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Regulation of E2F1 activity by acetylation

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During the G_1 phase of the cell cycle, an E2F–RB complex represses transcription, via the recruitment of histone deacetylase activity. Phosphorylation of RB at the G_1/S boundary generates a pool of 'free' E2F, which then stimulates transcription of S-phase genes. Given that E2F1 activity is stimulated by p300/CBP acetylase and repressed by an RB-associated deacetylase, we asked if E2F1 was subject to modification by acetylation. We show that the p300/CBP-associated factor P/CAF, and to a lesser extent p300/CBP itself, can acetylate E2F1 *in vitro* and that intracellular E2F1 is acetylated. The acetylation sites lie adjacent to the E2F1 DNA-binding domain and involve lysine residues highly conserved in E2F1, 2 and 3. Acetylation by P/CAF has three functional consequences on E2F1 activity: increased DNA-binding ability, activation potential and protein half-life. These results suggest that acetylation stimulates the functions of the non-RB bound 'free' form of E2F1. Consistent with this, we find that the RB-associated histone deacetylase can deacetylate E2F1. These results identify acetylation as a novel regulatory modification that stimulates E2F1's activation functions.

Keywords: acetylation/E2F1/histone deacetylase/p300/P/CAF/retinoblastoma protein

Introduction

The E2F family of transcription factors plays a critical role in organizing early cell cycle progression by coordinating early cell cycle events with the transcription of genes required for entry into S phase (Nevins, 1992; La Thangue, 1994; Ohtani *et al*., 1995). Aberrant expression of E2F in human cells leads to tumour formation (Hunter and Pines, 1994; Hall and Peters, 1996; Yamasaki *et al*., 1996), underscoring the importance of E2F in growth control.

E2F activity is regulated principally by association with different regulators. E2F has an activation domain located near its C-terminus that harbours the binding of several proteins that regulate its activity. Positively acting proteins include TATA box-binding protein (TBP; Hagemeier *et al*., 1993; Pearson and Greenblatt, 1997), the coactivator p300/CBP (Trouche and Kouzarides, 1996; Trouche *et al*., 1996) and the oncoprotein MDM2 (Martin *et al*., 1995). Negative regulators include the tumour supressor protein p53 (O'Connor *et al*., 1995) and the most important regulator, the retinoblastoma protein RB (Flemington *et al*., 1993; Hagemeier *et al*., 1993; Brehm *et al*., 1998; Luo *et al*., 1998; Magnaghi-Jaulin *et al*., 1998). The RB–E2F complexes, which predominate in quiescent cells (Bagchi *et al*., 1991; Cao *et al*., 1992), act as repressors of transcription. The E2F-bound RB represses transcription actively by contact with promoter-bound proteins (Weintraub *et al*., 1995; Zhang *et al*., 1999) and by recruiting a histone deacetylase complex (Brehm *et al*., 1998; Luo *et al*., 1998; Magnaghi-Jaulin *et al*., 1998). During G_1 phase, RB is phosphorylated, which blocks the binding of E2F–RB and releases transcriptionally active E2F at the G_1/S transition (reviewed by Mittnacht, 1998). Other levels of control that influence E2F activity include its own phosphorylation (Dynlacht *et al*., 1994, 1997; Xu *et al*., 1994; Krek *et al*., 1995; Vandel and Kouzarides, 1999), protein stabilization (Hateboer *et al*., 1996; Hofmann *et al*., 1996; Campero and Flemington, 1997; Martelli and Livingston, 1999) and regulation of its intracellular location (de la Luna *et al*., 1996; Allen *et al*., 1997; Lindeman *et al*., 1997).

There are five E2F family members that have highly conserved DNA-binding and activation domains (Helin *et al*., 1992; Kaelin *et al*., 1992; reviewed by Dyson, 1998; Helin, 1998) whereas a sixth protein, EMA, is only conserved in the DNA-binding domain. Three of these, E2F1, 2 and 3, have the ability to induce S phase (Johnson *et al*., 1993; DeGregori *et al*., 1995; Lukas *et al*., 1996). However, all E2F members are able to bind to a similar consensus sequence when heterodimerized with a member of the DP family (Girling *et al*., 1993; Wu *et al*., 1995).

The activity of E2F is increased by the CBP co-activator protein. Although the precise mechanism by which p300/CBP stimulates transcription remains unclear, the discovery that p300/CBP and an associated factor P/CAF have histone acetylase activities suggests that these cofactors may regulate transcription through acetylation (Bannister and Kouzarides, 1996; Ogryzko *et al*., 1996; Yang *et al*., 1996). Modification of histones by addition of acetyl groups occurs to a certain degree when the histones are associated with transcriptionally active DNA (Hebbes *et al*., 1988; Grunstein, 1997). Although the major targets of CBP/p300 and P/CAF are histones, these acetylases also modify certain transcription factors such as TFIIEβ, TFIIF, EKLF, p53, HMGI(Y) and GATA-1 (Gu and Roeder, 1997; Imhof *et al*., 1997; Boyes *et al*., 1998; Munshi *et al*., 1998; Zhang and Bieker, 1998).

Given that E2F1 activity is regulated positively by the p300/CBP acetylase and negatively by an RB-bound deacetylase, we have examined whether E2F1 is regulated by acetylation. Here we show that the acetylase P/CAF

and to a lesser extent CBP and p300 can acetylate E2F1 *in vitro* and that E2F1 is acetylated *in vivo*. We show that acetylation of E2F1 increases its DNA-binding ability, its transcriptional activation capacity and its protein half-life. The acetylation status of E2F1 is affected by the histone deacetylase associated with the RB–E2F1 complex. These data point to acetylation as a novel mechanism for the regulation of E2F1 activity.

Results

E2F1 is acetylated

The CBP acetylase can bind E2F1 and stimulate its ability to activate transcription (Trouche and Kouzarides, 1996; Trouche *et al*., 1996). We set out to establish if acetylation of E2F itself was part of the mechanism by which CBP functioned. We first tested if CBP or the closely related enzyme p300 could acetylate recombinant GST–E2F1 *in vitro*. Also included in this experiment was P/CAF (and the close homologue GCN5) since this enzyme is found associated with p300/CBP (Yang *et al*., 1996). The results in Figure 1A show that recombinantly expressed P/CAF acetylated E2F1 very potently. Longer exposure of the gel shows that E2F1 was also acetylated less efficiently by GCN5 and by the CBP and p300 HAT domains. The specificity of the acetylation is demonstrated in Figure 1B, where P/CAF is shown to acetylate GST–E2F1, but not GST or bovine serum albumin (BSA). We estimate that the proportion of recombinant E2F1 acetylated by P/CAF in our assays is ~25%.

To verify that E2F1 is acetylated *in vivo*, we used an antibody raised against acetylated histone H4 that has the ability to recognize acetylated proteins. Such antibodies have been used to establish whether the transcription factors GATA1 and EKLF are acetylated *in vivo*. The endogenous E2F1 protein was therefore precipitated with an anti-E2F1-specific antibody or with the unrelated haemagglutinin (HA)-tagged antibody. The anti-acetylated lysine antibody was then used in a Western blot analysis. Figure 1C shows that the specifically immunoprecipitated E2F1 protein is recognized by the anti-acetylated lysine antibody. Collectively, these results show that E2F1 is acetylated *in vivo* and that P/CAF can acetylate E2F1 efficiently *in vitro*.

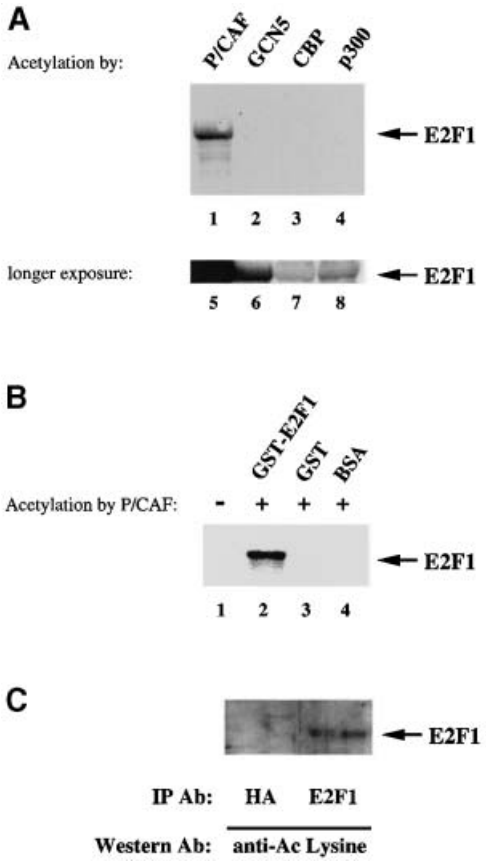

Fig. 1. Acetylation of E2F1 by P/CAF, GCN5, CBP and p300 acetylases. (**A**) *In vitro* acetylation of E2F1. Purified GST–E2F1 (amino acids 89–437) was incubated with [^{14}C]acetyl-CoA and recombinant GST–P/CAF (amino acids 352–832, lanes 1 and 5), His-GCN5 (amino acids 1–427, lanes 2 and 6), GST–CBP (amino acids 1098–1877, lanes 3 and 7) and His-p300 (amino acids 1071–1715, lanes 4 and 8). Reaction products were separated by SDS–PAGE and gels were autoradiographed. Lanes 5–8 are a longer exposure (8 days) of lanes 1–4 (6 h) to visualize acetylation of E2F1 by GCN5, CBP and p300. (**B**) Control acetylation reactions with GST–E2F1, GST and BSA were performed as described in (A) and show that E2F1 (lane 2) in the presence of [^{14}C]acetyl-CoA, but in the absence of P/CAF (lane 1) is not acetylated, and GST (lane 3) or BSA (lane 4), are not targets for P/CAF. (**C**) *In vivo* acetylation of E2F1. Extracts from U2OS cells were prepared and E2F1 was immunoprecipitated using an anti-E2F1 (C20) antibody or an anti-HA antibody as a control. Immunoprecipitates were analysed by SDS–PAGE and Western blot analysis using an antibody raised against acetylated histone H4.

E2F1 can associate with P/CAF

We wanted to establish whether the P/CAF enzyme can associate with E2F1. Western blot analysis indicates that intracellular levels of P/CAF are very low, or that P/CAF antibodies have low affinity for the substrate (data not shown). For these reasons, we transfected Flag-P/CAF into cells and asked if it can be co-immunoprecipitated with endogenous E2F1. Figure 2A shows that precipitation of Flag-P/CAF results in co-precipitation of E2F1, which was recognized in an anti-E2F1 Western blot. Figure 2B shows that the reverse is also true, namely that precipitation of E2F1 co-precipitates Flag-P/CAF.

Having established that the two proteins can interact *in vivo*, we set out to dissect the sites of interaction between E2F1 and P/CAF. Figure 2C shows that *in vitro* translated E2F1 can associate with GST–P/CAF (lane 3). The C-terminus of P/CAF (352–832), which contains the HAT, ADA2 and Bromo domains, is sufficient for the interaction with E2F1 (lane 4). Further dissection points to the ADA2 domain as the major determinant for the association (Figure 2C, lanes 5–9), although low affinity was exhibited by the HAT domain (lane 6). These results suggest that P/CAF may bind to E2F1 independently of p300/CBP, since the interaction site for p300/CBP lies in the N-terminus of P/CAF (Yang *et al*., 1996; Reid *et al*., 1998).

Identifying acetylation sites in E2F1

We wanted to establish how acetylation regulates E2F1 activity. We argued that identification of the lysines modified by P/CAF may give clues to the function regulated by acetylation. Deletion analysis indicated that acetylation sites resided within a region of E2F1 (89–287) that harbours the DNA-binding domain (Figure 3A and B). This region contains several lysines, some of which

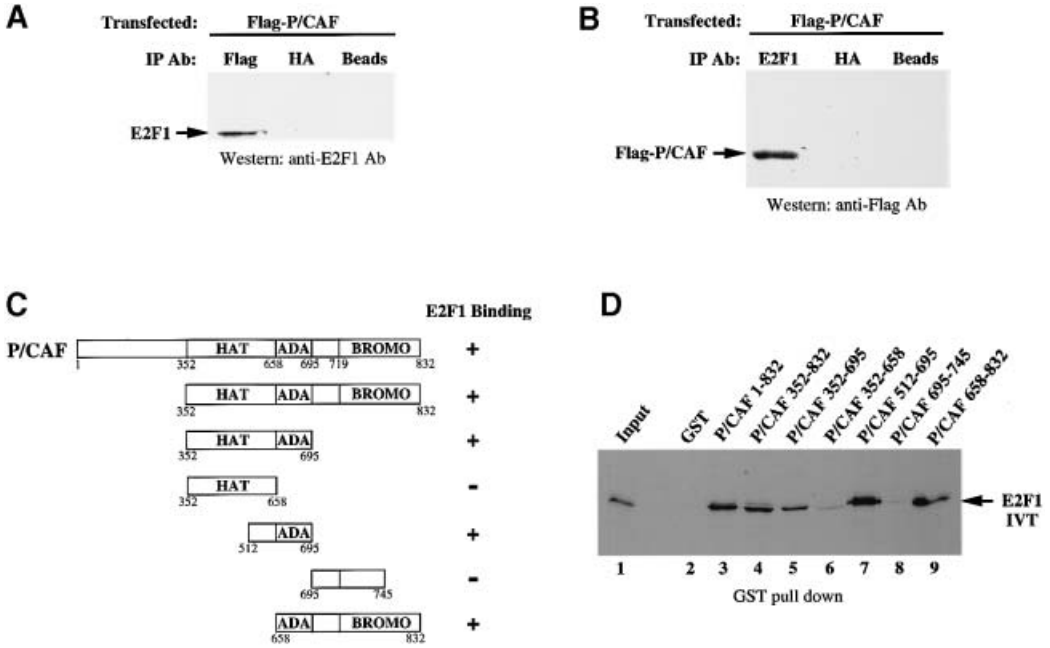

Fig. 2. E2F1 interacts with P/CAF. (**A**) U2OS cells were transfected with 8 μg of pCX-P/CAF (Flag-tagged), and afterwards whole-cell extracts were prepared and incubated with anti-Flag antibody and, as controls, either with anti-HA antibody or without any antibody. By incubation with protein A–Sepharose, immunoprecipitates were collected. The precipitates were then analysed by SDS–PAGE and Western blotting with the anti-E2F1 (C20) antibody for the presence of co-immunoprecipitated E2F1. (**B**) U2OS cells were transfected and extracted, as described in (A). In this experiment, immunoprecipitation of E2F1 was performed with an anti-E2F1 antibody (C20). The control reactions were the same as in (A). An anti-Flag Western blot was used for detection of co-immunoprecipitated P/CAF. (**C**) Schematic representation of P/CAF fragments used to identify the E2F interaction region in P/CAF. E2F1 binding to different P/CAF fragments is indicated in the scheme. (**D**) The P/CAF fragments were expressed as GST fusion proteins, purified and used in GST pull-down assays with *in vitro* translated, ^{35}S-labelled E2F1 protein. Equivalent P/CAF protein amounts of the different mutants were used. Interaction between E2F1 and P/CAF was analysed by SDS–PAGE of the pull-down reactions and autoradiography (lanes 3–9). The input (lane 1) contains 20% of the E2F1 IVT. GST protein (lane 2) was used as a control.

are implicated in DNA binding, according to the recent crystal structure of the E2F4–DP1–DNA complex (Zheng *et al.*, 1999). We mutated three clusters of lysines to arginine. Two sets of lysines are within the DNA-binding domain (KK 161,164 and KKK 182,183,185). All lysines (except Lys161) are conserved in all six E2F family members, and one of these, Lys182, makes direct contact with DNA. Another set of mutated lysines falls directly N-terminal and adjacent to the DNA-binding domain (KKK 117,120,125), and they are only conserved in E2F1, 2 and 3. The three mutant E2F1 proteins and wild-type E2F1 were expressed recombinantly, and equivalent concentrations of each protein were tested for acetylation by P/CAF. Figure 3C shows that mutating the two lysine clusters present within the DNA-binding domain (K161,164R and K182,183,185R) had no effect on acetylation. In contrast, mutating the lysines that lie outside the DNA-binding structure (K117,120,125R) generates an E2F1 mutant that can no longer be acetylated by P/CAF. This mutant is referred to from here on as the E2F1-R mutant.

We next asked if the E2F1-R mutant was defective for acetylation by P/CAF *in vivo*. P/CAF was therefore transfected into cells along with either wild-type E2F1 or with the E2F1-R mutant. The anti-acetylated lysine antibody was then used to precipitate acetylated E2F1, which was detected by Western blot analysis using an anti-E2F1 antibody. Figure 3D shows that the anti-acetylated lysine antibody can precipitate wild-type E2F1 but not the E2F-R mutant. An E2F1 Western blot of the transfected cells was used to make sure that equivalent protein levels of E2F1 and E2F1-R mutant were used (data not shown). These results indicate that P/CAF cannot acetylate E2F-R *in vivo*.

Acetylation of E2F1 augments DNA binding

Having identified an E2F1 mutant that cannot be acetylated by P/CAF, we set out to use this mutant to dissect the functional consequence of E2F1 acetylation. Given that the acetylated residues are adjacent to the E2F1 DNA-binding domain, we first tested whether acetylation affected the ability of E2F1 to recognize DNA.

In these experiments, we used recombinant E2F1 consisting of only the DNA-binding domain (residues 92–195). This domain contains lysines 117, 120 and 125 and can be acetylated by P/CAF (data not shown). Figure 4A shows that this E2F1 domain can weakly bind an E2F site DNA element (lanes 5–7), but when acetylated by P/CAF this domain recognizes DNA 6-fold more efficiently at the highest concentration (lanes 2–4).

In the cell, E2F1 binds to DNA as a dimer with the DP1 protein. We next tested whether the E2F1–DP1 heterodimer binds DNA more efficiently following the acetylation of E2F1. Figure 4B shows that acetylation of recombinant E2F1 by P/CAF increases DNA binding when dimerized with DP1 (lanes 7 and 8) compared with a control reaction in which E2F1 was incubated with a P/CAF mutant (ΔHAT) that has no HAT activity (lanes 5 and 6). To establish more definitely whether acetylation of E2F1 was causing the increase in DNA-binding potential, we used the E2F1-R mutant, which cannot be acetylated by P/CAF (Figure 3C). As seen in Figure 4C,

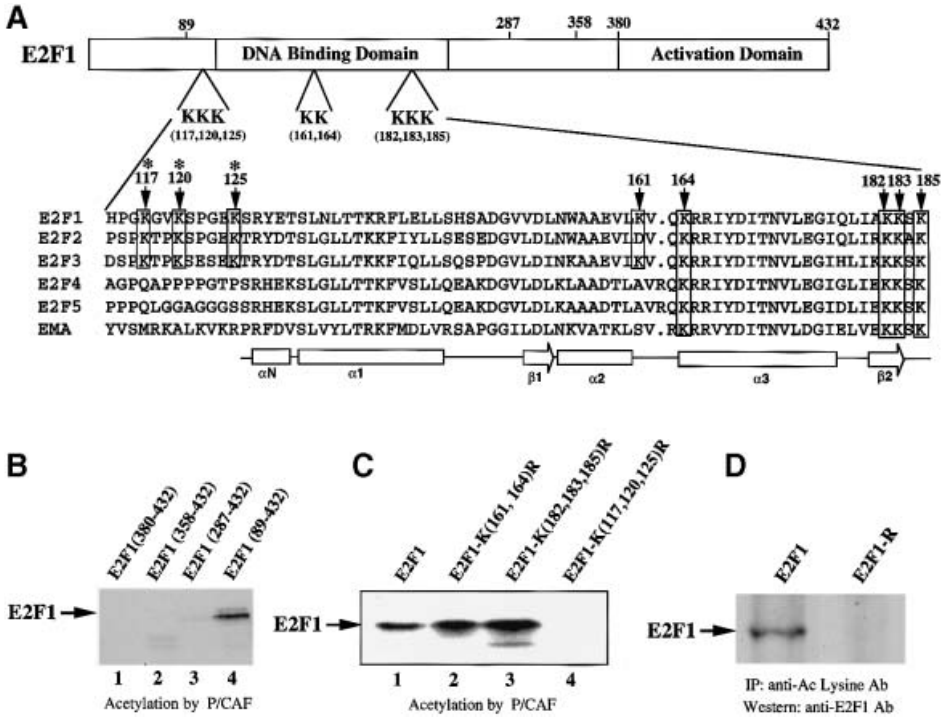

Fig. 3. The E2F1 acetylation sites are located directly adjacent to the DNA-binding domain. (**A**) Identification and evolutionary conservation of the acetylation sites in E2F1. Schematic representation of E2F1, its functional domains, the three lysine clusters that were mutated to arginines, and polypeptide sequence alignment of E2F1 (amino acids 114–185) with E2F2, E2F3, E2F4, E2F5 and EMA. The arrows indicate the mutated lysine residues, the conservation of which is highlighted by boxes. The corresponding secondary structure of the E2F4 DNA-binding domain (Zheng *et al.*, 1999) with its four α-helices and two β-sheets is shown underneath the sequence alignment. The acetylated lysines are indicated by asterisks. (**B**) The E2F1 fragments indicated (lanes 1–4) were expressed as GST fusion proteins, purified and 2 μg of each were assayed for acetylation by GST–P/CAF as described in Figure 1A. (**C**) Wild-type (lane 1) and mutated E2F1 proteins (lanes 2–4), which were mutated at the indicated lysines to arginines, were expressed, purified and 2 μg of each were assayed for acetylation by P/CAF. (**D**) To test *in vivo* acetylation of the E2F1-R mutant [E2F1-K(117,120,125)R, (C) lane 4], P/CAF (8 μg of pCX-P/CAF) was co-transfected with either E2F1 (3 μg of pcDNA3-E2F1) or E2F1-R (6 μg of pcDNA3-E2F1-R) into 293T cells. Acetylation of E2F1 and the mutant was investigated by immunoprecipitation of equivalent amounts of E2F1 and E2F1-R protein with an anti-acetylated lysine antibody covalently coupled to protein A–Sepharose and subsequent E2F1 Western blot analysis (with KH95 antibody) of the precipitates.

the E2F1-R mutant is still able to bind DNA perfectly well in combination with DP1 (lanes 1–3). The DNA-binding ability of the DP1–E2F1-R complex is not increased, however, when E2F1-R is incubated with an active P/CAF enzyme. This result demonstrates that the presence of acetylation sites within E2F1 is essential for the stimulatory effect of P/CAF on E2F1 DNA binding.

Acetylation by P/CAF stimulates E2F1 transactivation potential

Since acetylation increases DNA binding by E2F1, we asked if acetylation by P/CAF regulates the transactivation potential of E2F1. Figure 5A shows that overexpression of P/CAF stimulates the ability of an E2F1–DP1 heterodimer to activate transcription of a promoter bearing E2F1 elements. P/CAF had no effect on a promoter containing mutated E2F-binding sites (data not shown). The stimulation by P/CAF is dependent on P/CAF HAT activity since a P/CAF mutant (ΔHAT) that lacks acetylation capacity cannot augment E2F1–DP1 activity. The ability of P/CAF to act as a co-activator depended on its ability to acetylate E2F1. This is evident when we use the E2F1-R mutant, which cannot be acetylated by P/CAF, but which can still bind DNA efficiently. Figure 5B shows that this mutant is able to stimulate transcription in co-operation with DP1. However, the activity of this mutant E2F1-R–DP1 heterodimer is refractile to P/CAF stimulation. Taken together, these results suggest that acetylation increases the activation potential of E2F1.

P/CAF acetylation stabilizes E2F1 protein

During our analysis of E2F acetylation, a novel property of P/CAF was uncovered, namely its ability to increase the levels of E2F1 protein in the cell. Cycling cells possess low levels of E2F1 protein. Figure 6A shows that very little endogenous E2F1 is detected when whole cells are lysed and Western blotted with an anti-E2F1 antibody (lane 1). However, transfection of P/CAF into cells results in a significant increase in the levels of E2F1 (lane 2). P/CAF HAT activity seems essential for this increase in E2F1 since a HAT mutant of P/CAF (which is expressed at levels equal to the wild-type) does not show this effect (lane 3). Other acetylases, GCN5 and p300, do not increase the levels of E2F1 significantly in the cell (lanes 4 and 5). Similar observations regarding E2F1 stability have been reported for the RB protein (Martelli and Livingston, 1999). Under our conditions, transfection of RB leads to an increase in E2F1 protein levels, comparable with those seen with P/CAF (Figure 6A, lane 6).

If the increase in stability of E2F1 occurs as a result of P/CAF acetylation, we would expect the levels of endogenous acetylated E2F1 to rise following P/CAF transfection. To establish if this is the case, the anti-acetylated lysine antibody was used to immunoprecipitate

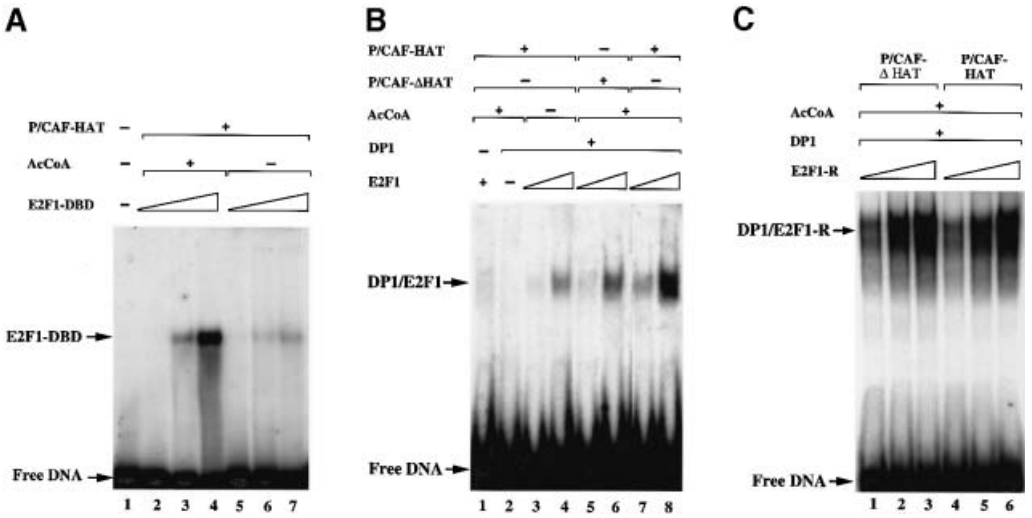

Fig. 4. DNA-binding activity of E2F1 is augmented by acetylation. (**A**) Acetylation of E2F1-DBD increases its DNA binding. The His-E2F1-DBD (amino acids 92–195) was bacterially expressed and purified. DNA binding of unmodified [in the presence of GST–P/CAF (amino acids 352–632), but the absence of AcCoA (lanes 5–7)] versus *in vitro* acetylated E2F1-DBD [in the presence of GST–P/CAF and AcCoA (lanes 2–4)] was tested by EMSA by incubation of increasing amounts of these proteins with ^{32}P-labelled E2F-binding site (50 fmol per reaction). Reaction products were resolved by electrophoresis and visualized by autoradiography. (**B**) E2F1–DP1 binds DNA more efficiently following acetylation of E2F1. His-E2F1 full-length protein was bacterially expressed, purified and *in vitro* acetylated [in the presence of P/CAF and AcCoA (lanes 7 and 8)] or not acetylated [in the presence of either P/CAF, but not AcCoA (lanes 3 and 4) or P/CAF-ΔHAT and AcCoA (lanes 5 and 6)]. DNA binding was investigated by incubation of these proteins with GST–DP1 and ^{32}P-labelled E2F-binding site (50 fmol per reaction). Complexes were separated by electrophoresis and autoradiographed. The DNA-binding ability of either E2F1 or DP1 alone is shown in lanes 1 and 2, respectively. (**C**) Increased DNA binding of acetylated E2F1 is due to acetylation. The E2F1-R mutant, which contains lysines 117, 120 and 125 mutated to arginines, and which cannot be acetylated (Figure 3C and D), was expressed in bacteria as a GST fusion protein, purified and tested for its DNA-binding ability after incubation with either P/CAF (lanes 4–6) or P/CAF-ΔHAT (lanes 1–3) as described in (A) and (B).

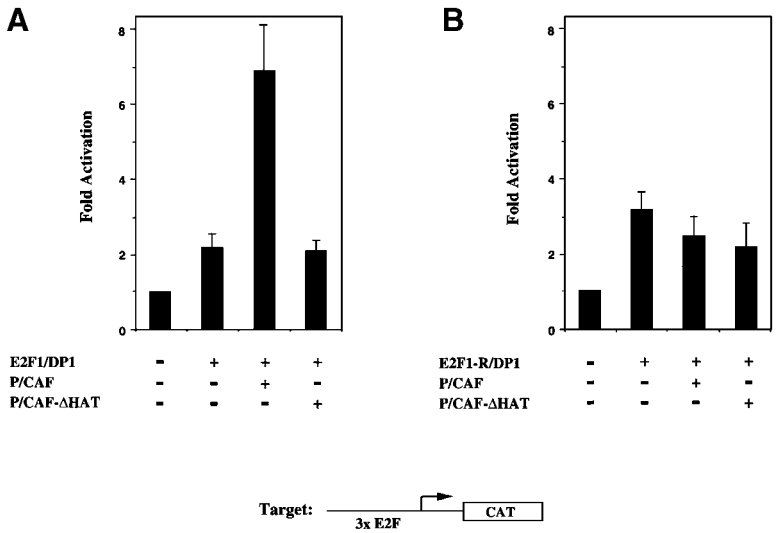

Fig. 5. Acetylation of E2F1 stimulates its transcriptional activity. (**A**) U2OS cells were transfected with 0.5 μg of the CAT reporter gene plasmid and 1 μg of the indicated expression plasmid for E2F1 and DP1, 5 μg of pCX-P/CAF and P/CAF-ΔHAT, respectively, or equivalent empty vector. Whole-cell extracts were used in CAT assays and the results were quantified on a PhosphoImager. The basal promoter activity of the CAT reporter (TK promoter with 3× E2F sites) in the presence of empty expression vector was normalized to 1.0, and the activities of the remaining transfection reactions were expressed relative to this, as fold activation of the basal promoter. The graph shows the average of three independent experiments. (**B**) The effects of P/CAF on the transactivation potential of E2F1-R is shown, as assayed under the same conditions as described in (A).

endogenous E2F1, and Western blotting with anti-E2F antibody was used to identify E2F1. In this experiment, the amount of endogenous E2F1 protein was first checked by Western blotting and equal amounts were used for the immunoprecipitations. Figure 6B shows that the levels of acetylated E2F1 are elevated following P/CAF transfection, but not P/CAF ΔHAT transfection, suggesting that the increase in E2F1 protein level is, at least partially, caused by *in vivo* acetylation of endogenous E2F1 by P/CAF.

An increase in E2F1 protein levels by P/CAF can also be detected on exogenously introduced E2F1 (Figure 6C, lanes 1 and 2). The effect of P/CAF on transfected E2F1 is not as great as that seen on endogenous E2F1 in whole cells (Figure 6A). Nevertheless, the increase in E2F1 levels observed requires acetylation activity, since a

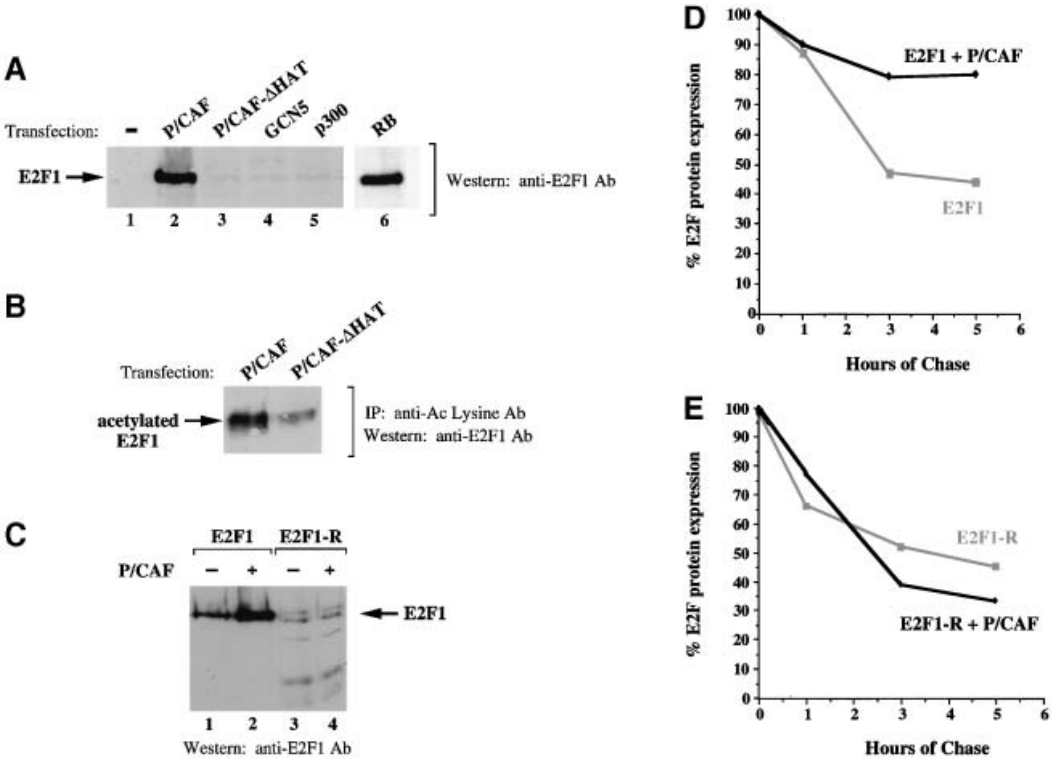

Fig. 6. Acetylation by P/CAF increases E2F1 protein level and prolonges its half-life. (**A**) 293T cells were either not transfected (lane 1) or transfected with 5 μg of the indicated eukaryotic expression plasmids for P/CAF (lane 2), P/CAF-ΔHAT (lane 3), GCN5 (lane 4) and p300 (lane 5), and 3 μg of RB (lane 6). At 24 h after the medium change, the cells were lysed in SDS-containing sample buffer and analysed by SDS–PAGE and anti-E2F1 Western blot (C20) for endogenous E2F1 expression levels. (**B**) To investigate if the increase in E2F1 protein level is accompanied by *in vivo* acetylation of endogenous E2F1, P/CAF- and P/CAF ΔHAT-transfected cells [as in (A)] were analysed by immunoprecipitation with anti-acetylated lysine antibody (covalently coupled) and Western blot of the precipitates with anti-E2F (KH95) antibody. Equivalent amounts of E2F1 protein (of both transfection) were used for the immunoprecipitations. (**C**) 293T cells were transfected with E2F1 (lanes 1 and 2) or E2F1-R (lanes 3 and 4) in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of P/CAF overexpression. SDS–PAGE and Western blot analysis with the anti-E2F1 (C20) antibody revealed the effect of acetylation by P/CAF on the exogenous E2F1 protein level. (**D**) To determine the protein half-life of E2F1 in the presence or absence of P/CAF, 293T cells were transfected with 2 μg of pcDNA3-E2F1 in the absence or presence of 8 μg of pCX-P/CAF. At 24 h after the medium change, cells were pulse-labelled for 2 h with a [^{35}S]methionine–cysteine mix. Chase was performed in medium supplemented with 10-fold excess of cold methionine and cysteine for the time periods indicated. Cells were lysed and immunoprecipitations were performed with the anti-E2F (C20) antibody. Immunoprecipitates were separated by SDS–PAGE, blotted and autoradiographed. The intensity of the ^{35}S-labelled proteins was measured densitometrically and calculated in comparison with the amount of E2F protein present at time point zero, which was set at 100%. (**E**) The effects of P/CAF on the half-life of E2F1-R were assayed under the same conditions as described in (D). Therefore, 6 μg of pcDNA3-E2F1-R were transfected with or without P/CAF into 293T cells.

P/CAF ΔHAT mutant cannot mediate this effect (data not shown). To show that E2F1 stability involves direct acetylation of E2F1, we used the E2F1-R mutant. Figure 6C (lanes 3 and 4) shows that this mutant is not stabilized after P/CAF transfection. It is worth noting that increased stability of endogenous E2F1 is never observed following transfection of exogenous E2F1, probably due to the shut-off of endogenous E2F gene transcription (Figure 6C). Collectively, these results indicate that acetylation of E2F1 is necessary for the observed increase in E2F1 protein levels. However, we cannot exclude the possibility that acetylation of other cellular components by P/CAF may also contribute to this stabilizing effect.

What is noticeable in Figure 6C (lanes 3 and 4) is that the E2F1-R mutant is expressed at lower levels than wild-type E2F1. The expression levels can be made equivalent if the concentration of E2F1-R transfected is increased. However, what is also evident is that specific degradation products are detectable when E2F1-R is transfected. This suggested to us that lysines 117, 120 and 125 may be involved in E2F1 stability and that acetylation by P/CAF may be stabilizing the E2F1 protein. The increase in E2F1 levels detected with RB (Figure 6A, lane 6) has already been demonstrated to result from an increase in E2F1 stability (Hateboer *et al*., 1996; Hofmann *et al*., 1996; Campero and Flemington, 1997; Martelli and Livingston, 1999). Since P/CAF expression does not change the levels of E2F1 RNA in the cell (data not shown), we explored the possibility that P/CAF acetylation of E2F1 affects its half-life. The levels of exogenously expressed E2F1 protein (detected following immunoprecipitation) were therefore monitored in a pulse–chase experiment, in either the presence or the absence of exogenous P/CAF. Figure 6D shows that in the presence of P/CAF, E2F1 has an increased half-life. In contrast, the stability of the E2F1-R mutant, which cannot be acetylated by P/CAF, is not affected by P/CAF expression (Figure 6E). This result suggests that acetylation of E2F1 is a necessary step for the stabilizing effect of P/CAF.

RB-associated HDAC deacetylates E2F1

Our results indicate that acetylation of E2F1 by P/CAF leads to an increase in the transactivation potential of E2F1. This would suggest that acetylation is relevant to

the 'free' state of E2F1, when it is not complexed with the RB repressor protein. We and others have shown that RB can repress the activity of E2F1, at least partly, by tethering E2F1 to a histone deacetylase. We therefore wanted to establish if the deacetylase associated with RB can deacetylate E2F1. To ask this question, we purified the RB-associated deacetylase present in nuclear extracts using GST–RB. This endogenous deacetylase preparation was then added to recombinant E2F1 that was acetylated *in vitro* by P/CAF. Figure 7A shows that the acetylated E2F1 protein can be deacetylated by the RB-bound deacetylase.

We next sought to establish whether RB facilitates the deacetylation of E2F1 by tethering the deacetylase to E2F1. To this end, we generated recombinant baculovirus-expressed HDAC1 deacetylase. Figure 7B shows that this recombinant enzyme is capable of deacetylating P/CAF-acetylated E2F1 (lane 1) and that this effect is stimulated by RB (compare lanes 2 and 3). This stimulation was greatly diminished when an RB pocket mutant (Δ22), which cannot bind E2F, was used (lane 4). This suggests that RB may facilitate deacetylation of E2F1, either by stimulating the enzymatic capacity of HDAC or by facilitating the interaction between HDAC1 and its substrate E2F1. To distinguish between the two possibilities, we used as a substrate for HDAC1 the DNA-binding domain of E2F1, which cannot bind RB. Deacetylation of this truncated E2F1 protein is inefficient and is not augmented by RB, supporting a tethering role for RB (Figure 7C, lanes 1–4). Taken together, these results indicate that the acetylation of E2F1 is likely to be relevant to the non-RB-bound, 'free' form of E2F1, since tethering of the deacetylase complex by RB can deacetylate E2F1.

Discussion

Here we show that E2F1 is an acetylated protein *in vivo* and can be modified by acetylases *in vitro*. The enzyme which most potently acetylates E2F1 is P/CAF. Other acetylases (GCN5, p300 and CBP) can also acetylate E2F1, though much less efficiently. It is still uncertain as to which of these enzymes is responsible for the acetylation of E2F1 *in vivo* but, given that P/CAF and p300/CBP can be found in a complex, acetylation by either enzyme has the potential to regulate E2F1. Here we have pursued the consequences of acetylation by P/CAF.

The residues acetylated by P/CAF lie adjacent to the DNA-binding domain of E2F1. An E2F1 mutant (E2F1-R) that cannot be acetylated by P/CAF has been used to establish how acetylation regulates E2F1 function. One consequence of acetylation is an increase in E2F1 DNA-binding potential. The acetylated residues do not take part directly in DNA binding, as suggested by the recent crystal structure of the E2F4–DP1–DNA complex (Zheng *et al.*, 1999). However, the close proximity of the acetylated residues to the N-terminal helical extension (a proposed specificity helix) of the E2F1 DNA-binding domain makes it likely that a conformational change mediated by acetylation allows better access to DNA.

Acetylation by P/CAF leads to an increase in the transcriptional activation capacity of E2F1. We can confirm that this 'coactivation' by P/CAF is due to its ability to acetylate E2F1 since the E2F1-R mutant is unresponsive

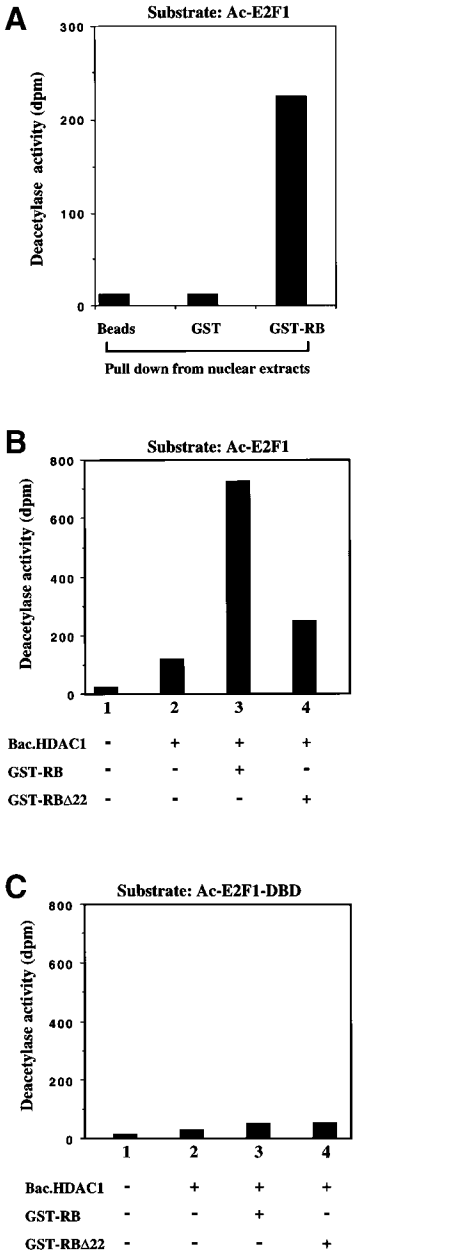

Fig. 7. RB-associated HDAC deacetylates E2F1. (**A**) The RB-associated deacetylase activity was purified from HeLa nuclear extract using GST–RB fusion protein bound to glutathione–Sepharose beads. As a control, only glutathione–Sepharose beads and GST protein were used. The recovered endogenous deacetylase complex was then added to 1 μg of recombinant acetylated His-E2F1 full-length fusion protein [previously *in vitro* acetylated by GST–P/CAF (amino acids 352–632) in the presence of [^{3}H]AcCoA]. The deacetylase assay was performed for 1 h at 37°C as described in Materials and methods. The histone deacetylase activity is given as d.p.m. of [^{3}H]acetate released from the full-length acetylated E2F1. (**B**) RB facilitates the deacetylation of E2F1. Active HDAC1 deacetylase was expressed from baculovirus, purified to homogeneity and assayed for its ability to deacetylate 1 μg of *in vitro* acetylated His-E2F1 full-length protein (lane 2) alone or in the presence of a saturating amount of either GST–RB fusion protein (lane 3) or a GST–RB pocket mutant (GST–RBΔ22), which cannot bind E2F (lane 4). (**C**) RB tethers the deacetylase to E2F1. A 1 μg aliquot of *in vitro* acetylated His-E2F1-DBD was subjected to deacetylation by purified recombinant, baculovirus-expressed HDAC1 in the absence of RB (lane 2) or in the presence of a saturating amount of either GST–RB recombinant protein (lane 3) or GST–RB pocket mutant (GST–RBΔ22). The histone deacetylase activity is given as d.p.m. of [^{3}H]acetate released from the acetylated His-E2F1-DBD.

to P/CAF. However, we cannot conclude at this point whether the increase in transactivation is a direct consequence of increased E2F1 DNA binding, or whether it is due to the increase in E2F1 stability caused by acetylation.

Acetylation is likely to regulate E2F1 when it is in its 'free' state, dissociated from RB. In this state, E2F1 can act as a transcriptional activator, stimulating the transcription of genes needed in S phase. Interestingly, the acetylated lysines are conserved only in the E2F family members that have the potential to induce S phase, suggesting that the acetylated form of E2F1 is relevant to this function. Acetylation may promote the activator functions of E2F1 by increasing its ability to recognize certain promoters. These promoters are likely to be those whose activity is stimulated by E2F1 since the RB repressor brings in a deacetylase complex that can deacetylate E2F1. Thus the E2F1–RB–HDAC repressor complex is unlikely to have its DNA-binding potential regulated by acetylation of E2F1. We therefore predict that the E2F–RB complex will contain unacetylated E2F1. We have not been able to verify this *in vivo* due to the low affinity of the anti-acetylated lysine antibody for acetylated E2F1 (data not shown). The generation of specific high affinity antibodies recognizing acetylated E2F1 is necessary to identify the complexes in which acetylated E2F exists.

What is still unclear is the necessity for an increase in DNA-binding functions of free E2F1, relative to RB-bound E2F1. One possibility is that acetylation not only increases DNA binding, but also allows E2F1 to recognize a subset of promoters distinct from those normally bound and repressed by E2F1–RB. Binding site selection experiments show that E2F1–RB may recognize a different consensus site from that recognized by free E2F1 (Tao *et al*., 1997). Also, there is evidence that certain E2F1 promoter elements act positively whereas others act negatively to regulate transcription (reviewed by Dyson, 1998). This difference may be, in part, a reflection of the different binding specificity and affinity of 'free' versus RB-bound E2F1. The close proximity of the acetylated lysines to the E2F1 DNA-binding domain specificity helix is suggestive of a role for site selection. Crystallization of full-length E2F1 bound to DNA will establish if these lysines are an integral part of the specificity helix.

Another consequence of acetylation uncovered by this analysis is an increase in the half-life of the E2F1 protein. This is clearly demonstrated by the comparison of E2F1 and the E2F1-R mutant. First, the E2F1-R protein accumulates to much lower levels than wild-type E2F1. This is not due to a general misfolding of the protein because E2F1-R can bind DNA *in vitro* and activate transcription *in vivo* as efficiently as wild-type E2F1 (Figures 4 and 5). Secondly, P/CAF overexpression stabilizes the E2F1 protein, but has no effect on the stability of the E2F1-R mutant (Figure 6). The mechanism by which acetylation protects from degradation is unknown. One possibility is that acetylation protects E2F1 from a ubiquitin-mediated degradation. Such a mechanism has been proposed for the stabilizing effect of RB. An alternative, but not mutually exclusive possibility is that acetylation protects E2F1 from a proteolytic cleavage.

The E2F1 protein is inherently unstable, so accumulation of functional E2F1 may be dependent on the protection from degradation mediated by proteins that associate with it. The stabilization of E2F1 by P/CAF acetylation may be necessary to allow the accumulation of a non-RB-bound form of E2F1. When E2F1 is bound to RB, its degradation is prevented, and the E2F–RB complex can then act to repress transcription. However, when E2F1 has to function as an activator, in an RB-free state, a protein distinct from RB has to mediate its stability. Such a protein may well be a co-activator protein such as P/CAF, which, by definition, is likely to associate with E2F1 when it is free from RB.

Thus, taken together, our data suggest the following model. The E2F1 protein present as part of the E2F1–RB complex is likely to be unacetylated. This complex has specific DNA-binding properties, is repressive for transcription and is stable because RB prevents E2F1 degradation by the ubiquitination pathway. In contrast, free E2F1 is, at least partly, acetylated, has DNA-binding properties distinct from the RB-bound complex, is able to activate transcription and is stable because co-activator proteins such as P/CAF can prevent its degradation via an acetylation-mediated pathway. What is still unclear is whether these two complexes co-exist in the cell temporally, or whether the E2F–RB complex is replaced by an E2F–P/CAF complex. From our current knowledge, the expectation would be that the E2F1–RB complex is active in G_1, and the free E2F protein, complexed with P/CAF, is active in S phase. The generation of specific high affinity antibody reagents that recognize the acetylated form of endogenous E2F1 will be useful in addressing this issue. What our results demonstrate is that acetylation represents a novel mechanism by which the activity of E2F1 is regulated.

Materials and methods

Cell culture, transfections and reporter gene assay

U2OS and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) and grown at 37°C and 5% CO_2. Cells were transfected by the calcium phosphate co-precipitation method (Hagemeier *et al*., 1993) for 14–18 h and then the medium was changed. After a 24–36 h incubation, the cells were washed in phosphate-buffered saline (PBS) and harvested.

For E2F1–P/CAF interaction, U2OS cells (14 cm dishes) were transfected with 8 μg of pCX-P/CAF. For immunoprecipitation of exogenous acetylated E2F1, 293T cells (14 cm dishes) were transfected with 8 μg of either pCX-P/CAF or pCX-P/CAFΔHAT in the presence or absence of either 3 μg of pcDNA3-E2F1 or 6 μg of pcDNA3-E2F1-R. For the reporter gene assay, U2OS cells (6 cm dishes) were transfected with 1 μg of pcDNA3-E2F1 or 0.5 μg of pcDNA3-E2F1-R, 1 μg of pCMV-DP1, 5 μg of pCX-P/CAF or pCX-P/CAF-ΔHAT, equivalent amounts of empty vector and 0.5 μg of CAT reporter gene plasmid. CAT assays were performed as described previously (Hagemeier *et al*., 1993). For the stability assay (by Western blot), 293T cells (6 cm dishes) were transfected with 5 μg of either pCX-P/CAF, pCX-P/CAFΔHAT, pCMV-sport2 mGCN5 FU or pCMVβ-p300, or with 3 μg of pCMV-RB. After transfection, the cells were resuspended in 100 μl of SDS-containing sample buffer (Bannister and Kouzarides, 1996). For pulse–chase experiments, 293T cells (one 80 cm^2 flask per time point) were transfected with 2 μg of pcDNA3-E2F1 or 6 μg of pcDNA3-E2F1-R, 8 μg of pCX-P/CAF and 1 μg of pCMV-luciferase. The transfection efficiency of the pulse–chase extracts was determined by a luciferase assay from Promega according to the supplier's instructions.

Plasmid and recombinant proteins

Mutations in the E2F1 cDNA (pGEX-E2F1 amino acids 89–437, a gift from Dr Kaelin) were introduced using the Quick change mutagenesis

kit (Stratagene) following the manufacturer's instructions and verified by DNA sequencing. Various domains of E2F1 and P/CAF were cloned into the pGEX-2TK vector (Pharmacia). The E2F1 full-length cDNA was cloned from pCMV-NeoBam-E2F1 (a gift from Dr Kaelin) as a *Bam*HI–*Eco*RV fragment into *Bam*HI–*Sma*I-digested pQE30 vector (Qiagen). P/CAF-ΔHAT (amino acids 352–658; Reid *et al*., 1998), CBP (amino acids 1098–1877), RB, RBΔ22 and DP1 (amino acids 59–410) were expressed as GST fusion proteins, whereas GCN5 (amino acids 1–427, a gift from Dr Berger) and p300 (amino acids 1071–1715) were expressed as His fusion proteins. The different GST and His fusion proteins were expressed in *Escherichia coli* strain XA90 or M15 and purified as previously described (Brehm *et al*., 1998). The His-E2F1-DNA-binding domain (DBD) was a gift of Steve Gamblin.

cDNAs of E2F1 and E2F1-R were introduced into *Bam*HI–*Eco*RI and *Bam*HI–*Eco*RV sites, respectively, of the pcDNA3 vector (Invitrogen). pCX-P/CAF vector was a gift from Dr Nakatani. pCX-P/CAF-ΔHAT, which contains a deletion of amino acids 497–526, was cloned by digesting pCX-P/CAF cDNA with the enzymes *Bbr*PI and *Pvu*II and religating. pCMV-sport2 mGCN5 FU vector was a gift from Dr Roth and pCMV-DP1 was from Dr Helin. The reporter gene vector used in the CAT assays contains three wild-type or mutated E2F-binding sites of the adenovirus E2A promoter cloned upstream of a thymidine kinase promoter (Zamanian and La Thangue, 1992). pCMV-luciferase was a gift from Dr Herget.

In vitro translations and pull-down assays

In vitro translations and GST pull-downs were performed essentially as described previously (Hagemeier *et al*., 1993). Equal amounts of the different P/CAF fusion proteins were assayed.

Protein acetylase and deacetylase assays

Protein acetylase assays were carried out as described (Bannister and Kouzarides, 1996). Liquid HAT assays were used to adjust the activities of the different acetylases. Therefore, crude core histones and enzyme samples were incubated in the presence of [^{3}H]acetyl-CoA (NEN), and histone acetylation was determined by liquid scintillation counting. For the analysis of HAT activity on E2F1, 20–200 ng of the different acetylase fusion proteins (20000 c.p.m. activity on histones) were incubated with 2 μg of purified E2F1 fusion proteins and [^{14}C]acetyl-CoA (NEN). The reaction products were resolved by SDS–PAGE and blotted on nitrocellulose membrane. Autoradiography was performed at –70°C for 6 h–8 days. For the preparations of acetylated E2F1 and E2F1-R for electrophoretic mobility shift assay (EMSA), [^{14}C]acetyl-CoA was replaced with unlabelled acetyl-CoA (Sigma).

The pull-down deacetylase assay was performed as described (Brehm *et al*., 1998). For the deacetylase assay with baculovirus-expressed HDAC1, 5 μl of HDAC1 (30 000–50 000 c.p.m. activity on acetylated H4 peptide) were incubated with 1 μg of either acetylated His-E2F1 or acetylated His-E2F1-DBD as a substrate in the presence or absence of 20 μg of either GST–RB or GST–RBΔ22 mutant.

Immunoprecipitations and Western blot analysis

Immunoprecipitations were carried out as previously described (Vandel and Kouzarides, 1999). Antibodies used were monoclonal anti-Flag antibody M2 (Kodak), polyclonal anti-E2F1 antibody C20 (Santa Cruz) or monoclonal anti-HA antibody 12CA5 (Boehringer Mannheim). In the case of E2F1 and E2F1-R overexpression, the expression levels of both proteins were checked by Western blot analysis, and equal amounts of protein were used for immunoprecipitation. For immunoprecipitation with polyclonal anti-acetylated lysine antibody (New England Biolabs), the antibody was covalently coupled to protein A–Sepharose using the cross-linker dimethylpimelimidate (Sigma) as described (Harlow and Lane, 1988).

Proteins were separated by SDS–PAGE, followed by liquid transfer to nitrocellulose and immunostaining of the blot according to standard procedures (Martin *et al*., 1995). Primary antibodies for Western blot analysis were anti-E2F1 C20 or KH95 (Santa Cruz) at 1:400 dilution, anti-Flag M2 (Kodak) at 1:1000 dilution and anti-acetylated H4 histone tail (a gift from Dr Crane-Robinson) at 1:500 dilution.

Electrophoretic mobility shift assay

EMSA was carried out as described (Kaelin *et al*., 1992). The sequence of the oligonucleotide containing the E2F-binding site of the DHFR promoter is as follows: 5′-ATTTAAGATTTCCCGCCTTTTCTCAA-3′. DNA–protein complexes containing E2F1-DBD were separated on an 8% 1× TAE polyacrylamide gel, whereas complexes containing full-length E2F1 were separated on 4% 0.25× TBE gels.

Pulse–chase experiment

Pulse–chase experiments were performed as previously described (Hofmann *et al*., 1996) with some modifications. After transfection, 293T cells were starved for 1 h in methionine/cysteine-free medium and then labelled with 0.2 mCi of [^{35}S]methionine/cysteine (NEN) per flask for 2 h. Cells were harvested (for time point zero) or incubated in the presence of a 10-fold excess of non-radioactive methionine and cysteine for 1, 3 and 5 h and harvested thereafter. Equal amounts of radioactive lysate were used for immunoprecipitation of E2F1 by adjusting differences in the transfection efficiency with the aid of the luciferase assay. Immunoprecipitates were separated by SDS–PAGE, blotted and autoradiographed. The intensity of ^{35}S-labelled E2F protein was calculated.

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