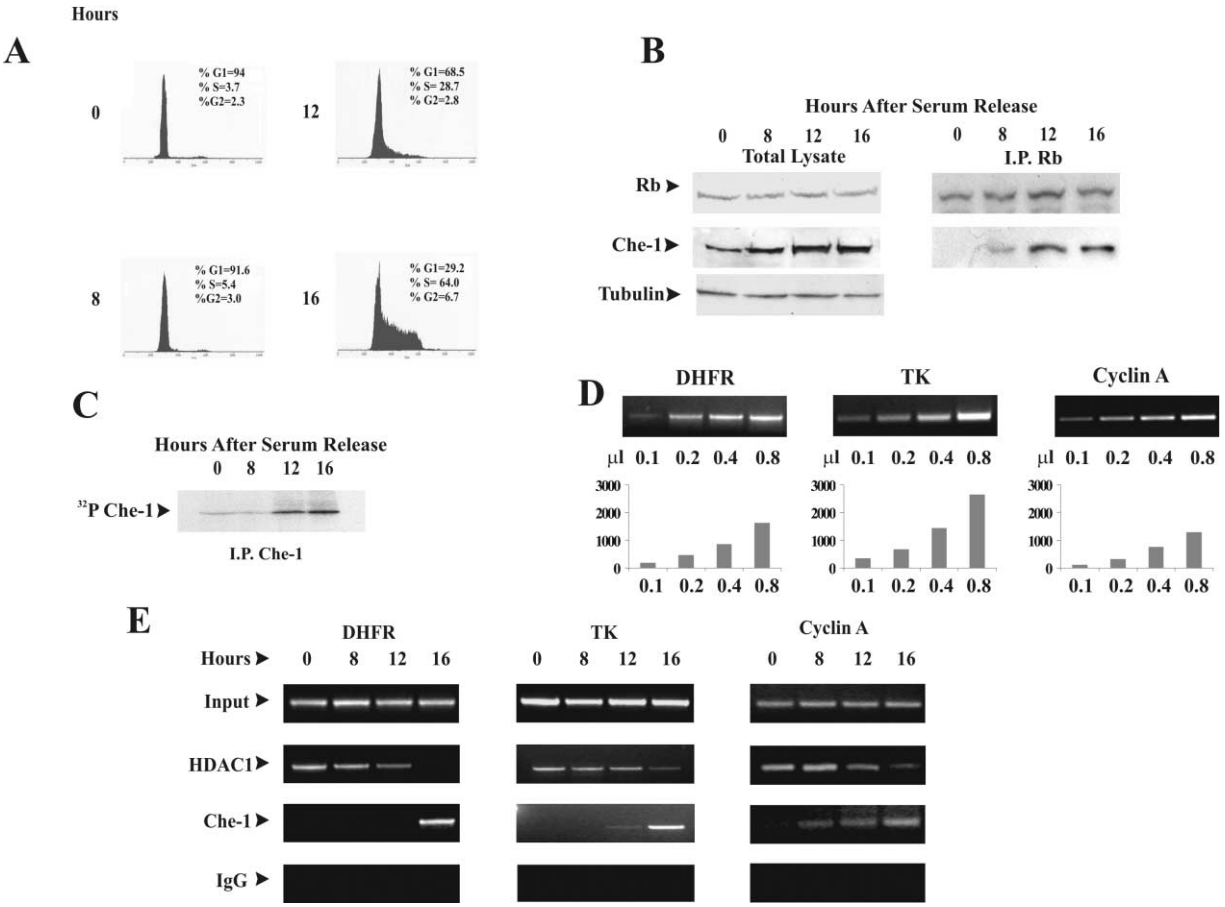


Figure 5. Che-1 binding to E2F target promoters is regulated during cell cycle

A: Flow cytometry cell cycle analysis of NIH 3T3 cells subjected to synchronization by serum starvation and stimulation. G_1, S, and G_2/M populations are indicated as percentages of the whole population. **B:** Synchronized NIH 3T3 cells were lysated at the indicated times, and whole cell extracts were immunoprecipitated with anti-Rb antibody and analyzed by Western blot using the indicated antibodies. **C:** Autoradiography of immunoprecipitates with anti-Che-1 antibody from synchronized NIH 3T3 cells incubated with ^{32}P inorganic phosphate and lysated at the indicated times. **D** and **E:** Synchronized NIH 3T3 cells were subjected to chromatin immunoprecipitation using specific polyclonal antibodies anti-HDAC1 or anti-Che-1. Increasing amounts of input samples (0.1, 0.2, 0.4, 0.8 µl) amplified using primers specific for the different promoters (**D**). Immunoprecipitates from each sample were analyzed by PCR (**E**). As a control, a sample representing linear amplification of the total input chromatin (Input) was included in the PCRs. Additional control included a precipitation lacking specific antibodies (IgG).

Altogether, these results indicate that, in physiological conditions, a competition between Che-1 and HDAC1 for Rb binding can occur, and this competition appears to be cell cycle-related.

E2F activity requires Che-1 in normal cells

The results described above show that exogenous Che-1 expression at physiological levels increases E2F activity and cell proliferation. Conversely, inhibition of Che-1 should lead to a decreased E2F-dependent transcription. To test this hypothesis, the small interfering RNA (siRNA) technique (Elbashir et al., 2001) was used to reduce Che-1 expression in HFF human fibroblasts. Cells transduced with double-stranded (dsRNA) oligonucleotides to Che-1 (siChe-1) showed a specific reduction of Che-1 expression compared to control, green fluorescent protein dsRNA (siGFP)-transduced cells (Figure 6A). Che-1 reduction resulted in a dramatic increase of Rb/HDAC1 association on DHFR promoter (Figure 6B), a decrease of E2F-dependent transcription (Figure 6C), and cell proliferation (Figure 6D). Furthermore, as expected, inhibition of Che-1 expression (Figure 6E) did not change its presence on the chromatin (Figure 6F), and did not affect either E2F activity or cell proliferation in cells lacking functional pocket proteins, such as 293 cells (Figures 6G and 6H). These data strongly indicate a requirement of Che-1 for E2F activity in normal proliferation, and this requirement is mediated by the pocket proteins.

Che-1 requires Rb but not RNA pol II to recruit E2F-responsive gene promoters

We have previously shown that Che-1 binds to subunit 11 of human RNA pol II (Fanciulli et al., 2000). It is possible that Che-1 may associate with E2F-responsive promoters through the binding to pol II and not to pocket proteins. Therefore, to test whether functional pocket proteins are required for E2F promoter binding, 293 cells were transfected either with Rb or empty vector (Figure 7A), and ChIP assays were performed with anti-Rb and anti-Che-1 specific antibodies. As shown in Figure 7B, Che-1 was almost undetectable on *Dhfr* promoter in control cells, but

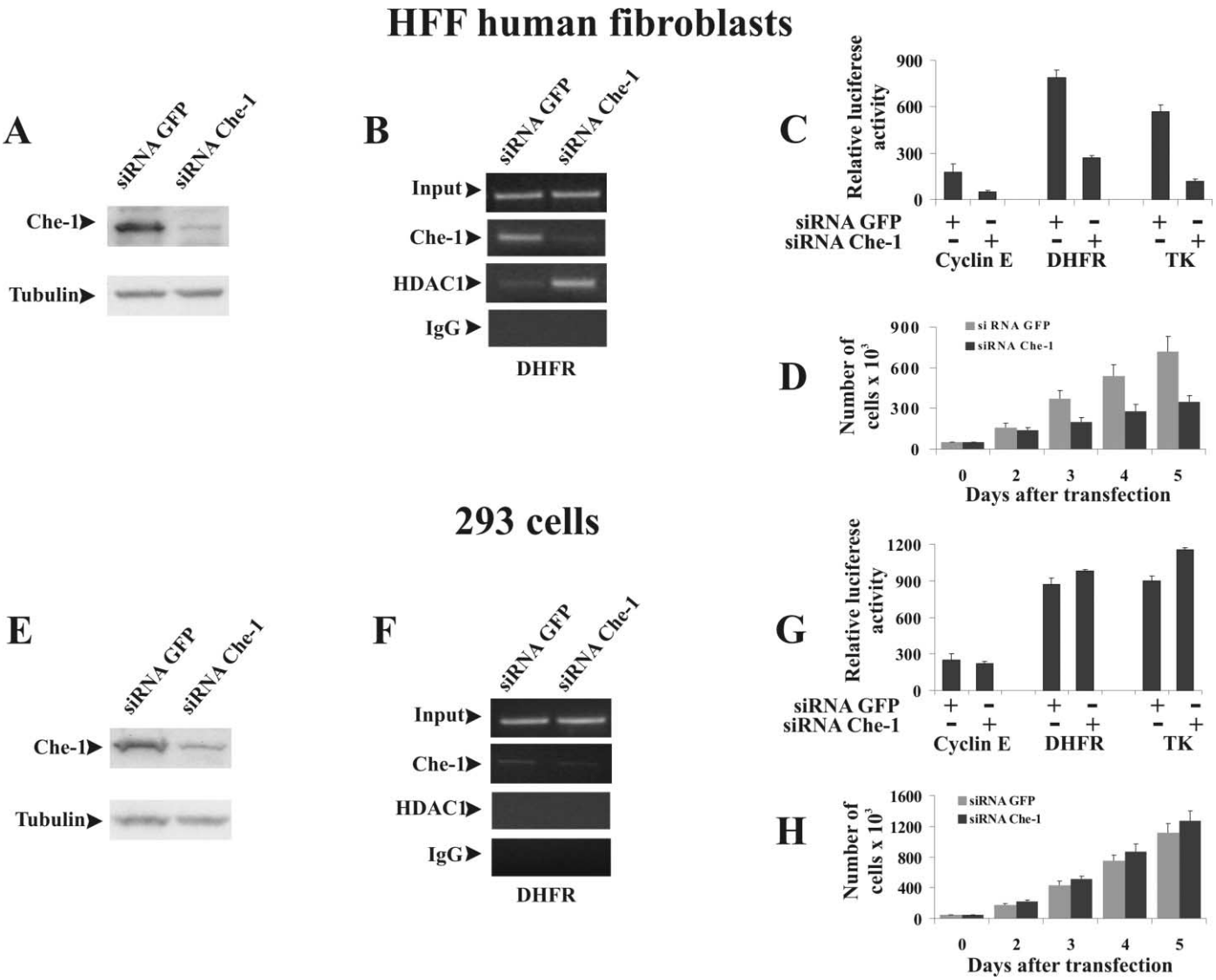


Figure 6. E2F activity requires Che-1 in normal cells

A and **E:** Silencing of Che-1 in HFF human fibroblasts (**A**) and 293 cells (**E**). Western blots of cells transfected with green fluorescent protein (GFP) siRNA or Che-1 siRNA probed with anti-Che-1 antibody. The blots were stripped and reprobed with anti-α-tubulin antibody. **B** and **F:** HFF human fibroblasts (**B**) and 293 cells (**F**) were transiently transfected with GFP siRNA or Che-1 siRNA and subjected to chromatin immunoprecipitation using specific antibodies anti-HDAC1 or anti-Che-1. Immunoprecipitates from each sample were analyzed by PCR using primers specific for the E2F binding sites of human *Dhfr* gene promoter. As a control, a sample representing linear amplification (see Figure 3B) of the total input chromatin (Input) was included in the PCRs. Additional control included a precipitation lacking specific antibodies (IgG). **C** and **G:** HFF human fibroblasts (**C**) and 293 cells (**G**) were transiently transfected with GFP siRNA or Che-1 siRNA and 1 μg of the indicated luciferase reporters. Data are presented as the mean ± SD from three independent experiments performed in duplicate. **D** and **H:** Cell proliferation analyses of HFF human fibroblasts (**D**) and 293 cells (**H**) transiently transfected with GFP siRNA or Che-1 siRNA. Data are presented as the mean ± SD from three independent experiments performed in duplicate.

became present in Rb overexpressing cells, indicating that the presence of Rb mediates Che-1 binding to E2F-dependent promoters. To evaluate whether Che-1 could be recruited on these promoters through its association with pol II, we generated a new Che-1 mutant, Δ-zip, lacking the whole leucin zipper (aa 270–298), the region responsible for RNA pol II binding (Fanciulli et al., 2000). When transfected into NIH-3T3 cells, this mutant was unable to bind pol II (Figure 7C) but still retained the ability to displace HDAC1 when recruited on a E2F-responsive promoter, (Figure 7D) and to counteract Rb activity when cotransfected in Saos-2 cells (Figure 7E). Surprisingly, although the Che-1 mutant ΔChe-1 bound GST-Rb in vitro, it was not able to associate with Rb on this promoter (Figure 7D) and did not affect HDAC1 recruitment (Figure 7D) and Rb activity (Figure 7E). These results indicate that Che-1 requires the pocket proteins, rather than pol II, to bind E2F target gene promoters.

Overexpression of Che-1 induces quiescent cells to enter S phase

Previous reports have shown that E2F1 overexpression can induce DNA synthesis in the absence of serum in otherwise quiescent cells (Johnson et al., 1993; Kowalik et al., 1995). Thus,

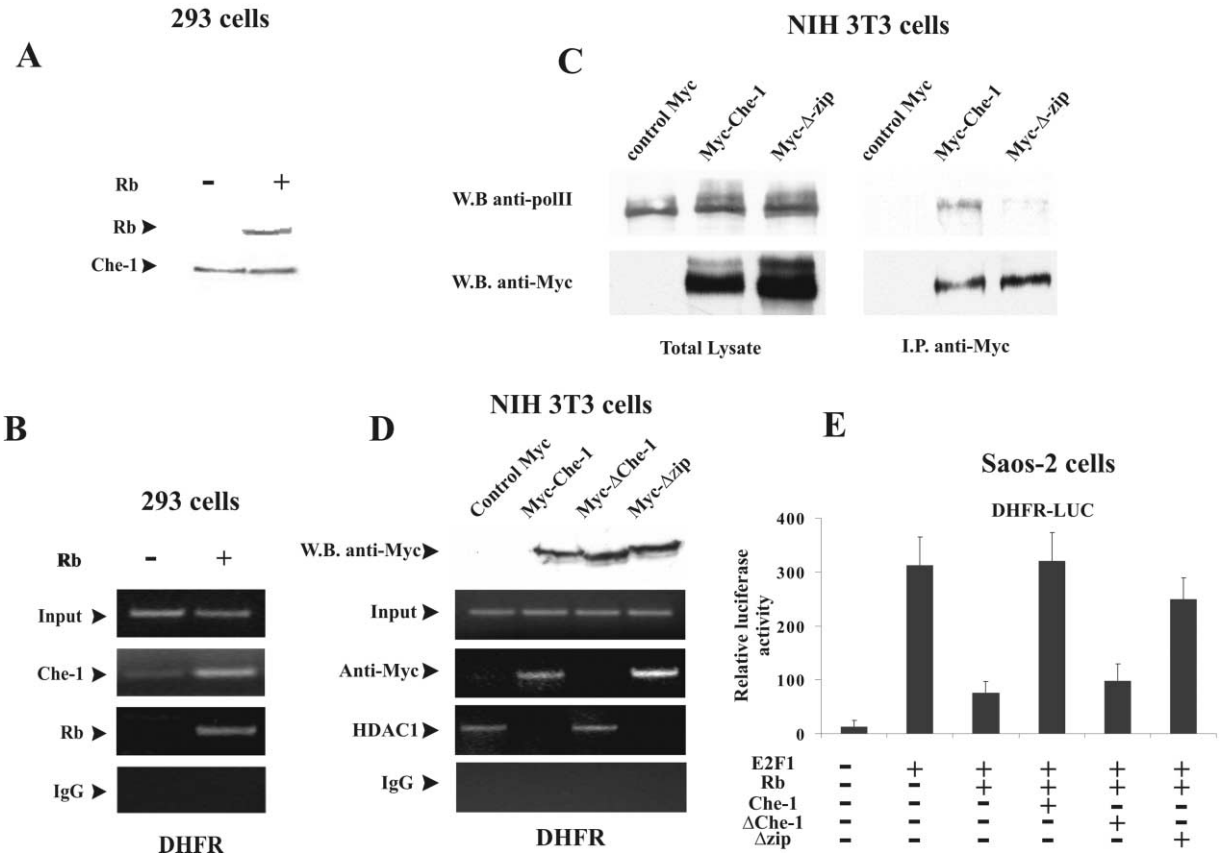


Figure 7. Che-1 requires Rb but not RNA pol II to recruit E2F-responsive gene promoters

A: Lysates from 293 cells transiently transfected with empty vector or pCMV Rb, respectively, were subjected to Western blot analysis with anti-Rb and anti-Che-1 antibodies to verify protein expression. **B:** Che-1 requires Rb to bind E2F target gene promoters. Lysates from 293 cells transfected as described in **A** were subjected to chromatin immunoprecipitation using specific antibodies anti-Rb or anti-Che-1. Immunoprecipitates from each sample were analyzed by PCR using primers specific for the E2F binding sites of human *Dhfr* gene promoter. As a control, a sample representing linear amplification (see Figure 3B) of the total input chromatin (Input) was included in the PCRs. Additional control included a precipitation lacking specific antibodies (IgG). **C:** NIH 3T3 cells transiently transfected with the indicated expression vectors were lysated, immunoprecipitated with anti-myc antibody, and analyzed by Western blot using the indicated antibodies. **D:** Che-1 does not require RNA pol II to bind E2F-target gene promoters. Lysates from NIH 3T3 cells transfected with the indicated expression vectors were subjected to chromatin immunoprecipitation using anti-HDAC1 and anti-myc antibodies. Immunoprecipitates from each sample were analyzed by PCR using primers specific for the E2F binding sites of murine *Dhfr* gene promoter. Controls were the same described in **B**. **E:** Δ-zip, but not ΔChe-1, inhibits Rb. Saos-2 cells were transiently transfected with 1 μg *Dhfr*-luciferase reporter and, where indicated, 100 ng pCMV E2F1 expression vector, 200 ng pCMV Rb, and 1 μg of myc-Che-1, myc-ΔChe-1, or myc-Δ-zip, respectively. The total amount of transfected DNA was normalized with pCMV empty vector (−). Data are presented as the mean ± SD from three independent experiments performed in duplicate.

we evaluated whether comparable effects could be obtained by overexpressing Che-1 or its mutants in serum-starved cells. NIH-3T3 cells were made quiescent as described above and transiently transfected with either myc-tagged Che-1, ΔChe-1, Δ-zip, or empty vector as negative control. The addition of serum to empty vector-transfected cells was used as positive control (Figure 8A). After 24 hr from transfection, BrdU was added to the media, and cells were incubated for additional 6 hr before fixation and immunofluorescence staining with anti-BrdU antibody (Figure 8B). Che-1 overexpression substantially increased the fraction BrdU positive cells (Figure 8C), indicating that Che-1 can stimulate DNA synthesis. Similar results were obtained with Δ-zip, but not with ΔChe-1 (Figure 8C) deletion mutants. Thus, Che-1 plays an important role in progression into S phase, and this capacity is associated with HDAC1 displacement from Rb (Figure 8D).

Discussion

We had previously provided evidence that a novel human protein, Che-1, interacts with Rb protein, and this binding affects the growth-suppressing function of Rb by relieving its inhibition of E2F1 transcriptional activity (Fanciulli et al., 2000). One of the important biological functions of the Rb family is to regulate the expression of genes required for cell cycle progression and DNA synthesis. Rb family members utilize the pocket domain to recruit HDAC activity and to repress transcription of genes in early G_1 phase, or when cells exit from the cell cycle (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998; Ferreira et al., 1998; Stiegler et al., 1998). During G_1, Rb phosphorylation by several different cyclin-Cdk combinations leads to successive intramolecular interactions that block HDAC binding to the pocket domain and transcriptional repression (Harbour et al., 1999). Similarly, interaction of Rb family members with viral transforming

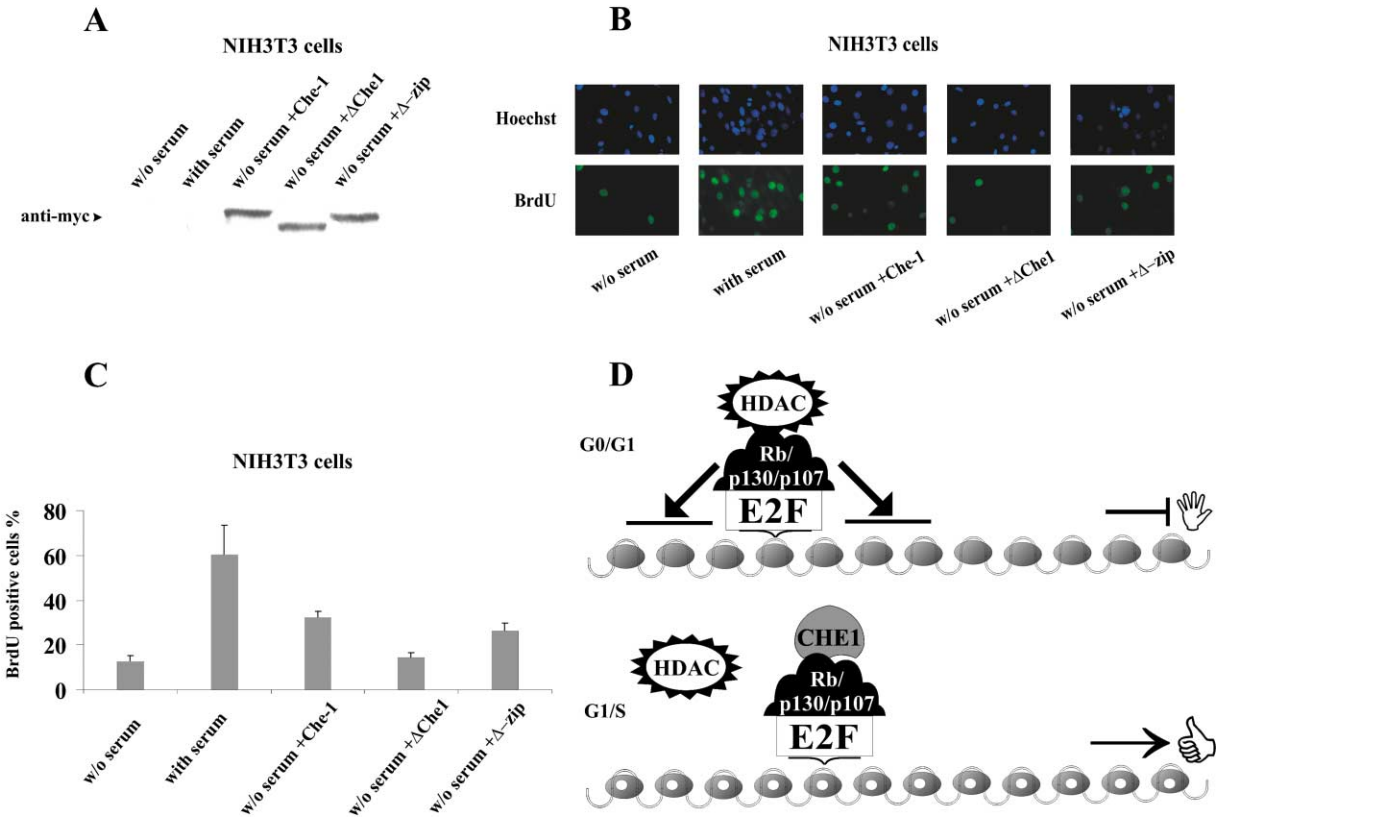


Figure 8. Che-1 induces DNA synthesis in serum-starved cells

A: Lysates from NIH 3T3 serum-starved cells transiently transfected with empty vector (w/o serum) or with the indicated myc-Che-1 constructs were subjected to Western blot analysis with anti-myc antibody to verify Che-1 expression. **B:** NIH 3T3 serum starved cells transiently transfected as in **A** visualized for BrdU incorporation by indirect immunofluorescence performed using an anti-BrdU monoclonal antibody. **C:** Quantification of BrdU-positive NIH 3T3 cells. Data are presented as the mean ± SD from three independent experiments. **D:** Model to explain Che-1-mediated Rb repression. Histone deacetylase is recruited during G_0/G_1 phases to promoters that contain E2F/pocket protein complexes through an interaction with pocket proteins. Histone deacetylase then modifies the histones proximal to the promoter, causing transcriptional silencing. During G_1/S progression, Che-1 displaces HDAC1 from pocket proteins, allowing transcriptional activation.

proteins results in loss of HDAC binding (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998). Here, we present evidence that the binding of Che-1 to Rb affects the growth suppression function of Rb by interfering with HDAC1 recruitment by Rb, and that Che-1 overexpression is capable of inducing S phase in otherwise quiescent cells. In this way, Che-1 appears to behave like DNA virus oncoproteins.

Che-1 is a ubiquitously expressed nuclear protein (Fanciulli et al., 2000; Thomas et al., 2000). The studies performed on this protein and its homologs in rat and mice strongly support the idea that Che-1 is involved in the regulation of gene transcription and cell proliferation. Che-1 directly interacts with Rb and the core of RNA pol II (Fanciulli et al., 2000), it is downregulated by TGF-β (Lindfors et al., 2000), and when fused to the GAL4 DNA binding domain, it transactivates GAL4 target reporters (Page et al., 1999). The knockout of Traube, the murine Che-1, blocks early embryonic development and reduces cell proliferation (Thomas et al., 2000). Che-1 contains a leucine zipper structure, several potential phosphorylation sites for different kinases, and three nuclear receptor binding LXXLL consensus sequences (Heery et al., 1997; Torchia et al., 1997). Although Che-1 does not posses a LXCXE motif, one of the two regions of interaction with Rb (aa 305–323) contains features that are conserved among viral Rb binding proteins and HDAC1-3, with importance predicted by crystallographic data and supported by peptide competition experiments (Jones et al., 1990; Lee et al., 1998). These features suggest that Che-1 would bind Rb with high affinity and could compete with other Rb binding proteins.

In order to analyze the pattern of Che-1 expression during the cell cycle, NIH-3T3 cells were synchronized by serum starvation. We found that during G_1/S transition, Che-1 is phosphorylated, and its expression levels increased. Importantly, this augment of expression produced a larger amount of Che-1 bound to Rb (Figure 5B), suggesting that these modifications can regulate HDAC1 displacement. Consistent with these results, the amount of Che-1 associated with E2F responsive gene promoters increased as cells progressed through the S phase, whereas a reciprocal result was obtained when the presence of HDAC1 on the same promoters was assessed. This supports the hypothesis that binding of HDAC1 and Che-1 to Rb are mutually exclusive events, regulated during the cell cycle progression, and that the negative role exerted by HDAC1 on the transcription of E2F-responsive genes can be counteracted by Che-1. As

shown Figure 5E, the association of Che-1 with cyclin A promoter is detectable during G_1 phase and increases during G_1/S transition, consistent with a gradual disappearance of HDAC1 on this promoter. Cyclin A is an important protein involved in the cell cycle progression, and its expression is tightly regulated during S phase; therefore, it is also possible that besides the presence of Che-1, some other factor might be required for the expression of this protein.

Two studies have recently demonstrated that E2F target genes are bound by distinct E2F-pocket protein complexes that change as cells progress through the cell cycle (Wells et al., 2000; Takahashi et al., 2000). Thus, it is possible that the association of HDAC1 and Che-1 detected on these promoters may involve all three members of the pocket family and not only Rb.

Overexpression of Che-1 into serum-starved NIH-3T3 cells induced S phase entry, and viewed from the perspective of a protein that can activate E2F1 by overcoming Rb block, an induction of cellular DNA synthetic capacity in quiescent cells is a rational effect. Nevertheless, we cannot exclude the possibility that Che-1 can exert this induction by other mechanisms.

The highly acidic amino-terminal region of Che-1 is found in several proteins associated with a chromatin remodeling function during transcription, including the MOZ family proteins (Borrow et al., 1996; Champagne et al., 1999) and BRCA1 (Hu et al., 1999). Consistent with this observation, preliminary research in our hands indicates that this region, but not the whole protein, even if it does not directly contact DNA, strongly activates transcription, suggesting a potential involvement in the modification of the chemical and structural composition of chromatin.

Finally, the pocket region of Rb mediates interaction with several chromatin remodeling factors (Ferreira et al., 2001b); thus, it is possible that, besides HDAC1, Che-1 may displace other proteins from Rb. Therefore, a deeper characterization of Che-1 functions could amplify this novel pathway of regulation of Rb activity, exerted by the control of the recruitment of chromatin remodeling enzymes, and in addition could shed light on the role played by Che-1 in the regulation of transcription and cell proliferation.

Experimental procedures

Plasmids

Myc-tagged Che-1, Rb, and E2F1 mammalian expression vectors, GST-Rb fusion protein, and DHFR-luciferase vector have been previously described (Fanciulli et al., 2000).

To generate pTRE-IRES-EGFP-Che-1, the region of pIRES2-EGFP vector (Clontech) corresponding to cytomegalovirus immediate early (CMV) promoter was restricted and substituted with the Tet-responsive *PhCMV-1* promoter region removed from pTRE vector (Clontech). The DNA fragment containing full-length ORF of human Che-1 and the Tag-Flag was then adapted in this resulting novel vector. Plasmid expressing bacterial recombinant 6xHis-Che-1 was produced by cloning the complete ORF of Che-1 into pQE30 bacterial expression vector (Quiagen). The complete ORF of human HDAC1 was generated by PCR and cloned into myc-tagged expression vector pCS2-MT. GST-Rb 1–379 was kindly provided by Dr. Wang (University of California, San Diego). Other deletions of Rb and its mutations fused to GST were generously provided by Dr. Caruso (CNR-IBC, Rome). Mutagenesis of myc-Che-1 were performed using the Quickchange Mutagenesis system (Stratagene).

Cell culture and transfections

Saos-2 human osteosarcoma cells, 293 human transformed embryonal kidney cells, HFF human fibroblasts (kind gift from Dr. Lauren Wood), and NIH 3T3 mouse fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. To produce a proliferation block, NIH 3T3 cells were cultured for 60 hr in medium containing 0.2% fetal bovine serum. DNA distribution analysis was performed according to standard procedures, measuring the DNA content by an Epics XL analyzer (Coulter Corporation). BES-calcium phosphate precipitation was used to transiently transfect cells as previously described (Fanciulli et al., 2000). Transfection efficiency ranged between 40% and 60% of cells.

Metabolic labeling

NIH 3T3 cells were synchronized by serum starvation, stimulated by serum for various times, and incubated for 3 hr with 0.5 mCi/ml [^{32}P] inorganic phosphate (Amersham) in phosphate-free medium (Sigma) before immunoprecipitation of Che-1.

Inducible Che-1 overexpression

Stable cell clones expressing Che1 in a conditional way (tetracycline-regulated transactivation system [Gossen and Bujard, 1992; Gossen et al., 1995]) were obtained by transfecting the construct pTRE-Ires-GFP-Che1 in an NIH 3T3 cell line stably expressing rtTA (3T3-tetOn) and carrying resistance to puromycin, obtained by Dr. Caruso and Dr. Tirone (INMM-CNR, Rome, Italy). Details of stable clones production are provided in the Supplemental Data at http://www.cancercell.org/cgi/content/full/2/5/387/DC1. Reentry of cells into the cell cycle after starvation was analyzed by flow cytometry using BrdU incorporation as previously described (Biroccio et al., 2002).

Immunoprecipitations and Western blot analysis

Cells were rinsed three times with ice-cold PBS, harvested, centrifuged at 4°C, and cell pellets lysed by incubation at 0°C for 30 min in 300 μl lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 10% glycerol, 0.5 mM EDTA, 0.5 mM EGTA, 100 mM NaF, 3 mM $NaOV_4$, 10 nM okadaic acid, 1 mM PMSF, 10 mg/ml leupeptin) supplemented with 1% NP40. Supernatants were cleared by centrifugation, precleared using 20 μl protein A/protein G beads (Santa Cruz), and immunoprecipitated by standard procedures using anti-Che-1, anti-Rb (C15, Santa Cruz) polyclonal antibodies or anti-myc (9e10, Invitrogen) monoclonal antibody. Western blots were prepared by standard procedures using the following rabbit polyclonal antibodies: anti-Che-1, anti-Rb, anti-p107 (C18, Santa Cruz), anti-p130 (C20, sc317, Santa Cruz), anti RNA Pol II (C-21, Santa Cruz), anti-cyclin D1 (C20, sc307, Santa Cruz), anti-cyclin A (C19, Santa Cruz), anti-HDAC1 (Upstate Biotechnology), or anti-α Tubulin (TU-02, Santa Cruz) and anti-myc monoclonal antibodies. Immunoreactivity was detected by ECL chemiluminescence reaction (Amersham).

BrdU incorporation analysis

NIH 3T3 cells were growth arrested as described above and transfected with myc-tagged wild-type Che-1, ΔChe-1, Δ-zip, or with empty vector and placed in DMEM medium containing 0.2% fetal bovine serum for additional 24 hr. Growth-arrested cells transfected with empty vector were placed in medium supplemented with 10% fetal calf serum and used as positive control. Immunofluorescence staining for BrdU was performed as previously described (Crescenzi et al., 1995).

Pull-down analysis

BL21 bacteria strains were transformed with GST fusion protein constructs and the proteins purified on glutathione-Sepharose resin (Pharmacia). For in vitro binding assays, comparable amounts of resin-bound GST fusion proteins were incubated with 500 μg of cell lysates in NETN buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, and 0.5% NP-40) for 1 hr at 4°C. The resins were then pelleted and extensively washed in the same buffer. The bound proteins were separated by SDS-PAGE, and the gels were analyzed by Western blot using anti-myc monoclonal antibody. Immunoreactivity was detected by ECL chemiluminescence reaction (Amersham). In vitro transcription and translation were carried out with TNT-coupled reticulocyte lysate systems (Promega) and L-[^{35}S]methionine (>1,000 Ci/mmol; Amersham) as previously described (Fanciulli et al., 2000).

Peptide synthesis

Peptides were prepared in a Vega Peptide Synthetizer Model 1000, using Fmoc chemistry with protected amino acids purchased from Inalco, Milan. At the end of the synthesis, peptides were cleaved and deprotected in a

mixture of trifluoroacetic acid/anisole (19:1) for 60 min, precipitated with cold ether, recovered by centrifugation, and purified by HPLC. The correct amino acid composition was confirmed by amino acid analysis by HPLC/fluorescence after complete hydrolysis of 50 μg of samples with 6 N HCl at 108°C for 24/48 hr. Peptides sequence: Che-1 305–323: DGTKPNTESEEIS SEDDEL; HDAC1: SDKRIACEEEFSDSEE; SV40 Large T: NEENLFCSEEMPS SDD; Control: LKGAKPIRPVVVKAPPA.

Deacetylase assay

Deacetylase assays were performed utilizing a Histone Deacetylase Assay Kit (Upstate Biotechnology) according to the manufacturer's instructions. All experiments were performed at least three times and samples were assayed in duplicate.

Chromatin immunoprecipation assay (ChIP)

ChIP assay was performed using the acetyl-histone H3 immunoprecipation assay kit (Upstate Biotechnology) according to the manufacturer's instructions. For ChIP experiments conducted with anti-HDAC1 and anti-Che-1 polyclonal antibodies, conditions were as reported by Nissen and Yamamoto (2000). In each experiment, linearity of the signal was insured by amplifying increasing amounts of the template DNA (see Figures 3B and 5D). Generally, DNA representing 0.005% to 0.01% of the total chromatin sample (input) or 1% to 10% of the immunoprecipitated was amplified using promoter-specific primers whose sequences are provided in Supplemental Data at http://www.cancercell.org/cgi/content/full/2/5/387/DC1. Immunoprecipitation with no specific immunoglobulins (Santa Cruz) was performed as a negative control. PCR conditions were: 1 × 95°C for 2 min, followed by 30 cycles at 95°C for 1 min, 60°C or 64°C for 1 min, and 72°C for 2 min.

RT-PCR analysis

For semiquantitative RT-PCR analysis, Saos-2 cells were transfected with the indicated expression vectors, and 36 hr after transfection, total RNA was isolated using TRIZOL reagent (Life Technologies). RT-PCR was performed using a Platinum quantitative RT-PCR kit (Invitrogen) following the manufacturer's instructions. PCR products were separated on a 2% agarose gel. PCR conditions were: 1 × 95°C for 5 min., followed by 25 cycles at 95°C for 1 min, 55°C for 1 min., and 72°C for 2 min. Sequences of primers employed in RT-PCR analysis are provided in the Supplemental Data.

RNA interference in human cells

The 22 nucleotide siRNA duplexes corresponding to nucleotides 191–212 of human Che-1 sequence and to nucleotides 122–143 of the negative control green fluorescent protein (GFP) sequence were synthesized by Xeragon. RNA interference was performed as described by Elbashir et al. (2001).

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