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High Mobility Group A1 (HMGA1) proteins interact with p53 and inhibit its apoptotic activity

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Abstract

HMGA gene overexpression and rearrangements are frequent in several tumours, but their oncogenic function is still unclear. Here we report of a physical and functional interaction between High Mobility Group A1 (HMGA1) protein and p53 oncosuppressor. We found that HMGA1 binds p53 *in vitro* and *in vivo*, and both proteins are present in the same complexes bound to the *Bax* gene promoter. HMGA1 interferes with the p53-mediated transcription of p53 effectors *Bax* and *p21^{waf1}* while cooperates with p53 in the transcriptional activation of the p53 inhibitor *mdm2*. This transcriptional modulation is associated with a reduced p53-dependent apoptosis in cells expressing exogenous HMGA1 and p53, or in cells expressing endogenously the proteins and in which p53 was activated by UV-irradiation. Furthermore, antisense inhibition of HMGA1b expression dramatically increases the UV-induced p53-mediated apoptosis. These data define a new physical and functional interaction between HMGA1 and p53 that modulates transcription of p53 target genes and inhibits apoptosis.

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Abbreviations: HMGA1, High Mobility Group A1; Mdm2, murine double minute 2; CMV, cytomegalovirus

Introduction

The high mobility group A (HMGA) non-histone chromosomal HMGA1a, HMGA1b and HMGA2 proteins constitute a subgroup of HMG accessory factors that play key roles in chromatin architecture and gene control.^{1,2} These proteins are low molecular weight nuclear factors that interact with the minor groove of many AT-rich promoters and enhancers.³ The HMGA proteins *per se* do not exert transcriptional activity, but orchestrate the assembly of nucleoprotein complexes involved in gene transcription, replication, and chromatin structure through a complex network of protein-DNA and protein-protein interactions.⁴ The expression of HMGA proteins is high during embryogenesis^{5,6} and low or undetectable in normal adult tissues. High HMGA expression is a frequent feature of tumour transformation. Indeed, increased HMGA levels correlate with the appearance of a malignant phenotype in rat thyroid cells and in experimental thyroid and skin tumours.^{7–9} HMGA1 levels are high in human thyroid,^{10,11} colon,^{12–14} prostate,¹⁵ pancreas,¹⁶ cervix,¹⁷ ovary¹⁸ and breast¹⁹ carcinomas. We had previously demonstrated that overexpression of HMGA proteins is required for cell transformation, since the blockage of their synthesis prevents tumorigenic transformation of rat thyroid cells by murine transforming retroviruses.^{20,21} Moreover, infection with a recombinant adenovirus carrying the *HMGA1b* cDNA in antisense orientation led several carcinoma cell lines to death.²²

Despite the wealth of data associating aberrant- or overexpression of HMGA1 proteins with cancer, little is known about the molecular roles by which HMGA1 proteins exert their tumorigenic effect.

The tumour suppressor protein p53 is the most frequently altered gene in human cancers. Besides contributing to tumorigenesis, loss or inactivation of p53 is also involved in the development of tumour resistance to chemo and radiotherapy. p53 was originally defined as the 'guardian of the genome' based on its functions in the prevention of genetic instability.²³ However, accumulating evidence indicates that the p53 apoptotic function is the most relevant for both tumorigenicity and induction of tumour-resistance to antineoplastic treatments.^{24–26} The apoptotic activity of p53 can be regulated independently of the cell cycle arrest function, and several proteins have been identified that are necessary for p53 to mediate the full apoptotic response, and so show efficient tumour suppressor activity.²⁴

Here we show that HMGA1 interacts with the p53 tumour suppressor and interferes with its proapoptotic activity. We demonstrate that HMGA1 and p53 are present in the same multiprotein complexes bound to the *Bax* promoter and that HMGA1 modifies the p53 transcriptional activity on p53 target genes (i.e. *Mdm2*, *Bax*, and *p21^{waf1}*). Furthermore, HMGA1 strongly interferes with p53-induced apoptosis, suggesting

that HMGA1 overexpression can contribute to tumorigenic transformation by counteracting the apoptotic function of the p53 tumour suppressor.

Results

HMGA1 physically interacts with p53

In order to identify new HMGA1-interacting proteins, we employed an antibody array containing hundreds of high quality antibodies against well-studied proteins, involved in cell cycle regulation, apoptosis, and signal transduction. The membrane-immobilized antibodies retain their capabilities of recognizing and capturing antigens. The array was incubated with a total cell extract from 293 cells transfected with the full-length *Hmga1b* cDNA tagged with the influenza virus HA epitope, and immunoblotted with anti-HA antibody. The results indicated interaction between HMGA1b and various proteins (data not shown); among these, we selected the

oncosuppressor p53 because of its relevance in tumour development.

To verify whether HMGA1b interacts with p53, we evaluated the binding between the two purified proteins *in vitro*. His-p53 protein was allowed to bind to the His-HMGA1b protein, and the complexes were immunoprecipitated with anti-p53 or anti-HMGA1 Abs and analysed by Western blotting with anti-HMGA1 or anti-p53 Abs, respectively. As shown in the Figure 1a, the two proteins can directly interact.

To examine further the specificity of this interaction and to map the HMGA1b regions required for binding to p53, we generated a series of NH₂- and COOH-terminal deletion mutants of *Hmga1b*. The resulting cDNAs were HA-tagged by cloning into the pCEFL-HA expression vector (Figure 1b). These vectors were transiently transfected into 293 cells that were harvested 48 h later. The protein extracts were tested for their interaction with bacterially expressed p53 fused to glutathione *S*-transferase (GST). The complexes were immobilized on glutathione-Sepharose matrix. The bound

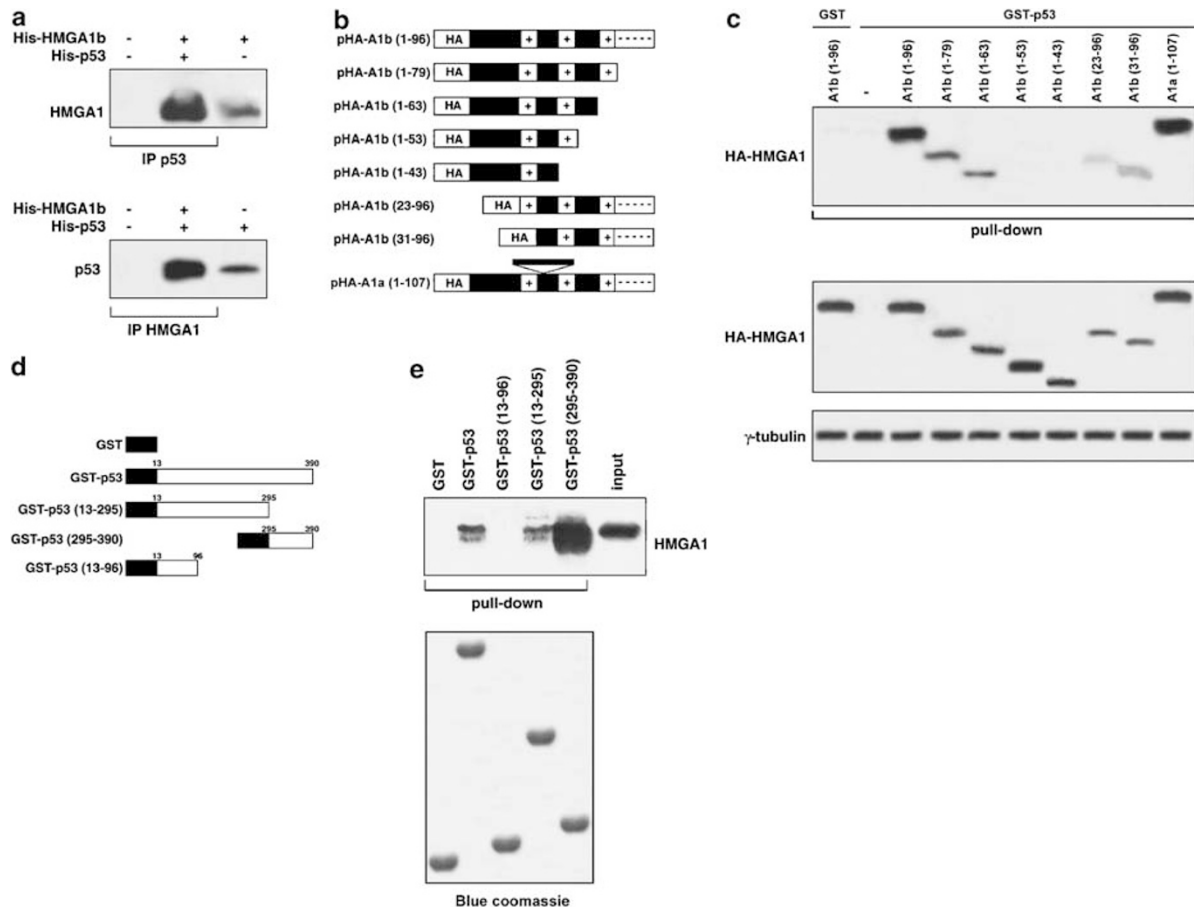


Figure 1 *In vitro* interaction between HMGA1 and p53. (a) The *in vitro* interaction between p53 and HMGA1b was assessed by immunoprecipitation assay with anti-p53 (upper panel) or anti-HMGA1 (lower panel) antibodies and blotting with the reciprocal antibodies after preincubation of the two proteins. The relative inputs are the His-HMGA1b and His-p53 recombinant proteins loaded as controls. (b) Diagram of the HA-tagged *Hmga1* deletion mutants used in co-immunoprecipitation experiments. The AT-hook domains (+) and the acidic tail (----) are indicated. (c) 293 cells were transiently co-transfected with the indicated plasmids carrying the HA-*Hmga1* wild type or deletion mutants. GST pull-down assays were performed between total cell extracts (TCEs) from pHA-*Hmga1* mutants transfected cells and the GST-p53 fusion protein. The bound complexes (upper panel) and TCEs (lower panel) were separated on SDS-PAGE and analysed by Western blotting with anti-HA antibody or anti- γ -tubulin antibody, as a loading control. (d) Schematic representation of GST-p53 fusion proteins used in this study. (e) GST pull-down assay of the indicated GST-fusion proteins with recombinant HMGA1b protein. The bound complexes were separated by SDS-PAGE, and the filter was incubated with anti-HMGA1 antibodies (upper panel). Gels were stained with blue-Coomassie to show equal amount of the GST-fusion protein used in the assay (lower panel)

proteins were separated by SDS-PAGE, blotted, and the filters were immunoreacted with anti-HA antibody. As expected, full-length HMGA1b protein binds to GST-wtp53 (Figure 1c). Deletion of the carboxy-terminal tail (pHA-A1b/1-79) and of the third AT-hook domain (pHA-A1b/1-63) did not impair the binding of HMGA1b to p53. In contrast, the HMGA1b/p53 interaction did not occur in the absence of the region between the second and the third AT-hook (amino acids 54–63, construct pHA-A1b/1-53) or in the presence of the first 43 amino acids encompassing only the first AT-hook (construct pHA-A1b/1-43). The NH₂-terminal truncations of HMGA1b (constructs pHA-A1b/23-96 and pHA-A1b/31-96) were also involved in the HMGA1b/p53 interaction, as demonstrated by the reduced binding to p53 of the HMGA1b mutants *versus* the wild-type protein (Figure 1c). Thus, the region between the second and the third AT-hook and the amino-terminus of HMGA1b protein take part in p53 binding. Finally, we have also analysed the interaction between the long HMGA1a isoform, which has an extra stretch of 11 aa between the first and the second AT-hook domains, and p53. To this aim, we transiently overexpressed the pHA-A1a/1-107 construct, and performed a pull-down assay with GST alone (data not shown) and GST-p53. As shown in Figure 1c, also the HMGA1a isoform is able to bind p53 protein.

To identify the p53 domain involved in the interaction with HMGA1b, pull-down assays were performed with recombinant HMGA1b protein and a series of p53 deletion mutants fused to GST (Figure 1d). Only the recombinant polypeptide GST-p53(13-96) does not bind HMGA1b protein (Figure 1e), whereas the other mutants tested, the GST-p53(295-390) and the GST-p53(13-295) proteins, bind HMGA1b. Noteworthy, the GST-p53(295-390) C-terminal domain binds more efficiently than the full-length or the GST-p53(13-295) proteins do. No HMGA1b was detectable in the complexes obtained with the GST protein alone (Figure 1e, upper panel). Gels were stained with blue-Coomassie to show the equal amount of GST-fusion protein used for the pull-down assay (Figure 1e, lower panel). These results suggest that the N-terminal domain of p53, which comprises the transactivation and the

proline-rich domains, is not involved in the binding, while the DNA binding (aa 102–292) and the C-terminal domain, which comprises the tetramerization (aa 323–356) and the regulatory (363–393) domains, are independently able to bind HMGA1b, with the C-terminal domain itself binding more efficiently than the full-length or the DNA binding domain alone.

HMGA1b interacts with p53 *in vivo*

To verify the interaction between HMGA1b and p53 *in vivo*, total cell extracts (TCE) from 293 cells transiently transfected with pCMV/*Hmga1b* and pCMV/*p53* were immunoprecipitated with anti-p53 monoclonal Ab and analysed by Western blotting with anti-HMGA1 polyclonal Ab. Larger amounts of HMGA1 protein were present in the immunocomplexes from cells transfected with the two expression plasmids (Figure 2a, upper panel). The reciprocal experiment performed immunoprecipitating with anti-HMGA1 Ab and revealing with anti-p53 Ab confirmed the interaction (Figure 2a, lower panel). We could detect this association also between the endogenous proteins (Figure 2b), demonstrating that HMGA1 and p53 form complexes *in vivo*.

To further verify the specificity of the interaction between HMGA1 and p53 proteins, we performed co-immunoprecipitation experiments also in p53^{-/-}H1299 cells transfected with the relative expression constructs. The extracted proteins were immunoprecipitated with anti-HMGA1 or anti-p53 antibodies, and the filters were incubated with the relative antibody. The results shown in Figure 2c demonstrate that the interaction occurs only in cells expressing both proteins.

HMGA1 and p53 are present in the protein-complexes bound to the *bax* promoter

p53 is a sequence-specific transcription factor whose DNA-binding consensus is present in a large number of promoters.²⁵ HMGA1 proteins are DNA-binding factors that interact

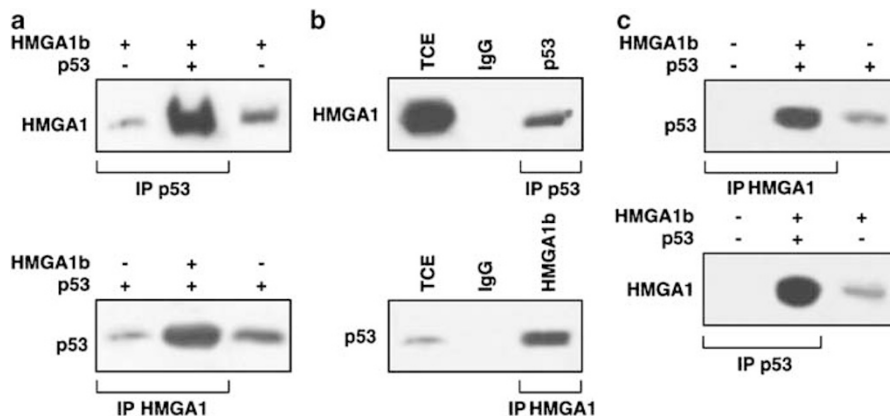


Figure 2 *In vivo* characterization of the HMGA1/p53 interaction. (a) 293 cells were transfected with pCMV/*Hmga1b* vector alone or together with pCMV-*p53*. After 48 h, total cell extracts were prepared and equal amounts of proteins were immunoprecipitated with anti-p53 (upper panel) or anti-HMGA1 (lower panel) antibodies, and the immunocomplexes analysed by Western blotting using the reciprocal antibodies. The relative inputs are total cell extracts derived from 293-transfected cells with the expression vector encoding HMGA1b or p53 protein, respectively. (b) The co-immunoprecipitation was performed as in (a) but on the endogenous p53 and HMGA1 proteins (TCE from parental 293 cells). IgG indicates the negative control of immunoprecipitation using an unrelated antibody. (c): Co-immunoprecipitation with the anti-HMGA1 or anti-p53 antibodies of untransfected or pCMV/*Hmga1b* and pCMV-*p53* transfected H1299 cells

with the minor groove of many promoters and enhancers.³ Thus, we asked whether the physical interaction between HMGA1 and p53 takes place on promoter regions of p53 targets. The human *Bax* gene promoter was selected because its region spanning nucleotides -441/-500 (hbaxpr) contains the p53-binding site and an AT-rich putative HMGA1-binding site. We first evaluated whether HMGA1 binds this 60 bp promoter region by EMSA. As shown in Figure 3a, 50 ng of recombinant His-HMGA1b protein were able to bind the ³²P-end-labelled double-strand *Bax* promoter oligonucleotide. Binding specificity was demonstrated by competition experiments showing loss of binding with the addition of a 100-fold molar excess of unlabelled *Bax* promoter oligonucleotide. We have also demonstrated that for the binding of HMGA1b protein to the *Bax* promoter region is not required the p53-binding site, as demonstrated by an EMSA assay performed with the same oligonucleotide deprived of the p53-binding site (hbaxprdel).

Next, we evaluated whether HMGA1 protein binds the *Bax* promoter *in vivo* by performing ChIP assays. The binding of p53 was used as a positive control. p53-null H1299 cells were transfected with p53 and/or HMGA1b, tested by Western blotting for protein expression (Figure 3b), crosslinked, and immunoprecipitated with anti-HMGA1, anti-p53, or anti-IgG antibodies. Immunoprecipitation of chromatin was then analysed by semiquantitative PCR, using primers spanning the -250/-530 region of the *Bax* promoter. As expected, anti-p53 precipitated this *Bax* region from H1299 cells transfected with p53 alone or with overexpressed-HMGA1b protein (Figure 3c). Occupancy of this *Bax* region by HMGA1 was clearly detectable in anti-HMGA1-precipitated chromatin from untransfected or from H1299 cells overexpressing p53 alone (Figure 3c), being endogenous HMGA1 protein expressed in H1299 cells, and even more in cells overexpressing both p53 and HMGA1b exogenous proteins. No precipitation was observed with anti-IgG precipitates, and when primers for

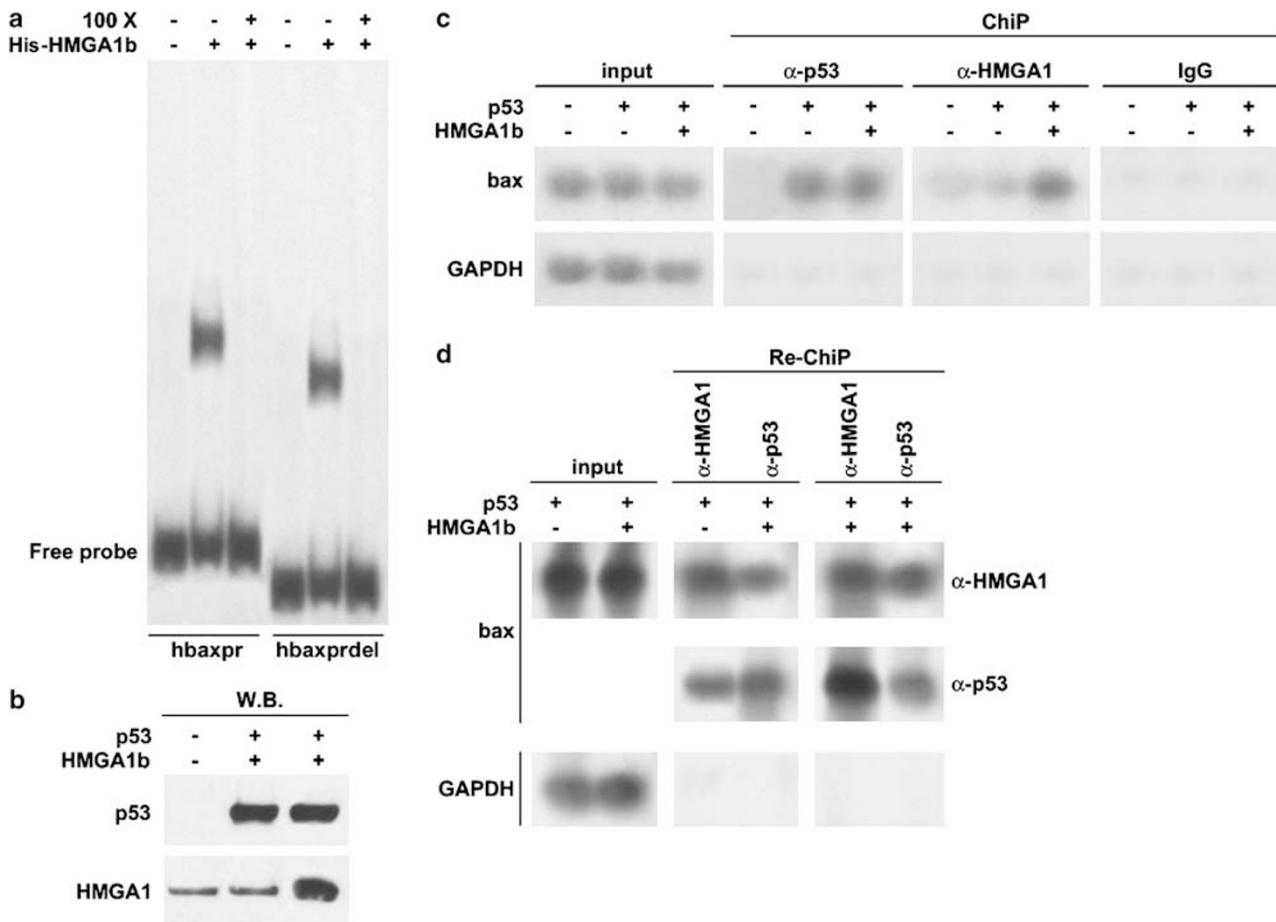


Figure 3 HMGA1 binds the human *Bax* promoter *in vitro* and *in vivo*. (a) EMSA performed with a radiolabelled oligonucleotide spanning from nt -441 to -500 of the human *Bax* promoter (hbaxpr) and the same region deprived of p53-binding site (hbaxprdel) incubated with 50 ng of recombinant HMGA1b protein. To assess the specificity of the binding, His-HMGA1b protein was incubated in the presence of a 100-fold excess of unlabelled oligonucleotide used as competitor. (b) Cellular extracts derived from untransfected or p53 and/or HMGA1b overexpressing H1299 cells used for ChIP experiments were analysed by Western blotting. (c) Soluble chromatin from H1299 cells transfected with p53 alone or in combination with HMGA1b was immunoprecipitated with anti-p53 or anti-HMGA1. The DNAs were then amplified by semiquantitative PCR using primers that cover a region of human *Bax* promoter (-250/-530), which contains the p53-binding sites. As an immunoprecipitation control, IgG was used. The panel shows PCR amplification of the immunoprecipitated DNA using primers for the *GAPDH* gene promoter. (d) In Re-ChIP experiments, soluble chromatin from transfected H1299 cells was immunoprecipitated with anti-p53, eluted, and reimmunoprecipitated with anti-HMGA1. The purified DNA was used as template for PCR with primers that amplify the -250/-530 human *Bax* promoter region

the control promoter *GAPDH* were used (Figure 3c, lower panel), indicating that the binding is specific for the *Bax* promoter. These results indicate that HMGA1 protein binds the *Bax* promoter region *in vivo*, and that the binding occurs also in absence of p53 protein.

To determine whether HMGA1 occupies the *Bax* promoter together with p53, the anti-HMGA1 complexes were released, reimmunoprecipitated with anti-p53, and then analysed by semiquantitative PCR (Re-ChIP). The results show that the antibodies against p53 precipitate the *Bax* promoter after their release from anti-HMGA1, indicating that HMGA1 occupies this region with p53 (Figure 3d). The reciprocal experiment provided comparable results (Figure 3d). Taken together, these results indicate that HMGA1 binds the human *Bax* promoter *in vitro* and *in vivo*, and participate to the same DNA-bound complexes that contain p53.

HMGA1b modulates p53-mediated transcriptional activity

p53 regulates the transcription of several genes whose products can trigger a variety of antitumour functions.^{27,28} To evaluate the effect of HMGA1 expression on p53-mediated transcription, p53-null H1299 cells were co-transfected with expression vectors encoding HMGA1b and p53, and with reporter vectors carrying the luciferase gene under the control of various p53-responsive promoters, that is, *mdm2*, *p21^{waf1}*, and *Bax*. As shown in Figure 4, in the presence of HMGA1b, p53 has increased transcriptional activity on the promoter of its inhibitor MDM2, whereas has showed a strongly reduced activity on the promoter of its effectors *Bax* and *p21^{waf1}*. The effects exerted on the *mdm2* and *Bax* promoters were dose-dependent, whereas the effect on *p21^{waf1}* was maximal even at the lowest HMGA1b concentration used. HMGA1b expression alone did not affect the activity of any promoter; hence, it acts in the regulation of these promoters only cooperating with p53 protein. We next tested whether the effects of HMGA1 overexpression in combination of p53 reflected in changes in the endogenous levels of *Bax*, *Mdm2*, and *p21* proteins. As shown in Figure 4d, *Bax* protein levels were significantly elevated in cells expressing p53 alone, compared with control cells, while HMGA1 alone had no effects on *Bax* levels. However, co-expression of HMGA1 with p53 resulted in a significant decrease in *Bax* levels compared with p53 only expressing cells. Similar results were obtained evaluating endogenous p21, while *Mdm2* is increased from p53 expression, and further increased when both HMGA1 and p53 are overexpressed. These results indicate that HMGA1b cooperates in p53-mediated transcription of the *mdm2* promoter while represses that of the *p21^{waf1}* and *Bax* promoters, suggesting that modulation of p53 transcriptional activity by HMGA1b is promoter-specific, at least in the case of H1299 cells.

HMGA1 represses p53 apoptotic function

To evaluate the effects exerted by HMGA1 on the biological activity of p53, we infected H1299 cells, which express HMGA1 but are null for p53 expression, with a recombinant

adenovirus carrying the *wtp53* gene (*Adp53*) or the empty control vector (*dl70.3*), with or without overexpressed HMGA1b. The percentages of TUNEL positive cells were measured 36 h postinfection/transfection. As expected, exogenous p53 expression induced apoptotic cell death in these cells (Figure 5a). However, when HMGA1b was concomitantly overexpressed with p53, the percentage of TUNEL positive cells was strongly reduced (Figure 5a), suggesting that HMGA1b might interfere with p53 apoptotic function. Interestingly, HMGA1b overexpression in the absence of p53 did not suppress the basal level of TUNEL positivity induced by the control adenovirus infection, which was rather mildly increased (Figure 5a), supporting the hypothesis that HMGA1b inhibits the p53-dependent apoptosis. Similar results were obtained by concomitantly overexpressing the *wtp53* gene (*Adp53*) and the long HMGA1a isoform, as evaluated measuring the percentage of cell death by Trypan blue exclusion test (Figure 5b).

To verify the antiapoptotic activity of HMGA1b in a more physiological context, we exposed the *wtp53*-carrying HCT116 cells, which express HMGA1 and p53, to UV irradiation to activate the endogenous p53 in the presence or absence of overexpressed HMGA1b. As shown in Figure 5c, HMGA1b overexpression strongly repressed UV-induced TUNEL positivity. Complementary results were observed when HCT116 or RKO cells, another colon cancer cell line also expressing HMGA1 and p53 similar to the HCT116 cells, were treated with specific antisense oligodeoxynucleotides (AS) to reduce the endogenous HMGA1b expression. As shown in Figure 5d, AS-treatment reduced HMGA1b expression in both cell lines (upper panels) and was associated with an increased sensitivity to UV-induced, p53-mediated apoptosis (lower panels).

To further assess the p53 dependency of the HMGA1b antiapoptotic activity, the isogenic HCT116 system, which includes the p53(+/+) and the p53(-/-) cells,²⁹ was employed. Cells from both lines were exposed to UV irradiation in the presence or absence of overexpressed HMGA1b, and their percentages of death measured by Trypan blue exclusion test. Similar to the results obtained by TUNEL assay (Figure 5c), HMGA1b overexpression significantly reduced the amount of cell death induced by UV irradiation in the p53(+/+) cells (Figure 5e, black bars). However, no modification of death rate by HMGA1b overexpression was observed in the p53(-/-) isogenic cells, although a small amount of UV-induced, p53-independent death was present in these latter cells (Figure 5e, grey bars), strongly suggesting that the antiapoptotic effect of HMGA1b depends on its activity on p53.

Taken together, these data indicate that HMGA1 can functionally interfere with the apoptotic function of the p53 oncosuppressor.

Discussion

HMGA protein overexpression and gene rearrangements are a frequent event in several human cancers. We employed an antibody array screening to get clue on the mechanisms of HMGA tumorigenesis. Here, we report a novel interaction

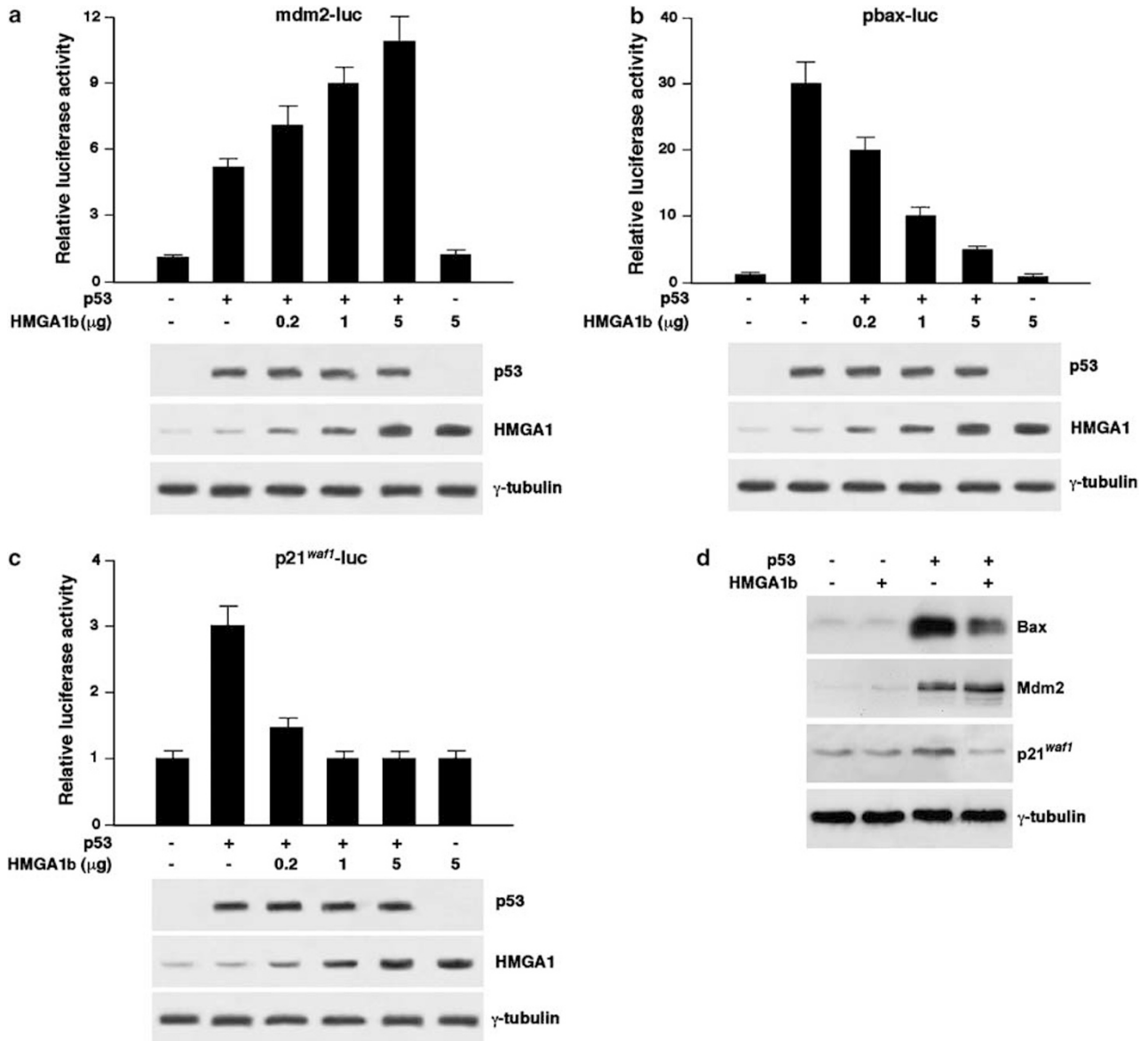


Figure 4 HMGA1b modulates p53 transcriptional activity. Dose–response analysis of increasing amounts of HMGA1b on p53 activity on the *mdm2* (a), *Bax* (b), and *p21^{waf1}* (c) luciferase-reporter vectors transiently transfected in p53-null H1299 cells. All transfections were performed in duplicate and the data are means \pm S.D. of five independent experiments. Insert-less vectors were used as control. Western blot analyses of p53 and HMGA1 proteins from one indicative experiment are shown in the lower panels. γ -Tubulin was used to equalize protein loading. Expression of the endogenous HMGA1 protein is detectable in the insert-less vector transfected H1299 cells. (d) Endogenous protein levels of Bax, Mdm2 and p21 in the same total cellular extracts used in one representative luciferase assays

between HMGA1 and the tumour suppressor p53 that results in the inhibition of p53 apoptotic activity.

We found that both the HMGA1a and HMGA1b isoforms bind p53 protein. Moreover we have mapped the regions of HMGA1 and p53 involved in the binding. We have identified that the 54–63 amino-acid region between the second and the third AT-hooks of HMGA1b is required for its interaction with p53, while the p53 domain principally involved in the binding to HMGA1b are the C-terminal region and the DNA-binding domain. Indeed, we observed that the C-terminal domain itself binds more efficiently than the full-length p53 or the DNA binding domain alone. Since an intramolecular regulatory

domain is included in the C-terminus of p53,³⁰ a possible interpretation of our results is that conformational changes, such as those mediated by the regulatory region over the DNA-binding domain, might modify the p53/HMGA1 interaction. Further analyses are required to elucidate this issue.

We have demonstrated that HMGA1 overexpression modifies the transcriptional activity of p53 by activating the transcription of the p53 inhibitor *Mdm2*, and repressing the p53 effector genes *Bax* and *p21^{waf1}*. At biological level, these transcriptional modifications were associated with a strong inhibition of the p53 apoptotic activity, strongly supporting the hypothesis that HMGA1 contributes to tumour transformation

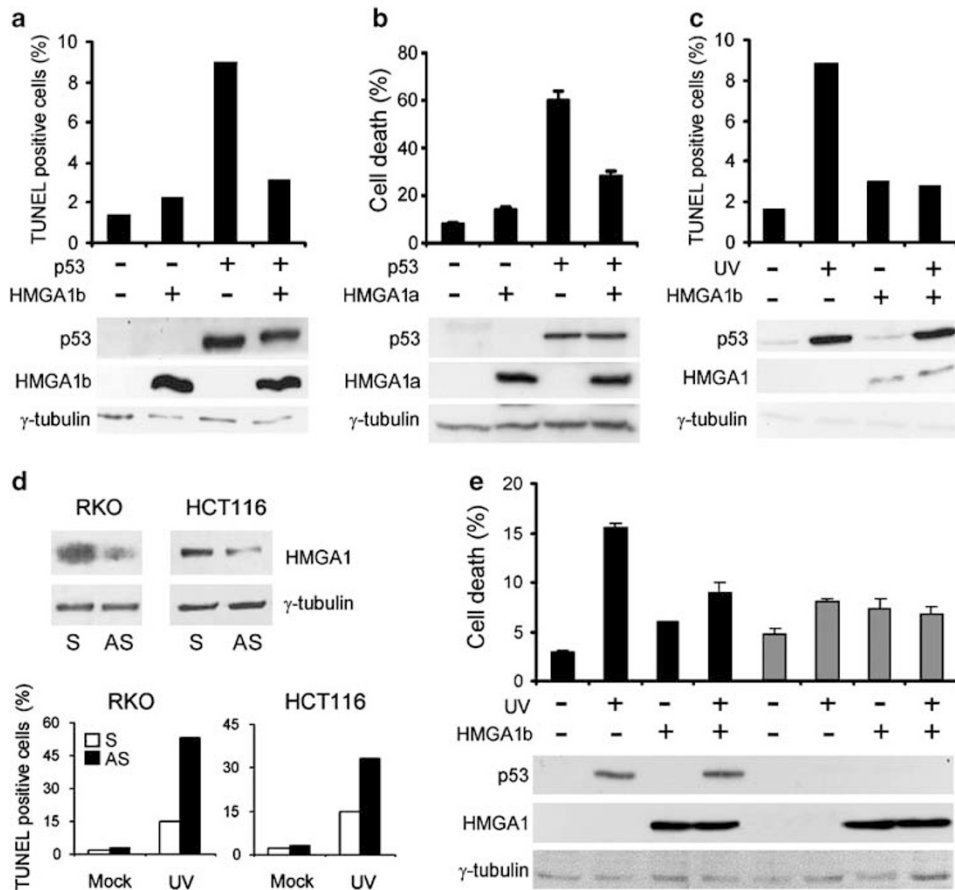


Figure 5 HMGA1 interferes with the apoptotic activity of p53. (a) p53 null H1299 cells were infected with the control vector dl70.3 (– in the p53 row) or with the wtp53-carrying Adp53 (+ in the p53 row). At 1 h postinfection, cells were transfected with the pCMV control vector (– in the HMGA1b row) or the pCMV/*Hmga1b* vector (+ in the HMGA1b row). After 48 h, both floating and adherent cells were spun onto slides and analysed by TUNEL assay. The percentage of TUNEL-positive cells of one indicative experiment out of four is reported. The expression of the indicated proteins was analysed by Western blotting. (b) p53-null H1299 cells were infected as in (a). At 1 h postinfection, cells were transfected with the pCEFL control vector (– in the HMGA1 row) or the pCEFL-*Hmga1a* vector (+ in the HMGA1 row). After 48 h, both floating and adherent cells were collected and analysed by Trypan blue exclusion. Expression of the indicated proteins was analysed by Western blotting; the result of one of three experiments performed is reported. (c) wtp53-carrying HCT116 cells were irradiated or not (+ and –, respectively, in the UV row) with 50 J m⁻² of UV to activate the endogenous p53. The percentage of cell death and the protein levels were analysed as in (a). Mean ± S.D. of three independent experiments are shown. Cells from the same sample were analysed by TUNEL as described in (a). (d) RKO and HCT116 cells were transduced with HMGA1b-specific sense (S) or antisense (AS) oligonucleotides. After 24 h, cells were mock or UV-irradiated (50 J m⁻²). TUNEL assay was performed 24 h postirradiation. After 48 h, TCEs were analysed by Western blotting for HMGA1 expression; γ -tubulin was used loading control. (e) wtp53-carrying HCT116 cells (black bars) and p53(–/–) isogenic cells (grey bars) were irradiated or not (+ and –, respectively, in the UV row) with 50 J m⁻² of UV to activate the endogenous p53. After 48 h, both floating and adherent cells were collected and analysed by Trypan blue exclusion. Means ± S.D. of four independent experiments are shown. The expression of the indicated proteins was analysed by Western blotting

by interfering with the apoptotic function of p53. We also observed that HMGA1 protein is present on the same complexes containing p53 and bound to the *Bax* promoter. Despite the negative action of HMGA1 on p53 transcriptional activity on this promoter, we found that HMGA1 does not decrease the occupancy of p53 to this region. At this time, we cannot completely explain the mechanism by which HMGA1 binding to the *Bax* promoter interferes with its p53-mediated activation. The possibility that binding of HMGA1 proteins to the DNA could somehow impair the activity of either positive regulatory proteins of the *Bax* promoter or basal transcription machinery is actually under investigation. An *in silico* analysis of the p53-responsive *mdm2* and *p21^{waf1}* promoters that we investigated in this study also revealed the presence of

several putative binding sites for HMGA1 protein, suggesting a regulation similar to that observed on the *Bax* promoter.

Defects in apoptosis are thought to play a major role in tumorigenesis as well as in the response of tumours to anticancer treatments. The finding that HMGA1 can bind to p53 and inhibit its apoptotic activity strongly suggests that HMGA1 contributes to tumorigenicity by inactivating the tumour-suppressing function of p53. Consistent with this hypothesis is the observation that at least two tumour histotypes with elevated HMGA expression (i.e. thyroid and prostate cancers)^{31,32} have a very low frequency of mutations in the *TP53* gene. This suggests that inhibition of p53 activity by the presence of HMGA1 proteins might diminish the pressure for direct *TP53* gene mutations.

Physical and functional interactions between p53 and members of other subfamilies of HMG proteins, such as the HMGB proteins, have been previously described.^{33,34} However, the different subfamilies possess divergent functions.³⁵ Indeed, the reported HMGB-mediated modulation of p53 transcriptional activity seems to be relevant in normal conditions while the HMGA1-mediated inhibition of p53 apoptotic function, which we are reporting here, might be mostly relevant for tumour formation. This hypothesis is consistent with the presence, in adult life, of HMGA1 only in tumour tissue.

In conclusion, our data strongly support the existence of a new mechanism of p53 inactivation through HMGA1-mediated modification of the p53 transcriptional activity. This mechanism might have important implications in tumorigenicity as well as in the development of tumour resistance to antineoplastic treatments.

Materials and Methods

Cell cultures, transfections and transactivation assays

HEK 293, HCT116, RKO and H1299 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal calf serum (GIBCO-BRL, Life Technologies, Gaithersburg, Maryland, United States of America), glutamine and antibiotics. Human HCT116 colorectal adenocarcinoma cells and the p53^{-/-} derivatives were kindly provided by B Vogelstein.²⁹ Cells were transfected with plasmids by lipofectamine-plus reagent or with oligonucleotides by oligofectamine reagent (Invitrogen), as suggested by the manufacturer. Cells were transiently transfected with the reporter vectors indicated and previously described,³⁶ and normalized with the use of a co-transfected β -galactosidase construct. Luciferase activity was analysed by Dual-Light System (Applied Biosystems, Massachusetts, USA).

For the inhibition of HMGA1b expression, antisense and the corresponding sense oligonucleotides have been designed and synthesized specifically by BIOGNOSTIK (Göttingen, Germany). Oligonucleotides were added once at a concentration of 2 μ M.

Expression constructs

The pCMV/*Hmga1b* is described elsewhere.³⁷ pHemagglutinin (pHA)-tagged *Hmga1* expression plasmids containing the entire or various portions of the *Hmga1* coding sequence were amplified and inserted into the pCEFL-HA expression vector (kindly provided by Dr. S Gutkind): pHA-A1b (amino acids 1–96) is constituted by the entire coding sequence of the HMGA1b isoform; pHA-A1b/T is constituted by the first 79 amino acids including the three AT-hook domains; pHA-A1b/1–63 is constituted by the first 63 amino acids including the first two AT-hook domains and the region between the second and the third AT-hook domains; pHA-A1b/1–53 is constituted by the first 53 amino acids including the first two AT-hook domains; pHA-A1b/1–43 is constituted by the first 43 amino acids including the first AT-hook domain and the region between the first and the second AT-hook domains; pHA-A1b/23–96 contains the *Hmga1b* coding sequence deprived of the first 23 amino acids; pHA-A1b/31–96 contains the *Hmga1b* coding sequence deprived of the first 31 amino acids that include the first AT-hook domain; pHA-A1a (amino acids 1107) is constituted by the entire coding sequence of the long HMGA1a isoform.

In vitro translation and protein–protein binding

The pCAG-p53, pGST-p53, pGST-p53(13–96), pGST-p53(13–295), pGST-p53(295–390) and pET2c-HMGA1b constructs are previously described (36 and 19, respectively). GST fusion proteins and His recombinant proteins were produced in *Escherichia coli* BL21 cells. Stationary phase cultures of *E. coli* cells transformed with the plasmid of interest were diluted 5–400 ml in LB with ampicillin (100 μ g/ml), grown at 30°C to an OD₆₀₀ of 0.6 and induced with 0.1 mM IPTG. After an additional 2 h at 30°C, the cultures were harvested and resuspended in 10 ml of cold PBS (140 mM NaCl, 20 mM sodium phosphate (pH 7.4)), 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors (Boehringer). The cells were broken by French Press. For the GST proteins, the supernatant was then incubated at 4°C for 1 h with 250 μ l of glutathione-Sepharose beads (Amersham Pharmacia Biotech). The resin was washed with PBS and protease inhibitors. The recombinant proteins were eluted with a buffer containing PBS, 10 mM reduced glutathione, and 10% (v/v) glycerol. For the His-HMGA1b protein, the supernatant was purified by using nickel-agarose beads supplied with the His-Trap purification kit (Amersham Pharmacia) following the manufacturer's instructions, eluted with 500 mM imidazol and dialysed in PBS. The purified p53 protein was purchased from Li StarFISH (Carugate, Milan). The recombinant proteins were subjected to *in vitro* protein–protein binding. The proteins were incubated with total cellular extracts (TCEs) or other recombinant protein in NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40) for 1 h at 4°C. The resins were then extensively washed in the same buffer. The bound proteins were separated by SDS-PAGE, and analysed by Western blotting.

Antibody array screening, Western blotting, and immunoprecipitation assay

TCE were prepared with lysis buffer (50 mM Tris Hcl pH 7.5, 5 mM EDTA, 300 mM NaCl, 150 mM KCl, 1 mM dithiothreitol, 1% Nonidet P40, and a mix of protease inhibitors). We used an antibody array filter (Hypermatrix Incorporation) in which 100 polyclonal or monoclonal antibodies, including antibodies against well-studied proteins involved in cell cycle regulation, apoptosis, and signal transduction pathways are immobilized on a membrane, at predetermined positions, and retain their capabilities of recognizing and capturing antigens. After incubation with a TCE from 293 cells overexpressing HA-HMGA1b, immunoblot assay was performed following the manufacturer's instructions using an HRP-conjugated anti-HA antibody (Santa Cruz Biotechnology Inc.), followed by enhanced chemiluminescence (ECL, Amersham Pharmacia) to detect the captured proteins.

For co-immunoprecipitation experiments, antigens and Abs were incubated for 3 h and then supplemented with protein A-sepharose or G-sepharose beads (Pharmacia Biotech). After 1 h, the beads were collected and washed five times with lysis buffer, and boiled in Laemmli sample buffer for immunoblotting analysis. Protein extracts and immunoprecipitated pellets were separated by SDS-PAGE, and then transferred onto Immobilon-P Transfer membranes (Millipore) or stained with blue Coomassie. Membranes were blocked with 5% non-fat milk proteins and incubated with Abs at the appropriate dilutions. The filters were incubated with horseradish peroxidase-conjugated secondary Abs, and the signals were detected with ECL. The Abs used for immunoprecipitation and Western blotting were: anti-FLAG monoclonal Ab (Sigma); anti-HA 12CA5 monoclonal Ab (Roche); anti-p53 F1393 polyclonal Ab and anti-p53 DO1 monoclonal Ab (Santa Cruz Biotechnology Inc.); anti-HMGA1 are polyclonal Ab raised against a synthetic peptide located in the

NH₂-terminal region. Anti- γ -tubulin (Santa Cruz Biotechnology Inc.) was used for loading control.

Electrophoretic mobility shift assay (EMSA)

DNA binding assays with the recombinant proteins were performed as previously described.⁴ Briefly, 50 ng of His-HMGA1b recombinant protein were incubated with radio-labelled double-strand oligonucleotide, corresponding to region spanning bases -441/-500 of the human *bax* promoter region (*hbaxpr*), and the mutated oligonucleotide, in which the p53-binding site has been deleted (*hbaxprdel*). His-HMGA1b protein was incubated in a solution made of 20 mM HEPES pH 7.9, 40 mM KCl, 0.1 mM EDTA, 0.5 mM MgCl₂, 0.5 mM DTT, 0.1 mM PMSF, 0.5 μ g poly(dC-dG), 2 μ g BSA and 10% glycerol, to a final volume of 20 μ l, for 10 min at room temperature. The samples were incubated for 15 min after addition of 2.5 fmol of a ³²P-end-labelled oligonucleotide (specific activity, 8000–20 000 cpm/fmol). A 100-fold molar excess of unlabelled oligonucleotide was added as specific competitor. The DNA-protein complexes were resolved on 6% nondenaturing acrylamide gels and visualized by exposure to autoradiographic films.

Chromatin immunoprecipitation (ChIP) and Re-ChIP assays

Briefly, 5×10^6 H1299 cells were cross-linked using 1% formaldehyde for 10 min at room temperature. The reaction was stopped with glycine 0.125 M for 5 min. The cells were washed twice with cold PBS, harvested, and lysed sequentially by 10 min in ice and 5 min centrifugation at $3000 \times g$ at 4°C with 1 ml buffer A (10 mM HEPES pH 8, 10 mM EDTA pH 8.0, 0.5 mM EGTA pH 8.0, 0.25% Triton X-100 and protease inhibitors), and then with 1 ml buffer B (10 mM HEPES pH 8.0, 200 mM NaCl 1 mM EDTA pH 8.0, 0.5 mM EGTA pH 8.0, 0.01% Triton X-100 and protease inhibitors). The pellets were then resuspended in 200 μ l of lysis buffer (10 mM EDTA, 50 mM Tris-HCl pH 8.0, 1% SDS and protease inhibitors), and sonicated five times for 30 s at maximum settings, obtaining fragments between 0.3 and 1.0 kb. The samples were cleared by centrifugation at 14 000 r.p.m. for 15 min. After centrifugation, 20 μ l of the supernatants were used as inputs, and the other part of the samples diluted 2.5-fold in Ip buffer (100 mM NaCl, 2 mM EDTA pH 8.0, 20 mM Tris-HCl pH 8.0, 0.5% Triton X-100 and protease inhibitors). The samples were subjected to immunoprecipitation with specific antibody (anti-HMGA1 described in the previous section, and anti-p53 sheep polyclonal Ab7 from Calbiochem) after 2 h preclearing at 4°C with Protein A Sepharose or Protein G Sepharose/BSA/Salmon Sperma (Upstate). Precipitates were washed sequentially with 1 ml Ip buffer (25 mM Tris-HCl pH 8, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS), 1 ml Wash buffer 1 (25 mM Tris-HCl pH 8.2 mM EDTA, 500 mM NaCl, 1% Triton X-100, 0.1% SDS), 1 ml Wash buffer 2 (0.25 M LiCl, 1% NP40, 1% Na deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8), and then twice with 1 mM EDTA, 10 mM Tris-HCl pH 8.0. Precipitated chromatin complexes were removed from the beads through 15 min incubation with 250 μ l of 1% SDS, 0.1 M NaHCO₃. This step was repeated twice.

In Re-ChIP experiments, complexes were eluted by incubation for 30 min at 37°C in 250 μ l of Re-IP buffer (2 mM DTT, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl pH 8.1) and then diluted four-fold in Re-IP dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl pH 8.1 and protease inhibitors), and subjected again to the ChIP procedure. Crosslink was reversed by an overnight incubation at 65°C with 20 μ l of 5 M NaCl. Then were added 10 μ l 0.5 mM EDTA, 20 μ l

1 M Tris-HCl pH 6.5 and 20 μ g of Proteinase K, and incubated for 1 h at 45°C. DNA was purified by Phenol/CHCl₃, and precipitated by two volumes of ethanol in the presence of tRNA. PCR was performed using specific primers: *h-bax-pr-up* 5'-TAATCCCAGCGCTTTGGAAG-3'; *h-bax-pr-dw* 5'-GTCCAATCGCAGCTCTAATG-3'; *h-GAPDH-pr-up* 5'-GTAT TCCCCAGGTTTACATG-3'; *h-GAPDH-pr-dw* 5'-TTCTCCATGGTGGT GAAGAC-3'.

Recombinant adenoviruses and adenoviral infection

The E1/E3 defective recombinant adenovirus dl70.3 (dl) and its Adp53 derivative, in which the human wtp53 cDNA was cloned,³⁸ were amplified and titrated on 293 cells. Adenoviral infection was performed using 60 plaque forming units/cell of recombinant adenovirus.³⁹

Cell viability and TUNEL assay

Both floating and adherent cells were collected and counted in a hemocytometer after the addition of Trypan blue. The percentage of dead cells, that is, number of blue cells/total cell number, was determined by scoring 100 cells per chamber three times. Cell numbers were determined in duplicate.

For TUNEL assay, both floating and adherent cells were spun onto slides by cytocentrifugation. After fixing in 4% formaldehyde in PBS, cells were incubated with fluorescein-conjugated dUTP terminal deoxynucleotide transferase mixture for TUNEL reaction (Roche), according to the manufacturer's instructions, counterstained with 1 μ g/ml of Hoechst 33258 for 2 min and mounted with a coverslip in 25% glycerol in PBS. At least 400 cells were counted in each plate.

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Retraction

Retraction: High Mobility Group A1 (HMGA1) proteins interact with p53 and inhibit its apoptotic activity

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It has come to our attention that multiple bands in Figure 1e and lanes in Figure 3a have been inappropriately duplicated.

The authors are unable to provide the original source files that were used to generate these data and they have all agreed to retract this article. The conclusions of the paper have been confirmed by subsequent papers.