HMGA2 induces pituitary tumorigenesis by enhancing E2F1 activity

Monica Fedele,[1,8] Rosa Visone,[1,2,8] Ivana De Martino,[1] Giancarlo Troncone,[3] Dario Palmieri,[1] Sabrina Battista,[1] Andrea Ciarmiello,[4] Pierlorenzo Pallante,[1] Claudio Arra,[4] Rosa Marina Melillo,[1] Kristian Helin,[5,6] Carlo Maria Croce,[2] and Alfredo Fusco[1,7,*]

[1] Istituto di Endocrinologia ed Oncologia Sperimentale del CNR and Dipartimento di Biologia e Patologia Cellulare e Molecolare, Università di Napoli ''Federico II'', 80131 Naples, Italy
[2] Division of Human Cancer Genetics, Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio 43210
[3] Dipartimento di Anatomia Patologica, Università di Napoli ''Federico II'', 80131 Naples, Italy
[4] Istituto dei Tumori di Napoli Fondazione ''G. Pascale'', via Semmola, 80131 Naples, Italy
[5] Biotech Research & Innovation Centre, Fruebjergvej 3, 2100 Copenhagen, Denmark
[6] European Institute of Oncology, Department of Experimental Oncology, 20141 Milan, Italy
[7] NOGEC (Naples Oncogenomic Center) – CEINGE, Biotecnologie Avanzate, Naples, Italy
[8] These authors contributed equally to this work.
*Correspondence: afusco@napoli.com

Summary

***HMGA2* gene amplification and overexpression in human prolactinomas and the development of pituitary adenomas in *HMGA2* transgenic mice showed that HMGA2 plays a crucial role in pituitary tumorigenesis. We have explored the pRB/E2F1 pathway to investigate the mechanism by which HMGA2 acts. Here we show that HMGA2 interacts with pRB and induces E2F1 activity in mouse pituitary adenomas by displacing HDAC1 from the pRB/E2F1 complex—a process that results in E2F1 acetylation. We found that loss of E2F1 function (obtained by mating *HMGA2* and *E2F1*$^{-/-}$ mice) suppressed pituitary tumorigenesis in *HMGA2* mice. Thus, HMGA2-mediated E2F1 activation is a crucial event in the onset of these tumors in transgenic mice and probably also in human prolactinomas.**

Introduction

High mobility group A (HMGA) proteins are nonhistone nuclear compounds known as architectural transcriptional factors because they mediate the assembly of multiprotein complexes involved in gene transcription. In fact, by interacting with the minor groove of many AT-rich promoters and enhancers, HMGA proteins do not per se exert transcriptional activity, but organize chromatin into the structure required by the transcription machinery to allow gene transcription. There are four HMGA proteins: HMGA1a, HMGA1b, HMGA1c, and HMGA2. The first three are encoded by the gene *HMGA1*, whereas HMGA2 is encoded by *HMGA2. HMGA2* is almost ubiquitously expressed at high levels during embryogenesis, whereas in adult tissues it occurs only in CD34-positive hematopoietic stem cells, uterine myoblasts, testicular cells, and proliferating preadipocytes. Its function is critical for growth and adipocytic cell differentiation. In fact, its impairment results in pygmy mice with greatly reduced fat tissue, whereas its activation by truncation leads to giant mice affected by lipomatosis. *HMGA2* overexpression was first demonstrated in rat thyroid transformed cells and experimental thyroid tumors. Subsequent studies showed that *HMGA2* expression is required for thyroid cell neoplastic transformation and that HMGA2 expression is correlated with human malignant tumors. HMGA2 rearrangements are frequent in benign tumors of mesenchymal origin (reviewed by Fedele et al., 2001). We previously found that the *HMGA2* gene is amplified and overexpressed in most human prolactinomas characterized by chromosome 12 trisomy and tetrasomy (Finelli et al., 2002) and that transgenic mice expressing high levels of the *HMGA2* transgene in all tissues develop pituitary adenomas secreting prolactin and growth hormone, thereby confirming that *HMGA2* plays a critical role in human pituitary adenomas (Fedele et al., 2002).

The aim of our study was to investigate the mechanisms by which HMGA2 leads to the onset of pituitary adenomas in *HMGA2* transgenic mice and probably also in humans.

There are striking phenotypic similarities between the pRB and HMGA2 animal models. In fact, transgenic mice

SIGNIFICANCE

The molecular pathway leading to pituitary tumorigenesis is one of the challenges of endocrine oncology. Here, we describe a mechanism by which HMGA2 induces pituitary adenomas in mice. This mechanism entails an HMGA2-dependent process that relieves pRB-mediated repression of E2F1. It is likely that the same mechanism is involved in most of the human prolactinomas in which the *HMGA2* gene is amplified and overexpressed. Elucidation of the mechanism by which HMGA2 induces pituitary adenomas opens up the possibility of intervening in this mechanism to prevent or halt the onset of these tumors.

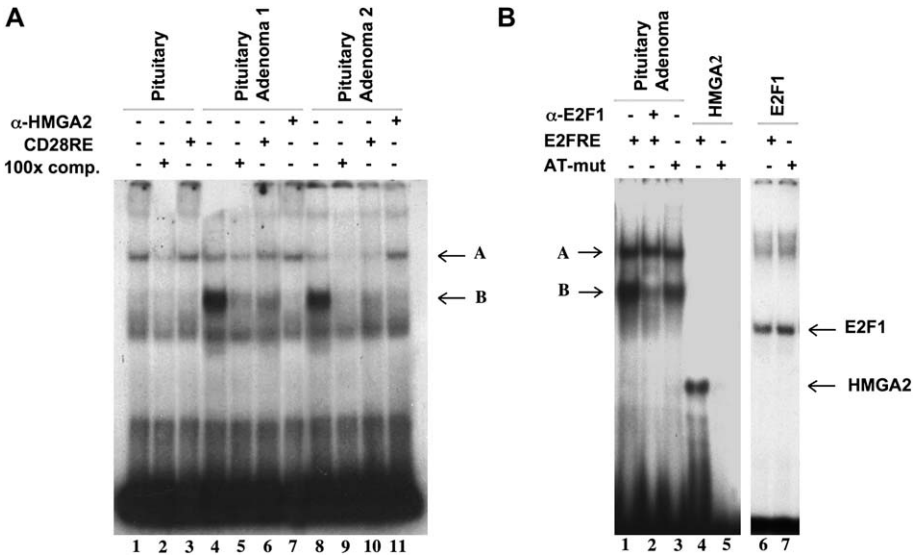


Figure 1. HMGA2 increases E2F1 DNA binding activity in pituitary adenomas

A: EMSA with extracts from a pool of pituitary glands (lanes 1–3) and two pituitary adenomas (lanes 4–11) of *HMGA2* transgenic mice using an E2F-responsive element sequence (E2FRE) as a probe. Samples were incubated with E2FRE (lanes 1, 4, and 8) or were preincubated with 100× CD28 responsive element (CD28RE), which is a specific competitor for HMGA2 binding (lanes 3, 6, and 10), with 100× E2FRE unlabeled probe (lanes 2–5 and 9), and with specific polyclonal anti-HMGA2 antibody (lanes 7 and 11), before the addition of the probe. Arrows indicate specifc complexes DNA/proteins.

B: EMSA of pituitary extracts of adenomas from *HMGA2* transgenic mice with E2FRE (lanes 1 and 2) or E2FRE mutated in the region rich in AT bases (AT-mut; lane 3). The samples in lanes 1 and 2 were incubated without (lane 1) or with anti-E2F1 antibody (lane 2), before the addition of the probe. His-HMGA2 (lanes 4–5) or GST-E2F1 (lanes 6–7) recombinant proteins were incubated with E2FRE or AT-mut, as indicated above.

overexpressing pRB show a dwarf phenotype because of defective cell proliferation (Bignon et al., 1993) as do *HMGA2* null mice. Conversely, $Rb^{+/-}$ mice are larger than their littermates (Jacks et al., 1992), as are transgenic mice carrying an activated *HMGA2* gene (Battista et al., 1999). Moreover, mice carrying a germline mutation of one *Rb* allele are highly predisposed to develop pituitary tumors (Jacks et al., 1992), and this occurs also in mice with impaired functioning of p27 or p18, both of which converge on pRB (Franklin et al., 1998).

The finding that loss of E2F1 reduced pituitary tumorigenesis in *Rb* knockout mice suggests that loss of pRB induces the onset of pituitary tumors by activating E2F1 (Yamasaki et al., 1998). We therefore investigated the role of the pRB pathway in the onset of pituitary adenomas in transgenic mice overexpressing HMGA2.

pRB and the related proteins p130 and p107 control cell cycle progression through their interactions with the E2F family of transcription factors. E2F1 is known to activate transcription of a number of genes required for the S phase of the cell cycle. Transcriptional repression of E2F1 target genes entails recruitment of pRB by E2F1 to the gene promoters. This recruitment masks the activation domain of E2F1 and prevents its interaction with the general transcriptional machinery. Before cells enter S phase, pRB is phosphorylated at multiple sites by cyclin-dependent kinases. This phosphorylation leads to pRB inactivation, release of E2F1, and transcriptional activation of its target genes (reviewed by Seville et al., 2005). Moreover, pRB recruits class I histone deacetylase proteins that repress transcription by removing acetyl groups from the histones. Removal of the acetyl groups facilitates the condensation of nucleosomes into chromatin, which in turn blocks access to transcription factors and leads to gene repression (Magnaghi-Jaulin et al., 1998).

Here we report that HMGA2 binds to pRB and prevents it from repressing the E2F1-responsive promoters mainly by displacing HDAC1 from pRB. This results in enhanced acetylation of both E2F1 and associated histones, which could account for the enhanced E2F1 activity observed in HMGA2-induced pituitary adenomas and associated with the HMGA2 overexpression. To verify the crucial role of enhanced E2F activity in the onset of pituitary adenomas, we mated transgenic mice overexpressing HMGA2 with *E2F1* knockout mice (Field et al., 1996). The adenomatous phenotype was almost totally rescued in the double mutant mice, which demonstrates that HMGA2-mediated E2F1 activation is a prerequisite for pituitary tumorigenesis.

Results

E2F1 DNA binding activity is increased in pituitary adenomas of *HMGA2* transgenic mice

To investigate whether the pRB/E2F1 pathway is involved in pituitary tumorigenesis in *HMGA2* transgenic mice, we analyzed E2F DNA binding activity in HMGA2 pituitary adenomas and normal pituitary glands from control mice by electrophoretic mobility shift assay (EMSA) using an E2F-responsive element (E2FRE) as probe. As shown in Figure 1A, E2F1 DNA binding activity was dramatically higher in HMGA2 pituitary adenomas than in pituitary glands from control mice. A specific complex containing E2F (complex A) was present in normal pituitary gland (lane 1). Another, faster-migrating complex (complex B) was associated with pituitary adenomas from HMGA2 mice (lanes 4 and 8). Competition analyses with specific anti-E2F1 antibodies showed that this band corresponds to the free E2F1-DNA complex (Figure 1B, lane 2). Band specificity was assessed by adding a 100× molar excess of unlabeled probe (Figure 1A, lanes 2, 5, and 9). Preincubation with an oligonucleotide (CD28RE) that specifically binds to HMGA2 (Baldassarre et al., 2001) prevented the formation of this complex (Figure 1A, lanes 6 and 10). Moreover, an antibody against HMGA2 significantly reduced the band corresponding to complex B (Figure 1A, lanes 7 and 11). These data show that HMGA2 plays a crucial role in the formation of complex B.

HMGA2 binds to the E2F1 responsive element and enhances the binding of E2F1 to DNA

HMGA proteins allow the assembly of multiprotein complexes by directly binding to the DNA in AT-rich sequences. We used an E2F consensus oligonucleotide (E2FRE) that has an AT stretch compatible with HMGA binding in an EMSA assay with

a recombinant HMGA2 protein. As shown in lane 4 of Figure 1B, HMGA2 was able to bind to the E2F-responsive oligonucleotide, but not to the same oligonucleotide mutated in the AT-stretch flanking the E2F-consensus sequence (AT-mut). Interestingly, the binding of free E2F1 to DNA in pituitary adenomas from HMGA2 transgenic mice was considerably decreased on the AT-mut probe (Figure 1B, lane 3). Because the binding of the recombinant E2F1 to its consensus DNA was not impaired by the AT mutation (Figure 1B, lanes 6 and 7), we suggest that the binding of HMGA2 to its consensus sequence, flanking the E2F binding site, is crucial for efficient E2F1/DNA binding.

HMGA2 interacts with the pRB protein

Since free E2F1/DNA binding depends essentially on E2F1 release from pRB, HMGA2 may interfere with the pRB pathway. To investigate whether HMGA2 interacts with pRB, we coimmunoprecipitated protein lysates from two HMGA2 pituitary adenomas and from a pool of pituitary glands from control mice. Immunoprecipitation of the protein lysates with anti-pRB antibodies resulted in the coimmunoprecipitation of HMGA2 from pituitary adenomas (Figure 2A, left panels). Western blot analysis showed that pRB was equally expressed in pituitary glands and adenomas, whereas the HMGA2 protein was expressed only in HMGA2 pituitary adenomas.

We next verified the HMGA2/pRB interaction in a heterologous cell system. HEK293 cells were transiently cotransfected with hemagglutinin (HA)-tagged-HMGA2 and pRB expression vectors. Protein lysates were immunoprecipitated with anti-pRB or anti-HA antibodies and immunoblotted with anti-HA or anti-pRB, respectively (Figure 2A, right panels). Coexpression of pRB and HMGA2 resulted in coimmunoprecipitation of the two proteins. Conversely, there was no coimmunoprecipitation when HEK293 cells were transfected with HA-HMGA2 or pRB expression vectors alone. Indeed, consistent with the endogenous expression of pRB in HEK293 cells, longer exposure times resulted in a faint band in HA-HMGA2 transfected cells (data not shown).

The HMGA2/pRB interaction is direct and involves either the pocket region or the N terminus of pRB

To determine whether HMGA2 interacts directly with pRB, we carried out Far-Western analyses in which we probed HMGA2 on pRB immunoprecipitates from cell lysates overexpressing wild-type and different mutant forms of the pRB protein (Figures 2B and 2C). Interestingly, HMGA2 was found to bind to the pRB mutant expressing only the pocket region (lane 5) as efficiently as to the wild-type pRB (lane 1). It did not bind to the naturally occurring mutant pRBΔ21 that carries a deletion of exon 21 and encodes a portion of the pocket region (lane 4). pRB mutants 661 and 13S, which carry a point mutation in the pRB pocket region and amino acid substitutions and insertions in the C-terminal region, respectively (lanes 2 and 3), did not affect binding between pRB and HMGA2.

These results are supported by in vitro binding experiments in which GST-pRB fusion proteins containing various pRB domains (Figure 2B) were incubated with a lysate from cells overexpressing HMGA2 (Figure 2D). pRB(379-928), which contains the entire wild-type pocket region of pRB plus the C-terminal region, was able to bind efficiently to HMGA2 (lane 1). In contrast, pRB(379-928)Δ21, which lacks the region corresponding to exon 21, only weakly associated with HMGA2 (lane 3). pRB(768-928) and pRB(834-928), which carry the C-terminal region plus a small region of the pocket and the C-terminal region only, respectively, did not bind to HMGA2 at all (lanes 5 and 6). Finally, pRB(1-379) and pRB(379-792), carrying the N-terminal region and the pocket region, respectively, were able to bind efficiently to HMGA2 (lanes 2 and 4). These results indicate that HMGA2 binds to pRB in two regions: the N terminus and the pocket region between amino acids 703 and 737.

The second AT-hook of HMGA2 is required for interaction with pRB

We next investigated the regions of HMGA2 that are required to form a complex with pRB. HMGA2 contains three AT-hook domains that are involved in both DNA and protein-protein interactions, and an acidic C-terminal tail preceded by a spacer region (Figure 2E). We used cell lysates expressing HA-HMGA2 mutants carrying serial deletions at the N and C terminus regions (Figure 2E) in a pull-down assay with a recombinant GST-pRB protein (Figure 2F, lanes 1–6). The only mutant that lost the capacity to bind to pRB was A2(1-44), which contains the N terminus, the first AT-hook, and the region preceding the second AT-hook. Because all the other mutants, including A2(1-56), which encodes the same protein portion as mutant A2(1-44) plus the second AT-hook, were able to bind to pRB as efficiently as the wild-type HMGA2, we suggest that the second AT-hook domain of HMGA2 is principally involved in binding to pRB. To map more precisely the HMGA2 region involved in forming a complex with pRB, we generated an HMGA2 mutant lacking only the second AT-hook [A2(Δ^{45-56})] (Figure 2E) and used it in a pull-down assay with the GST-pRB protein (Figure 2F, lane 7). The A2(Δ^{45-56}) mutant was less able than wild-type HMGA2 to bind to pRB. This confirms that the region of HMGA2 coding for the second AT-hook is principally involved in binding pRB and suggests, however, that other regions of the HMGA2 protein might also be involved in this binding.

HMGA2 overexpression counteracts pRB-mediated inhibition of E2F activity and cell proliferation

To define better the role of the pRB/HMGA2 interaction in the regulation of E2F1 transcriptional activity, we examined the activity of two E2F1-responsive promoter genes, i.e., CDC25A and CDC6, fused to a luciferase reporter gene, in the HEK293 cells transiently transfected with plasmids expressing the pRB and HMGA2 proteins. As shown in Figure 3A, pRB repressed CDC6 and CDC25A promoter activity (3-fold decrease), whereas HMGA2 increased these activities (up to 3-fold) in a dose-dependent manner. When pRB was cotransfected with increasing levels of HMGA2, pRB repression of CDC25A and CDC6 promoter activity was significantly antagonized. In particular, 5 μg of transfected HMGA2 vector abolished repression, and 10 μg of HMGA2 caused a switch from repression to activation (2.5-fold increase). Similar experiments were carried out in pRB null Saos-2 cells by transfecting pRB, HMGA2, and mutants A2(1-44) or A2(Δ^{45-56}), as indicated in Figure 3B. In the absence of pRB, HMGA2 does not affect E2F target gene activation, and neither of the mutants antagonized the pRB-mediated inhibition of the promoter-response activity, which suggests that HMGA2 binding to pRB is required for this effect.

The results of a colony-forming assay of Saos-2 cells demonstrate that HMGA2 antagonizes the pRB-mediated inhibition of cell proliferation (Figure 3C). The expression of transfected

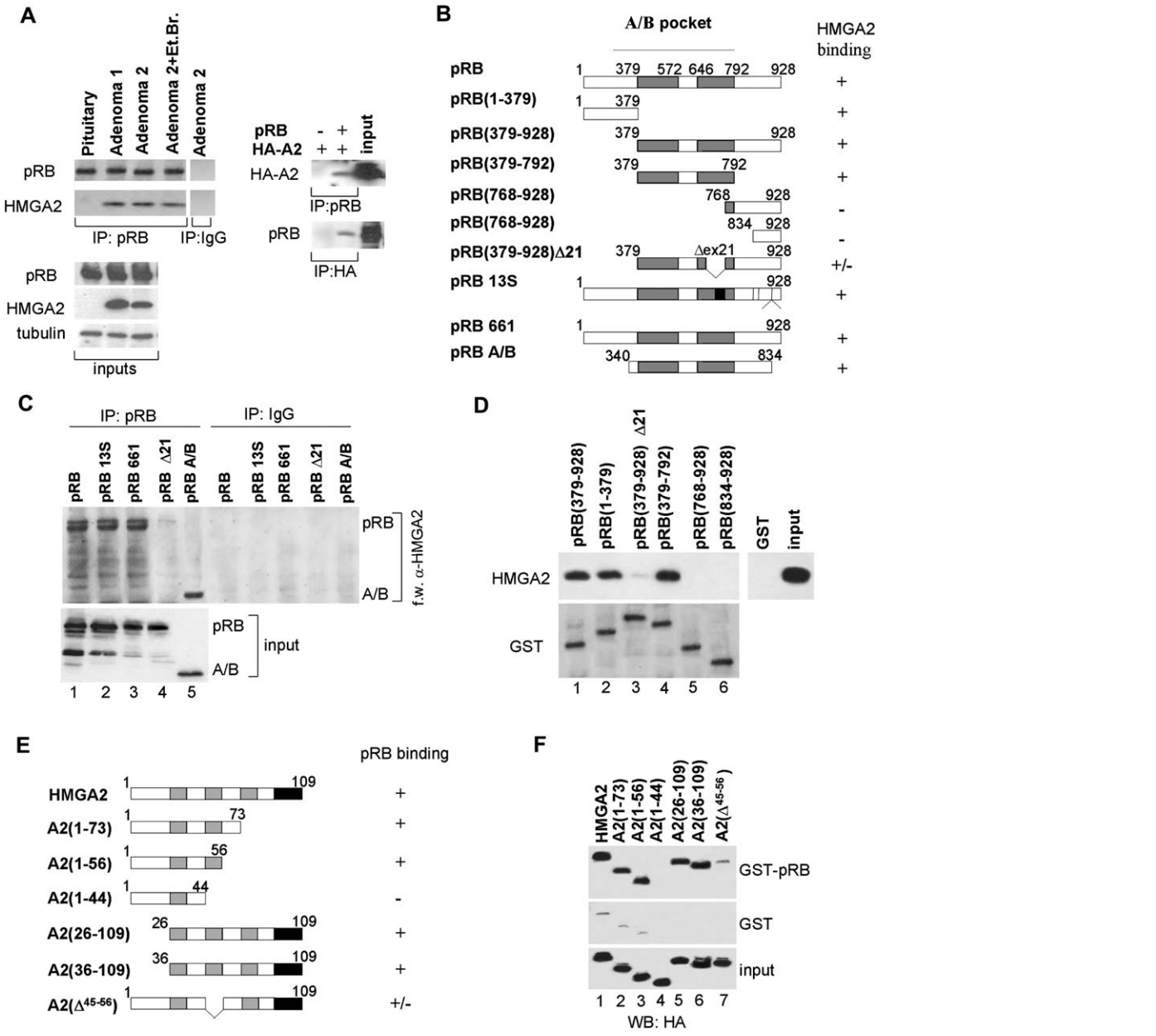


Figure 2. HMGA2 interacts with pRB

A: Left panels. Lysates from a pool of pituitary glands of control mice and from the pituitary adenomas shown in Figure 1 were subjected to Western blot analysis with either anti-pRB or anti-HMGA2 antibodies to verify protein expression. The expression of γ-tubulin served to verify equal loading of proteins. The same lysates were immunoprecipitated with anti-pRB antibody, resolved by SDS-PAGE, and analyzed by Western blot with anti-HMGA2 antibody. Immunoprecipitation in the presence of EtBr and with IgG were also performed as control of the specificity of the interaction. Right panels. Whole-cell lysates from HEK293 cells untransfected or transfected with the indicated expression plasmids were immunoprecipitated with anti-pRB (upper panel) or anti-HA (lower panel) antibodies, run on an SDS-PAGE gel, and transferred to nitrocellulose. The blot was probed with anti-HA or anti-pRB antibodies, respectively.

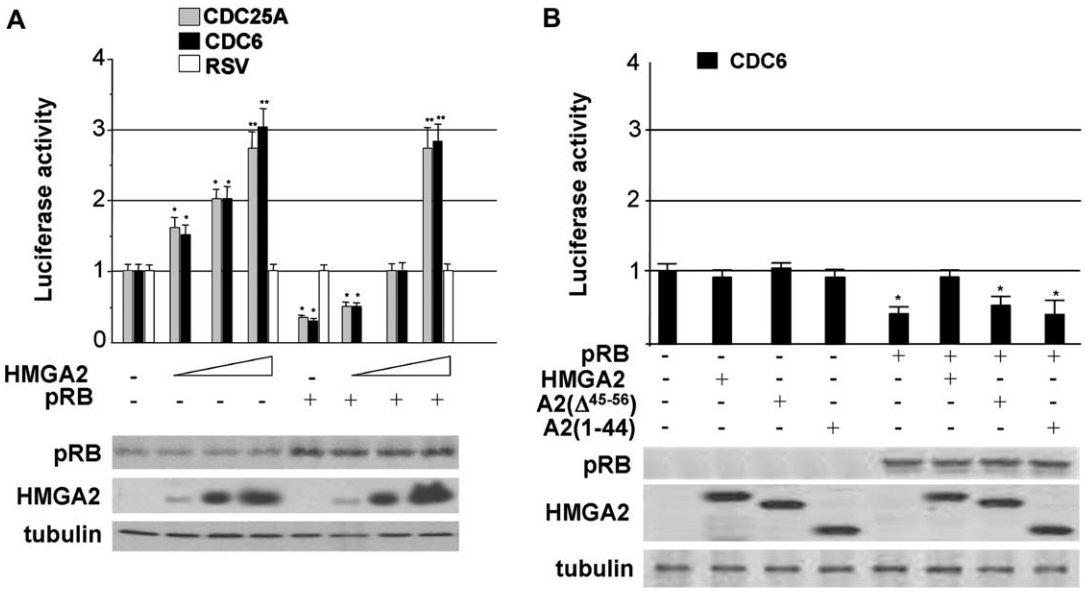
B: Schematic representation of GST-pRB fusion proteins and pRB expressing plasmids used for the analysis.

C: Whole-cell lysates from HEK293 cells transfected with plasmids expressing pRB proteins, as described in (**B**) were immunoprecipitated with either anti-pRB polyclonal antibody or preimmune serum. After being washed, samples were run on an SDS-PAGE gel and transferred to nitrocellulose. The blot was probed with His-HMGA2 in a Far-Western assay as described in Experimental Procedures (upper panel). The membrane was stripped and probed with an anti-pRB antibody (lower panel).

D: Whole-cell lysate from HEK293 cells transfected with a plasmid expressing HA-HMGA2 (5 μg) was subjected to GST pull-down analysis using the GST or GST-pRB fusion proteins reported in (**B**). Binding reaction products were washed, and proteins were separated by SDS-PAGE. The membrane was probed with both anti-HA and GST antibodies.

E: Schematic representation of plasmids expressing wild-type and mutant HA-HMGA2 proteins used for the analysis.

F: Whole-cell lysates from HEK293 cells transfected with the plasmids reported in (**E**) were subjected to GST pull-down analysis using GST-pRB protein beads (upper panel) or GST protein beads (medium panel) as control. Aliquots of the same lysates were probed with anti-HA antibody to evaluate the comparable expression of the transfected plasmids (lower panel).



C **Colony-forming assay**

Transfected plasmids	No. of clones
Empty vector	201 ± 12.0
HMGA2	187 ± 9.0
pRB	52 ± 3.2
pRB + HMGA2	212 ± 19.3
pRB + A2(1-44)	49 ± 2.5
pRB + A2(Δ^{45-56})	56 ± 5.0

D **Focus assay**

Transfected plasmids	Transforming efficiency (f.f.u./pmol)
Empty vector	0
RasV12	$3x10^4$
HMGA2	$2x10^2$
A2(1-44)	0
A2(Δ^{45-56})	0

Figure 3. HMGA2 counteracts the pRB-mediated inhibition of E2F activity and cell proliferation

A: Luciferase activities of extracts from HEK293 cells cotransfected with CDC25A-luc or CDC6-luc reporter plasmids and increasing amounts of HMGA2 with or without a pRB expression vector. The RSV-luc plasmid served as a negative control. Relative activities were calculated by dividing normalized activities with the activity of cells transfected with the reporter plasmid alone. Aliquots of the same lysates were resolved by SDS-PAGE, transferred to Immobilion-P, and immunoblotted with the indicate antibodies (lower panels).

B: Saos-2 cells were transiently transfected with plasmids expressing HA-HMGA2 wild-type and mutated proteins and subjected to luciferase assay on the CDC6-luc reporter promoter. Relative activities were calculated as reported in (**A**). Aliquots of the same lysates were resolved by SDS-PAGE and immunoblotted with the indicate antibodies (lower panels).

C: Saos-2 cells were transiently transfected with the expression plasmids indicated on the left and selected for 14 days in the appropriate antibiotic. Cell clones were counted following staining with crystal violet.

D: *Focus* assay on Rat-2 cells transfected with the plasmids indicated on the left. The transforming efficiency was expressed as foci-forming units (f.f.u.)/pmol of transfected DNA. All the data reported are the mean ± SE of three independent experiments. *$p < 0.05$; **$p < 0.01$.

pRB greatly reduced the colony-forming ability. Cell growth inhibition was significantly reduced in the presence of a coexpressed HMGA2 construct and was not affected by either the A2(1-44) or A2(Δ^{45-56}) mutant. Conversely, HMGA2 alone had no significant effects on colony-forming ability. These results suggest that HMGA2, by interacting with pRB, inhibits the pRB negative regulation of E2F1 activity, thereby blocking its growth-suppressing potential.

The binding of HMGA2 to pRB is crucial for its transforming activity

It has been previously reported that HMGA2 induces neoplastic transformation of cultured rat fibroblasts (Wood et al., 2000), demonstrating its oncogenic potential. In order to verify whether the binding between HMGA2 and pRB, with the resulting pRB inactivation, might be responsible for the oncogenic properties of HMGA2, we evaluated the *focus*-forming ability of HMGA2 and pRB binding mutants of HMGA2. To do this, we transfected Rat-2 cells with either HMGA2 or each of the HMGA2 mutants unable to efficiently bind to pRB. As shown in Figure 3D, the wild-type HMGA2 was able to induce *foci*, whereas no *foci* were observed in transfected cells with both the HMGA2 mutants. High transforming efficiency was obtained by transfecting the activated Ha-Ras gene (Ras 12V) that we used as a positive control of the assay. These results suggest that the binding between HMGA2 and pRB is necessary for the HMGA2 oncogenicity and that the mechanism by which HMGA2 is involved in pituitary tumorigenesis may be generally involved in HMGA2-mediated cell transformation.

HMGA2 displaces HDAC1 from the pRB/E2F complex

Many cellular and viral proteins have been shown to bind pRB within the pocket region of pRB (Hu et al., 1990). Consequent to this process, E2F1 is released from the binding to pRB and becomes active. Because HMGA2 binds to pRB in this same pocket region, we tested the hypothesis that the HMGA2/pRB

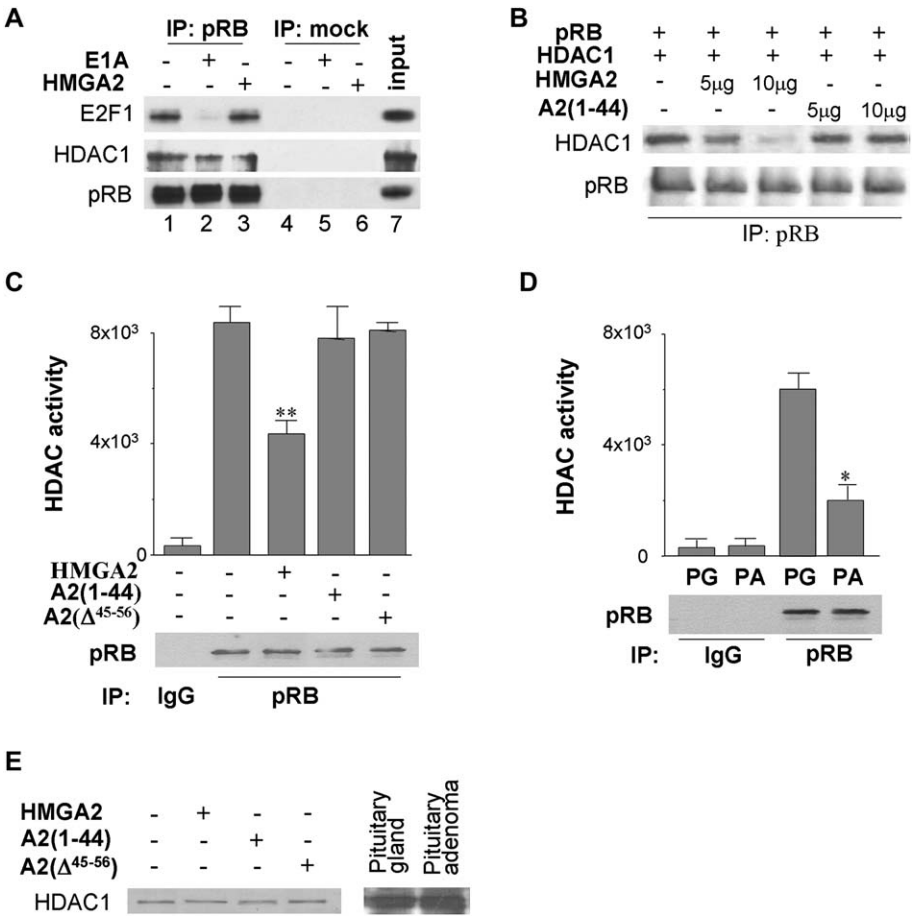


Figure 4. HMGA2 competes with HDAC1 but not with E2F1 for pRb binding

A: HEK293 cell lysates coexpressing both E2F1 and pRB transiently transfected plasmids were subjected to immunoprecipitation with anti-pRB antibody after incubation with either HMGA2 or E1A recombinant proteins, as indicated above, and then blotted against either E2F1 or HDAC1, as indicated on the left. pRB Western blot served as a control of the equal loading of the immunoprecipitated pRB protein.

B: Labeled HDAC1 and recombinant pRB were coimmunoprecipitated with or without 5 or 10 μg of either recombinant HMGA2 or A2(1-44) proteins. The samples were then blotted and probed with either HDAC1 or pRB antibody, as indicated on the left.

C: Lysates from Saos-2 cells, transiently transfected with the plasmids indicated on the left or with the empty vector, were incubated with a recombinant pRB protein and immunoprecipitated with anti-pRB antibodies. The resulted immunoprecipitates were in part subjected to histone deacetylase assay as described under Experimental Procedures and in part run on a SDS-PAGE for evaluation of the equal amount of pRB immunoprecitated in each sample.

D: Lysates from a pool of pituitary glands of wild-type mice and one pituitary adenoma of *HMGA2* transgenic mice were assayed as in (**C**). The results reported in (**C**) and (**D**) are the mean ± SE of three independent experiments. *$p < 0.05$; **$p < 0.01$.

E: Cell inputs from the experiments shown in (**C**) and (**D**) were assayed by Western blot for the expression of the HDAC1 protein.

interaction interferes with the binding between pRB and E2F1, thereby inducing E2F1 activation. To this aim, we performed coimmunoprecipitation experiments with HEK293 cells transiently cotransfected with pRB and E2F1 expression plasmids in the presence of E1A or HMGA2 recombinant proteins (Figure 4A). As previously reported (Putzer et al., 1997), E1A prevented the binding between pRB and E2F1 (lane 2), whereas HMGA2 did not (lane 3). Thus, HMGA2 does not activate E2F1 by competing with it for binding to pRB.

Another mechanism by which the pRB/E2F complex represses transcription entails recruitment of a histone deacetylase, the HDAC1 protein, to the E2F binding sites by pRB (Magnaghi-Jaulin et al., 1998). Histone acetyl transferases and histone deacetylases acetylate and deacetylate, respectively, core histone tails that protrude from the nucleosome. Histone acetylation is thought to weaken the interaction between histone N-terminal tails and DNA, thus opening up the chromatin and increasing accessibility for activating transcription factors. Therefore, the binding of the viral transforming proteins to the pRB-pocket region results in the loss of E2F and HDAC1 binding.

We thus asked whether the HMGA2/pRB interaction could displace HDAC1 from the pRB/E2F complex, and so account for E2F1 activation. To this aim, we used the same immunoprecipitates described above to also analyze the binding of HDAC1 to pRB. As shown in Figure 4A, both E1A (lane 2) and HMGA2 (lane 3) reduced the binding of pRB to HDAC1. This shows that, similarly to the E1A/pRB interaction, the HMGA2/pRB complex can affect HDAC1 recruitment.

To verify this result, we used a cell-free system in which pRB and HDAC1 recombinant proteins were incubated with or without increasing amounts of a recombinant HMGA2 protein. As shown in Figure 4B, HDAC1 was partially or completely displaced by the binding to pRB in the presence of 5 μg and 10 μg of HMGA2, respectively. This result demonstrates that HMGA2 directly interferes with the binding between HDAC1 and pRB. Moreover, to correlate the effect of HMGA2 on HDAC1 displacement from pRB with the HMGA2/pRB interaction, we also performed the experiment by using the A2(1-44) mutant, unable to bind pRB. As shown in the same Figure 4B, the binding between HDAC1 and pRB was not affected at all by the HMGA2 mutant, suggesting that the interaction between HMGA2 and pRB plays a crucial role in the displacement of HDAC1 from pRB.

Because recruitment of HDAC1 by pRB to gene promoters decreases their level of histone acetylation (Magnaghi-Jaulin et al., 1998), we evaluated whether HMGA2 overexpression was associated with decreased HDAC activity associated with the pRB complexes. To this aim, lysates from Saos-2 cells, transiently transfected with either HMGA2 or each of the two mutants A2(1-44) and A2(Δ^{45-56}), were incubated with a recombinant pRB protein and then immunoprecipitated with antibodies against pRB. These immunoprecipitates were incubated with a ^{3}H-acetylated histone H4 peptide, and the released ^{3}H-acetate was measured (Figure 4C). The same assay was carried out with mouse tissue lysates from wild-type pituitaries and *HMGA2* transgenic pituitary adenomas (Figure 4D). Consistent with the HMGA2-induced displacement of HDAC1 from the

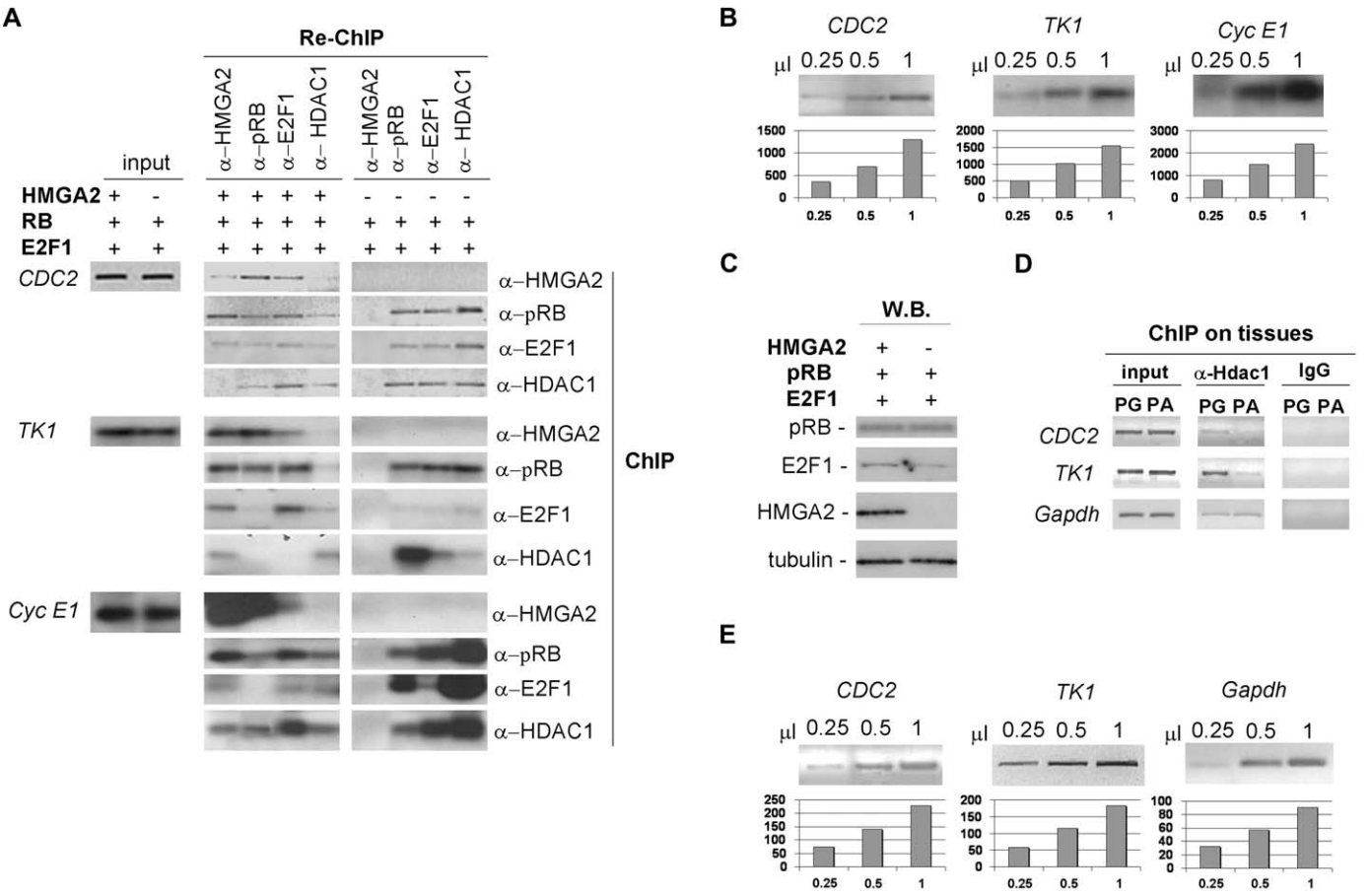


Figure 5. HMGA2 displaces HDAC1 from E2F1 target promoters

A: Lysates from cells transfected with plasmids expressing pRB, E2F1, and HMGA2 or pRb, E2F1, and empty vector, as indicated above the panel, were subjected to ChIP using specific polyclonal antibody, as indicated on the right. Before reversal of formaldehyde cross-linking, each precipitate was washed, resuspended, and subjected to re-ChIP using specific antibody as indicated on the top. Immunoprecipitates from each sample were analyzed by PCR, and a sample representing linear amplification (0.25–1 μl) of the total input chromatin (Input) was included in the PCRs as a control.

B: Increasing amounts of input samples as described in (**A**) were used as template in PCR amplifications performed using primers specific for the different promoters including E2F binding sites: *CDC2*, *TK1*, and *cyclin E1*.

C: Lysates from cells transiently transfected as described in (**A**) and indicated above the panel were subjected to Western blot analysis with anti-pRB, anti-Ha (for HMGA2), or anti-E2F1 antibodies to verify protein expression. Tubulin expression served as a control of equal protein loading.

D: Tissue extracts from a pool of normal pituitary glands (PG) and a representative HMGA2-induced pituitary adenoma (PA) were assayed by ChIP using a specific polyclonal antibody anti-HDAC1. Immunoprecipitates from each sample were analyzed by PCR, and a sample representing linear amplification (0.25–1 μl) of the Input was included in the PCRs as a control. Another control included precipitation performed without specific IgG.

E: Increasing amounts of input samples as described in (**D**) were used as template in PCR amplifications carried out with primers specific for the different promoters: *CDC2*, *TK1*, and *Gapdh*.

pRB active repressor complex, HDAC activity was significantly lower in cells and pituitary adenomas overexpressing HMGA2 than in mock-transfected cells and normal pituitary, respectively. To exclude that this effect could be due to a downregulation of HDAC1 expression following HMGA2 overexpression, we also monitored HDAC1 expression in the inputs of the experiments shown in Figures 4C and 4D. As shown in Figure 4E, no differences in HDAC1 expression were observed between HMGA2-transfected and parental cells or between pituitary adenoma and normal gland.

HMGA2 binds to E2F target promoters in vivo and interferes with the association of the single subcomplexes

We next evaluated whether HMGA2 is part of the complexes, including pRB, E2F1, and HDAC1 that form at the E2F binding sites of the E2F-responsive promoters in vivo, and whether it can displace HDAC1. In a combination of chromatin immunoprecipitation (ChIP) and Re-ChIP analyses, HEK293 cells were transfected with pRB and E2F1 or pRB and E2F1 plus HMGA2 (Figure 5C), and crosslinked genomic DNA was immunoprecipitated in two rounds with two specific antibodies directed against protein components of the complex (Figure 5A). Re-ChIPed DNA was analyzed by PCR using promoter-specific primers that encompass the E2F binding sites, under conditions of linear amplification (Figure 5B). Figure 5A shows that HMGA2 can associate in vivo with three E2F-responsive promoters, i.e., those driving *CDC2*, *TK1*, and *cyclin E1* gene transcription, and is a component of a promoter-bound multimeric complex containing E2F1, pRB, and HDAC1. A comparison of the subcomplexes with and without HMGA2 revealed three mechanisms whereby HMGA2 can activate E2F1 in vivo. First, in agreement

with our in vitro results, HDAC1/pRB binding was decreased in the presence of HMGA2 on all the promoters analyzed, especially the TK1 promoter. Second, pRB/E2F binding is decreased in the presence of HMGA2 on the cyclin E1 promoter, suggesting that HMGA2 can displace pRB from E2F1, at least on this promoter. Finally, E2F1 binding on the TK1 promoter was significantly increased in the presence of HMGA2, suggesting that, at least on this promoter, HMGA2 can directly enhance the binding of free E2F1 to the DNA target. This effect was also observed in the EMSA with an E2F consensus oligonucleotide (Figure 1). Quantitative data of this experiment, evaluated by densitometric analyses, are shown in the Figure S1 in the Supplemental Data available online.

To improve the significance of the HMGA2-mediated displacement of HDAC1 from the E2F-responsive promoters, we performed ChIP analyses on tissue extracts from normal and neoplastic pituitary tissues from wild-type and transgenic mice, respectively, using specific anti-HDAC1 antibodies. The DNA that was immunoprecipitated was analyzed by PCR using promoter-specific primers that encompass the E2F binding sites under conditions of linear amplification (Figure 5E). As shown in Figure 5D, differently from what occurs in normal pituitary glands from control mice, the binding of HDAC1 to the protein complex bound to the E2F-responsive promoters is much more reduced compared to normal tissue.

Overexpression of HMGA2 promotes acetylation of histones and E2F1 protein on E2F target promoters in vivo

We have shown that HMGA2 can displace HDAC1 from pRB at the E2F binding sites—a process that could account for the decreased HDAC activity associated with HMGA2 overexpression. HDAC can also affect proteins that can be acetylated. An example of this is E2F1, whose acetylation augments DNA binding and stabilizes the protein (Martinez-Balbas et al., 2000).

To analyze the acetylation status of histones and E2F1 on E2F1 target promoters in vivo, we transfected Saos-2 cells with pRB or pRB and HMGA2 (Figure 6A) and subjected the lysates to a ChIP assay using specific anti-acetylated histone H3 antibodies (Figure 6B). Immunoprecipitated DNA was analyzed by PCR using promoter-specific primers that encompass the E2F binding sites under conditions of linear amplification (Figure 6C). Control vector-transfected cells had high levels of acetylated histone H3 in association with the E2F binding sites of the analyzed promoters (Figure 6B). Overexpression of pRB decreased the association of the acetylated histone H3 with the same promoters, but not with the glyceraldehyde-3-phosphate dehydrogenase (Gapdh) unrelated promoter. In contrast, coexpression of pRB and HMGA2 abolished the effects of pRB. In order to investigate E2F1 acetylation on the same promoters and correlate it with the HMGA2/pRB interaction, we transfected HEK293 cells with either HMGA2 or A2(1-44) and subjected the lysates to a ChIP assay with a specific anti-acetylated E2F1 antibody. As shown in Figure 6D, E2F1 acetylation was enhanced by the HMGA2 overexpression, whereas it did not change in cells transfected with the A2(1-44) mutant. This result, consistent with our previous data demonstrating the role of the HMGA2/pRB interaction in displacing HDAC1 from the E2F-responsive promoters, was also confirmed by an additional experiment in which total lysates from cells transfected as in Figure 6D were immunoprecipitated with the anti-acetylated E2F1 antibody and then blotted for E2F1 (Figure 6F).

Finally, to evaluate whether these results correspond to real regulation of the expression of these genes in vivo, we carried out a semiquantitative reverse transcriptase-PCR. The expression levels of *CDC2* and *TK1* mRNA were measured in Saos-2 cells upon overexpression of pRB or pRB and HMGA2. In agreement with the results reported above, these genes were specifically downregulated upon pRB expression, but not upon coexpression of pRB and HMGA2 (Figure 6G).

Loss of E2F1 suppresses the development of pituitary tumors in HMGA2 transgenic mice

The afore-reported data demonstrate that HMGA2 induces E2F1 activity in pituitary adenomas by enhancing E2F1 acetylation and the binding of free E2F to the target promoter. This suggests that E2F1 activity is a critical event in pituitary tumorigenesis of HMGA2 mice.

To address this hypothesis, we crossed *HMGA2* transgenic mice ($HMGA2^{TG}$) with $E2f1^{-/-}$ mice to generate double mutants. We obtained $HMGA2^{TG};E2f1^{+/-}$ and $HMGA2^{TG};E2f1^{-/-}$ in a mixed 129/Sv × C57BL/6 genetic background. We monitored the female double mutants for the development of pituitary tumors, using $HMGA2^{TG};E2f1^{+/+}$ female mice as controls. All control animals (9/9) and nearly all $HMGA2^{TG};E2f1^{+/-}$ (13/14) double mutants developed grossly detectable pituitary tumors by the age of 15 months (see arrow in Figure 7A). In contrast, only 4 of the 16 (25%) $HMGA2^{TG};E2f1^{-/-}$ mice developed pituitary tumors (Figures 7A and 7B) at the same age. The loss of E2F1 function significantly affected the penetrance of pituitary tumors in $HMGA2^{TG}$ mice ($p = 0.0121$; log rank test). In addition, the tumors in the $HMGA2^{TG};E2f1^{+/+}$ mice featured multiple neoplastic nodules constituted by cords and solid nests of monomorphous polygonal tumor that had a high mitotic activity and intense PCNA labeling (Figures 7Cd and 7Ce); the nodules enlarged, distorted or completely replaced the anterior pituitary lobe. In contrast, the pituitary adenomas in the $HMGA2^{TG};E2f1^{-/-}$ animals were neither grossly enlarged nor distorted, and the anterior lobe showed a normal acinar pattern, except for a small nodule containing a few blood-filled spaces and rare mitotic figures and PCNA labeling (Figures 7Cj and 7Ck). The Gordon-Sweet silver method was used for the histological diagnosis of these nodules (Figure 7Cl) and showed focal disruption of the reticulin fiber network, which is pathognomic for the adenomatous transformation of pituitary cells (Figures 7Cc and 7Cf). These results provide strong evidence that E2F1 activation is required for the development of pituitary adenomas in HMGA2 transgenic mice.

In order to further investigate the molecular mechanisms involved in the pituitary alterations of the few cases of $HMGA2^{TG};E2F1^{-/-}$ mice that develop small adenomas, we analyzed the status of pRB/HMGA2 complexes and E2F "free" activation in these E2F1 null tumors. As shown in Figure 7D, HMGA2 and pRB still retain their capacity to interact with each other. However, the E2F "free" DNA binding activity does not show any significant increase in these tumors compared to control wild-type pituitary glands (Figure 7E, lane 3). Conversely, a strong increase in E2F free DNA binding was always observed in pituitaries from $HMGA2^{TG};E2f1^{+/+}$ mice even before the appearance of the pituitary tumor (Figure 7E, lane 2). Thus, even though HMGA2 is still able to bind pRB in absence of E2F1, there are

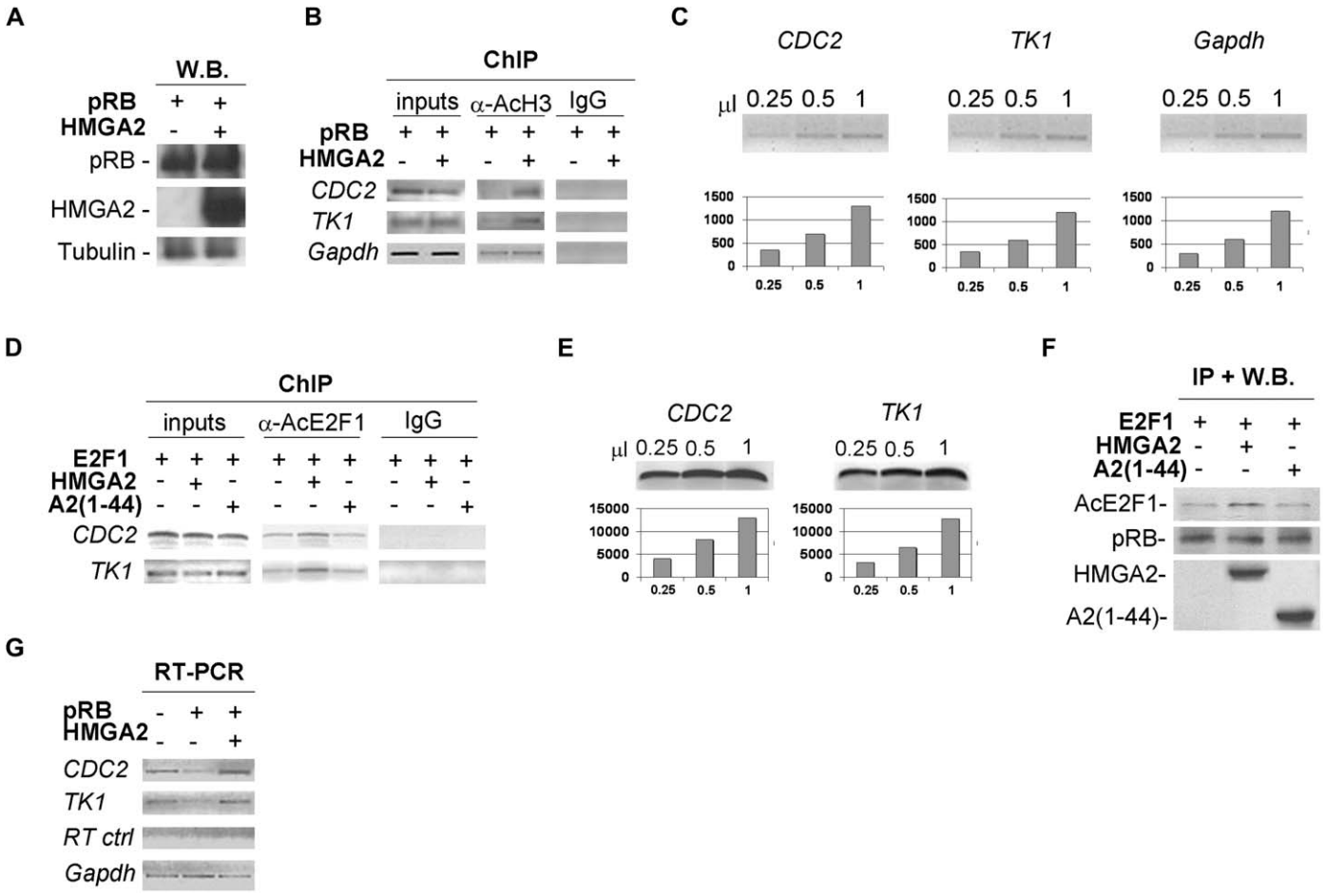


Figure 6. HMGA2 overexpression increases acetylation of both histones and E2F1 protein on E2F target promoters

A: Western blot analysis showing the protein expression of pRB and HMGA2 in Saos-2 cells transiently transfected with the relative expression plasmids, as indicated on the top. Tubulin expression served as a control of equal protein loading.

B and C: Lysates from Saos-2 cells transfected as described in (**A**) were subjected to ChIP using specific polyclonal antibodies anti-acetylated histone H3 (**B**). Immunoprecipitates were analyzed by PCR, and a sample representing linear amplification (0.25–1 μl) of the Input was included in the PCRs as a control. Another control included precipitation performed without specific IgG. Increasing amounts of input samples (**C**) were used as template to verify the linear range of amplification.

D: HEK293 cells transfected with E2F1, HMGA2, A2(1-44), or the backbone vector alone were assayed by ChIP using a specific polyclonal antibody anti-acetylated E2F1 under the same conditions as described in (**B**).

E: Increasing amount of input samples from the experiment described in (**D**) were used as template to verify the linear range of amplification.

F: HEK293 cells transfected as described in (**D**) were immunoprecipitated with anti-acetylated E2F1 antibody and assayed by Western blot for E2F1 (upper panel). The inputs were assayed by Western blot for the expression of endogenous pRB and transfected HMGA2 and A2(1-44) proteins (middle and lower panels).

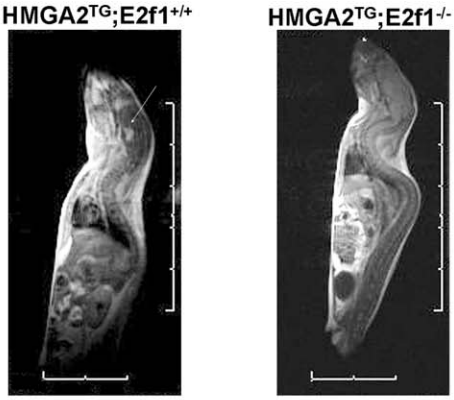
G: RNA from Saos-2 cells transiently transfected as described in (**A**) was analyzed by RT-PCR for the expression of *CDC2*, *TK1*, and *Gapdh*. The RT control lanes represent RT-PCR without reverse transcription.

no alternative E2F proteins whose DNA binding activity is enhanced following this interaction, suggesting that other E2F-independent mechanisms may be responsible for the pituitary alterations in the minority of these mice.

Discussion

Tumors of the pituitary gland can be found in 20% of the population, and at least one-third of these cause health complications. In fact, although not classified as malignant, most pituitary adenomas grow rapidly and can invade downward into the paranasal sinuses, laterally into the cavernous sinuses, and upward into the parenchyma of the brain. Little is known about the molecular events responsible for pituitary transformation (reviewed by Asa and Ezzat, 2002). A large body of evidence implicates HMGA2 in pituitary tumorigenesis (Fedele et al., 2002; Finelli et al., 2002). In an attempt to identify the mechanism by which HMGA2 acts, we explored the pRB pathway. We selected this strategy because of experimental data indicating that the *HMGA2* gene interferes with this pathway (Bignon et al., 1993; Fedele et al., 2002; Jacks et al., 1992). Moreover, the finding that mice with heterozygous *RB* mutations develop pituitary tumors (Jacks et al., 1992) suggests that impairment of the pRB/E2F pathway is involved in pituitary tumorigenesis. Thus far, *RB* mutations have not been identified in human pituitary adenomas (Cryns et al., 1993). However, methylation of the *RB* gene-promoter region at a CpG island in human pituitary tumor cells resulted in loss of protein expression (Simpson et al., 2000),

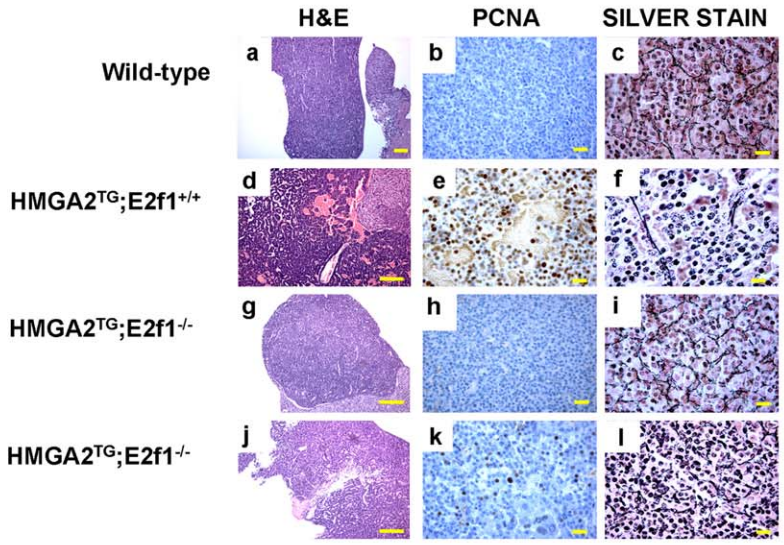
A



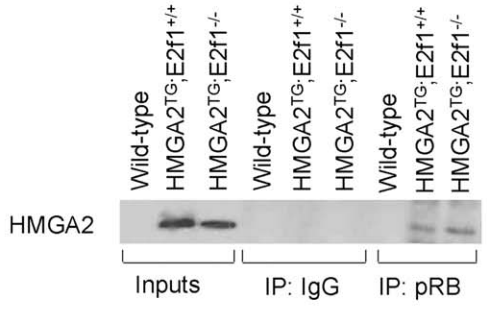
B

	HMGA2^{TG};E2f1 $^{+/+}$	HMGA2^{TG};E2f1 $^{+/-}$	HMGA2^{TG};E2f1 $^{-/-}$
Normal	0/9	0/14	8/16
Hyperplasia	0/9	1/14	4/16
Adenoma	9/9	13/14	4/16

C



D



E

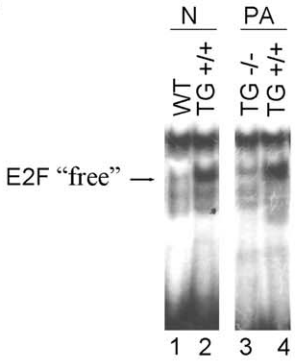


Figure 7. The absence of E2F1 suppresses pituitary tumorigenesis in *HMGA2* transgenic mice

A: MRI analysis of a representative 12-month-old transgenic mouse homozygous for the knockout mutation of *E2F1* (right: *HMGA2^{TG};E2f1$^{-/-}$*), compared with a sex-age matched control transgenic mouse (left: *HMGA2^{TG}; E2f1$^{+/+}$*). The arrow indicates the pituitary tumor that is absent in the *E2f1$^{-/-}$* mouse.

B: *HMGA2* transgenic mice and *E2f1$^{-/-}$* mice were crossed to generate *HMGA2^{TG};E2f1$^{+/+}$*, *HMGA2^{TG};E2f1$^{+/-}$*, and *HMGA2^{TG};E2f1$^{-/-}$* mice. The total number of pituitary tumors developed by each genotype is shown.

C: Histological analysis (hematoxylin and eosin staining) of the anterior pituitary from a wild-type animal showing a normal architecture (Ca), absence of PCNA labeling (Cb), and a normal acinar pattern and reticulin network (Gordon-Sweet silver stain) (Cc). *HMGA2^{TG};E2f1$^{+/+}$* mice had a very large tumor that enlarged and completely replaced the anterior lobe (Cd); most neoplastic cells showed PCNA labeling (Ce), and the reticulin network was lost (Cf). *HMGA2^{TG};E2f1$^{-/-}$* mice showing either a morphology (Cg), PCNA labeling (Ch), and reticulin network (Ci) similar to wild-type mice, or neoplastic nodules

which confirms that pRB is important in human pituitary tumorigenesis.

By repressing E2F1 activity, the pRB protein prevents cells from progressing beyond the G1 phase of the cell cycle. Phosphorylation of pRB by cyclin-cdk complexes or expression of viral transforming proteins relieves this repression and allows cells to progress toward S phase (reviewed by Seville et al., 2005). Interaction of pRB with histone deacetylase HDAC1 is involved in pRB repression of E2F1 activity, and this interaction is disrupted by both phosphorylation and viral transforming proteins (Magnaghi-Jaulin et al., 1998).

Here we demonstrate that HMGA2 induces the development of pituitary adenomas by enhancing E2F1 activity. In fact, E2F1 activity was drastically increased in all pituitary tumors analyzed. Moreover, the absence of E2F1, obtained by mating *HMGA2* transgenic with *E2f1* knockout mice, greatly inhibited the formation of pituitary tumors. In fact, most *HMGA2^{TG};E2f1$^{-/-}$* mice did not develop tumors, and only a few had small, slow growing adenomas. The reduced severity of pituitary lesions in *HMGA2^{TG};E2f1$^{-/-}$* animals suggests that loss of *E2F1* may delay the onset or progression of these tumors. It is noteworthy that the loss of one wild-type *E2F1* allele did not alter the frequency of pituitary tumors in *HMGA2^{TG}* mice.

The mechanism by which the HMGA2 protein affects the pRB/E2F pathway and enhances E2F1 activity is quite unique. In fact, although HMGA2 binds to the pRB pocket, it does not compete with the E2F protein in pRB binding, unlike proteins encoded by the adenovirus *E1A* gene. Conversely, our data show that HMGA2 activates E2F by interfering with HDAC1 recruitment by pRB on various E2F-responsive promoters (Figure 8). Displacement of HDAC1 would result in acetylation of both E2F1 and DNA-associated histones, thereby promoting E2F1 activation.

It would be worthwhile to investigate whether HMGA2 is able to displace also the other HDAC members (as suggested by our preliminary experiments) and any other protein associated to the pRB/E2F complex.

HDAC1 displacement from pRB is not the only way by which HMGA2 activates E2F1. HMGA2 also acts directly on the E2F-responsive DNA elements, and at least on the *TK1* promoter causes enhanced E2F1 DNA binding (Figure 5A). Although we do not demonstrate in vitro that HMGA2/pRB binding caused displacement of E2F1 from pRB, we show that HMGA2, at least on the *cyclin E1* promoter, displaced pRB from the E2F1-DNA complex (Figure 5A). On the basis of our results it is reasonable to suppose that displacement of pRB from E2F1 does not occur simply through competition between E2F1 and HMGA2 for the A/B pocket of pRB, but through a more indirect mechanism due to the enhanced E2F1 acetylation dependent upon the HMGA2 overexpression. In fact, it has been proposed (Martinez-Balbas et al., 2000) that acetylation stimulates the functions of the non-RB bound "free" form of E2F1, including DNA binding and its protein stability.

In conclusion, HMGA2 induces pituitary adenomas by enhancing E2F1 activity through displacement of HDAC1 from pRB and consequent induction of E2F1 acetylation. It is likely that the same mechanism may be involved in most human prolactinomas in which the *HMGA2* gene is amplified and overexpressed. Should this be the case, one may envisage a therapeutic strategy aimed at disrupting this mechanism.

Experimental procedures

Electrophoretic mobility shift assay

Protein/DNA binding was determined by electrophoretic mobility shift assay (EMSA), as previously described (Battista et al., 2005). The E2F1 oligonucleotide (Santa Cruz, CA; sc-2507) was mutated as follows (mutated bases in bold) in the AT-mut oligonucleotide: 5′-A**C**TT**GG**GTTTCGCGC CCTTTCTCAA-3′. The antibodies used were anti-HMGA2 (polyclonal Ab raised against a synthetic peptide located in the NH2-terminal region) and anti-E2F1 (sc-193, Santa-Cruz). The DNA-protein complexes were resolved on 6% (w/v) nondenaturing acrylamide gels and visualized by exposure to autoradiographic films. Binding reactions with the purified proteins were carried out as previously described (Baldassarre et al., 2001).

Protein extraction, immunoblot analysis, and immunoprecipitation

Tissues were rapidly dissected, frozen on dry ice, and stored at −80°C. Protein extraction, Western blot, and immunoprecipitation were carried out as previously described (Pierantoni et al., 2001). The antibodies used were anti-HMGA2 (polyclonal antibody raised against a synthetic peptide located in the NH2-terminal region), anti-pRB (C-15, Santa Cruz, CA), anti-HA (F-7; Santa Cruz), anti-GST (B-14, Santa Cruz), anti-E2F1 (C-20, Santa Cruz), and anti-HDAC1 (06-720, Upstate Biotechnology Inc., Lake Placid, NY).

Plasmids and recombinant proteins

HA-tagged HMGA2 expression plasmids containing the entire and various separate portions of the HMGA2 coding sequence were obtained by PCR amplification and subcloned into the pCEFL-HA expression vector. The expression vectors for pRB and all pRB mutants (each subcloned into the CMV-neo-Bam vector) were kindly donated by G. Condorelli. CDC25A-luc and CDC6-luc plasmids were already described (Hateboer et al., 1998; Vigo et al., 1999). pRB(379-928), pRB(1-379), pRB(379-928)Δ21, pRB(379-792), pRB(768-928), pRB(834-928), and E2F1 were expressed as GST fusion proteins, whereas HMGA2 and E1A-12S were expressed as His fusion proteins. pGEX2T plasmids carrying the different deletion mutants of pRB were kindly provided by S. Soddu. The pCMVHAE2F1 and pGST20TE2F-1 vectors are described elsewhere (Helin et al., 1993; Helin and Harlow, 1994). The GST and His fusion proteins were expressed in *Escherichia coli* strain BL21 (DE3) (Stratagene) and purified using glutathione sepharose or nickel beads as previously described (Baldassarre et al., 2001; Pierantoni et al., 2001). The pRB full-length recombinant protein was from Abcam (ab 1111).

Pull-down and Far-Western experiments

Pull-down analysis was carried out as previously described (Pierantoni et al., 2001). For Far-Western analysis, 50 μg of cell lysates expressing pRB and different pRB mutants were separated by SDS PAGE and transferred to a nitrocellulose membrane. The membrane was air-dried, and the proteins were denaturated for 10 min in 6 M guanidine hydrochloride in HBB buffer (10 mM HEPES, pH 7.5, 60 mM KCl, 1 mM EDTA, and 1 mM DTT). The proteins were renaturated sequentially in 3, 1.5, 0.75, 0.38, and 0.19 M GuHCl in the same buffer (10 min each step). The membrane was extensively rinsed with HBB and blocked with HBB supplemented with 5% nonfat milk and 0.5% NP40 for 1 hr at 4°C and for 30 min in HBB with 1% milk. The His-HMGA2 protein was incubated with the membrane in PIB buffer (10 mM HEPES, pH 7.5, 13 mM NaCl, 50 mM KCl, 1% milk, and 0.5% NP40, 1 mM DTT) for 4 hr at 4°C. The unbound material was removed with extensive

that differed from those in *HMGA2^{TG};E2f1$^{+/+}$* mice in that they were smaller (Cj), had less intense PCNA labeling (Ck), and only partial loss of the reticulin network (Cl). Scale bars (in yellow): 100 μm H&E, 75 μm PCNA staining, 25 μm Gordon-Sweet silver stain.

D: Coimmunoprecipitation of HMGA2 and pRB in tissue extracts from pituitary glands and tumors from mice as indicated on the top.

E: EMSA analysis of wild-type (WT), *HMGA2^{TG};E2f1$^{+/+}$* (TG +/+), and *HMGA2^{TG};E2f1$^{-/-}$* (TG −/−) pituitary samples, either normal (N) or adenomatous (PA), using a consensus E2F as probe.

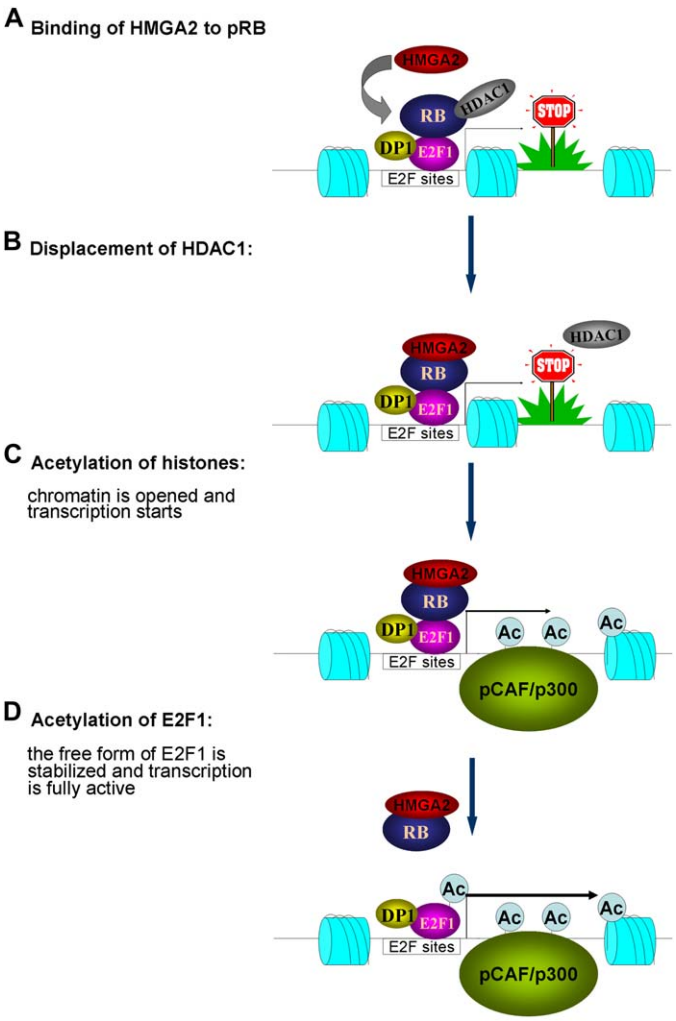


Figure 8. Model of E2F1 activation by HMGA2

Following HMGA2 overexpression, transcription through E2F1 sites switches from repression to activation throughout four steps.

A: HMGA2 binds to pRB, which is complexed with E2F1 and HDAC1 to form the active repression.

B: The interaction between HMGA2 and pRB displaces HDAC1.

C: In the absence of HDAC1, the histone acetylase enzymes are recruited and, by acetylating histones, relieve transcriptional repression.

D: Histone acetylases also acetylate E2F1 causing the stabilization of its ''free'' active form.

washes for 30 min in the same buffer. Binding was detected with Western blot analysis and anti-His antibody (Santa Cruz).

Cell lines and transfections

The HEK293, Rat-2, and Saos-2 cell lines were grown in Dulbecco's modified Eagle medium (DMEM) (Gibco-BRL) supplemented with 10% fetal-calf serum (Gibco-BRL). HEK293 cells were transfected by calcium phosphate precipitation by standard procedures. Rat-2 and Saos-2 cells were transfected by lipofectamine-plus reagent (Invitrogen) according to the manufacturer's instructions. For the luciferase activity assay, cells were transfected with 0.2 μg of CDC25A-luc or CDC6-luc reporter plasmids with or without 0.2 μg of pRB expression vector and increasing amounts (1 μg, 5 μg, and 10 μg) of HA-HMGA2 expression vector or 1 μg of HA-A2(1-44) or HA-A2(Δ^{45-56}) plasmid. One microgram of pCMVβgal plasmid was cotransfected to verify equal transfection efficiency in the cell lines tested. Cells were harvested 36 hr after transfection, and luciferase and β-galactosidase activities were measured with a luminometer (Lumat LB9507, Berthold) with the Dual light kit (Tropix). The data represent the average of three independent experiments, performed in duplicate, with standard errors. Colony and Focus assays were carried out as previously described (Pierantoni et al., 2001; Fedele et al., 1998).

Competition experiments

Whole-cell lysates from HEK293 cells transiently cotransfected with HA-E2F1 and pCMV-pRB were incubated for 1 hr with His-HMGA2 or His-E1A recombinant proteins and subjected to immunoprecipitation with anti-pRB antibody (C-15, Santa Cruz, CA), resolved by SDS-PAGE on a 12% gel, and analyzed by Western blot with anti-E2F1 (C-20, Santa-Cruz), anti-HDAC1 (06-720, Upstate Biotechnology), or anti-pRB antibodies (C-15, Santa Cruz). In another experiment, labeled HDAC1 and a recombinant GST protein were subjected to immunoprecipitation with or without 5 or 10 μg of recombinant His-HMGA2 or His-A2(1-44). The samples were resolved by 12% SDS-PAGE gel and probed with anti-pRB antybody (C-15, Santa Cruz).

Deacetylase assay

Deacetylase assays were carried out with a histone deacetylase assay kit (Upstate Biotechnology) according to the manufacturer's instructions. All experiments were carried out three times, and samples were assayed in duplicate.

Chromatin immunoprecipitation and reprecipitation

Chromatin immunoprecipitation (ChIP) was carried out with an acetyl-histone H3 immunoprecipitation assay kit (Upstate Biotechnology) according to the manufacturer's instruction. For ChIp experiments with the polyclonal antibody anti-HDAC1 (Upstate Biotechnology), anti-E2F1 (C-20 Santa Cruz), anti-pRB (C-15 Santa Cruz), and anti-HMGA2 (polyclonal antibody raised against a synthetic peptide located in the NH2-terminal region), conditions were as previously reported (Shang et al., 2000). For Re-ChIP experiments, complexes were eluted by incubation for 30 min at 37°C in 250 μl of Re-ChIP elution buffer (2 mM DTT, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1) and then diluted 4-fold in Re-ChIP dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1) and subject again to the ChIP procedure. Crosslinking was reversed by incubating samples overnight at 65°C with 20 μl of 5 M NaCl. Samples were then incubated in proteinase K solution (10 mM EDTA, 40 mM Tris-HCl, pH 6.5, 40 μg/ml of Proteinase K) for 1 hr at 45°C. DNA was purified with phenol/chloroform/isoamyl alcohol and precipitated by adding 2 volumes of ethanol and tRNA. PCR reactions were carried out by standard procedures, for a number of cycles optimized to ensure product intensity within the linear phase of amplification. The PCR products were separated on a 2% agarose gel, stained with ethidium bromide, and either scanned using a Typhoon 9200 scanner or blotted and hybridized with specific probes. Primer sequences are available as Supplemental Data.

Reverse transcriptase-PCR

Total RNA was isolated using TRI-reagent solution (Sigma) according to the manufacturer's protocol and treated with DNase (Invitrogen). Reverse transcription was performed by standard procedures. Primer sequences are available as Supplemental Data.

Generation and genotyping of mutant mice

E2f1-deficient animals of mixed 129/SvxC57BL/6 genetic background (Jackson Laboratory) were mated to *HMGA2* transgenic (*HMGA2*^{TG}) animals (Fedele et al., 2002) to generate *HMGA2*^{TG};*E2f1*$^{+/-}$ mice. Subsequent intermating of *HMGA2*^{TG};*E2f1*$^{+/-}$ males and females produced *HMGA2*^{TG};*E2f1*$^{-/-}$ mice. Animals were genotyped from tail DNA using Southern blot and PCR as previously described (Fedele et al., 2002) and according to the Jackson protocol. Mice were maintained under specific pathogen-free conditions in our Laboratory Animal Facility (Istituto dei Tumori di Napoli, Naples, Italy), and all studies were conducted in accordance with Italian regulations for experimentations on animals.

Histological analyses

Paraffin-embedded sections 4–5 μm thick were stained with hematoxylin and eosin and with the Gordon-Sweet silver method for the reticulin matrix. Immunohistochemical staining to identify proliferating cells was carried out

with the PCNA antibody (Novocastra) and the streptavidin-biotin peroxidase technique (DAKO Corp., CA).

Magnetic resonance

Magnetic resonance imaging was carried out at the Istituto dei Tumori di Napoli G. Pascale with a 1.5-T magnet system using local receiver coils and an 8 cm field of view. Sagittal slices 3 mm thick were obtained with T1 weighting (TR/TE = 400/11 ms).

Supplemental Data

The Supplemental data for this article can be found online at http://www.cancercell.org/cgi/content/full/9/6/459/DC1/.

Acknowledgments

This work was supported by grants from the Associazione Italiana Ricerca sul Cancro (AIRC) to A.F. and Regione Campania to M.F. We are grateful to Jean Ann Gilder (Scientific Communication) for editing the text. A.F. thanks Professor G. Della Porta for his continuous support.

Received: September 28, 2005
Revised: February 23, 2006
Accepted: April 18, 2006
Published: June 12, 2006

References

Asa, S.L., and Ezzat, S. (2002). The pathogenesis of pituitary tumours. Nat. Rev. Cancer *2*, 836–849.

Baldassarre, G., Fedele, M., Battista, S., Vecchione, A., Klein-Szanto, A.J., Santoro, M., Waldmann, T.A., Azimi, N., Croce, C.M., and Fusco, A. (2001). Onset of natural killer cell lymphomas in transgenic mice carrying a truncated HMGI-C gene by the chronic stimulation of the IL-2 and IL-15 pathway. Proc. Natl. Acad. Sci. USA *98*, 7970–7975.

Battista, S., Fidanza, V., Fedele, M., Klein-Szanto, A.J., Outwater, E., Brunner, H., Santoro, M., Croce, C.M., and Fusco, A. (1999). The expression of a truncated HMGI-C gene induces gigantism associated with lipomatosis. Cancer Res. *59*, 4793–4797.

Battista, S., Fedele, M., Martinez-Hoyos, J., Pentimalli, F., Pierantoni, G.M., Visone, R., De Martino, I., Croce, C.M., and Fusco, A. (2005). High-mobility-group A1 (HMGA1) proteins down-regulate the expression of the recombination activating gene 2 (RAG2). Biochem. J. *389*, 91–97.

Bignon, Y.J., Chen, Y., Chang, C.Y., Riley, D.J., Windle, J.J., Mellon, P.L., and Lee, W.H. (1993). Expression of a retinoblastoma transgene results in dwarf mice. Genes Dev. *7*, 1654–1662.

Cryns, V.L., Alexander, J.M., Kilbanski, A., and Arnold, A. (1993). The retinoblastoma gene in human pituitary tumors. J. Clin. Endocrinol. Metab. *77*, 644–646.

Fedele, M., Berlingieri, M.T., Scala, S., Chiariotti, L., Viglietto, G., Rippel, V., Bullerdiek, J., Santoro, M., and Fusco, A. (1998). Truncated and chimeric HMGI-C genes induce neoplastic transformation of NIH3T3 murine fibroblasts. Oncogene *17*, 413–418.

Fedele, M., Battista, S., Manfioletti, G., Croce, C.M., Giancotti, V., and Fusco, A. (2001). Role of the high mobility group A proteins in human lipomas. Carcinogenesis *22*, 1583–1591.

Fedele, M., Battista, S., Kenyon, L., Baldassarre, G., Fidanza, V., Klein-Szanto, A.J., Parlow, A.F., Visone, R., Pierantoni, G.M., Outwater, E., et al. (2002). Overexpression of the HMGA2 gene in transgenic mice leads to the onset of pituitary adenomas. Oncogene *21*, 3190–3198.

Field, S.J., Tsai, F.Y., Kuo, F., Zubiaga, A.M., Kaelin, W.G., Jr., Livingston, D.M., Orkin, S.H., and Greenberg, M.E. (1996). E2F-1 functions in mice to promote apoptosis and suppress proliferation. Cell *85*, 549–561.

Finelli, P., Pierantoni, G.M., Giardino, D., Losa, M., Rodeschini, O., Fedele, M., Valtorta, E., Mortini, P., Croce, C.M., Larizza, L., et al. (2002). The High Mobility Group A2 gene is amplified and overexpressed in human prolactinomas. Cancer Res. *62*, 2398–2405.

Franklin, D.S., Godfrey, V.L., Lee, H., Kovalev, G.I., Schoonhoven, R., Chen-Kiang, S., Su, L., and Xiong, Y. (1998). CDK inhibitors p18(INK4c) and p27(Kip1) mediate two separate pathways to collaboratively suppress pituitary tumorigenesis. Genes Dev. *12*, 2899–2911.

Hateboer, G., Wobst, A., Petersen, B.O., Le Cam, L., Vigo, E., Sardet, C., and Helin, K. (1998). Cell cycle-regulated expression of mammalian *CDC6* is dependent on E2F. Mol. Cell. Biol. *18*, 6679–6697.

Helin, K., and Harlow, E. (1994). Heterodimerization of the transcription factors E2F-1 and DP-1 is required for efficient binding to the adenovirus E4 (ORF6/7) protein. J. Virol. *68*, 5027–5035.

Helin, K., Wu, C.-L., Fattaey, A.R., Lees, J.A., Dynlacht, B.D., Ngwu, C., and Harlow, E. (1993). Heterodimerization of the transcription factors E2F-1 and DP-1 leads to cooperative transactivation. Genes Dev. *7*, 1850–1861.

Hu, Q.J., Dyson, N., and Harlow, E. (1990). The regions of the retinoblastoma protein needed for binding to adenovirus E1A or SV40 large T antigen are common sites for mutations. EMBO J. *9*, 1147–1155.

Jacks, T., Fazeli, A., Schmitt, E.M., Bronson, R.T., Goodell, M.A., and Weinberg, R.A. (1992). Effects of an PRB mutation in the mouse. Nature *359*, 295–300.

Magnaghi-Jaulin, L., Groisman, R., Naguibneva, I., Robin, P., Lorain, S., Le Villain, J.P., Troalen, F., Trouche, D., and Harel-Bellan, A. (1998). Retinoblastoma protein represses transcription by recruiting a histone deacetylase. Nature *391*, 601–605.

Martinez-Balbas, M.A., Bauer, U.M., Nielsen, S.J., Brehm, A., and Kouzarides, T. (2000). Regulation of E2F1 activity by acetylation. EMBO J. *19*, 662–671.

Pierantoni, G.M., Fedele, M., Pentimalli, F., Benvenuto, G., Pero, R., Viglietto, G., Santoro, M., Chiariotti, L., and Fusco, A. (2001). High mobility group I (Y) proteins bind HIPK2, a serine-threonine kinase protein which inhibits cell growth. Oncogene *20*, 6132–6141.

Putzer, B.M., Rumpf, H., Rega, S., Brockmann, D., and Esche, H. (1997). E1A 12S and 13S of the transformation-defective adenovirus type 12 strain CS-1 inactivate proteins of the PRB family, permitting transactivation of the E2F-dependent promoter. J. Virol. *71*, 9538–9548.

Seville, L.L., Shah, N., Westwell, A.D., and Chan, W.C. (2005). Modulation of pRB/E2F functions in the regulation of cell cycle and in cancer. Curr. Cancer Drug Targets *5*, 159–170.

Shang, Y., Hu, X., DiRenzo, J., Lazar, M.A., and Brown, M. (2000). Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. Cell *103*, 843–852.

Simpson, D.J., Hibberts, N.A., Mc Nicol, A.M., Clayton, R.N., and Farrell, W.E. (2000). Loss of pPRB expression in pituitary adenomas is associated with metylation of the PRB1 CpG island. Cancer Res. *60*, 1211–1216.

Vigo, E., Müller, H., Prosperini, E., Hateboer, G., Cartwright, P., Moroni, M.C., and Helin, K. (1999). CDC25A phosphatase is a target of E2F and is required for efficient E2F-1 induced S phase. Mol. Cell. Biol. *19*, 6379–6395.

Wood, L.J., Maher, J.F., Bunton, T.E., and Resar, L.M.S. (2000). The oncogenic properties of the HMG-I gene family. Cancer Res. *60*, 4256–4261.

Yamasaki, L., Bronson, R., Williams, B.O., Dyson, N.J., Harlow, E., and Jacks, T. (1998). Loss of E2F-1 reduces tumorigenesis and extends the lifespan of Rb1(+/−) mice. Nat. Genet. *18*, 360–364.