

HMGA1 Inhibits the Function of p53 Family Members in Thyroid Cancer Cells

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Abstract

HMGA1 is an architectural transcription factor expressed at high levels in transformed cells and tumors. Several lines of evidence indicate that HMGA1 up-regulation is involved in the malignant transformation of thyroid epithelial cells. However, the mechanisms underlying the effect of HMGA1 on thyroid cancer cell phenotype are not fully understood. We now show that in thyroid cancer cells, HMGA1 down-regulation by small interfering RNA and antisense techniques results in enhanced transcriptional activity of p53, TAp63 α , TAp73 α , and, consequently, increased apoptosis. Coimmunoprecipitation and pull-down experiments with deletion mutants showed that the COOH-terminal oligomerization domain of p53 family members is required for direct interaction with HMGA1. Moreover, inhibition of HMGA1 expression in thyroid cancer cells resulted in increased p53 oligomerization in response to the DNA-damaging agent doxorubicin. Finally, electrophoretic mobility shift assay experiments showed that the p53-HMGA1 interaction results in reduced DNA-binding activity. These results indicate a new function of HMGA1 in the regulation of p53 family members, thus providing new mechanistic insights in tumor progression. (Cancer Res 2006; 66(6): 2980-89)

Introduction

The high-mobility group A factors (HMGA1a, HMGA1b, and HMGA2) are nonhistone proteins, which participate in a variety of cellular processes, including gene transcription, integration of retroviruses into chromatin, induction of neoplastic transformation, and promotion of metastatic progression (1, 2). HMGA1a and HMGA1b are together referred to as HMGA1 because they result from alternative splicing of the same gene and differ for an internal region of 11 amino acids (3). The highly homologous HMGA2 derives from a different but related gene (4). All members of the HMGA family are characterized by the presence of multiple copies of a DNA-binding peptide motif called the "AT hook" that preferentially binds to the narrow minor groove of stretches of AT-rich DNA sequences. Because of their intrinsic flexibility, the HMGA proteins participate in specific protein-DNA and protein-protein interactions that induce both structural changes in

chromatin and formation of stereospecific complexes called "enhanceosomes" on the promoter/enhancer regions of genes. The formation of such regulatory complexes is characterized by reciprocal induction of conformational changes in both the HMGA proteins themselves and in their interacting substrates (1).

The expression level of HMGA genes is maximal during embryonic development but is low in fully differentiated adult cells (5–7). In addition, HMGA proteins are rapidly induced in cells exposed to growth factor stimulation and are involved in the control of cell proliferation (8). Consistent with such a growth regulatory role, homozygous mutations in the *Hmga2* gene result in the *pygmy* or "mini-mouse" phenotype in mice (7).

In several tumor cells, including colorectal, prostate, breast, cervical, lung, and thyroid, the HMGA1 protein level is high and correlated to the increasing degree of malignancy or metastatic potential (2). Increased expression of HMGA proteins was indeed shown to promote a transformed phenotype in different cell lines (1, 9–12), and transgenic mice overexpressing HMGA proteins develop tumors (13–15). With respect to thyroid cancer, the use of an HMGA2 antisense vector resulted in the suppression of both HMGA1 and HMGA2 synthesis and in the prevention of the retrovirus-mediated neoplastic transformation of rat thyroid cells (16). The requirement for HMGA1 expression during rat thyroid cell transformation was further assessed by an antisense methodology specific for HMGA1 (17). Moreover, an adenovirus-mediated suppression of HMGA1 protein synthesis based on antisense strategy caused apoptosis in anaplastic human thyroid carcinoma cell lines, leading to a drastic reduction of tumor growth *in vivo* (18). However, despite these lines of evidence regarding the protumorigenic role of HMGA, the mechanisms underlying this effect are still poorly understood.

p53 is an oncosuppressor activated by DNA damage. It elicits cell cycle arrest and apoptosis, thereby preventing the formation of tumors (19). Loss of p53 function is a common finding in most human cancers (20), including thyroid cancer. Although the well-differentiated thyroid carcinomas (papillary and follicular) rarely display p53 inactivation (21), >80% of poorly differentiated (anaplastic) thyroid carcinomas have p53-inactivating mutations (22). Loss of p53 function is believed to be an important event in thyroid tumor progression from papillary to anaplastic cancer (22).

p53 belongs to a family of proteins comprising two additional members: p63 (23) and p73 (24), which share extensive homologies with p53. *In vitro* experiments have shown that p63 and p73 are able to transactivate p53-responsive genes, including *p21*, *Bax*, and *Mdm2*, and to induce cell cycle arrest and apoptosis (23, 25, 26). *p63* and *p73* genes undergo multiple COOH-terminal splicing, skipping one or more exons and giving rise to six isoforms for p73 (α , β , γ , δ , ϵ , and ϕ ; refs. 24, 27, 28) and three isoforms for p63

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(α , β , and γ ; ref. 23). Very recently, p53 variants have been described (29). The use of alternative starting codons adds more complexity to p53, p63, and p73 networks, with the generation of isoforms lacking the transactivation domain that can function in a "dominant-negative" fashion on their corresponding full-length protein (26, 30). Regulation of p53 family activity is a complex and multifaceted process involving post-translational modifications, protein interactions, and modulation of subcellular localization (31, 32).

We and others have previously shown that although p53 family members are expressed in thyroid cancer cells (33–35), their tumor suppressor activity is kept latent in these malignant cells. Because HMGA proteins have been shown to associate with and modulate the activity of several nuclear factors (1, 2, 36), here, we have explored whether HMGA may also influence the function of p53 family members. We found that HMGA1 associates *in vivo* with p53 family members, and that this interaction results in the inhibition of their tumor suppressor activity in thyroid cancer cells. These data indicate that HMGA1 up-regulation may promote progression of thyroid cancer via the inhibition of p53 family proteins.

Materials and Methods

Cells. Papillary (TPC-1 and BC-PAP) and anaplastic (ARO) thyroid cancer cells were provided by Drs. A. Fusco and M. Santoro (Naples, Italy); anaplastic thyroid cancer cells C-643 were provided by Dr. N.E. Heldin (Uppsala, Sweden). These cell lines were grown in complete 10% fetal bovine serum (FBS) RPMI 1640. The human osteosarcoma cell line Saos-2 and simian kidney cell line COS-1 were provided by Dr. J.Y. Wang (La Jolla, CA) and cultured in complete 10% FBS DMEM (Sigma, St. Louis, MO). The human breast cancer cell line MCF-7 (American Type Culture Collection, Manassas, VA) was grown in complete 10% FBS MEM.

Plasmids. Plasmid pGEX HMGA1b expressing the human HMGA1b in fusion with the glutathione *S*-transferase (GST) and pcDNA3HA-HMGA1a have already been described (37, 38). Human pcDNAHA-p53, mouse pcDNA-myc-p63 α , and human pcDNA-HA-p73 α , pcDNA-HA-p73 β , pcDNA-HA-p73 γ , and pcDNA-HA-p73 δ , as well as the deletion mutants of pcDNA-HA-p53 (1-363, 1-355, 1-338, 1-298, 57-393, 90-393) have also been described (39). pcDNA3.1-p53-GFP was a gift of Geoffrey Wahl and Jane Stommel (The Salk Institute, La Jolla, CA). Deletion constructs pcDNA-myc-p63 α 1-369 and 1-264 were obtained by cutting in the coding region at the unique restriction sites *Bsm*BI and *Bsr*GI, respectively, and with *Xba*I, which cuts in the polylinker of pcDNA, at the 3' end followed by blunt-ending and ligation. Deletion mutant pcDNA-HA-p73 α 1-348 was obtained by digesting with *Eco*RI and religating the plasmid, while pcDNA-HA-p73 α 1-248 digesting with *Bsm*BI and *Xho*I, which cuts in the polylinker, blunting and religating. pcDNA3.1-HA- Δ Np73 α was kindly provided by Dr. J. Wang (UCSD, La Jolla, CA); pcDNA3.0-Myc-TAp63 α , pcDNA3.0-Myc-TAp63 γ p21Luc, BaxLuc, and Mdm2Luc were donated by Dr. G. Blandino (Regina Elena Cancer Institute, Rome, Italy). pWWP (containing the p53-responsive element of the p21 promoter), pWP124 (devoid of the p53-responsive element), and PGVB2 luciferase plasmids were kindly donated by Prof. T. Sakai (Kyoto Prefectural University of Medicine, Kyoto, Japan). pcDNA-HATNV 322-355, pcDNA-HATNV 355-363, and pcDNA-HATNV 363-384 containing oligonucleotides encoding p53 peptides cloned within the *Escherichia coli* thioredoxin cDNA have been described elsewhere (40).

Plasmids pAR HMGA1a, pAR HMGA1a 1-80, pAR HMGA1a 1-52, pAR HMGA1a 35-107, and pAR HMGA1a 46-107 used for the expression of recombinant proteins used in Far Western experiments have also been previously described (38).

GST pull-down assays and Far Western blot analysis. Expression and purification of GST fusion proteins for GST pull-down assays were carried out using standard protocols. Proteins were translated *in vitro* using the commercial *in vitro* transcription/translation kit (TNT, Promega Corp., Madison, WI), with ³⁵S-methionine (NEN Life Science, Boston, MA) following the manufacturer's instructions. GST pull-down assays were

carried out essentially as previously described (37). When included, ethidium bromide was used at a final concentration of 200 μ g/mL. Recombinant HMGA1a proteins used in Far Western experiments were expressed and purified as previously described (36) HMGA1a 45-75 was a degradation product obtained during the preparation of HMGA1a 45-106. One microgram of each of the HMGA1 recombinant proteins was separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane. Far Westerns were then carried out as previously described (36).

Electrophoretic mobility shift assay. Nuclear extracts from human MCF-7 epithelial breast cancer cells were prepared as already described (41), and protein concentration was determined by the Bradford assay. 2 μ g of nuclear extract from MCF-7 cells, treated or untreated with 10 μ mol/L doxorubicin for 7 hours, were incubated in 20 μ L binding buffer [25 mmol/L Tris-HCl (pH 7.5), 50 mmol/L KCl, 50 mmol/L DTT, 20 μ g bovine serum albumin, 0.1% Triton X-100, 20% glycerol, and 1.5 μ g poly(deoxyguanylic-deoxycytidylic acid)] and, when indicated, high-performance liquid chromatography-purified recombinant full-length (wt) and truncated (1-51) HMGA1a proteins were included (20 and 40 pmol). For supershift experiment, α -p53 monoclonal antibody 421 (Oncogene Research, Uniondale, NY) was included in the reaction mixture. After incubation for 20 minutes at room temperature, ³²P-labeled DNA was added and incubated at room temperature for an additional 15 minutes. Samples were separated in a native 4% polyacrylamide gel in 45 mmol/L Tris-borate, 1 mmol/L EDTA buffer (pH 8.3, 4°C, 10 V/cm), and the gel was fixed, dried, and exposed to X-ray film (Hyperfilm MP, Amersham Biosciences, Uppsala, Sweden). The following oligonucleotide was used (only upper strand is shown): p21, 5'-AGCTTAGGCATGTCTAGGCATGTCTA-3'.

Immunoprecipitation and immunoblot analysis. Cell lysates were prepared in complete radioimmunoprecipitation assay. For immunoprecipitation experiments, 1 mg of cell lysate was incubated for 2 hours with 2 μ g of antibody, and protein A-Sepharose was then added (Amersham Biosciences). Samples were then subjected to SDS-PAGE, and membranes were incubated with primary antibodies in 5% milk-TBST (1 μ g/mL). Horseradish peroxidase-conjugated secondary antibodies were used for protein detection by enhanced chemiluminescence (Pierce, Rockford, IL).

The following antibodies were used for immunoprecipitation: polyclonal anti-hemagglutinin (HA) antibody (CRP, Berkeley, CA), polyclonal anti-p63 α antibody H129 (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal antibody DO-1 against the NH₂ terminus of p53 (Santa Cruz Biotechnology, Santa Cruz, CA), mixture of anti-p73 monoclonal antibodies (Ab-4; Neomarkers, Fremont, CA).

The following antibodies were used for Western blotting: anti-HA monoclonal antibody (CRP), anti-pan-p63 monoclonal antibody 4A4 (Santa Cruz Biotechnology), anti-p53 monoclonal antibody DO-1 (Santa Cruz Biotechnology), anti-p21^{Cip1} polyclonal antibody (Santa Cruz Biotechnology), anti- β -actin monoclonal antibody (Sigma), anti-GFP monoclonal antibody (CRP), anti-Myc monoclonal antibody 9E10 (Santa Cruz Biotechnology). The anti-HMGA1 polyclonal antibody has been raised against a peptide derived from the NH₂-terminal region and therefore recognizes both HMGA1a and HMGA1b isoforms (42).

Transfections. All transfections were done by the Fugene 6 method (Roche Biochemical, Inc., Basel, Switzerland) according to the manufacturer's instructions, and cells were processed 24 hours after transfection.

Luciferase assay. The p21Luc, BaxLuc, and Mdm2Luc constructs were cotransfected with pcDNA3.1, pcDNA3.0-Myc-TAp63 α , pcDNA3.0-Myc-TAp63 γ , and pcDNA3.1-p53 (DNA ratio, 1:1). A vector coding for the *Renilla* luciferase was also cotransfected in all conditions (DNA, ratio 1:20). Twenty-four hours after transfection, the cells were processed with the Dual Luciferase Assay (Promega) according to the manufacturer's instructions. Luciferase activity was normalized for transfection efficiency (*Renilla* activity).

Gene silencing by small interfering RNA. Cells were plated onto six-well plates (10⁵ per well), maintained in antibiotic-free medium for 24 hours, and transfected with a mixture containing Opti-MEM, 8 μ L/well LipofectAMINE 2000 (Invitrogen, San Diego, CA), and either 0.5 μ g/well scramble small interfering RNA (siRNA) or a mixture of four HMGA1 siRNA (Dharmacon Research, Inc., Lafayette, CO) for 5 hours. The sequence of these siRNAs is available from the manufacturer. Cells were then incubated

with fresh medium for 48 hours and transfected with either p53, Tap63 α , or Tap73 α (0.5-1.0 μ g/well) along with either p21Luc, BaxLuc, or Mdm2Luc (1.0 μ g) and *Renilla* (0.2 μ g), using Fugene 6 reagent (Roche Biochemical). Cells were processed 24 hours after transfection.

Fluorescence-activated cell sorting analysis. For cell cycle evaluation, cells were fixed and permeabilized with 70% ethanol in PBS and incubated with propidium iodide and RNase for 30 minutes at room temperature in the dark. Cells were then subjected to fluorescence-activated cell sorting (FACS) analysis for DNA content (FL2). Apoptosis measurement was done by staining intact cells with FITC-Annexin and propidium iodide (BD, Franklin Lakes, NJ), according to the manufacturer's protocol. Cells were then evaluated by FACS analysis (FITC = FL1, propidium iodide = FL2). Annexin-positive cells were considered to be in early apoptosis, whereas cells stained with both Annexin and propidium iodide were considered to be in late apoptosis.

In experiments where the green fluorescent protein (GFP) vector was cotransfected as marker, apoptosis measurement was done by staining with phycoerythrin-Annexin (BD) and evaluating Annexin-positive cells among the GFP-positive cell population.

Statistical analysis. FACS analysis results were compared by two-way ANOVA. Significance was obtained by *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Statistical analysis was carried out with Microsoft Excel Software.

Results

HMG1A1 inhibits the transcriptional activity of p53 family members in thyroid cancer cells. It has been previously reported that HMG1A1 overexpression correlates with malignant transformation of thyroid epithelial cells (42, 43). However, the exact mechanism underlying this HMG1A1-transforming potential is still poorly understood. To investigate this, we asked whether in thyroid cancer cells HMG1A1 may somehow influence the function of p53, p63, and p73. For this purpose, we selected the thyroid cancer cell line TPC-1, which expresses HMG1A1 to a high level (refs. 42, 43; data not shown). In these cells, the expression of HMG1A1 was silenced using siRNA (A1-si) and tagged p53 family members (GFP-p53, Myc-TAp63 α , and HA-TAp73 α) were cotransfected along with the p53-responsive promoters (p21, Bax, and Mdm2). Luciferase assays revealed that the transcriptional activity of ectopically expressed p53 family members was higher in HMG1A1-depleted thyroid cancer cells compared with controls transfected with scramble siRNA (scr-si), although to a variable degree (Fig. 1). Western blot analysis (Fig. 1, bottom) shows the expression of tagged p53 family members in transfected cells and moreover that the levels of endogenous HMG1A1 (both HMG1A1a and HMG1A1b isoforms) were efficiently depleted using A1-si.

To extend these results, we selected other thyroid cancer cell lines, ARO and BC-PAP, that express a high level of HMG1A1. In these cells, we transfected either HMG1A1 sense or antisense expressing vectors and evaluated the transcriptional activity of the tagged versions of p53 family members (GFP-p53, Myc-TAp63 α , and HA-TAp73 α), by measuring the activation of the p21-Luciferase promoter (Table 1). Luciferase assays revealed that the HMG1A1 antisense (A1-AS) significantly enhanced the transcriptional activity of p53 family members toward the p21Luc promoter to a variable degree. However, experiments done with HMG1A1 sense did not reveal any inhibition of p53 family members (Table 1). In accordance with previous reports (11, 44), we hypothesized that the high level of the endogenous HMG1A1 protein expressed in thyroid cancer cells was already exerting a maximal inhibition of p53 family members and that, therefore, a further increase of HMG1A1, obtained by transient transfection, could not cause a further inhibition. To overcome this problem, we selected the p53

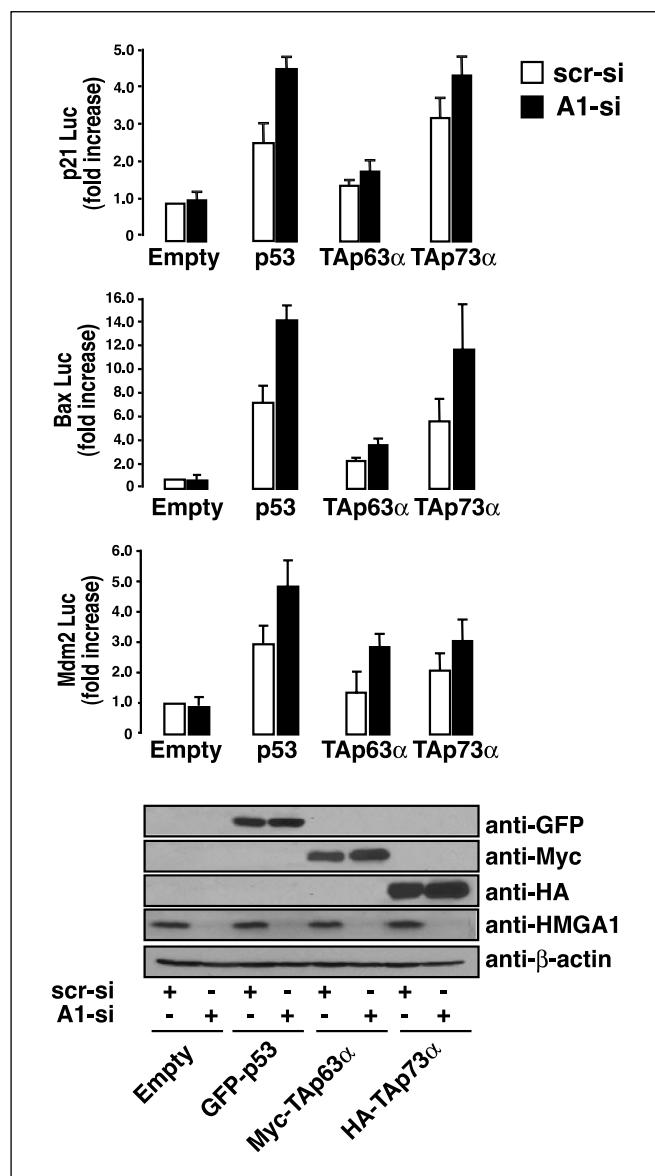


Figure 1. Effect of siRNA against HMGA1 on the transcriptional activity of p53 family members in TPC-1 thyroid cancer cells. TPC-1 cells were transfected with either scrambled (scr-si; white columns) or HMGA1-siRNA (A1-si; black columns) as indicated in Materials and Methods. Forty-eight hours after transfection with siRNAs, cells were transfected with either empty vector, GFP-p53, Myc-TAp63 α , or HA-TAp73 α along with either p21 Luc, Bax Luc, or Mdm2 Luc as reporter genes. Lysates were used for luciferase assays. Columns, mean fold increase over empty-transfected cells of three separate experiments done in triplicate; bars, SE. Aliquots of these lysates were processed by Western blot analysis and representative blots, displaying the expression level of HMGA1 and ectopic p53, TAp63 α , and TAp73 α , are shown.

null human osteosarcoma cell line Saos-2 (45), which expresses HMG1A1 at a very low level (ref. 37; data not shown). In these cells, transfection of HMG1A1 sense was effective in inhibiting ectopic p53 family member transcriptional activity, whereas HMG1A1 antisense had no effect (Table 1). Similar experiments done with a p21 promoter devoid of the p53-binding site (pwwP124) indicated that this enhancement was specific and dependent on p53 family activity (data not shown).

Taken together, these results indicate that HMG1A1 may act as an inhibitor of p53 family members.

Table 1. Effect of HMGA1 sense and antisense on transcriptional activity of p53 family members (p21Luc promoter)

	Empty	p53	TAp63 α	Tap73 α
TPC-1				
Empty	1.0 \pm 0.0	3.0 \pm 0.9	1.3 \pm 0.8	3.5 \pm 0.7
HMGA1 sense	1.1 \pm 0.2	2.8 \pm 1.1	1.2 \pm 0.5	3.2 \pm 0.9
HMGA1 antisense	1.2 \pm 0.6	5.0 \pm 0.5*	2.0 \pm 0.3 [†]	4.0 \pm 0.8
ARO				
Empty	1.0 \pm 0.0	22.2 \pm 2.4	5.4 \pm 0.9	8.3 \pm 0.2
HMGA1 sense	1.3 \pm 0.9	19.9 \pm 3.7	6.2 \pm 1.4	9.9 \pm 1.5
HMGA1 antisense	1.1 \pm 0.4	38.7 \pm 2.8*	9.7 \pm 4.3 [†]	12.2 \pm 2.8
BC-PAP				
Empty	1.0 \pm 0.0	4.2 \pm 2.1	2.5 \pm 1.0	4.6 \pm 0.9
HMGA1 sense	1.4 \pm 0.3	3.8 \pm 2.7	2.6 \pm 0.8	4.1 \pm 1.3
HMGA1 antisense	0.9 \pm 0.3	13.6 \pm 5.1*	3.0 \pm 1.3	5.9 \pm 0.8
Saos-2				
Empty	1.0 \pm 0.0	14.5 \pm 2.1	12.2 \pm 1.3	13.6 \pm 1.9
HMGA1 sense	1.1 \pm 0.4	5.5 \pm 1.3*	3.9 \pm 2.8 [†]	5.1 \pm 1.6*
HMGA1 antisense	0.8 \pm 0.8	13.8 \pm 4.3	13.8 \pm 1.3	13.1 \pm 1.5

**P* < 0.01.
[†]*P* < 0.05.

HMGA1 depletion enhances the tumor suppressor activity of p53 in thyroid cancer cells. We then tested whether the HMGA1 inhibitory effect, observed in transient transfection experiments, could also occur with endogenous proteins. To this end, we exposed the well-differentiated thyroid cancer cell line TPC-1, which expresses wild-type p53 (46), to the DNA-damaging agent doxorubicin for 4 hours (Fig. 2A). In these cells, HMGA1 silencing by siRNA resulted in increased levels of p21, Bax, and Mdm2 protein in response to doxorubicin (Fig. 2A). Moreover, the addition of p53 siRNA confirmed that the induction of target proteins was p53 dependent (Fig. 2A).

In cells treated as above, the effect of HMGA1 depletion was analyzed both on cell cycle and apoptosis by FACS analysis. Exposure to doxorubicin for 4 and 8 hours induced a predominant S-G₂ arrest in TPC-1 cells transfected with scramble siRNA, whereas it induced a robust G₁ arrest in cells transfected with HMGA1 siRNA (Fig. 2B). Cotransfection with p53 siRNA prevented the cellular G₁ arrest in response to doxorubicin, suggesting that the effect of HMGA1 depletion on G₁ arrest was due, at least in part, to the enhancement of p53 function. Parallel experiments, done with FITC-Annexin- and propidium iodide-stained cells, confirmed the same results with apoptosis (Fig. 2C).

To further characterize the HMGA1 effect on the proapoptotic activity of p53, we transfected the thyroid cancer cell line TPC-1, C-643, and ARO cells with either an empty vector, p53, TAp63 α , or TAp73 α along with H2B-GFP to mark the transfected population. FACS analysis was done in transfected (GFP positive) thyroid cancer cells. We found that cotransfection with HMGA1 antisense (AI-AS) increased the apoptotic effect of all p53 family members, as assessed by phycoerythrin-Annexin staining, in all the cancer cell lines tested (Fig. 2D).

Taken together, these results indicate that depletion of HMGA1 results in the enhancement of p53 family member function.

HMGA1 interacts *in vivo* and *in vitro* with p53 family proteins. To unravel the mechanism(s) underlying the effect of HMGA1 on p53 family members, we did coimmunoprecipitation

experiments aimed to detect possible interactions of HMGA1 with p53 family proteins.

TPC-1 thyroid cancer cell lysates were subjected to immunoprecipitation with either an anti-p53 antibody or an anti-HMGA1 polyclonal antibody (Fig. 3A). As a control a nonimmune serum was used (NIS) for immunoprecipitation. Western blot analysis detected the presence of HMGA1 in anti-p53 immunoprecipitates (Fig. 3A, *top*) and vice versa (Fig. 3A, *bottom*). These results indicate an interaction between endogenous HMGA1 and p53 *in vivo* in TPC-1 thyroid cancer cells.

To assess whether HMGA1 may interact with all p53 family proteins, we cotransfected 293 human kidney cancer cells with HA-HMGA1a and either GFP-p53, Myc-TAp63 α , or HA-TAp73 α . In these experiments, anti-p53, anti-p63 or anti-p73 were used to immunoprecipitate p53 family members, whereas an anti-HA antibody was used to detect HMGA1a. As a control a non immune serum was used (NIS). Coimmunoprecipitation experiments were able to detect the presence of HMGA1a in anti-p53, anti-p63 and anti-p73 immunoprecipitates (Fig. 3B).

To assess whether the *in vivo* interaction was direct, a pull-down assay using a GST-HMGA1b fusion protein and *in vitro* translated p53 family members was employed. *In vitro* translated ³⁵S-labeled p53, TAp63 α and TAp73 α were retained by GST-HMGA1b even in the presence of high concentrations of ethidium bromide, that disrupts DNA-dependent protein-protein interaction (data not shown).

These results suggest that HMGA1 is able to interact directly with all p53 family members, thus modulating their functional properties.

Binding of HMGA1 requires the oligomerization domain of p53 family members. As a first step to gaining an insight into the HMGA1 interaction with p53 family proteins, we mapped the regions of the p53 family proteins to identify those involved in the interaction with HMGA1. To this end, various deletion mutants of p53, cloned in the pcDNA3HA vector, were *in vitro* translated and tested for their ability to bind recombinant GST-HMGA1b in pull-down experiments (Fig. 4A). As summarized in Fig. 4A (*bottom*), a deletion from the COOH-terminal end [construct

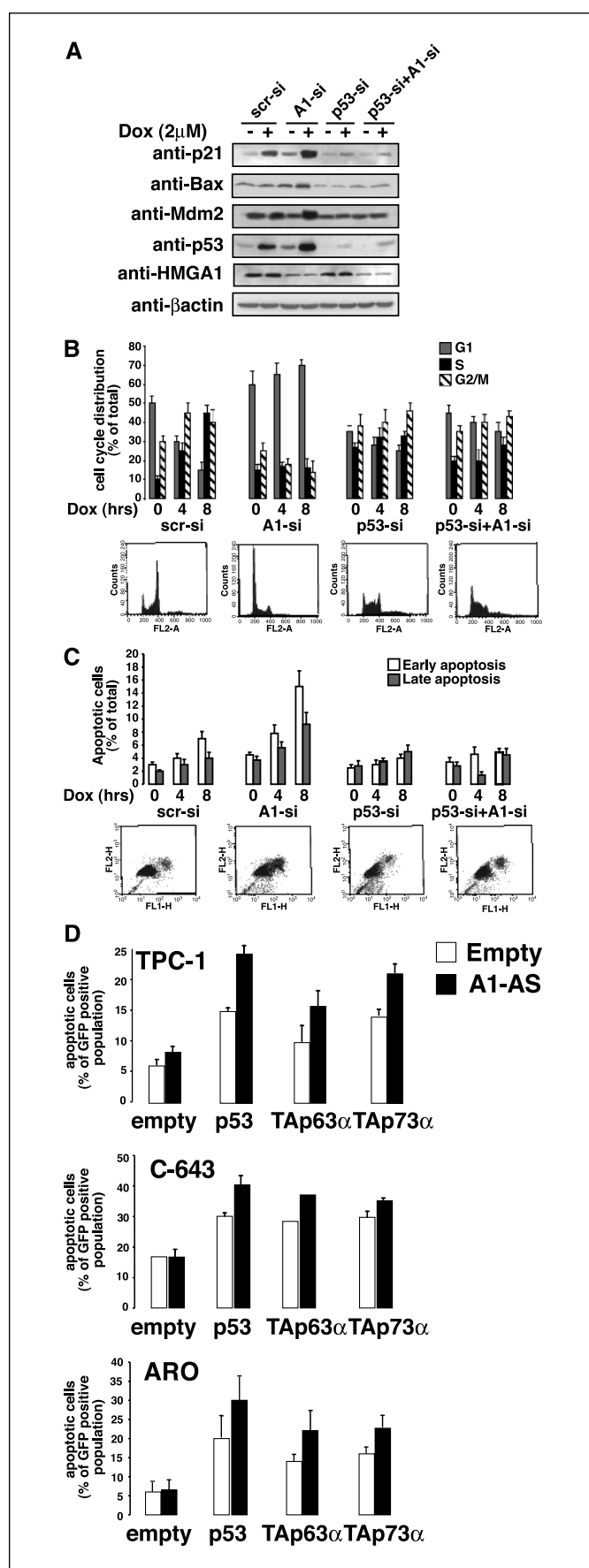
p53(1-363), lanes 4-6], removing the p53 basic domain, did not have a marked effect on HMGA1b binding. Further deletions [constructs p53(1-355), p53(1-338), and p53(1-298), lanes 7-15] abolished the HMGA1b binding, suggesting that the oligomerization domain is critical for HMGA1b binding. A deletion from the NH₂-terminal end [construct p53(90-393), lanes 16-18], which removes the transcriptional activator domain (TA), had no effect on HMGA1b binding.

The other two members of the p53 family, p63 and p73, have a similar domain organization as p53; however, unlike p53, both p63 and p73 harbor a sterile α -motif (SAM) domain at their COOH terminus. To extend this analysis to the entire p53 family, deletion mutants and different isoforms of p63 and p73 were also tested in GST pull-down experiments (Fig. 4B and C). p63 isoforms (TAp63 α , Δ Np63 α , and TAp63 γ , lanes 4-9), having different COOH and NH₂ terminus domains but retaining the DBD and the oligomerization domain, all associated with GST-HMGA1b. Conversely, deletion constructs of TAp63 α partially or completely lacking the oligomerization domain [constructs TAp63 α (1-369) and TAp63 α (1-264), lanes 10-15] failed to interact with GST-HMGA1b.

Similarly to p53 and p63, the oligomerization domain of p73 was also required for the HMGA1b interaction. Figure 4C shows that p73 isoforms Δ Np73 α , TAp73 β , and TAp73 γ , which maintain the DBD and the oligomerization domain, interact with HMGA1b. In contrast, TAp73 α (1-348) and TAp73 α (1-248), which are devoid of the oligomerization domain, did not associate with GST-HMGA1b.

These results clearly show that HMGA1b binding to p53, p63, and p73 requires the oligomerization domain. From these experiments, it seems also that the p53 COOH-terminal basic residues participate in HMGA1b binding (see Fig. 4A). To map more precisely the region in p53, we used constructs encoding different peptides derived from the p53 COOH-terminal region, inserted within the scaffold of the thioredoxin protein. These constructs, corresponding to regions 322-355, 355-363, and 361-393, were *in vitro* translated and tested in pull-down experiments with GST-HMGA1b fusion protein. As shown in Fig. 4D, the peptide corresponding to the oligomerization domain (amino acid 322-355) and the COOH-terminal basic domain (amino acid 361-393) showed a specific interaction with HMGA1b, whereas no binding

Figure 2. Effect of HMGA1 depletion on the tumor suppressor function of p53 family members in thyroid cancer cells. **A**, papillary thyroid cancer cells TPC-1 carrying wild-type p53, were transfected with either scrambled-siRNA (scr-si), HMGA1-siRNA (A1-si), or p53-siRNA (p53-si) as indicated in Materials and Methods. After 48 hours, cells were exposed to 2 μ mol/L doxorubicin (Dox) for 4 hours and subjected to Western blot analysis for p21, Bax, Mdm2, HMGA1, p53, and β -actin, as a loading control. Representative blots are shown. **B**, TPC-1 cells were transfected as above and either left untreated (*time 0*) or exposed to 2 μ mol/L doxorubicin for 4 and 8 hours (*time 4* and *8*). Cell cycle profile was evaluated by propidium iodide staining and FACS analysis (as indicated in Materials and Methods). *Gray columns*, cells in G₁; *black columns*, cells in S; *hatched columns*, cells in G₂-M. *Columns*, mean percentage of total cells of three separate experiments done in triplicate; *bars*, SE. Representative cell cycle profiles of TPC-1 cells exposed to doxorubicin for 4 hrs are also shown at the bottom. **C**, TPC-1 cells treated as above were stained with both propidium iodide and FITC-Annexin and subjected to FACS analysis (as indicated in Materials and Methods). *White columns*, early apoptosis (Annexin-positive cells); *gray columns*, late apoptosis (cells stained by both Annexin and propidium iodide). *Columns*, mean percentage of total cells of three separate experiments done in triplicate; *bars*, SE. Representative FACS analyses of TPC-1 cells exposed to doxorubicin for 4 hours (*bottom*). **D**, TPC-1, C-643, and ARO thyroid cancer cells were transfected with the indicated p53 family member constructs in combination with HMGA1 antisense (A1-AS) along with H2B-GFP to mark transfected population. Cells were then stained with phycoerythrin-Annexin and subjected to FACS analysis as indicated in Materials and Methods. GFP-positive cells were gated, and the percentage of apoptotic cells measured as indicated in Materials and Methods. *Columns*, mean of three separate experiments done in triplicate; *bars*, SE.



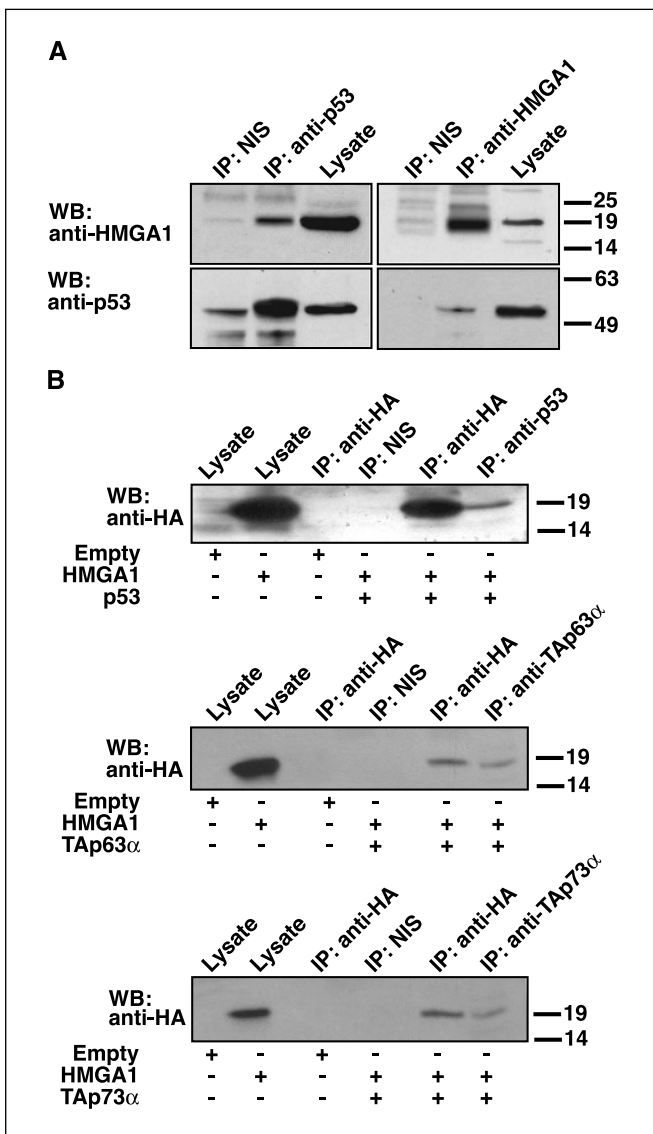


Figure 3. Interaction of HMGA1 with p53 family members. *A*, TPC-1 thyroid cancer cells were lysed, and proteins were immunoprecipitated with either anti-p53 or anti-HMGA1 antibodies. NIS was used as a control for immunoprecipitation, and crude cell lysates were used as a control protein transfer. Membranes were probed with either anti-HMGA1 (*top*) or anti-p53 antibody (*bottom*). *B*, 293 cells were transfected with either GFP-p53 (*top*), Myc-TAp63 α (*middle*), or HA-TAp73 α (*bottom*) along with HA-HMGA1a or the empty HA vector as indicated. Lysates were then immunoprecipitated with either anti-p53 (*top*), anti-p63 (*middle*), or anti-p73 (*bottom*) antibodies. NIS and anti-HA antibodies were used as controls. Membranes were probed with anti-HA antibody.

was detectable with the other peptide or with thioredoxin. It is possible therefore that because the basic COOH-terminal region in p53 is very close to the oligomerization domain, it might also be involved in the HMGA1b interaction.

Two regions in HMGA1 are involved in binding to p53 family members. To identify the HMGA1 domain that interacts with p53 family members, we generated deletions of the HMGA1a protein and tested their ability to interact with *in vitro* translated p53, Tap63 α , and Tap73 α in a Far Western assay. The two HMGA1 isoforms, HMGA1a and HMGA1b, are very similar; the HMGA1a isoform was used because it also contains the 11-amino-acid region

between the first and second AT-hook that is absent in the HMGA1b isoform. Data obtained from the experiments shown in Fig. 5*A* are similar for all three p53 family proteins in that the interaction is maintained when the acidic COOH-terminal tail and the third AT-hook are removed (HMGA1a 1-79), whereas a further deletion (HMGA1a 1-51), removing the second AT-hook, completely abolished the binding. Deletion of the NH₂-terminal tail (HMGA1a 34-106 and HMGA1a 45-106), removing the first AT-hook, shows that the proteins are still able to bind p53 proteins, although not as efficiently as the wild type HMGA1a. Mutant HMGA1a 45-75, comprising the central part of the protein and containing only the second AT-hook, shows a binding capacity similar to HMGA1a 34-106 and HMGA1a 45-106. These data are summarized in Fig. 5*B* and indicate that the HMGA1 region from amino acid 52-80 is critical for protein-protein interaction with p53 proteins, whereas region 1-33 is important for optimal binding efficiency.

HMGA1 interferes with p53 binding to DNA. We then investigated how the interaction of HMGA1 with p53 could influence p53 functional activity. To this end, we tested by electrophoretic mobility shift assay (EMSA) the effect of purified recombinant HMGA1a protein on p53 binding to the p21 promoter. Nuclear extracts from MCF-7 cells, that express wild-type p53 and low levels of HMGA1 (11), were used. Only nuclear extracts from cells treated with doxorubicin to induce p53 gave rise to a complex (Fig. 6*A*, *lane 6*) that was completely supershifted using a monoclonal antibody specific for p53 (Fig. 6*A*, *lane 11*). The p53-DNA complex was then tested with increasing amounts of HMGA1a wild type recombinant protein. As can be seen, increasing amounts of HMGA1a wild type progressively inhibited (up to about 50%) the formation of the p53-DNA complex (compare *lanes 7* and *8* with *lane 6*). This HMGA1a-mediated effect is specific because using the HMGA1a 1-51 deletion mutant, which is unable to associate with p53 (see Fig. 5*A*), there is no decrease of p53-DNA complex formation (compare *lanes 9* and *10* with *lanes 7* and *8*). Based on these results, therefore, we hypothesize that HMGA1 has a negative effect on p53 binding to the p21 target site. In addition, because no HMGA1a binding to the probe was detected when adding recombinant HMGA1a wild type to nuclear extracts of cells untreated with doxorubicin (compare *lanes 3* and *4* with *lane 2*), the effect shown on the p21 probe is likely due to protein-protein interaction.

HMGA1 interferes with p53 oligomerization *in vivo*. Because HMGA1 inhibits p53 binding to the p21 promoter and binds to p53 family members via their oligomerization domains, it is reasonable to suppose that HMGA1 may interfere with the p53 oligomerization. To explore this hypothesis, TPC-1 cells, treated with either scr-si or A1-si, were transfected with GFP-p53 (Fig. 6*B*, *bottom*). To track oligomer formation, we also cotransfected HA- Δ p53(57-393) (Fig. 6*B*, *bottom*), which is devoid of a NH₂-terminal fragment, retains the oligomerization domain, and has lower molecular weight, thus migrating at a different level on the Western blot. To activate p53 function, transfected cells were then incubated with 2 μ mol/L doxorubicin for the indicated time points, and protein extracts were subjected to immunoprecipitation with either anti-HA polyclonal antibody or nonimmune serum (Fig. 6*B*, *top*). Nontransfected cells were also used as a control (Fig. 6*B*). Western blots were then done with a mixture of both anti-p53 and anti-HA monoclonal antibodies (Fig. 6*B*, *top*). In scramble-transfected cells both endogenous and GFP-p53 were observed in anti HA immunoprecipitates but not in NIS immunoprecipitates, indicating that both endogenous and GFP-p53 interacted with HA- Δ p53(57-393) (Fig. 6*B*, *top*). In HMGA1-depleted cells, an increased

recruitment of both endogenous and GFP-p53 was observed in anti-HA immunoprecipitates, indicating an increased oligomerization with HA-Δp53(57-393) (Fig. 6B, top).

Because all these results were obtained by HMGA1 depletion, an opposite approach was done in Saos-2 cells, which express HMGA1

at very low level. Cotransfection of GFP-p53 and His-p53 plasmids together with increasing doses of HA-HMGA1a plasmid resulted in the attenuation of p53 oligomerization in response to doxorubicin (Fig. 6C). Taken together, these results suggest that HMGA1 binding to p53 interferes with its oligomerization.

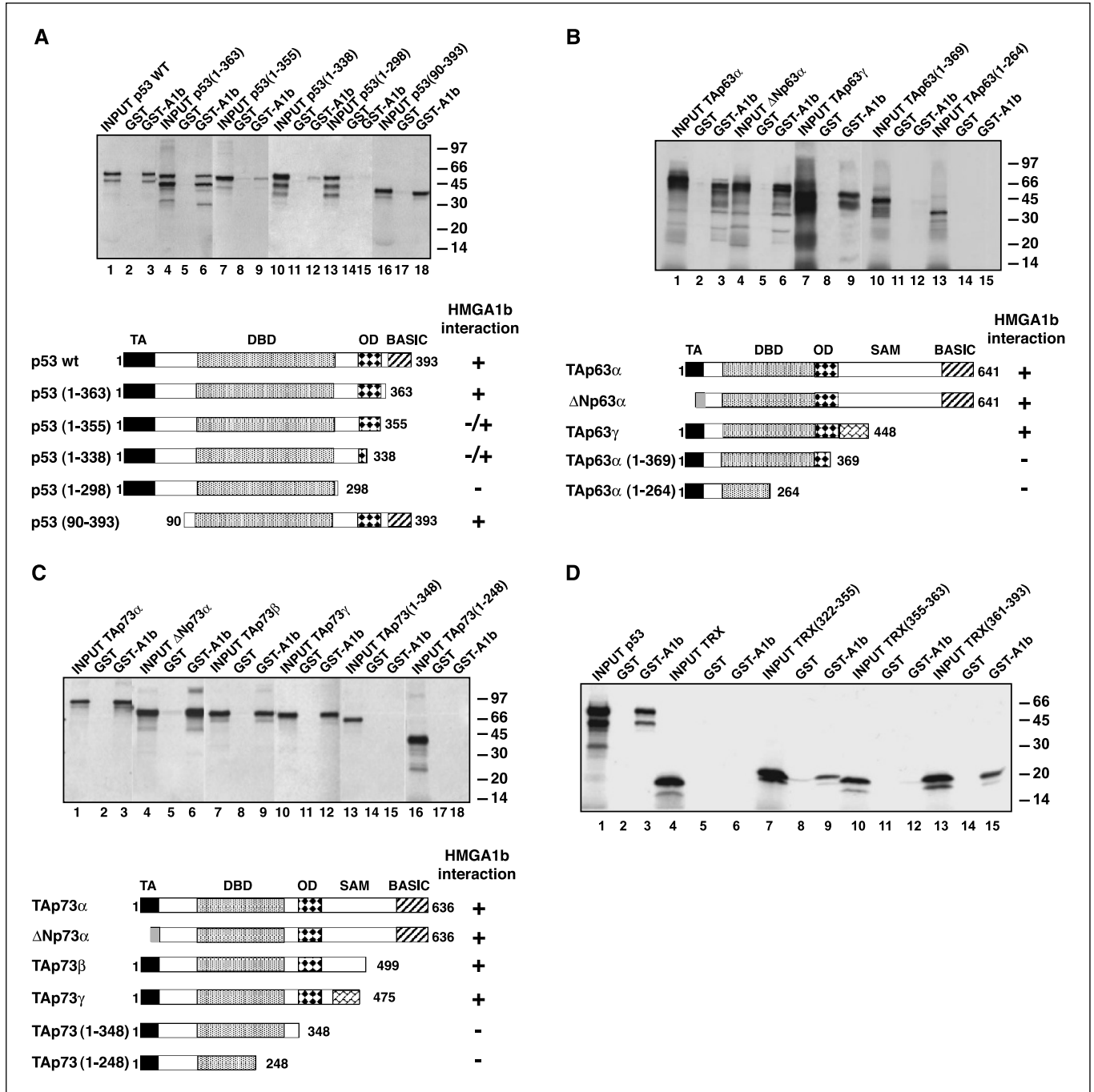


Figure 4. Binding to HMGA1 requires the oligomerization domain (OD) of p53 family members. **A**, ³⁵S-radiolabeled, *in vitro* translated p53 wild type and various p53 COOH-terminal and NH₂-terminal deletion mutants were incubated with GST-HMGA1b or GST immobilized on Sepharose beads in GST pull-down assays. The bound proteins were resolved on SDS-PAGE and visualized by autoradiography. Twenty-five percent of the *in vitro* translated reactions used in the pull-down experiments were included (*input*). Schematic representation of the various p53 constructs used in the pull-down assays. Summary of their ability to bind HMGA1b (*bottom*). Numbers refer to the amino acid sequence. **B**, ³⁵S-radiolabeled, *in vitro* translated TAp63α wild type along with different p63 isoforms and various TAp63α COOH-terminal deletion mutants were incubated with GST-HMGA1b or GST immobilized on Sepharose beads in GST pull-down assays as described in (A). **C**, ³⁵S-radiolabeled, *in vitro* translated TAp73α wild type along with different p73 isoforms and various TAp73α COOH-terminal deletion mutants were incubated with GST-HMGA1b or GST immobilized to Sepharose beads in GST pull-down assays as described in (A). **D**, ³⁵S-radiolabeled, *in vitro* translated peptides, corresponding to different p53 regions, within the thioredoxin (TRX) scaffold were incubated with GST-HMGA1b or GST immobilized on Sepharose beads in GST pull-down assays as described in (A).

Discussion

Previous reports have shown that HMGA2 and HMGA1 silencing results in resistance to retrovirus-mediated transformation in rat thyroid cells (16) and that in human thyroid cancer cells, which usually express HMGA1 at high levels, it may trigger apoptosis (18). These data suggest that, as already documented for other tumors, HMGA proteins participate in the malignant transformation of follicular thyroid cells. Here, we show that in human thyroid cancer cells HMGA1 is able to inhibit the activity of p53 family members. Indeed, in thyroid cancer cells HMGA1 down-regulation by either siRNA or antisense techniques enhanced the response of p21, Bax, and Mdm2 promoters to both endogenous and ectopic p53 family proteins. Moreover, this increased transcriptional activity of p53 family members was accompanied by an increased tumor suppressor activity. The inhibitory effect of HMGA1 on p53 family members was also confirmed in nonthyroid cancer cells, like the low-HMGA1 expressing Saos-2 cells.

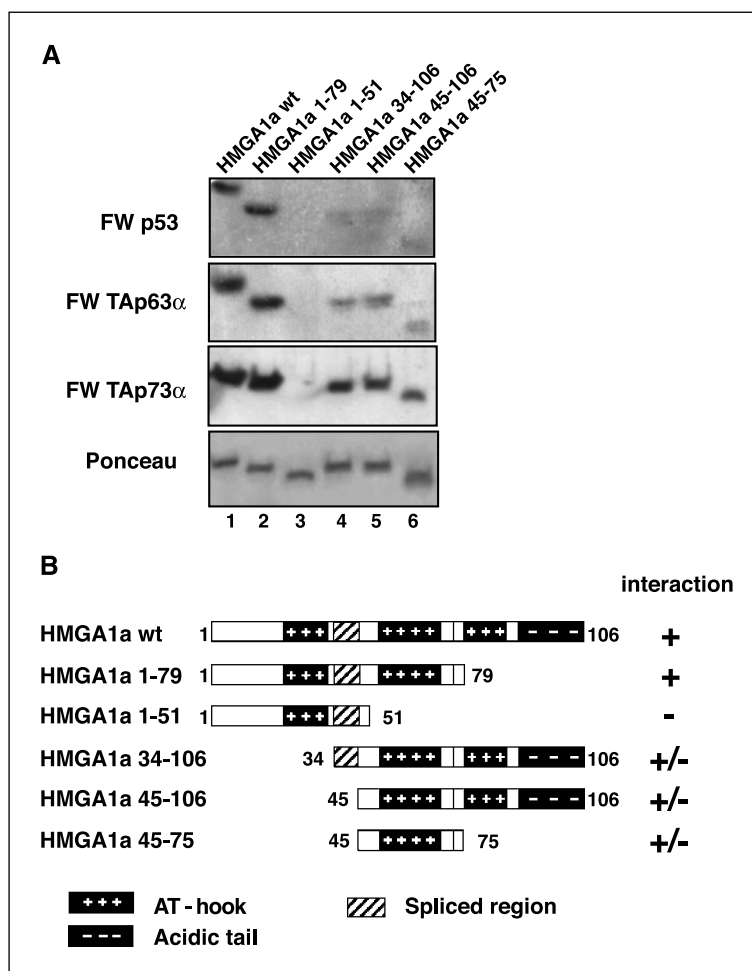
Further insights into the relationship between HMGA1 and p53 family proteins were obtained by coimmunoprecipitation and pull-down experiments: by this approach, a direct interaction between HMGA1 and p53, TAp73 α , and TAp63 α was shown. Subsequent experiments with deletion mutants of the p53 family members showed that HMGA1 interacts with p53 family proteins within a region, including the oligomerization domain at the COOH-terminal of the protein. This region (amino acid 326-355, in the

p53 sequence) is highly conserved among the p53 family proteins (47) and allows oligomerization of the protein. The p53 protein, in fact, is active when it is tetrameric and binds to DNA with high affinity and stimulates transcription of various genes involved in cell cycle arrest or apoptosis. Various experimental studies have shown evidence that the oligomerization domain is essential for DNA binding, protein-protein interaction, post-translational modifications, and p53 degradation (47).

Our data show that HMGA1 reduced p53 oligomerization, suggesting that by this mechanism it is able to blunt p53 transcriptional activity. Further support for this hypothesis came from the EMSA experiments showing the impairment of p53 binding to DNA by HMGA1.

Another not mutually exclusive mechanism to explain the effects of HMGA1 on p53 function might rely on the interference of enzymes that activate p53 through binding to the oligomerization domain. The oligomerization domain is in fact a binding site for PCAF and HIPK2, enzymes that modify and activate the p53 family proteins. PCAF is a potent coactivator of p73, and its interaction with p73 is essential for p73-mediated transactivation (48). HIPK2 phosphorylates p53, after DNA damage, resulting in p53 activation and stabilization (49, 50). Intriguingly, it has been reported that HIPK2 is able to phosphorylate HMGA1 (51), and it is therefore possible that HMGA1 could compete with p53 for HIPK2-mediated phosphorylation. The increased p53 level in HMGA1-depleted TPC-1 cells (shown in Fig. 2A) may support this hypothesis.

Figure 5. Regions 1-33 and 52-79 in HMGA1a interact with p53 family members. *A*, top, purified recombinant wild-type (*wt*) HMGA1a and several HMGA1a deletion mutants were probed in a Far Western blot analysis with ³⁵S-radiolabeled *in vitro* translated p53, TAp63 α , and TAp73 α . *Bottom*, a typical red Ponceau-stained membrane. *B*, schematic representation of the various HMGA1a constructs used in the Far Western experiments and summary of their ability to bind p53, TAp63 α , and TAp73 α . The functional domains of HMGA1a are indicated. Numbers refer to amino acids.



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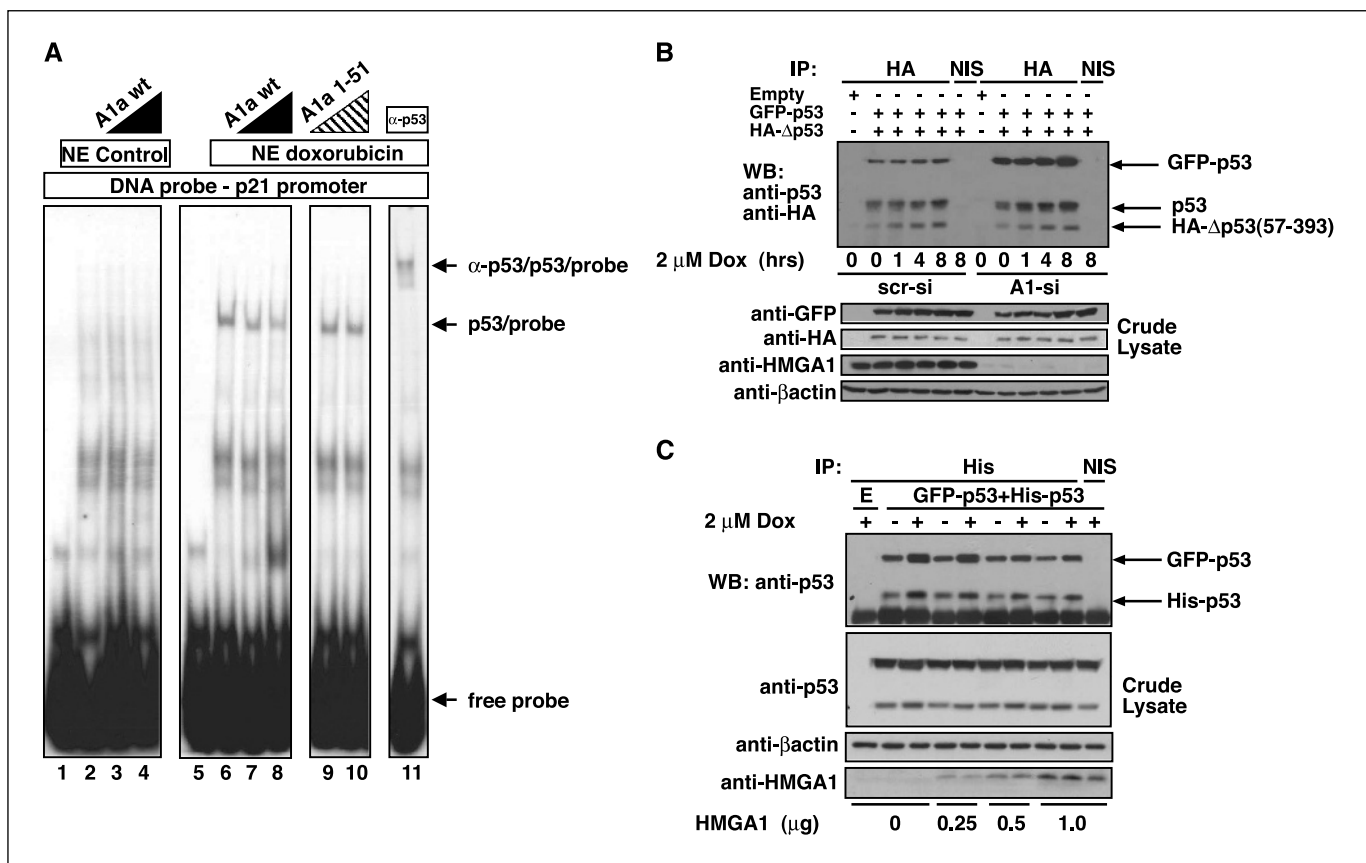


Figure 6. HMGA1 interferes with p53 binding to the p21 promoter and oligomerization. **A**, nuclear extracts (2 μ g) from doxorubicin untreated (*lanes 2-4*) and treated (*lanes 6-11*) MCF-7 cells were incubated with the 32 P-radiolabeled p21 oligonucleotide in an EMSA experiment. *Lanes 1* and *5*, free probe. Increasing amounts (20 and 40 pmol) of recombinant HMGA1 wild type (*wt*; *lanes 3* and *4*; *lanes 7* and *8*) and HMGA1a 1-51 (*lanes 9* and *10*) were included. *Lane 11*, α -p53 monoclonal antibody 421 was added. **B**, TPC-1 thyroid cancer cells were transfected with either scramble siRNA or HMGA1 siRNA. After 48 hours, cells were transfected with either empty vector or a mixture of GFP-p53 and HA- Δ p53(57-393). Twenty-four hours after transfection, cells were incubated for the indicated time points with 2 μ mol/L doxorubicin. Lysates were then immunoprecipitated with anti-HA or NIS. Membranes were probed with both anti-HA and anti-p53 antibody (*top*). Total crude lysates were probed with anti-GFP, anti-HA, anti-HMGA1, and anti- β -actin antibodies to control for transfection efficiencies (*bottom*). **C**, Saos-2 cells were transfected with either empty vector or GFP-p53 and His-p53 vectors. Cells were also cotransfected with either empty vector or increasing doses of HA-HMGA1a vector: 0.25, 0.5, and 1.0 μ g. After 24 hours, cells were incubated for 8 hours in the presence or the absence of 2 μ M doxorubicin. Lysates were then immunoprecipitated with anti-His or NIS. Membranes were probed for GFP-p53 and His-p53 (*top*). Total crude lysates were probed for transfection efficiencies (*bottom*).

In the light of these results, it is interesting to note that HMGB1 (a member of the HMGB family having a different structure compared with HMGA proteins) has an influence on p53 family members activity (52–56). The ubiquitously expressed HMGB1 is in fact able to associate with the COOH-terminal region of p53 and to enhance p53 DNA binding *in vitro* and p53-transactivating capabilities *in vivo* (52, 55). Intriguingly, HMGB1 and the highly related HMGB2 can stimulate the binding of both p73 α and p73 β isoforms to different p53-responsive elements, resulting in either down-regulation or up-regulation, depending on cell types suggesting differential promoter-specific and cell-specific effects (56). In this scenario, it is reasonable to suppose that p53 family member activity may be regulated by the balanced interaction between inhibitors (among these HMGA1) and activators that target p53 family members in different physiologic and pathologic conditions.

Although the molecular mechanism(s) by which HMGA1 is induced in thyroid tumors are not known, HMGA1 protein up-regulation is considered a molecular hallmark of these tumors (43) and is related to the degree of tumor aggressiveness (42). In well-differentiated thyroid cancer p53 mutations are not frequent (22),

suggesting that other mechanisms may be responsible for p53 inactivation in these tumors. Direct evidence of the functional inhibition of p53 tumor suppressor activity in thyroid cancer was obtained from cultured cells isolated from both normal and malignant rat thyroid. These experiments indicated that, compared with normal thyroid cells, thyroid cancer cells displayed a higher level of wild-type p53 protein expression and a concomitant defect in the ability to undergo G₁ and G₂-M growth arrest in response to DNA damage (35).

A similar impairment of tumor suppressor activity of the other members of the p53 family (i.e., Tap63 α and Tap73 α) has been observed in human thyroid cancer (33, 34), suggesting that in these tumors, an inactivating mechanism, common to all the members of p53 family, is present.

Our demonstration that p53 family member activity may be inhibited by HMGA1, which is overexpressed in thyroid cancer, unravels a novel mechanism for thyroid cancer progression and may provide targets for new anticancer therapies. The observation of an enhanced response to doxorubicin in HMGA1-depleted thyroid cancer cells suggests that siRNA adenovirus treatment may sensitize thyroid cancer cells to p53-activating agents. This

approach may also be successful in the presence of p53 mutations, because Tap63 α and Tap73 α , which are also targeted by HMGA1, may compensate for some of the functions lost through p53 mutation.

The novel function that we have described for HMGA1 in the regulation of the oncosuppressor function of p53 family members may be important not only for thyroid cancer but also for other tumors overexpressing the HMGA proteins that have, as a consequence, impaired p53 family protein oncosuppressor function.

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