Doctorate Program in Molecular Oncology
and Endocrinology
Doctorate School in Molecular Medicine

XXI cycle - 2005–2008
Coordinator: Prof. Giancarlo Vecchio

"High Mobility Group A1 (HMGA1) proteins regulate p53-mediated transcription of *Bcl-2* gene"

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“High Mobility Group A1 (HMGA1) proteins regulate p53-mediated transcription of *Bcl-2* gene”

TABLE OF CONTENTS

LIST OF PUBLICATIONS

This dissertation is based upon the following publications:

Pierantoni GM, Finelli P, Valtorta E, Giardino D, Rodeschini O, **Esposito F**, Losa M, Fusco A, Larizza L.
High-mobility group A2 gene expression is frequently induced in non-functioning pituitary adenomas (NFPAs), even in the absence of chromosome 12 polysomy.
Endocr Relat Cancer. 2005 Dec;12(4):867-74.

Pierantoni GM, Rinaldo C, **Esposito F**, Mottolese M, Soddu S, Fusco A.
High Mobility Group A1 (HMGA1) proteins interact with p53 and inhibit its apoptotic activity.
Cell Death Differ. 2006 Sep;13(9):1554-63**.**

Pierantoni GM, Rinaldo C, Mottolese M, Di Benedetto A, **Esposito F**, Soddu S, Fusco A.
High-mobility group A1 inhibits p53 by cytoplasmic relocalization of its proapoptotic activator HIPK2.
J Clin Invest. 2007 Mar;117(3):693-702.

Pierantoni GM, **Esposito F**, Giraud S, Bienvenut WV, Diaz JJ and Fusco A.
Identification of new high mobility group A1 associated proteins
Proteomics. 2007 Oct;7(20):3735-42.

Esposito F, Franco R, Fedele M, Liguori G, Pierantoni GM, Botti G, Tramontano D, Fusco A, Chieffi P.
Detection of high-mobility group proteins A1 and A2 represents a valid diagnostic marker in post-pubertal testicular germ cell tumours.
J Pathol. 2008 Jan;214(1):58-64.

ABSTRACT

The high mobility group A (HMGA) non-histone chromosomal proteins constitute a subgroup of HMG accessory factors that play key roles in chromatin architecture and 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. HMGA overexpression and gene rearrangements are frequent events in human cancer, but the molecular basis of HMGA oncogenic activity remains partially unclear. Recently, we have defined a new physical and functional interaction between HMGA1 and p53. This interaction modulates the transcription of p53 target genes such as *Mdm2*, $p21^{waf1}$, *Bax*, and inhibits p53-mediated apoptosis. Moreover, we have described a new mechanism through which HMGA1 inhibits p53-mediated apoptosis by counteracting the p53 proapoptotic activator homeodomain-interacting protein kinase 2 (HIPK2), which binds to and activates p53 by phosphorylating it at Ser46. We found that HMGA1 overexpression promoted HIPK2 relocalization from the nucleus to the cytoplasm, with consequent inhibition of p53 apoptotic function. During my doctorate course, I looked for other target genes that could be regulated by the HMGA1-p53 complex. So, I focused my attention on the apoptosis inhibitor gene *Bcl-2*, because this gene belongs to *Bax* family, is regulated by p53, and its promoter has several possible target regions for the HMGA1 proteins binding. I demonstrated that HMGA1 binds *Bcl-2* promoter *in vitro* and *in vivo*, and that this binding exerts regulatory effects on *Bcl-2* transcription. In fact, HMGA1 is able to abolish the repression promoted by p53 on *Bcl-2* expression. This effect, as for the *Bax* promoter regulation, is due to HIPK2 delocalization, from the nucleus to the cytoplasm. Therefore, also *Bcl-2* is a gene regulated by HMGA1-p53-HIPK2 complex. Subsequently, my studies were focalized in finding other possible components of this multiprotein complex. Using the neuronal cell line ND7, I found that the transcription factor Brn-3a belongs to this complex. Brn-3a is one of the most important activators of *Bcl-2* transcription but, when p53 is overexpressed, it acts as a corepressor for this gene. Moreover, the binding of Brn-3a to its consensus sequences is positively regulated by HIPK2. I demonstrated that HMGA1 overexpression, in this cellular context, promotes not only HIPK2 delocalization from nucleus to the cytoplasm, but also the reduction of Brn-3a binding to the *Bcl-2* promoter, removing Brn-3a from its role of corepressor following p53 overexpression. These data support the causal role suggested for the HMGA1 proteins in the neoplastic transformation and provide another mechanism of inactivation of p53 apoptotic function.

1. BACKGROUND

1.1 HMGA protein family

Chromatin is an organized structure which undergoes dynamic changes, both in composition and conformation, during DNA replication and transcription. Such dynamic chromatin structural changes are mediated by high-order nucleoprotein complexes in which a variety of proteins are involved other than histones. The heterogeneous high mobility group (HMG) protein family is one class of these proteins, and has the common feature of being smaller than 30 kDa and rich in both acidic and basic amino acids. However, unlike histones, HMGA proteins are not constitutive components of chromatin. In mammals, the HMG proteins have been classified into three distinct families: the HMGB (formerly HMG-1/-2) family, the HMGN (formerly HMG-14/-17) family, and the HMGA (formerly HMG-I/Y/C) family. Each HMG family has a characteristic functional sequence motif: the "HMG-box", the "nucleosomal binding domain" and the "AT-hook" are respectively the motifs of the HMGB, HMGN and HMGA families.

HMGA proteins are involved in many biological processes such as cell growth, differentiation and cancer, and our laboratory has been studying this class of proteins for several years.

At the present time, the mammalian HMGA gene family consists of two functional members, HMGA1 (HMGI/Y) and HMGA2 (HMGI-C). *HMGA1* is located at chromosomal locus 6p21 in humans (Friedmann *et al.* 1993), whereas *HMGA2* is located at chromosomal locus 12q14-15 in humans (Chau *et al.* 1995). In addition, there are also numerous inactive pseudogenes in both mice and humans. Alternative splicing of transcripts from *HMGA1* gene gives rise to three protein products: HMGA1a (formerly HMG-I), HMGA1b (formerly HMG-Y) (Johnson *et al.* 1989; Friedmann *et al.* 1993) and HMGA1c (formerly HMG-I/R) (Nagpal *et al.* 1999). The HMGA1a and HMGA1b proteins are identical in sequence except for an internal deletion of 11 amino acids in the latter, and are also the most abundant of these splice variants in mammalian cells (Figure 1.1).

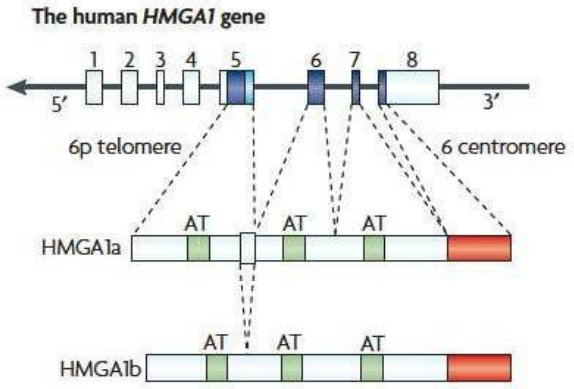


Figure 1.1. Schematic representation of HMGA1a and HMGA1b proteins. Each protein contains three basic domain, named AT hook (green box), with which they bind DNA, and an acidic carboxy-terminal region (red box).

Recently, a new HMGA1 isoform, HMGA1c, has been isolated, and consists of a 67 nucleotides deletion at RNA level in comparison to the HMGA1 sequence. This deletion results in a frameshift so that the two proteins are identical in their first 65 amino acids and differ thereafter. *HMGA1c* encodes a protein of 172 amino acids with molecular weight of 26-27 kDa (Nagpal *et al.* 1999). Little is known about this isoform, however, it appears to be the only *HMGA1* transcript present in normal human and mouse testis (Chieffi *et al.* 2002). In normal cells, transcripts from the *HMGA2* gene code primarily for the full-length HMGA2 protein but alternatively spliced mRNAs from this gene have also recently been detected in leiomyoma tumors (Hauke *et al.* 2001; Kurose *et al.* 2001) (Figure 1.2).

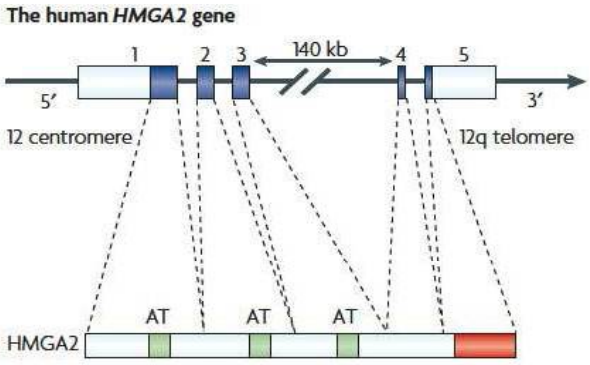


Figure 1.2. Schematic representation of HMGA2 protein. HMGA2 protein contains three basic domain, named AT hook (green box), with which they bind DNA, and an acidic carboxy-terminal region (red box).

1.2 Biochemical and structural characteristics of HMGA proteins

Mammalian HMGA proteins share a number of common, but distinctive, characteristics that collectively distinguish them from all other HMG (Goodwin *et al.* 1973) and known eukaryotic proteins. Originally the HMGA proteins were characterized by their small sizes (10.6–12 kDa), their solubility in dilute (5%) acids, their unusually high concentration of basic, acidic and proline amino acid residues, their highly phosphorylated state *in vivo* and their rapid mobility during electrophoresis (Lund *et al.* 1983). Subsequently, HMGA proteins were shown to bind to the minor groove of short stretches of AT-rich DNA (Reeves *et al.* 1987). Given these DNA-binding properties, it was somewhat surprising when biophysical techniques such as circular dichroism (CD) (Lehn *et al.* 1988) and nuclear magnetic resonance (NMR) spectroscopy, indicated that the HMGA proteins, as free molecules, had very little, if any, secondary structure. Nevertheless, when specifically bound to other molecules, such as DNA or protein substrates, the HMGA proteins assume induced structural features. For example, the DNA-binding regions of the HMGA proteins assume a planar, crescent-shaped configuration called the 'AT-hook' when specifically bound to the minor groove of short stretches of AT-rich DNA (Reeves and Nissen, 1990; Huth *et al.* 1997). Each HMGA protein has three similar, but independent, AT-hook peptides with the consensus sequence of Pro-Arg-Gly-Arg-Pro (with R-G-R-P being invariant) flanked on either side by a number of positively charged lysine/arginine residues. The core of the AT-hook peptide motif is highly conserved in evolution from bacteria to humans and is found in one or more copies in a large number of other, non-HMGA, proteins (Aravind and Landsman 1998), many of which are transcription factors or components of chromatin remodeling complexes. The HMGA proteins recognize DNA structure, rather than nucleotide sequence and, as a consequence, are able to preferentially bind to substrates with distorted or unusual features. These include bent and supercoiled DNAs (Nissen and Reeves 1995; Bustin and Reeves 1996), synthetic fourway junctions (Hill *et al.* 1999), base-unpaired regions of AT-rich DNA (Liu *et al.* 1999) and restricted regions of DNA on the surface of nucleosome core particles (Reeves and Nissen 1993; Reeves and Wolffe 1996). Furthermore, HMGA binding can induce structural changes in bound DNA substrates. Depending on the sequence, the organization, the topology or the length of the substrate itself, HMG binding can bend, straighten, unwind and induce looping in linear DNA molecules.

Interestingly, HMGA proteins also participate in protein–protein interactions and induce structural changes in the bound protein substrates. In their role as architectural transcription factors, the HMGA proteins specifically interact with a large number of other proteins, many of which are gene regulatory factors.

1.3 *In vivo* modifications of HMGA proteins

In addition to their other distinguishing characteristics, the HMGA proteins are among the most extensively modified proteins found in the mammalian nucleus. A variety of techniques have been employed to demonstrate that the HMGA proteins are subject to *in vivo* post-translational phosphorylation, acetylation, methylation and poly-ADP-ribosylation (reviewed in Reeves and Beckerbauer 2001). These secondary biochemical modifications (summarized in Figure 1.3) are dynamic, rapidly responding to both intra- and extracellular signaling events, and influence both the substrate-binding properties of the HMGA proteins and their biological activities. Earlier work by Reeves and coworkers showed that cdc2 could induce the phosphorylation of HMGA1 proteins and the phosphorylation resulted in decrease in binding of the protein toward DNA (Nissen *et al.*1991; Reeves *et al.*1991; Zhang and Wang 2007). Moreover, it has been shown that Homeodomain-interacting protein kinase 2 (HIPK2) could induce the phosphorylation of the same sites phosphorylated by cdc2, though these two kinases exhibit different site preferences, suggesting that phosphorylation of HMGA1 may be highly regulated, which in turn may control the expression of its target genes (Zhang and Wang 2007). It was also found that the HIPK2- and cdc2-phosphorylated HMGA1a showed much lower binding affinity toward DNA than the unphosphorylated counterpart, which is consistent with the fact that the major phosphorylation sites, i.e., Thr-52 and Thr-77, reside close to the second and third AT-hooks of HMGA1a, respectively (Zhang and Wang 2007). A number of studies have demonstrated that HMGA proteins are also the *in vivo* downstream targets for a number of signal transduction pathways that lead to phosphorylation of specific amino acid residues as a result of exposure to extracellular environmental stimuli. Two particularly well studied examples of the intimate connection between signaling events emanating from the cell surface and the state of phosphorylation of HMGA proteins in the nucleus are modifications resulting from the activation of casein kinase 2 (CK2) and protein kinase C (PKC) (Boothby *et al.* 1997; Wang *et al.* 1995, 1997; Banks *et al.* 2000; Xiao *et al.* 2000).

As illustrated in Figure 1.3, more recent MALDI/TOF mass spectrometry analyses of *in vivo* modified HMGA1 proteins in human mammary epithelial cells have demonstrated that, in addition to phosphorylation (Ferranti *et al.* 1992), the HMGA proteins exhibit several other types of secondary modifications including internal acetylation, methylation and possibly poly-ADP ribosylation (Banks *et al.* 2000). Nevertheless, the MALDI/MS results are consistent with earlier metabolic labeling studies (Elton and Reeves 1986) and other types of *in vitro* analyses (Munshi *et al.* 1998). Most importantly, however, these latest studies have also demonstrated that the complex patterns of *in vivo* modifications significantly reduce the affinity of binding of the HMGA proteins to both DNA and nucleosome substrates (Reeves *et al.* 1991; Banks *et al.* 2000). Although not yet conclusively demonstrated, these dynamic

and reversible secondary modifications are most likely regulatory in nature and control the biological activity of these proteins *in vivo* (Reeves 1992; Siino *et al.* 1995; Munshi *et al.* 1998).

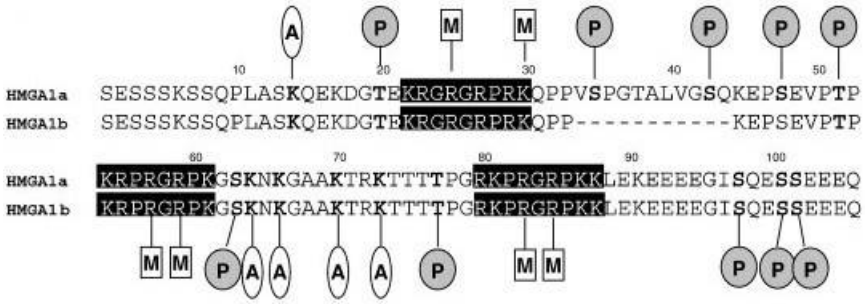


Figure 1.3. HMGA1 post-translational modification. Known post-translational modifications of human HMGA1a and HMGA1b proteins, with the modified residues highlighted in bold. The phosphorylation, methylation and acetylation are denoted by shaded circle, open oval and rectangle, respectively. The first methionine is removed post-translationally and not included in the sequence.

1.4 HMGA proteins, chromatin, and gene regulation

Chromatin structure plays a dominant role in the regulation of gene transcription in eukaryotic cells. In most instances, chromatin exerts a repressive effect on gene transcription if either nucleosomes or other inhibitory chromatin proteins (as histone H1) are associated with critical regulatory regions of gene promoters or enhancers. Elaborate mechanisms, such as ATP-dependent chromatin remodeling machines and precisely controlled biochemical modifications of histones and other regulatory protein complexes, have evolved to alter or modulate such repressive chromatin structures and allow for active gene transcription (Fry and Peterson 2001). Several different proposals have been advanced to explain how the HMGA proteins might participate in the regulation of gene transcription by influencing chromatin structure. These suggestions generally fall into two distinct categories: one category of models suggests that the HMGA proteins act to influence the structure of large regions or domains of chromatin, whereas a second type envisions a much more restricted or localized effect on chromatin and nucleosome structure. It should be stressed that these are not necessarily mutually exclusive models.

There are now reports of over 50 different eukaryotic and viral genes whose transcriptional expression is regulated by HMGA proteins *in vivo* (see Table 1.1; Reeves and Beckerbauer 2001; Martinez Hoyos *et al.* 2004) including those recently identified. The vast majority of these (>35) are positively regulated and their inducible expression is controlled by a variety of biological and environmental stimuli. The promoter regions of many of the positively

regulated genes contain multiple, yet individualistically arranged, stretches of AT-rich sequence that have been proposed to represent a gene-specific 'bar code' that is 'read' by the AT-hooks of the HMGA proteins during the process of transcriptional activation (Reeves and Beckerbauer 2001). Transcriptional activation of these types of promoters often involves the formation of an "enhanceosome", a stereo-specific, multi-protein complex that includes HMGA proteins and other transcription factors making specific protein–DNA and protein–protein contacts in intricate, but precise ways (reviewed in Merika and Thanos 2001; Chau *et al*. 2005). The mechanistic details of enhanceosome formation have been most extensively studied for the virus inducible β-interferon enhancer where the DNA–protein complex formed on the gene's promoter serves as a stereo-specific platform involved in recruiting RNA polymerase II and other co-factors necessary for transcription initiation (Kim and Maniatis, 1997; Merika *et al.* 1998; Munshi *et al.* 1998; Yie *et al.* 1999; Agalioti *et al.* 2000). In the cases where HMGA proteins act as negative regulators of gene transcription, they often serve as inhibitors of enhanceosome formation, usually by sterically blocking the functional binding of other crucial transcription factors to their recognition sites in gene promoters (Klein-Hessling *et al.* 1996; Arlotta *et al.* 1997; Zentner *et al.* 2001).

Table 1.1 Genes regulated by HMGA proteins

POSITIVE REGULATION

Vascular endothelial tissue related
E-selectin (Lewis *et al.* 1994)
IGFBP-1 (Allander *et al.* 1997)
COX-2 (Ji *et al.* 1998)
SM22α (Chin *et al.* 1998)
INOS (Perrella *et al.* 1999)
CD44 (Foster *et al.* 2000)

Immune system related
IL-2 (Reeves *et al.* 1987)
TNF-β (Fashena *et al.* 1992)
INF-β (Thanos *et al.* 1992)
IL-2Rα (John *et al.* 1995)
HLA-II (Abdulkadir *et al.* 1995)
MSGA/GROα (Wood *et al.* 1995)
GM-CSF (Himes *et al.* 1996)
IgG heavy chain (Sobasjima *et al.* 1997)
c-fos (Chin *et al.* 1998)
CXCL1 (Nirodi *et al.* 2001)

Viral genes
JV virus early and late genes (Leger *et al.* 1995)
HSV-1 IE3 (French *et al.* 1996)
HIV-1 LTR (Farnet *et al.* 1997)
HSV-1 EBNA1 (Sears *et al.* 2003)
BV EBNA1 (Sears *et al.* 2003)

Cell cycle related
Cyclin A (Tessari *et al.* 2003)
Cyclin E (Fedele *et al.* 2006)
CDC2 (Fedele *et al.* 2006)
CDC6 (Fedele *et al.* 2006)
CDC25A (Fedele *et al.* 2006)

Others
Tyrosinase (Sato *et al.* 1994)
PKCγ (Xiao *et al.* 1996)
Rhodopsin (Chau *et al.* 2000)
Neurogranin IRC3 (Xiao *et al.* 2000)
Leptin (Melillo *et al.* 2001)
Mdm2 (Pierantoni *et al.* 2006)

NEGATIVE REGULATION

GP 91-phox (Skalnik *et al.* 1992)
IL-4 (Chuvpilo *et al.* 1993)
IgE (Kim *et al.* 1995)
TCRα (Bagga *et al*. 1997)
β-globin (Chase *et al.* 1999)
α-EnaC (Zentner *et al.* 2001)
BRCA1 (Baldassarre *et al.* 2003)
RAG2 (Battista *et al.* 2005)
Bax (Pierantoni *et al.* 2006)
p21 (Pierantoni *et al.* 2006)
Hand1 (Martinez Hoyos *et al.* 2008)

1.5 HMGA proteins have many partners

In addition to their unique DNA-binding characteristics, another principal reason why the HMGA proteins are able to participate in the *in vivo* regulation of so many different genes is because of their ability to physically interact with a large number of other proteins, most of which are transcription factors. As listed in Table 1.2, at least 21 different transcription factors have so far been reported to specifically associate with HMGA proteins as determined by co-immunoprecipitation assays, yeast two-hybrid screening methods, protein "pull down" procedures and other techniques. Regardless of their extent of physical association with other proteins, however, two points are notable. First, the sites of interaction include part, or all, of one or more AT-hook peptide motifs plus some flanking regions. Second, these sites include amino acid residues that are known to be extensively modified *in vivo* by either phosphorylation, acetylation or methylation. As noted earlier, secondary modifications are known to significantly alter the interaction of the HMGA proteins with DNA and chromatin substrates. Labile biochemical modifications are, therefore, likely to modulate other specific HMGA–protein interactions *in vivo* and influence, for example, enhanceosome formation and gene transcriptional activity and/or a wide variety of other biological processes.

Table 1.2 Transcription factors that physically interact with HMGA proteins

NF-kB p50 homodimer (Lewis *et al.* 1994)	IRF-1 (Schaefer *et al.* 1997)
NF-kB p65 homodimer (Lewis *et al.* 1994)	NF-Y (Louis *et al.* 1997)
NF-kB p50/p65 heterodimer (Lewis *et al.* 1994)	Oct-1 (Abdulkadir *et al.* 1998)
AP-1 (Ogram *et al.* 1995)	Oct-2A (Abdulkadir *et al.* 1998)
ATF-2/c-jun heterodimer (Falvo *et al.* 1995)	SRF (Chin *et al.* 1998)
PU.1 (Nagulapalli *et al.* 1995)	RNF4 (Fedele *et al.* 2000)
Tst-1/Oct-6 (Leger *et al.* 1995)	PATZ (Fedele *et al.* 2000)
c-jun (Falvo *et al.* 1995)	PIAS3 (Zentner *et al.* 2001)
C/EBPβ (Nagulapalli *et al.* 1995)	p53 (Pierantoni *et al.* 2006)
Elf-1 (John *et al.* 1995)	Rb (Fedele *et al.* 2006)
NF-AT (Klein *et al.* 1996)	

1.6 Role of HMGA proteins in cell proliferation and cancer

HMGA gene expression is maximal during embryonic development (Chiappetta *et al.* 1996) and has long been thought to be involved in the control of cell growth and differentiation (Bustin and Reeves 1996). Considerable support for this comes both from experiments demonstrating that microinjection of HMGA proteins induces premature gene transcription in early mouse embryos (Beaujean *et al.* 2000) and from our findings that HMGA proteins are critical players in controlling the growth and differentiation of pre-

adipocytes 3T3-L1 cells (Melillo *et al.* 2001). The expression of HMGA proteins drops off markedly in most tissues following birth and is low, or undetectable, in fully differentiated or non-dividing adult cells (Lundberg *et al.* 1989; Bustin and Reeves 1996). Nevertheless, *HMGA* genes and their proteins are rapidly induced in quiescent normal cells following exposure to factors that stimulate metabolic activation and growth providing additional evidence that these proteins are involved in the regulation of normal cell proliferation (Friedmann *et al.* 1993; Johnson *et al.* 1990; Ogram and Reeves 1995; Holth *et al.* 1997). The high expression of HMGA proteins during embryogenesis suggests that they have important roles in development.
Also the phenotypic characterization of knockout mice for each of the *HMGA* genes confirms crucial roles for these proteins in different aspects of development. In fact, cardiac hypertrophy and type 2 diabetes were observed in *hmga1*-null and heterozygous mice (Foti *et al.* 2005; Fedele *et al.* 2006) suggesting that quantitatively appropriate expression of the HMGA1 proteins are required for cardiomyocytic cell growth and function of the insulin pathway. Instead, *hmga2*-null and heterozygous mice showed a pygmy phenotype with a decreased body size of 20% in heterozygous and 60% in homozygous mice, as well as a drastic reduction of the fat tissue (Zhou *et al.* 1995), suggesting an important role of the *Hmga2* gene in the control of body growth and adipocyte proliferation and differentiation. Therefore, although HMGA1 and HMGA2 may have overlapping functions, they seem to have different roles in development.

Overexpression of HMGA proteins is quite toxic to both bacterial (Reeves and Nissen 1999) and normal mammalian cells (Fedele *et al.* 2001). Our group demonstrated that forced overexpression of the HMGA1b protein in a line of normal rat thyroid epithelial cells induces apoptosis as a result of deregulating the S phase of the cell cycle and delaying entry of cells into the G2/M phase (Fedele *et al.* 2001). Regardless of the specific mechanisms involved, the biochemistry, physiology and phenotype of the cells that survive such HMGA-induced apoptotic events are radically altered. This is evident in neoplastically transformed cells that have survived apoptosis and become immortalized: the constitutive levels of HMGA proteins are often quite high, with increasing concentrations being correlated with increasing degrees of malignancy and metastatic potential (reviewed in Tallini and Dal Cin 1999). This correlation is so widespread and consistent that it has been suggested that elevated levels of HMGA proteins are diagnostic markers of both neoplastic transformation (Giancotti *et al.* 1989, 1991; Liu *et al.* 2001) and increased metastatic potential of a large number of different types of cancers. These include cancers of breast, prostate, colon, cervix, lung and thyroid (see Table 1.3). In all of these epithelial/endothelial cell derived malignant tumors, the overexpressed proteins are full-length non-mutant forms of the HMGA proteins. Our findings demonstrated that HMGA proteins have a causal role in the neoplastic transformation of normal thyroid epithelial cells since the antisense-mediated inhibition of HMGA protein synthesis suppresses their malignant phenotype

(Berlingieri *et al.* 1995; Berlingieri *et al.* 2002), induces apoptotic death in thyroid carcinoma cells but not in normal thyroid cells (Scala *et al.* 2000), and inhibits proliferation of some breast carcinoma cell lines (Reeves *et al.* 2001). In contrast to carcinomas, benign tumors of mesenchymal origin (lipomas, endometrial polyps, etc.) often contain chromosomal rearrangements that result in the creation of fusion genes coding for chimeric proteins in which the AT-hooks of the HMGA proteins are fused to ectopic peptides sequences (Hess 1998). The roles, if any, played by these AT-hook-containing chimeric fusion proteins in the etiology of benign tumors is still uncertain (Hess 1998; Tallini and Dal Cin 1999).

Table 1.3 Cancers associated with aberrant expression of HMGA proteins

Overexpression of full-length proteins

Lewis lung carcinoma (Giancotti *et al.* 1989)
Prostrate (Tamimi *et al.* 1993)
Thyroid neoplasias (Chiappetta *et al.* 1995)
Colorectal (Fedele et al.1996)
Squamous carcinoma of uterine cervix (Bandiera *et al.* 1998)
Non-small cell lung carcinoma (Rogalla *et al.* 1998)
Neuroblastomas (Giannini *et al.* 1999)
Burkitt's lymphoma (Wood *et al.* 2000)
Lipomas (Fedele *et al.* 2001)
Pancreatic duct cell carcinoma (Abe *et al.* 2002)
Breast (Baldassarre *et al.* 2003)
Lymphoblastic leukemia (Pierantoni *et al.* 2003)
Ovarian carcinoma (Masciullo *et al.* 2003)
Testicular germ cell tumours (Esposito *et al.* 2008)

Chromosomal translocations/AT hook rearrangements

Myeloid leukemias (Elton *et al.* 1986)
Thyroid neoplasias (Chiappetta *et al.* 1995)
Pulmonary chondroid hamartomas (Kazmierczak *et al.* 1996)
Uterine leiomyomas (Hennig *et al.* 1996)
Endometrial polyps (Hennig *et al.* 1996)
Breast hamartoma (Dal Cin *et al.* 1997)
Lipomas (Fedele *et al.* 2001)

Several mechanisms have been proposed to account for the transforming ability of the HMGA proteins. Because of their main function of regulating gene transcription, most of these mechanisms are based on the ability of the HMGA proteins to downregulate or upregulate the expression of genes that have a crucial role in the control of cell proliferation and invasion.

Recently, it has been reported that HMGA2 induces pituitary adenomas in *Hmga2*-transgenic mice by binding to pRB and enhancing E2F1 activity (Fedele *et al.* 2006). In fact, HMGA2 is able to displace histone deacetylase 1 from the pRB-E2F1 complex, resulting in enhanced acetylation of both E2F1 and DNA-associated histones, thereby promoting E2F1 activation. The crucial role of HMGA2-mediated E2F1 activation for pituitary

tumorigenesis was confirmed by crossing *Hmga2*-overexpressing mice with *E2f1* knock-out mice, which suppressed tumorigenesis (Fedele *et al.* 2006). It is reasonable to argue that increased E2F1 activity might also have an important role in other neoplasias, or aberrant cell-proliferation conditions, where *HMGA2* is overexpressed. The pygmy phenotype of the *Hmga2*-null mice (Zhou *et al.* 1995) may result from decreased E2F1 activity that would eventually lead to decreased embryonic cell proliferation.

Another mechanism by which the HMGA proteins might have a role in cancer progression is through their interaction with p53. It has been found that HMGA1 binds p53 protein *in vitro* and *in vivo*, and interferes with the p53-mediated transcription of p53 effectors Bcl2-associated X protein (Bax) and cyclin-dependent kinase inhibitor 1A ($p21^{Waf1/Cip1}$), as well as cooperating with p53 to activate transcription of the p53 inhibitor MDM2. This results in a reduction in p53-dependent apoptosis in cells expressing exogenous HMGA1 and p53 (Frasca *et al.* 2006; Pierantoni *et al.* 2006). Moreover, HMGA1 can also interfere with the apoptotic function of p53 by another mechanism that increases the interaction with the proapoptotic p53 activator homeodomain-interacting protein kinase 2 (HIPK2) (Pierantoni *et al.* 2007). HMGA1 overexpression promotes endogenous HIPK2 relocalization in the cytoplasm and inhibition of p53 apoptotic function, whereas HIPK2 overexpression re-establishes HIPK2 nuclear localization and sensitivity to apoptosis (Pierantoni *et al.* 2007) (Figure 1.4). Consistent with this process, strong correlations among HMGA1 overexpression, HIPK2 cytoplasmic localization and a low spontaneous apoptosis index (comparable to that observed in tumours with mutated p53) were observed in human breast carcinomas expressing wild-type p53 (Pierantoni *et al.* 2007). Therefore, HMGA1 inhibits p53-dependent apoptosis by modulating both transcription of p53 target genes and cytoplasmic relocalization of HIPK2.

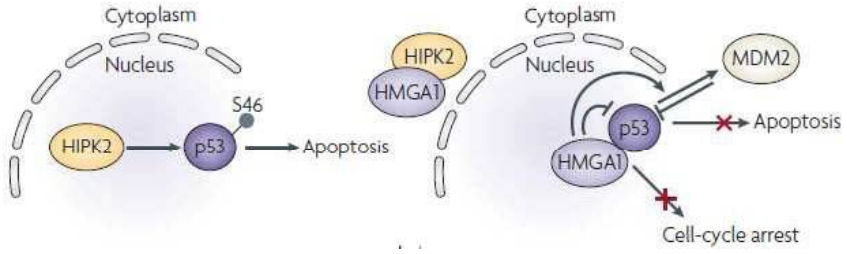


Figure 1.4. Schematic representation of p53 inhibition by high mobility group A (HMGA)1. In response to DNA damage, p53 induces either cell-cycle arrest or apoptosis. p53 phosphorylation at S46 by homeodomain-interacting protein kinase 2 (HIPK2) is one determinant of the outcome because it occurs only after severe, nonrepairable DNA damage that irreversibly drives cells to apoptosis. HMGA1 overexpression inhibits the p53 apoptotic function by two main mechanisms: it promotes HIPK2 relocalization in the cytoplasm, and it directly binds to p53 and interferes with the p53-mediated transcription of apoptotic and cell-cycle arrest effectors, while it cooperates with p53 in the transcriptional activation of the p53 inhibitor MDM2.

1.7 The tumor suppressor p53

Human p53 is a nuclear phosphoprotein of MW 53 kDa, encoded by a 20-Kb gene containing 11 exons and 10 introns (Lamb and Crawford 1986) which is located on the small arm of chromosome 17 (Isobe *et al.* 1986). This gene belongs to a highly conserved gene family containing at least two other members, p63 and p73. Wild-type p53 protein contains 393 amino acids and is composed of several structural and functional domains (Figure1.5): a N-terminus containing an amino-terminal domain (residues 1-42) and a proline-rich region with multiple copies of the PXXP sequence (residues 61-94, where X is any amino acid), a central core domain (residues 102-292), and a C-terminal region (residues 301-393) containing an oligomerization domain (residues 324-355), a strongly basic carboxyl-terminal regulatory domain (residues 363-393), a nuclear localization signal sequence and 3 nuclear export signal sequence (Vousden and Lu 2002).

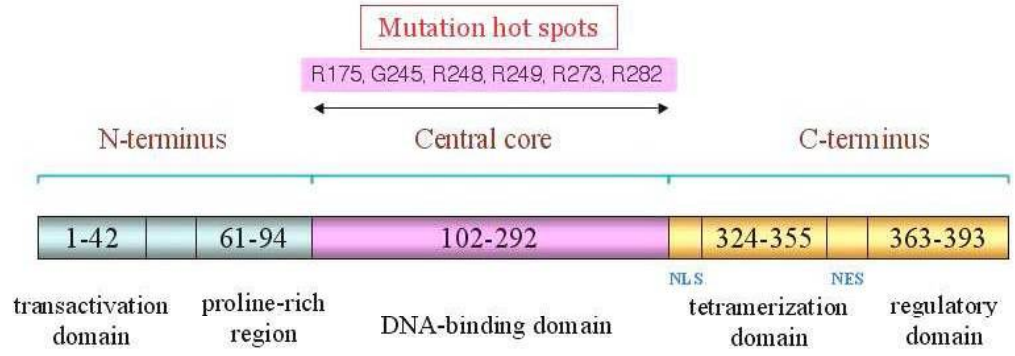


Figure 1.5. Schematic representation of the p53 structure. p53 contains 393 amino acids, consisting of three functional domains, i.e. an Nterminal activation domain, DNA binding domain and C-terminal tetramerization domain. The N-terminal domain includes transactivation subdomain and a PXXP region that is a proline-rich fragment. The central DNA binding domain is required for sequence-specific DNA binding and amino acid residues within this domain are frequently mutated in human cancer cells and tumor tissues. The Arg175, Gly245, Arg248, Arg249, Arg273, and Arg282 are reported to be mutation hot spots in various human cancers. The C-terminal region is considered to perform a regulatory function. Residues on this basic C-terminal domain undergo posttranslational modifications including phosphorylation and acetylation. Numbers indicate residue number. NLS, nuclear localization signal sequence; NES, nuclear export signal sequence.

The amino-terminal domain is required for transactivation activity and interacts with various transcription factors including acetyltransferases and MDM2 (murine double minute 2, which in humans is identified as Hdm2) (Fields and Jang 1990; Lin *et al.* 1994). The proline-rich region plays a role in p53 stability regulated by MDM2, where in p53 becomes more susceptible to degradation by MDM2 if this region is deleted (Sakamuro *et al.* 1997). The central core of this protein is made up, primarily, of the DNA-binding domain required for sequence-specific DNA binding (the consensus sequence contains two copies of the 10-bp motif 5'-PuPuPuC(A/T)-(T/A)GPyPyPy-3', separated by 0-13 bp)

(Kern *et al.* 1991). The basic C-terminus of p53 also functions as a negative regulatory domain (Vousden and Lu 2002), and has been implicated in induction of cell death (Chen *et al.* 1996). According to the allosteric model, in which C-terminal tail of p53 was considered as a negative regulator and may regulate the ability of its core DNA binding domain to lock the DNA binding domain as a latent conformation. If the interaction between the C-terminus and the core DNA binding domain is disrupted by post-translational modification (such as phosphorylation and acetylation), the DNA binding domain will become active, thus induce an enhanced transcriptional activity.

The central region of p53 is its most highly conserved region, not only when p53 is compared with its homologues from Drosophila and Caenorhabditis elegans, but also as compared with its mammalian family members, p63 and p73 (Kaelin 1999). Structural studies of p53 have revealed that the majority of p53 mutations found in cancers are missense mutations that are mostly located in the central DNA-binding domain, and more than 80% of p53 mutation studies have focused on residues between 126–306 (Cho *et al.* 1994). Acquired mutations (more than 18,000 mutations have been identified) in the p53 gene are found in all major types of human cancers. Approximately half of all human tumors have a mutation or loss in the p53 gene leading to inactivation of its function (Soussi and Beroud 2001; Bode and Dong 2004). For example, p53 mutation frequency is 70% in lung cancer, 60% in cancers of colon, head and neck, ovary, and bladder, and 45% in stomach cancer (Figure 1.6). In many of the others, approximately 50% human tumors in which p53 is not functionally inactive, p53 function is impaired owing to mutations in proteins operating either upstream or downstream of p53 targets, such as MDM2 or the E6 protein of HPV, or deletion of key p53 co-activators such as the ARF gene (Hollstein *et al.* 1991; Sherr 1998; Vogelstein *et al.* 2000).

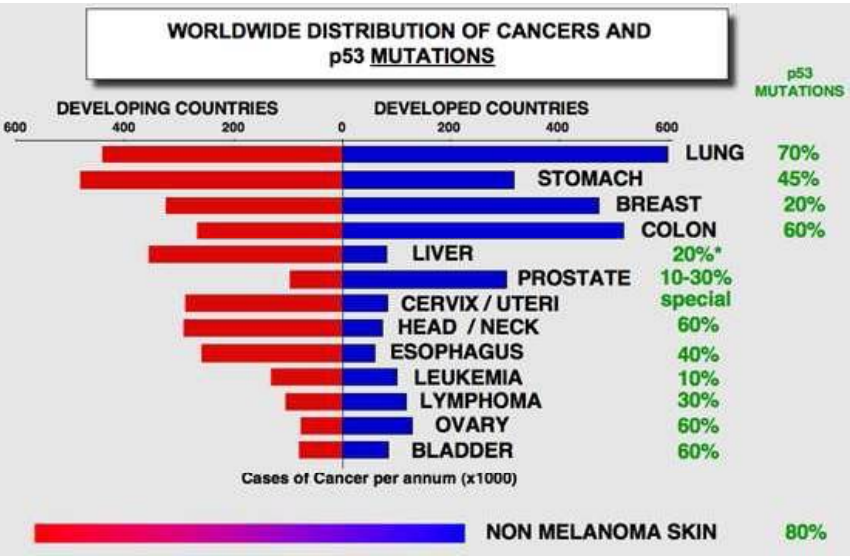


Figure 1.6. p53 mutation frequency. Inactivation of the p53 gene is essentially due to small mutations (missense and nonsense mutations or insertions/deletions of several nucleotides), which lead to either expression of a mutant protein (90% of cases) or absence of protein (10% of cases).

1.8 p53 functions

As a tumor suppressor, p53 is essential for preventing inappropriate cell proliferation and maintaining genome integrity following genotoxic stress (Vogelstein *et al.* 2000; Vousden and Lu 2002). Following various intracellular and extracellular stimuli such as DNA damage (by means including ionizing radiation, UV radiation, application of cytotoxic drugs or chemotherapeutic agents, and infectious virus), heat shock, hypoxia, and oncogene overexpression, wt p53 is activated and emerges as a pivotal regulatory protein which triggers diverse biological responses, both at the level of a single cell as well as in the whole organism (Levine 1997; Vogelstain *et al.* 2000; Vousden and Lu 2002). p53 activation involves an increase in overall p53 protein level and qualitative changes in the protein through extensive post-translational modification, thus resulting in activation of p53-targeted genes (Fritsche *et al.* 1993). For example, in response to DNA damage which leads to double strand breaks in DNA (DSBs), ATM (ataxia-telangiectasia mutated) protein kinase is activated and in turn activates Chk2 kinase (Matsuoka *et al.* 1998). ATM and Chk2 then both phosphorylate p53 at distinct sites leading to p53-dependent cell cycle arrest or apoptosis (Banin *et al.* 1998; Canman *et al.* 1998). In addition, DNA damage can also lead to replication blockage, thus activating the ATR (ATM and Rad3-related) kinase. Consequently, both activated ATR and subsequently activated Chk1 phosphorylate and activate p53 (Tibbetts *et al.* 1999; Shieh *et al.* 2000). Moreover, induction of the transcription dependent cell death program requires p53 phosphorylation at Ser 46, which is necessary for the development of apoptosis (Oda *et al.* 2000) and determines whether apoptosis is attenuated or amplified. Phosphorylation of p53 at Ser 46 critically depends on the serine/threonine kinase HIPK2 (D'Orazi *et al*, 2002) which enhances the transcriptional activity of p53 to promote apoptosis (D'Orazi *et al.* 2002; Hofmann *et al.* 2002). Genes activated by wt p53 are functionally diverse and constitute downstream effectors of signaling pathways that elicit diverse responses such as cell-cycle checkpoints, cell survival, apoptosis, and senescence (Hofseth *et al.* 2004). Many of the multiple functions of p53 including the primary role of p53 in tumor suppression, can be attributed to its ability to act as a sequence-specific transcription factor which regulates expression of different cellular genes to modulate various cellular processes (Farmer *et al.* 1992), although protein-protein interactions may also play a role. In response to various types of stress, p53 is accumulated in the nucleus, binds to specific sites in the regulatory regions of p53-responsive genes, and then strongly promotes the transcription of such genes (Kern *et al.* 1991). The p53 downstream targets are differentially activated depending on the cell type, extent of the damage which has influenced p53 activation, and various others as yet unidentified parameters (Oren 2003). Many approaches have been employed to identify the targets of p53 in various experimental systems (Yang *et al.* 2004). As a result of these efforts, hundreds of physiologically p53 responsive genes have been reported. These genes are principally involved in

cell cycle arrest and DNA repair, as well as apoptosis and senescence-related genes, such as genes for $p21^{Waf1/Cip1}$, *Gadd45* (growth arrest and DNA-damageinducible protein 45) and genes of the *Bcl-2* family (Vousden and Lu 2002; Fridman and Lowe 2003). Genes which may be repressed by p53 include *Bcl-2*, *Bcl-x*, *Cyclin B1*, *MAP4* and *Survivin* some of which are negative regulators of apoptosis (Vousden and Lu 2002; Hofman *et al.* 2002). Intriguingly, using ovarian cancer cells infected with p53-expressing adenovirus indicated that approximately 80% of the putative p53-responsive genes are, in fact, repressed by p53 (Mirza *et al.* 2003). The functions of p53 target genes are diverse, corresponding to p53's activity as a multifunctional protein.

1.9 p53 and apoptosis

As a cellular gatekeeper (Levine 1997; Kinzler and Vogelstein 1997), one of the roles of p53 is to monitor cellular stress and to induce apoptosis as necessary (Hofseth *et al.* 2004). In tissues where stressors generate severe and irrevocable damage, p53 can initiate apoptosis, thereby eliminating damaged cells. Apoptotic gene products which are induced by p53 include Bax (Bcl2-associated X protein) (Miyashita and Reed 1995; Thornborrow *et al.* 2002), DR5/KILLER (death receptor 5) (Takimoto and El-Deiry 2000), DRAL (Scholl *et al.* 2000), Fas/CD95 (cell-death signaling receptor) (O'Connor *et al.* 2000), PIG3 (p53-inducible gene 3) (Flatt *et al.* 2000), Puma (p53-upregulated modulator of apoptosis) (Nakano and Vousden, 2001; Yu *et al.* 2001), Noxa (from the Latin word for "harm" or "damage") (Oda *et al.* 2000), PIDD (p53-induced protein with death domain) (Lin *et al.* 2000), PERP (p53 apoptosis effector related to PMP-22) (Attardi *et al.* 2000), Apaf-1 (apoptotic protease-activating factor 1) (Cecconi *et al.* 1998), Scotin (Bourdon *et al.* 2002), p53AIP1 (p53-regulated apoptosis-inducing protein 1) (Matsuda *et al.* 2002), and others. The p53 associated apoptotic targets can be divided into several groups based on their functions and their executed pathways (Figure 1.7).

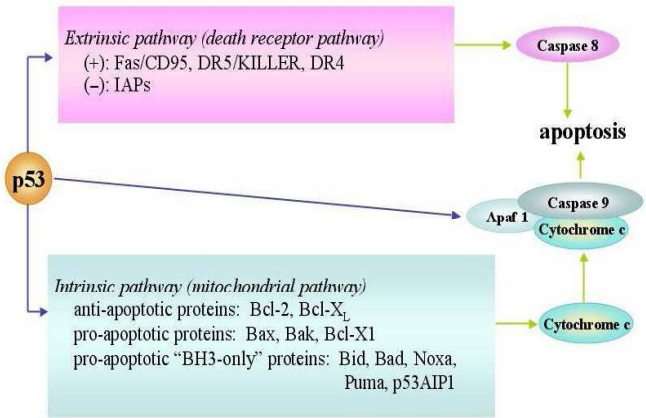


Figure1.7. p53-associated genes and pathways involved in apoptotic cell death.
p53 induces apoptosis mainly via two pathways: extrinsic and intrinsic pathways. The p53-associated extrinsic pathway is mainly executed by activating caspase 8 to induce apoptosis, whereas the p53-associated intrinsic pathway is almost executed by influencing mitochondrial proteins, by which activate caspase 9 to induce apoptosis. In addition, p53 may directly activate Apaf-1 to induce apoptosis.

The products of these genes may induce apoptosis through either an extrinsic or an intrinsic pathway, namely the death receptor pathway and the mitochondrial pathway, respectively.

The intrinsic apoptotic pathway is engaged when cells are challenged by stress and is dominated by the Bcl-2 family proteins (Cory and Adams 2002; Korsmeyer 1999). The Bcl-2 family proteins are composed of three classes: antiapoptotic proteins Bcl-2 and Bcl-x_L, pro-apoptotic proteins Bax, Bak and Bcl-x_s , and pro-apoptotic "BH3-only" proteins Bid (BH3-interacting death agonist), Bad, Noxa, and Puma (Haupt *et al.* 2003). In the regulation of the intrinsic pathway, pro-apoptotic gene products such as Bax, Bid, Puma, Noxa, and p53AIP1 localize to the mitochondria and promote the loss of mitochondrial membrane potential and release of cytochrome c, resulting in the formation of the apoptosome complex with Apaf-1 and caspase 9 (Nakano and Vousden, 2001; Yu *et al.* 2001; Oda *et al.* 2000; Matsuda *et al.* 2002). These apoptosis-related gene products mentioned above are closely associated with p53 function. Bax was the first identified p53-regulated pro-apoptotic Bcl-2 family member (Miyashita and Reed, 1995), and p53-responsive elements have been unequivocally identified in the *Bax* gene (Thornborrow *et al.* 2002). Several Bcl-2 family proteins and mitochondrial proteins such as Puma, Noxa, p53AIP1, and PIGs are implicated in p53-dependent apoptosis. They are activated in a p53-dependent manner following DNA damage. Puma induces very rapid apoptosis, which occurs within hours following its expression (Nakano and Vousden, 2001; Yu *et al.* 2001; Oda *et al.* 2000). p53AIP1 can cause mitochondrial membrane potential dissipation by interacting with Bcl-2 (Matsuda *et al.* 2002). p53 also regulates the genes encoding Apaf-1, a key component of the apoptosome (Cecconi *et al.* 1998), and PIG3, which may cause mitochondrial depolarization (Flatt *et al.* 2000). Nevertheless, activated p53 can directly or indirectly modulate the expression of its targeted proteins and other proteins that control mitochondrial membrane permeability, and can therefore modulate the release of mitochondrial proteins which further carry out apoptosis.

Another p53-related class of pro-apoptotic gene products is the components of the death receptor-mediated extrinsic pathway. In this cell death pathway, p53 can promote apoptosis through activation of the death receptors located at the plasma membrane, including Fas/CD95 (O'Connor *et al.* 2000), DR4 (Liu *et al.* 2004) and DR5 (Takimoto and El-Deiry 2000), and lead to inhibition of the production of IAPs (inhibitor of apoptosis proteins) (Takimoto and El-Deiry 2000). Both DR5 and DR4 can trigger or induce apoptosis by TRAIL (tumor necrosis factorrelated apoptosis-inducing ligand), Fas ligand and chemotherapeutic agents (Sheikh *et al.* 1998; Liu *et al.* 2004), with Fas being indispensable for p53-dependent apoptosis in most tissues (O'Connor *et al.* 2000). It has been suggested that the intrinsic apoptotic pathway is primarily utilized in p53-mediated apoptosis, whereas the extrinsic pathway is used to augment the apoptotic response (Fridman and Lowe 2003).

The ability of p53 to induce apoptosis appears to be well correlated with its ability to suppress malignant transformation. Loss of p53-dependent apoptosis accelerates mouse brain tumorigenesis (Symonds *et al.* 1994). Similarly, the observation that mice harboring the p53 R172P mutant develop many tumors may be due to lack of p53-induced apoptosis (Liu *et al.* 2004). These results reveal that regulation of apoptosis is an important and evolutionarily conserved tumor suppressor function of p53.

2. AIMS OF THE STUDY

The aim of the present study is to investigate the molecular mechanisms by which the *HMGA* genes contribute to neoplastic transformation by identifying genes directly or indirectly regulated by HMGA proteins and their physical interactors. On the basis of the our previous data, described in the background section, I consider of great interest to study the possible role of the HMGA1-p53 complex in the transcriptional regulation of other genes. Looking for new genes under the transcriptional control of the HMGA1 proteins, among p53 target genes, I focused my attention on B-cell lymphoma gene 2 (*Bcl-2*). The Bcl-2 protein exerts an antiapototic function. In fact, it inhibits the release into the cytosol of Cytochrome C which, in turn, activates caspase-9 and caspase-3, leading to apoptosis. The *Bcl-2* gene has been implicated in a number of cancers, including melanoma, breast, prostate, and lung carcinomas, as well as schizophrenia and autoimmunity. It is also thought to be involved in resistance to conventional cancer treatment. In *Bcl-2* promoter regions some AT-rich DNA sequences, which are the preferred binding sites for HMGA1, are present. Based on this evidence, I could assume that *Bcl-2* appears a good candidate as target for the HMGA1 proteins.

3. MATERIALS AND METHODS

3.1 Cell culture, transfections, and transactivation assays

ND7, HEK293, MCF7, MEFs and SAOS-2 cells were maintained in DMEM supplemented with 10% fetal calf serum (GIBCO; Invitrogen), glutamine, and antibiotics. FRTL5, FRTL5 KiMSV, FRTL5 αs HMGA1 KiMSV cells were maintained in F12 supplemented with 5% calf serum (GIBCO; Invitrogen), glutamine, and antibiotics. Cells were transfected with plasmids by lipofectamine-plus reagent (Invitrogen) as suggested by the manufacturer. Cells were transiently transfected with previously described reporter vectors (Budhram-Mahadeo *et al*. 1999; D'Orazi *et al*. 2002; Pierantoni *et al*. 2006) and normalized with the use of a cotransfected β-gal construct. Luciferase activity was analyzed by Dual-Light System (Applied Biosystems). For inhibition of HMGA1 expression, siRNAs and corresponding scramble siRNAs (Santa Cruz Biotechnology Inc.) were used as suggested by the manufacturer.

3.2 Expression constructs

The pCAG-p53, pCMV-Hmga1, pCEFL-HA-HMGA1, pFLAG-HIPK2, pEGFP-HIPK2, and pLTR-Brn-3a constructs have been described previously (Budhram-Mahadeo *et al*. 1999; D'Orazi *et al*. 2002; Pierantoni *et al*. 2006).

3.3 Western blotting and coimmunoprecipitation

Total cell extracts (TCEs) were prepared with lysis buffer (50 mM Tris HCl, pH 7.5; 5 mM EDTA; 300 mMNaCl; 150 mM KCl; 1 mM dithiothreitol; 1% Nonidet P40; and a mix of protease inhibitors) or in non denaturing buffer (50 mM Tris-HCl, pH 7.5;150 mM NaCl; 0.5% Triton; and 5 mM EDTA) for coimmunoprecipitation. Immunoprecipitation was carried out by incubating 1 mg of TCEs with Abs preadsorbed to protein G-agarose (Pierce Biotechnology). Immunocomplexes were collected by centrifugation, separated by SDS-PAGE, and blotted onto nitrocellulose membrane (Bio-Rad). Differential nuclear and cytoplasmic cell lysates were obtained as reported previously (Schreiber *et al*., 1989). Protein extracts were separated by SDS-PAGE and transferred to Immobilon-P Transfer membranes (Millipore). Membranes were blocked with 5% nonfat milk proteins and incubated with Abs at the appropriate dilutions. Filters were incubated with horseradish peroxidase-conjugated secondary Abs, and signals were detected with ECL (Amersham). The Abs used for Western blotting were as follows: anti-FLAG

M5 mAb (Sigma-Aldrich); anti-HA 12CA5 mAb (Roche); anti-p53 DO1 mAb, anti-Brn-3a 14A6 mAb, anti-Sp1 H225 policlonal Ab and anti-γ-tubulin C11 mAb (Santa Cruz Biotechnology Inc.); anti-Bcl-2 50E3 mAb (Cell Signaling); anti-HMGA1 polyclonal Ab raised against a synthetic peptide located in the NH2-terminal region (Pierantoni *et al*. 2006); and anti-HIPK2 M01 mAb (Abnova).

3.4 *In vitro* protein translation

pET2c-HMGA1b construct has been previously described (Pierantoni *et al*. 2006). His-HMGA1b recombinant protein was 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 mg/ml), grown at 30°C to an OD_{600} of 0.6 and induced with 0.1 mM IPTG. After an additional 2 h at 30°C, 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. The supernatant was then incubated at 41°C for 1 h with 250 ml of glutathione-Sepharose beads (Amersham Pharmacia Biotech). Resin was washed with PBS and protease inhibitors. Recombinant protein was eluted with a buffer containing PBS, 10 mM reduced glutathione, and 10% (v/v) glycerol and the supernatant was purified by using nickelagarose beads supplied with the His-Trap purification kit (Amersham Pharmacia) following the manufacturer's instructions, eluted with 500 mM imidazol and dialysed in PBS.

3.5 Electrophoretic mobility shift assay (EMSA)

DNA binding assays with the recombinant proteins were performed as previously described (Thanos and Maniatis 1995) Briefly, 5 ng and 10 ng of His-HMGA1b recombinant protein were incubated with radio-labelled double-strand oligonucleotide, corresponding to region spanning bases -526 to -477 of the human *Bcl-2* promoter region (Budhram-Mahadeo *et al*. 1999). 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 mg poly (dC-dG), 2 mg BSA and 10% glycerol, to a final volume of 20 ml, for 10 min at room temperature. The samples were incubated for 15 min after addition of 2.5 fmol of a ^{32}P-end-labelled oligonucleotide (specific activity, 8000-20000 cpm/fmol). A 200-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.

3.6 Cell viability and TUNEL assays

Both floating and adherent cells were collected and counted in a hemocytometer after the addition of trypan blue. The percentage of dead cells (e.g., number of blue cells per total number of cells) was determined by scoring 100 cells per chamber 3 times. Cell numbers were determined in duplicate. For TUNEL assay on in vitro cell lines, 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 mg/ml Hoechst 33258 for 2 minutes, and mounted with coverslip in 25% glycerol in PBS. At least 400 cells were counted in each plate.

3.7 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 on 0.5 μg of RNA using random exanucleotides as primers (100 mM) and MuLV reverse trancriptase (Applied Biosystems) according to standard procedures. cDNA was amplified by PCR using the following primers: Mu-Bcl-2-up 5'-ggTCATgTgTgTggAgCg-3'; Mu-Bcl-2-dw 5'-gCAgAgTCTTCAgAgACAgC-3'; the assay was normalized by amplifying *GAPDH* (Clontech, Palo Alto, CA).

3.8 Indirect immunofluorescence

Cells plated in 35-mm dishes were fixed in 2% formaldehyde in PBS and permeabilized in a solution of 0.25% Triton X-100 in PBS. Immunofluorescence was obtained with anti-HA 12CA5 mAb (Roche) and tetramethylrhodamine isothiocyanate–conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.). Cells were stained simultaneously for DNA with Hoechst 33342 before observation with a fluorescent microscope (Zeiss).

3.9 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 antibody anti-53 (Ab-7; Calbiochem), anti-Brn-3a (14A6 Santa Cruz), anti-HIPK2 (M01

Abnova), and anti-HMGA1 (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 first eluted by incubation for 30 min at 37ºC in 250 ml 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 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 subjected again to the ChIP procedure. Crosslinking was reversed by incubating samples overnight at 65ºC with 20 ml of 5 M NaCl. Samples were then incubated in proteinase K solution (10 mM EDTA, 40 mM Tris- HCl, pH 6.5, 40 mg/ml of Proteinase K) for 1 hr at 45ºC. 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. Immunoprecipitated chromatin was amplified by PCR using the following primers: Hu-Bcl-2-pr-up 5'-gAAgCAgAAgTCTggg AATC-3'; Hu-Bcl-2-pr-dw 5'-gCgTTTCCCTgTA CACACTg-3'; Hu-GAPDH -pr-up 5'-gTATTCCCCCAggTTTACATg-3'; Hu-GAPDH-pr-dw 5'-TTC TCCATggTggTgAAgAC-3'; Mu-Bcl-2-pr-up 5'-ACACACAgAgCgACTgT gTA-3'; Mu-Bcl-2-pr-dw 5'-gAggCACCTggATCTT TTCT-3'; Mu-GAPDH-pr-up 5'-TACTCgCggCTTTACggg-3'; GAPDH-pr-dw 5'-TggAACAgggAg gAgCAg-3'.

4. RESULTS AND DISCUSSION

4.1 Correlation between HMGA1 and Bcl-2 protein expression

Analyzing the possible correlation between HMGA1 proteins and other p53 target genes involved in apoptosis, we found a strong positive correlation between HMGA1 and Bcl-2 protein expression.

To this aim, several clones of MCF-7 breast cancer cell line stably transfected with a construct expressing HMGA1b, tagged with the Hemagglutinin protein (HA), were tested for apoptotic gene expression. Surprisingly, all the MCF7 clones showed higher Bcl-2 protein levels than the untransfected cells (Figure 4.1, panel A). This correlation was found in other cell lines examined. For example, in mouse embryonic fibroblast knock-out for the *hmga1* gene is evident the reduction of Bcl-2 expression, already present in the single knock out, compared to the wild type (Figure 4.1, panel B). Moreover, a rat thyroid cell line (FRTL-5), transformed with the Kirsten murine multiproliferative virus, expressing high levels of HMGA1 proteins, lost Bcl-2 expression when stably transfected with an antisense construct for HMGA1 (Figure 4.1, panel C). In addition, neuronal cell line (ND7) transiently interfered for the expression of HMGA1 proteins showed a reduction in Bcl-2 protein level compared with the scramble-transfected cells (Figure 4.1, panel D).

Recently, we have characterized a new physical and functional interaction between HMGA1 and p53. This interaction modulates the transcription of p53 target genes, such as *mdm2*, $p21^{waf1}$, *Bax*, and inhibits p53-mediated apoptosis.

Considering these data, we decided to verify whether *Bcl-2*, another p53 target gene, could be regulated by HMGA1-p53 interaction. To evaluate the effect of HMGA1 expression on p53-mediated transcription of Bcl-2, ND7 cells, expressing endogenous HMGA1 and p53, were co-transfected with expression vectors encoding p53 and/or HMGA1b. As expected from literature, p53 reduces Bcl-2 transcription about 50% (Vousden and Lu 2002; Hofman *et al.* 2002). Surprisingly, Western blot and RT-PCR analysis demonstrated that HMGA1 overexpression was able to revert p53 effect on *Bcl-2* transcription (Figure 4.2, panel A and B).

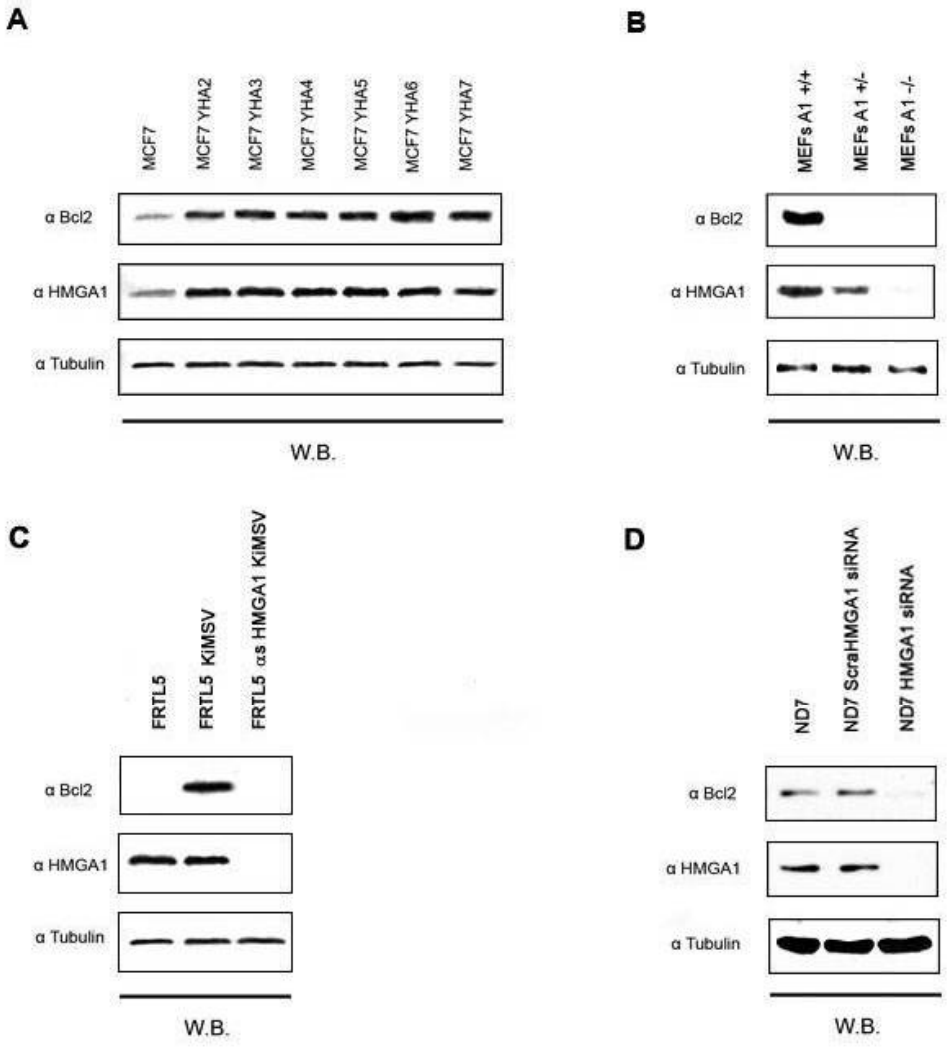


Figure 4.1. HMGA1correlates with Bcl2 protein expression. Immunoblot analysis of Bcl-2 and HMGA1 expression in MCF7 stable overexpressing HMGA1 proteins **(A)**, MEFs knock-out for the *hmga1* gene **(B)**, FRTL5 KiMSV stably transfected with an antisense construct for HMGA1 **(C)** and in ND7 cells transiently interfered for the expression of HMGA1 by siRNAs **(D)**. γ-tubulin was used to equalize protein loading.

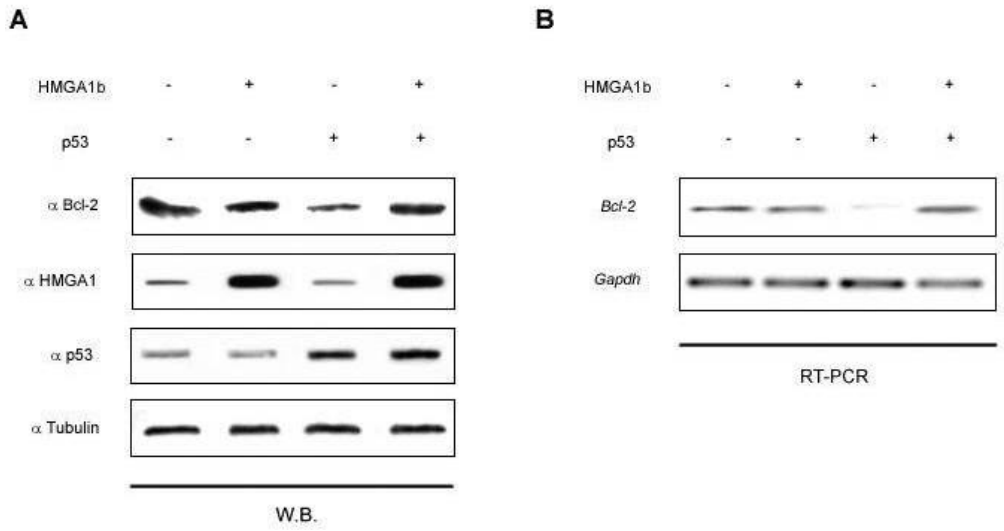


Figure 4.2. HMGA1 reverts p53 effect on *Bcl-2* transcription. (A) Immunoblot analysis of *Bcl-2* expression in ND7 cells co-transfected with expression vectors encoding p53 and/or HMGA1. γ-tubulin was used to equalize protein loading. **(B)** RNA from ND7 cells transiently transfected as described in A was analyzed by RT-PCR for *Bcl-2* expression. All cDNAs were co-amplified with GAPDH as an internal control.

4.2 HMGA1 proteins bind to *Bcl-2* promoter *in vitro* and *in vivo*

HMGA1 proteins are DNA-binding factors that interact with the minor groove of many promoters and enhancers. To evaluate if the correlation between HMGA1 and Bcl-2 protein expression was a direct effect of HMGA1 binding to *Bcl-2* gene regulatory regions, we performed electrophoretic mobility shift assays (EMSA) and Chromatin Immunoprecipitations (ChIP). First, by EMSA, we analyzed a region spanning nucleotides from -526 to -477 related to transcription start site (TSS) of the human *Bcl-2* gene (Budhram-Mahadeo *et al.* 1999) which contains AT-rich putative HMGA1 binding sites and two p53 consensus sequences. As shown in Figure 4.3, panel A, His-HMGA1b recombinant protein was able to bind the ^{32}P-end-labelled double-strand *Bcl-2* promoter oligonucleotide. Binding specificity was demonstrated by competition experiments showing loss of binding with the addition of a 200-fold molar excess of unlabelled *Bcl-2* promoter oligonucleotide. Next, we evaluated whether HMGA1 proteins bind the human *Bcl-2* promoter *in vivo* by performing ChIP assays. HEK293 cells were transfected with an expression vector encoding HMGA1b, crosslinked, and immunoprecipitated with anti-HMGA1 or anti-IgG antibodies as control. Immunoprecipitation of chromatin was then analysed by semiquantitative PCR, using primers spanning the -676/-320 region of the *Bcl-2* promoter. Occupancy of human *Bcl-2* promoter region by HMGA1 was detected in anti-HMGA1-precipitated chromatin from HEK293 cells.

Since HMGA1 proteins are lowly expressed in HEK293, a weak signal was detected in untransfected cells whereas, a stronger signal was detected in HEK293 transfected with HMGA1 (Figure 4.3, panel B). To verify whether HMGA1 proteins bind also the murine *Bcl-2* promoter *in vivo*, we performed chromatin immunoprecipitation experiments in a murine cell line. Anti-HMGA1 antibodies precipitated murine *Bcl-2* promoter from untransfected or transfected ND7 being endogenous HMGA1 protein expressed in these cells (Figure 3, panel C). No amplification was observed in samples immunoprecipitated with an unrelated antibody. The control promoter *GAPDH* was used to indicate that the binding is specific for the *Bcl2* promoter (Figure 4.3 B and C, lower panels).

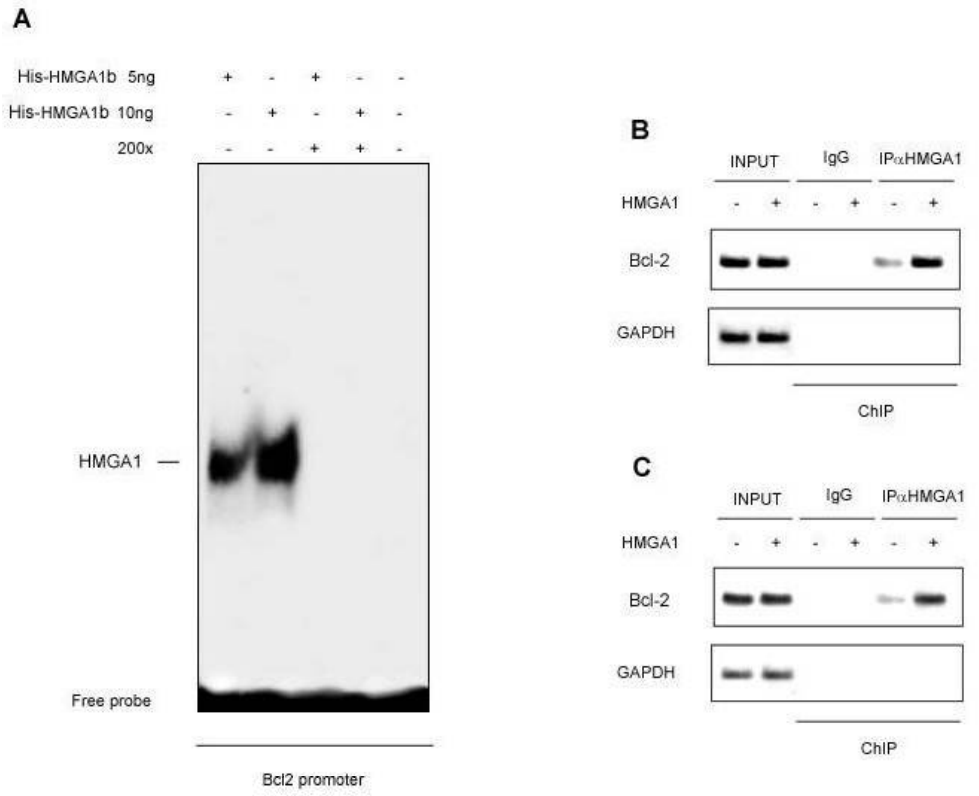


Figure 4.3. HMGA1 proteins bind to *Bcl-2* promoter *in vitro* and *in vivo*. (A) EMSA performed with a radiolabelled oligonucleotide spanning from -526 to -477 of the human *Bcl-2* promoter incubated with 5 ng and 10 ng of recombinant HMGA1b protein. To assess the specificity of the binding, His-HMGA1b protein was incubated in the presence of a 200-fold excess of unlabelled oligonucleotide used as competitor. **(B)** Soluble chromatin from HEK293 cells transfected with HMGA1b was immunoprecipitated with anti-HMGA1. The DNAs were then amplified by semiquantitative PCR using primers that cover a region of human *Bcl-2* promoter (-676/-320), 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. **(C)** Chromatin immunoprecipitation performed in a murine cell line (ND7), as described in B.

4.3 HMGA1 modulates p53-mediated transcriptional activity on *Bcl-2* promoter

p53 is a sequence-specific transcription factor that regulates the expression of several genes involved in cell cycle arrest or apoptosis in response to genotoxic damage or cell stress. To evaluate the effect of HMGA1 expression on *Bcl-2* transcription mediated by p53, ND7 cells were co-transfected with expression vectors encoding HMGA1b and/or p53, and with a reporter vector carrying the luciferase gene under the control of *Bcl-2* promoter activity. As shown in Figure 4.4, HMGA1 overexpression alone had no effects on *Bcl2* promoter, while *Bcl-2* expression was drastically reduced in cells overexpressing p53 alone compared with control cells. Surprisingly, co-overexpression of HMGA1 and p53 resulted in a significant reduction of p53 activity on *Bcl-2* promoter compared with p53 only overexpressing cells. Moreover, when HMGA1 proteins expression was transiently blocked by siRNA, Bcl2 expression level was sensibly reduced compared with untransfected cells. These results indicate that HMGA1 was able to abolish p53-mediated transcription of *Bcl-2* gene.

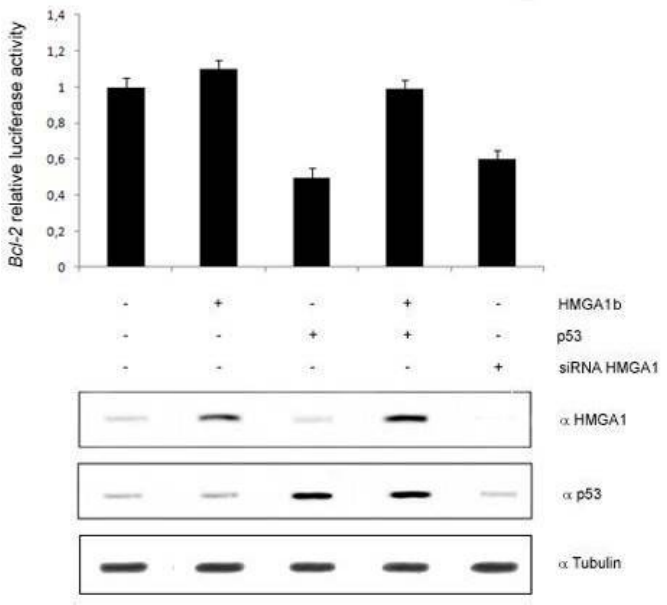


Figure 4.4. HMGA1 proteins revert p53-mediated transcription of *Bcl-2* gene. Analysis of HMGA1 overexpression on p53 activity on the *Bcl-2* luciferase-reporter vector transiently transfected in ND7 cells. All transfections were performed in duplicate and the data are means ± S.D. of five independent 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 and p53 protein is detectable in the insert-less vector transfected ND7 cells.

4.4 HMGA1 represses p53 apoptotic function exerted by *Bcl-2* down-regulation

To evaluate the biological effects exerted by HMGA1 on the abolished p53-mediated transcription of *Bcl-2* gene, we transfected SAOS-2 cells, which express HMGA1 but are null for p53 expression, with a vector encoding p53 or the empty control vector, with or without overexpressed HMGA1. The percentages of cell death were measured by Trypan blue exclusion test. As expected, exogenous p53 expression induced cell death in these cells (Figure 4.5, panel A). However, when HMGA1 was concomitantly overexpressed with p53, the percentage of cell death was strongly reduced (Figure 4.5, panel A), suggesting that HMGA1 might interfere with p53 apoptotic function.

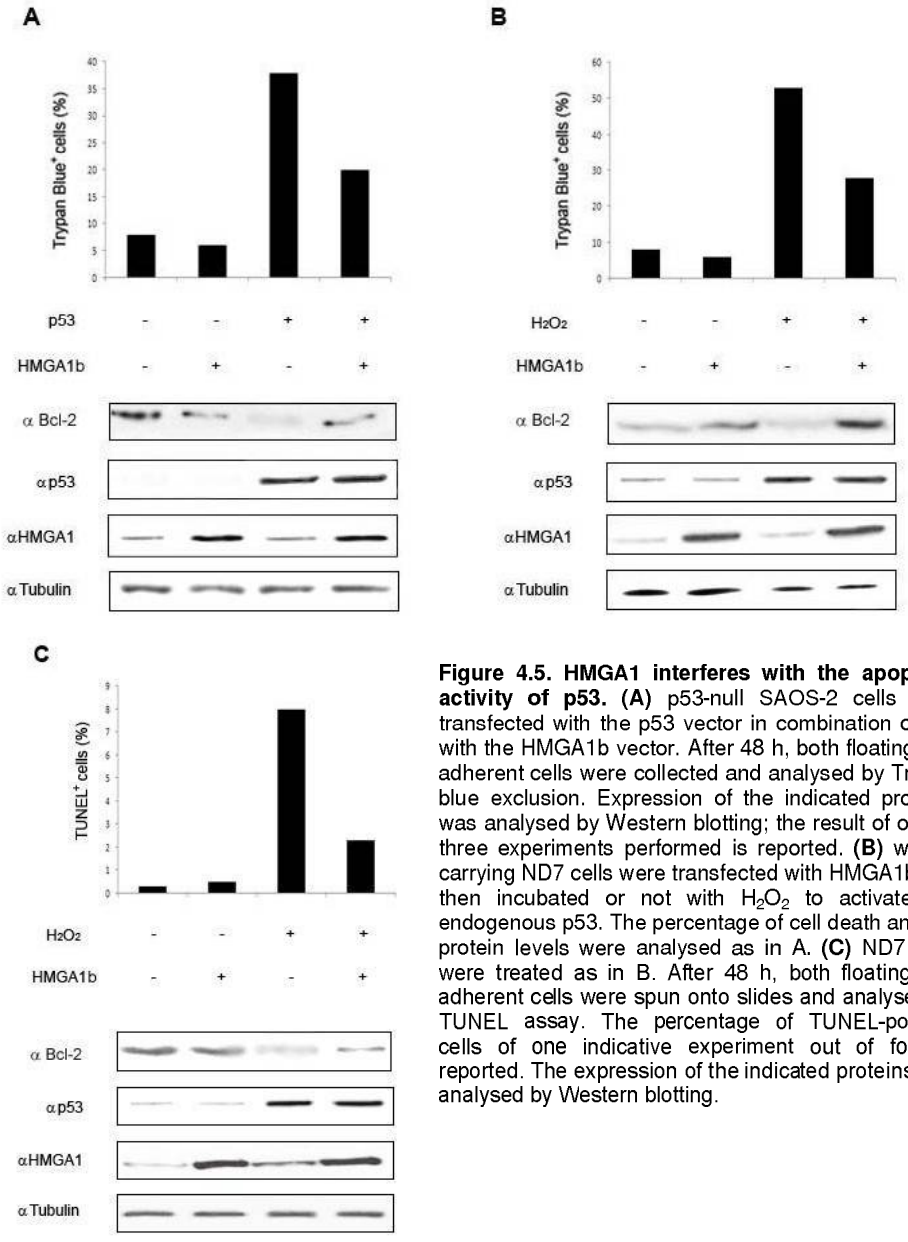


Figure 4.5. HMGA1 interferes with the apoptotic activity of p53. (A) p53-null SAOS-2 cells were transfected with the p53 vector in combination or not with the HMGA1b vector. After 48 h, both floating and adherent cells were collected and analysed by Trypan blue exclusion. Expression of the indicated proteins was analysed by Western blotting; the result of one of three experiments performed is reported. **(B)** wtp53-carrying ND7 cells were transfected with HMGA1b and then incubated or not with H_2O_2 to activate the endogenous p53. The percentage of cell death and the protein levels were analysed as in A. **(C)** ND7 cells were treated as in B. 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.

To verify the antiapoptotic activity of HMGA1 in a more physiological context, we incubated the wtp53-carrying ND7 cells, which express HMGA1 and p53, with H_2O_2 to activate the endogenous p53 in the presence or absence of overexpressed HMGA1 proteins. As shown in Figure 4.5, panel B, HMGA1 overexpression strongly repressed the number of Trypan blue positive cells. Similar to the result obtained by Trypan blue assay, HMGA1 overexpression significantly reduced the amount of TUNEL positive cells induced by H_2O_2 (Figure 4.5, panel C).

Taken together, these data indicate that HMGA1 can functionally interfere with the apoptotic function of the p53 oncosuppressor exerted by *Bcl-2* down regulation.

4.5 HMGA1 proteins bind to Brn-3a transcription factor

Another aim of our study was to investigate the possible role of Brn-3a expression on HMGA1-p53 regulation of the *Bcl-2* promoter. Brn-3a is a POU domain transcription factor which controls sensory neuron survival by regulating the expression of Trk receptors and the members of the Bcl-2 family (Wiggins *et al.* 2004). It is also a potent transcriptional regulator of the *Bcl-2* promoter, able to bind *in vitro* and *in vivo* both p53 and the serine/threonine nuclear kinase HIPK2 (Wiggins *et al.* 2004). These data strongly suggest the existence of a multiprotein complex that regulates the transcription levels of *Bcl-2* because our previous studies demonstrated that also HMGA1 is able to constitute a complex with p53 and HIPK2 that regulates p53 apoptotic function (Pierantoni *et al.*2007). Therefore, we determined whether HMGA1 binds Brn-3a through immunoprecipitation assays. To verify the interaction between HMGA1 and Brn-3a *in vivo*, total cell extracts (TCE) from ND7 cells transiently transfected with pHA/A1b and with pLTR/Brn-3a were immunoprecipitated with anti-Brn-3a monoclonal Ab and analysed by Western blotting with anti-HA monoclonal Ab. Larger amount of HMGA1 protein was present in the immunocomplexes from cells transfected with the two expression plasmids (Figure 4.6, panel A). The reciprocal experiment performed immunoprecipitating with anti-HA Ab and revealing with anti-Brn-3a Ab confirmed the interaction between the two proteins (Figure 4.6, panel B). Western blot analysis showed that the transfected cells expressed adequate levels of the Brn-3a and HA-HMGA1 proteins (Figure 4.6, panel C).

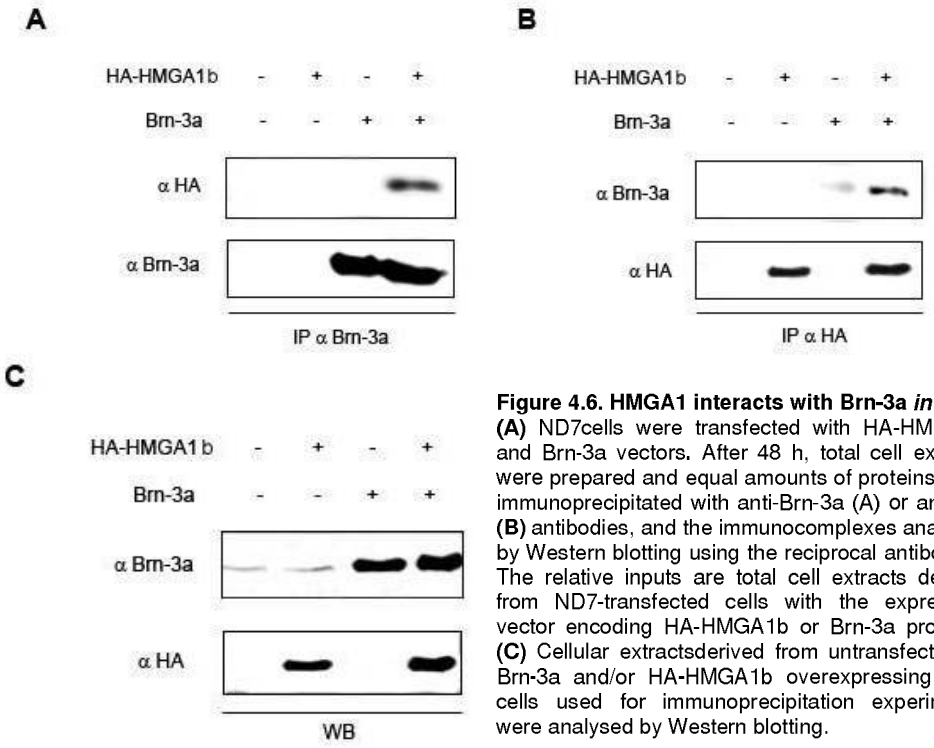


Figure 4.6. HMGA1 interacts with Brn-3a *in vivo.* **(A)** ND7cells were transfected with HA-HMGA1b and Brn-3a vectors. After 48 h, total cell extracts were prepared and equal amounts of proteins were immunoprecipitated with anti-Brn-3a (A) or anti-HA **(B)** antibodies, and the immunocomplexes analysed by Western blotting using the reciprocal antibodies. The relative inputs are total cell extracts derived from ND7-transfected cells with the expression vector encoding HA-HMGA1b or Brn-3a proteins. **(C)** Cellular extractsderived from untransfected or Brn-3a and/or HA-HMGA1b overexpressing ND7 cells used for immunoprecipitation experiments were analysed by Western blotting.

4.6 HMGA1-p53-Brn-3a-HIPK2: fine regulators of *Bcl-2* promoter

The Brn-3a POU family transcription factor has been shown to strongly activate the expression of *Bcl-2* and protect cells from programmed cell death (apoptosis). However, when p53 is overexpressed, Brn-3a works as a corepressor sustaining p53 repression action on *Bcl-2* promoter (Budhram-Mahadeo *et al.* 1999). Moreover, HIPK2 interacts with Brn-3a, promoting its binding to DNA, and suppressing Brn3a-dependent transcription of some Bcl-2 family members like Prox3, TrkA, Bcl-x_L (Wiggins *et al.* 2004). In addition, our previous results indicate that HIPK2 reverts the inhibitory activity of HMGA1 on the p53 effector promoters and points to a complex interplay among p53, HIPK2, and HMGA1 in the regulation of p53-target genes expression (Pierantoni *et al.* 2007).

To better define the role of the HMGA1-p53-Brn-3a-HIPK2 multicomplex in the regulation of *Bcl-2* transcription, we examined its effect on a vector carrying the luciferase gene under the control of *Bcl-2* promoter, in the SAOS-2 cells transiently transfected with different combination of plasmids expressing the complex proteins. As expected from literature, p53 represses *Bcl-2* transcription and this effect is increased by Brn-3a (Budhram-Mahadeo *et al.* 1999). When HMGA1 was co-transfected with p53 and Brn-3a, the repression on *Bcl-2* promoter was antagonized, in particular the repression

activity exerted by Brn-3a was abolished (Figure 4.7). To test whether HIPK2 contributes to this transcriptional regulation, we co-expressed HIPK2 with p53, HMGA1 and Brn-3a. HIPK2 was able to restore the transcriptional repression of Bcl-2 performed by p53 and Brn-3a counteracting HMGA1 activity (Figure 4.7).

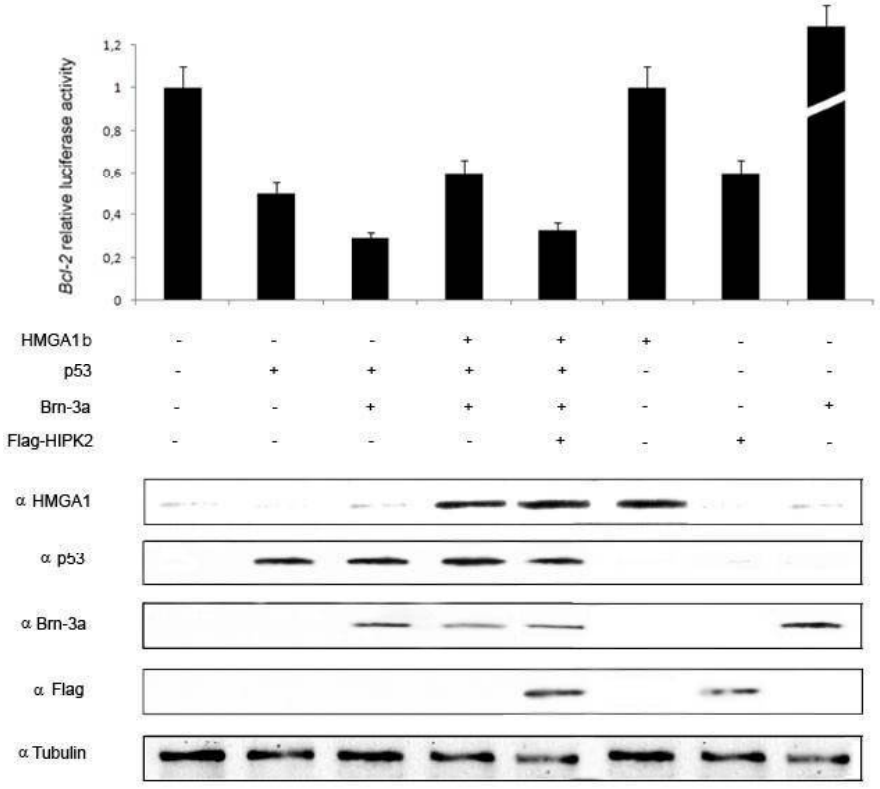


Figure 4.7. HMGA1, p53, Brn-3a and HIPK2 coexpression regulates *Bcl-2* gene. Effect of HMGA1 and HIPK2 on p53-Brn-3a super-repression exerted on *Bcl-2* reporter vector. p53-null SAOS-2 cells were used as recipient. The pCMV-p53 vector encoding WT p53 protein was transfected alone or with the indicated plasmids. All transfections were performed in duplicate; data are mean ± SD of 5 independent experiments. Empty vectors were used as a control. Western blot analyses of p53, HMGA1, Brn-3a and FLAG-HIPK2 proteins expression from one indicative experiment are shown in the lower panel.

4.7 HMGA1 exerts its antiapoptotic function by promoting HIPK2 cytoplasmic localization in ND7 cells

Previously, we demonstrated that HMGA1 inhibits p53-induced apoptosis by acting on HIPK2 delocalization from nucleus to the cytoplasm in H1299, RKO, HCT116 cell lines and in a significant number of samples from breast cancer patients (Pierantoni *et al.* 2007). Thus, we asked whether this mechanism occurs also following HMGA1 overexpression in the ND7 cell line. To this aim, we transfected ND7 cells with vectors encoding EGFP-HIPK2 protein to follow its localization when HMGA1 proteins are overexpressed. By immunofluorescence, we observed a nuclear localization of EGFP-HIPK2 overexpressing cells while, when HMGA1 proteins were overexpressed, we observed a cytoplasmic localization of HMGA1 (Figure 4.8, panel A) that was strongly associated with cytoplasmic relocalization of EGFP-HIPK2 (Figure 4.8, panel A). HIPK2 delocalization was confirmed by Western blotting analysis on nuclear/cytoplasmic cellular extracts. As shown in Figure 4.8, panel B, in ND7-HMGA1 overexpressing cells, the amount of cytoplasmic HIPK2 is higher than in cells expressing endogenous levels of HMGA1 proteins. Sp1 and γ-tubulin were used as markers of nuclear/cytoplasmic separation as well as loading controls (Figure 4.8, panel B). In Figure 4.8, panel C, HIPK2 and HMGA1 protein levels from ND7 total cell extracts are shown.

4.8 HIPK2 reverts the effects of HMGA1 on p53-Brn-3a apoptotic function

Data from luciferase assays demonstrate that HMGA1 proteins are able to abolish the super-repression exerted by p53-Brn-3a cooperation on *Bcl-2* promoter. Moreover, HIPK2 overexpression reverts HMGA1 effect on the *Bcl-2* transcription. Consequently, we analyzed the biological effects of these observations on cell death. To this aim, we transfected ND7 cells, which express HMGA1 and p53 endogenously, with vectors encoding Brn-3a alone or in combination with HMGA1 proteins overexpression, and incubated them with H_2O_2 to activate the endogenous p53. and them As expected from literature, by Trypan Blue exclusion tests and TUNEL assays, we observed an increase of apoptosis cell death due to the p53-Brn-3a cooperating repression on *Bcl-2* promoter (Budhram-Mahadeo *et al.* 1999) (Figure 4.9, panel A and B). Surprisingly, following HMGA1 overexpression, the cooperation between Brn-3a and p53 was deleted and apoptosis level was comparable to that exerted by p53 alone. In addition, HIPK2 overexpression inhibited the antiapoptotic activity of HMGA1 and induced a percentage of cell death comparable to that promoted by p53-Brn-3a cooperation (Figure 4.9, panel A and B).

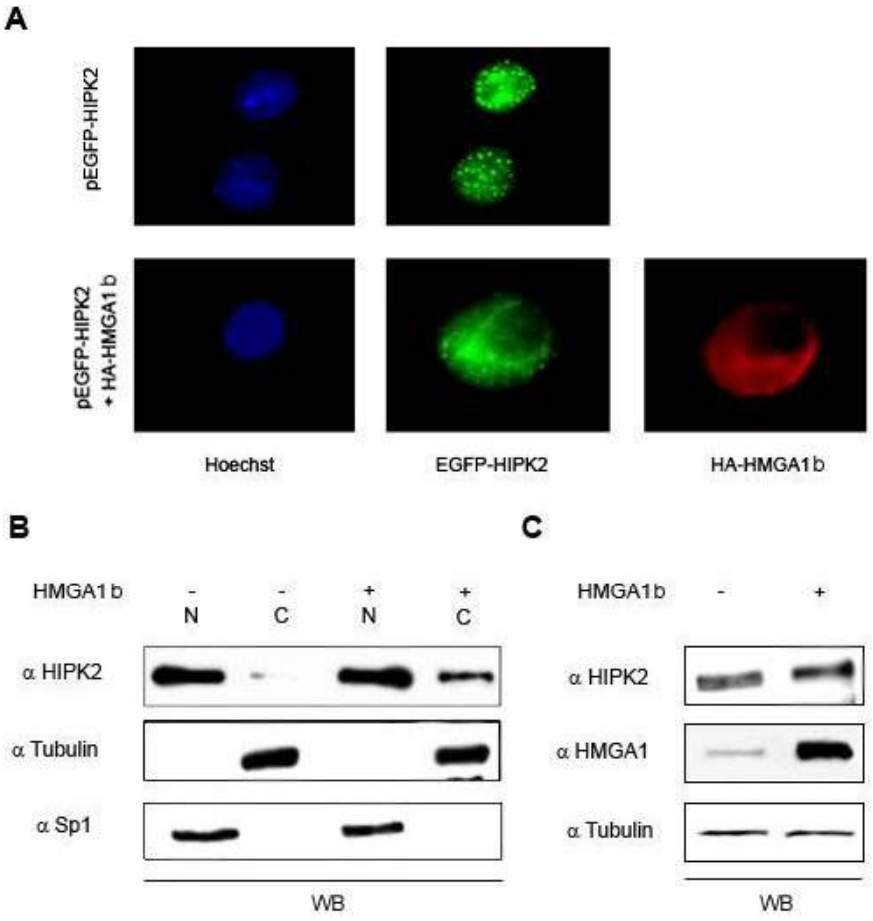


Figure 4.8. HMGA1 promote HIPK2 cytoplasmic localization in ND7 cells. (A) EGFP-HIPK2 subcellular localization in ND7 cells transfected with the indicated vectors. EGFP-HIPK2 is visible by its intrinsic green fluorescence. HA-HMGA1 expression was analyzed by immunofluorescence with anti-HA and tetramethylrhodamine isothiocyanate–conjugated Abs. Nuclei were stained with Hoechst. Images are from one representative experiment of the three performed. **(B)** From ND7 cells overexpressing or not HMGA1, nuclear (N) and cytoplasmic (C) extracts were prepared 48 hours after transfection and analyzed by Western blotting for the indicated proteins. Sp1 and γ-tubulin were used as markers of nuclear/cytoplasmic separation as well as loading controls. **(C)** From the same cells as in B, Total cell extracts were analyzed by Western blotting for endogenous HIPK2 and exogenous HMGA1 protein expression. γ-Tubulin was used to equalize protein loading.

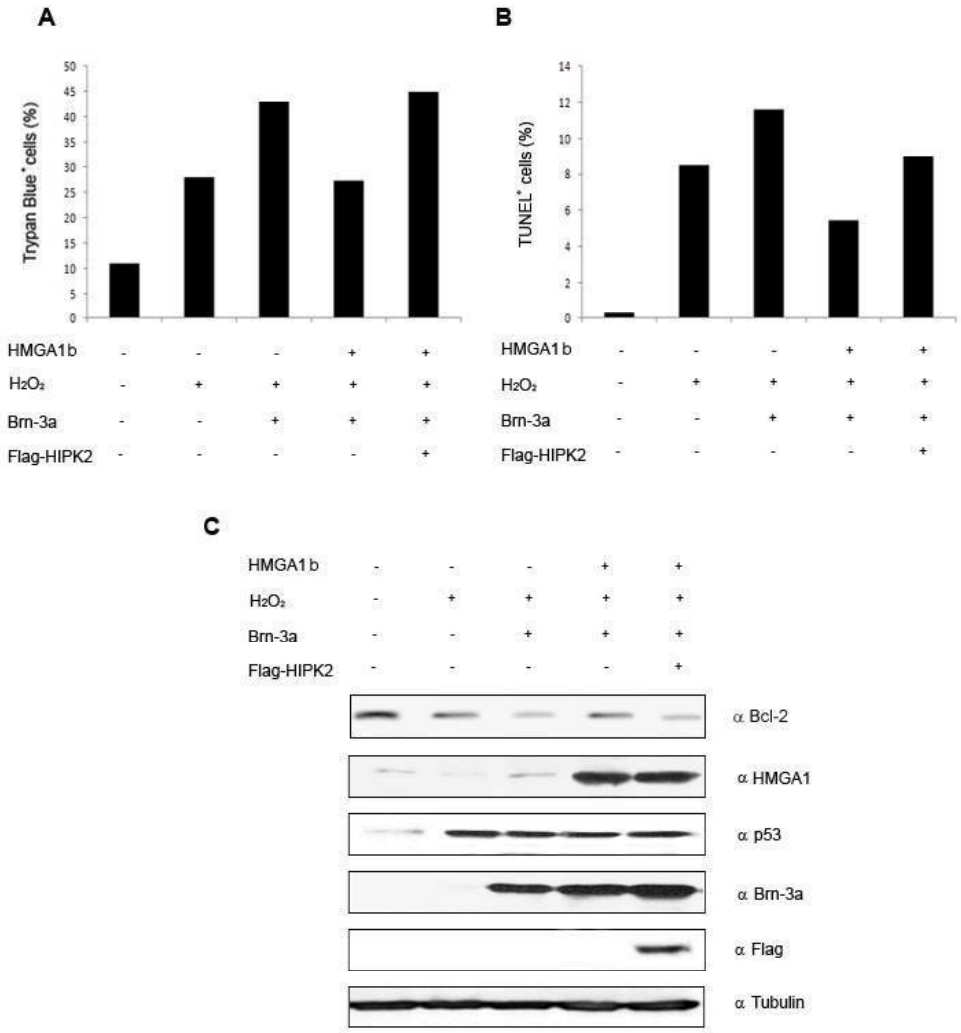


Figure 4.9. HIPK2 rescues the HMGA1 antiapoptotic effect. (**A**) ND7 cells were transfected with HMGA1b, Brn-3a, HIPK2 vectors or control vectors and then incubated or not with H_2O_2 to activate the endogenous p53. After 48 hours, floating and adherent cells were collected and analyzed by trypan blue exclusion. Mean ± SD of 3 independent experiments are shown. The results of 1 indicative experiment of the 3 performed are reported. (**B**) The same cells reported in A were analyzed by TUNEL assay. One indicative experiment is reported. **(C)** Expression of the indicated proteins was analyzed by Western blot. Expression of γ-tubulin shows equal loading of samples.

4.9 HMGA1 displaces HIPK2 and Brn-3a from *Bcl-2* promoter

We next evaluated whether HMGA1 is part of the complex, including p53, Brn-3a, and HIPK2, that forms at the p53 binding sites of the *Bcl-2* promoter *in vivo* (Budhram-Mahadeo *et al.* 1999). In a combination of chromatin immunoprecipitation (ChIP) and Re-ChIP analyses, ND7 cells were transfected with vectors encoding p53 and Brn-3a plus HMGA1 alone or in combination with HIPK2 (Figure 10, panel A). The crosslinked genomic DNAs were immunoprecipitated in two rounds with two specific antibodies directed against the examined proteins of the complex (Figure 10, panel B). Re-ChIPed DNA was analyzed by PCR using promoter-specific primers that encompass the p53 binding sites. As shown in Figure 10, panel B, HMGA1 is a component of the promoter-bound multimeric complex containing p53, Brn-3a, and HIPK2. A comparison of the subcomplexes with or without HMGA1 revealed a mechanism whereby HMGA1 can abolish the *Bcl-2* repression exerted by p53 and Brn-3a. First, when HMGA1 is overexpressed, following HIPK2 delocalization from nucleus to the cytoplasm, the amount of HIPK2 on *Bcl-2* promoter was decreased. Second, Brn-3a was not still able to bind its DNA consensus in ND7 cells transfected with a vector encoding HMGA1. These two effects were reverted when HIPK2 was cotransfected with the other vectors. HIPK2 overexpression re-establishes its nuclear localization, exerted by HMGA1, and its biological function. In fact, HIPK2 has two main roles in apoptotic regulation of this cell line: the activation of p53 by ser46 phosphorilation (D'Orazi *et al.* 2001) and the increase of Brn-3a binding activity to DNA consensus (Wiggins *et al.* 2004). By ChIP and Re-ChIP assays we demonstrate that HMGA1 impairs p53 proapoptotic activity by a sensible reduction of HIPK2 and Brn-3a binding on *Bcl-2* promoter.

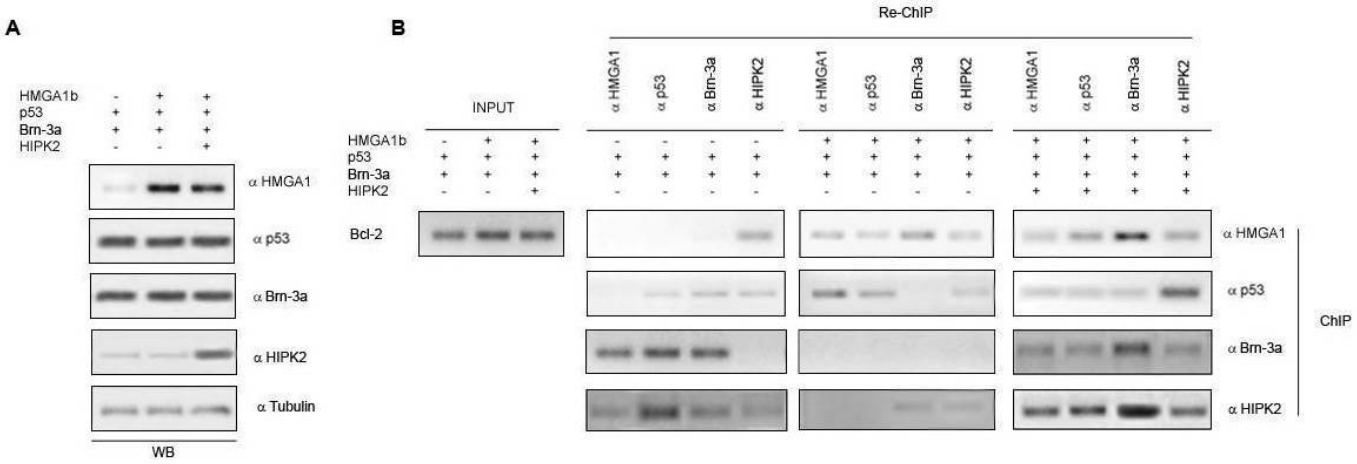
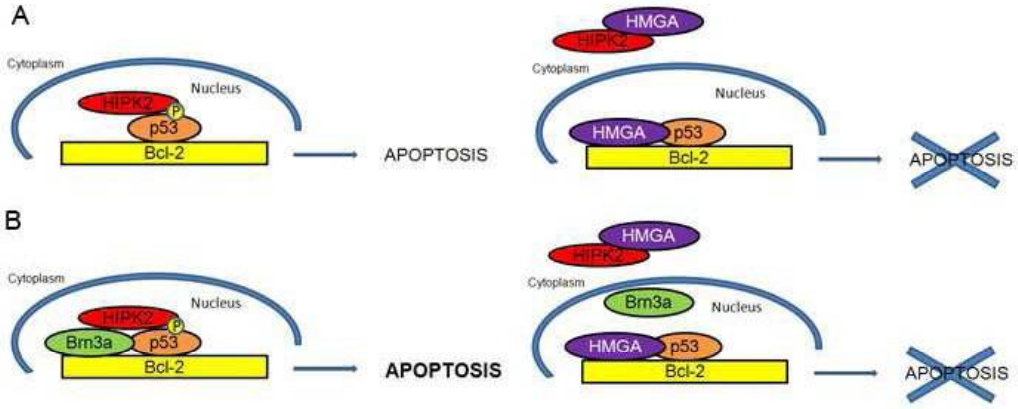


Figure 4.10. HMGA1 displaces HIPK2 and Brn -3a from *Bcl-2* promoter. (A) Lysates from ND7 cells transiently transfected with HMGA1b, p5 3, Brn -3a, HIPK2 as indicated, were subjected to Western blot analysis to verify protein expression. γ-Tubulin expression served as a control of equal protein loading. **(B)** Lysates from cells transfected with plasmids as in A, were subjected to ChIP using s pecific 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 by semiquantitative PCR using primers that cover a region of murine *Bcl-2* promoter, and a sample representing the total input chromatin (Input) was included in the PCRs as a control.

5. CONCLUSIONS

High mobility group A1 (HMGA1) overexpression and gene rearrangement are frequent events in human cancer, but the molecular basis of HMGA1 oncogenic activity still remains to be determined. Here I describe a new mechanism through which HMGA1 proteins regulate p53-mediated transcription of *Bcl-2* gene. In particular, I demonstrated that HMGA1 overexpression promoted HIPK2 relocalization in the cytoplasm and inhibition of p53 transcriptional repression exerted on the *Bcl-2* promoter, while HIPK2 overexpression reestablished HIPK2 nuclear localization and sensitivity to apoptosis (A).



Moreover, HIPK2 is not only able to phosphorylate and activate p53 but also to enhance Brn3a DNA binding that, when p53 is active, works as a corepressor of *Bcl-2* transcription. My results demonstrate that HMGA1 overexpression promotes not only HIPK2 delocalization from nucleus to the cytoplasm, but also the reduction of Brn-3a binding to the *Bcl-2* promoter removing Brn-3a from its role of corepressor following p53 overexpression (B).

In conclusion, my data strongly support the existence of a new mechanism of p53 inactivation through HMGA1-mediated cytoplasmic localization of HIPK2 and Brn-3a decreased affinity for its DNA binding sites. This mechanism might have important implications in tumorigenicity as well as in the development of tumor resistance to antineoplastic treatments.

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Endocrine-Related Cancer (2005) **12** 867–874

High-mobility group A2 gene expression is frequently induced in non-functioning pituitary adenomas (NFPAs), even in the absence of chromosome 12 polysomy

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Abstract

The high-mobility group A2 (*HMGA2*) gene has a critical role in benign tumors where it is frequently rearranged, and in malignant tumors, where it is overexpressed in the absence of structural modification of the *HMGA2* locus. By previous fluorescence *in situ* hybridization (FISH) and reverse transcriptase PCR analyses on human prolactin-secreting pituitary adenomas we detected rearrangement of the *HMGA2* gene and amplification of its native region associated with activated expression. These data indicated a role for the *HMGA2* gene in the development of human pituitary prolactinomas, since they are consistent with the appearance of prolactin/growth hormone adenomas in transgenic mice overexpressing the *HMGA2* gene. To assess a more general role for HMGA2 in pituitary oncogenesis, we investigated *HMGA2* amplification and expression in a panel of non-functioning pituitary adenomas (NFPAs) which account for 25% of all pituitary adenomas. We provide evidence that out of 18 NFPA tumors tested, 12 expressed HMGA2, but, different from prolactinomas, only in two cases the upregulation of the gene could be associated with amplification and/or rearrangement of the *HMGA2* locus. Increased dosage of chromosome 12 was found in the expressing and non-expressing NFPAs, confirming that this sole event is insufficient to drive up activation of the *HMGA2* gene. A role for chromosome 12 polysomy to promote structural instability of *HMGA2* is confirmed, but the mechanism via trisomy is less prevalent in the frequently diploid NFPAs than in the usually hyperdiploid prolactinomas. Micro-rearrangements of *HMGA2* gene not detectable by FISH analysis and/or sequence alterations could contribute to upregulation of *HMGA2* gene in pituitary adenomas of the NFPA subtype. However, it cannot be excluded that the *HMGA2* overexpression may be due, in some NFPA patients, to the same, still mainly unknown, mechanisms responsible for *HMGA2* overexpression in malignant neoplasias.

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Introduction

Pituitary adenomas are common benign, monoclonal neoplasms accounting for approximately 15% of intracranial tumors (Kovacs & Horvath 1986, Monson *et al*. 2000). Despite benign proliferations of adenohypophysial cells, they cause significant morbidity due to critical location, expanding size and/or inappropriate

DOI:10.1677/erc.1.01049
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pituitary hormone expression. Various subtypes have been recognized on the basis of clinical presentation, as well as immunocytological and ultrastructural characteristics. About one-third of pituitary adenomas are not associated with clinical hypersecretory syndromes, but with symptoms of an intracranial mass such as headaches, hypopituitarism or visual-field disturbances, and are classified as non-functioning pituitary adenomas (NFPAs). The genesis of pituitary tumors is still under investigation since the genetic alterations of the pituicytes themselves, hypothalamic dysregulation and locally produced growth factors have not been integrated in a multistep model of carcinogenesis. According to this model, genetic alterations represent the initializing event that transforms cells, whereas hormones and/or growth factors play a role in promoting cell proliferation. However, apart from activating mutations of GNSA1, which have been associated with 40% of sporadic somatotrophic adenomas and with 10% of NFPAs, none of the candidate cell-cycle, receptor, second-messenger or related genes examined thus far appear to be individually responsible for more than a few percent of sporadic pituitary adenomas. Somatic mutations identified in other malignancies such as MEN1A mutations, commonly found in patients affected by the MEN-1 syndrome, have been rarely found in sporadic pituitary adenomas, although a decreased expression of menin has been demonstrated (Theodoropoulou *et al*. 2004). Equally, even though $p27^{kip1}$ and Rb inactivation is associated with the development of pituitary adenomas of the intermediate lobe in mice, no such mutations have been identified in human pituitary adenomas (for a review see Levy & Lightman 2003). Increased expression of pituitary tumor transforming gene (*PTTG*), encoding a securin protein, has been found in sporadic pituitary adenomas, and the development of multifocal plurihormonal focal pituitary adenomas in male transgenic mice overexpressing PTTG supports its role in pituitary cell proliferation (Levy & Lightman 2003, Abbud *et al*. 2005).

Recently, our group suggested a critical role for high-mobility group A2 (*HMGA2*) in pituitary oncogenesis. It has been shown that transgenic mice overexpressing the *HMGA2* gene develop growth hormone- and prolactin-secreting adenomas (Fedele *et al*. 2002). The HMGA protein family consists of a group of small nuclear non-histone chromatinic proteins. They are involved in the regulation of chromatin structure (Fashena *et al*. 1992) and play an important role in the assembly of a multi-protein transcriptional complex that regulates the transcription of the target genes (Grosschedl *et al*. 1994). HMGA proteins play a crucial role in the process of cancerogenesis. In fact, chromosomal translocations of 12q13-15, involving the *HMGA2* gene, leading to rearrangements and dysregulated expression of the *HMGA2* gene, have been frequently detected in benign human tumors of mesenchymal origin (Ashar *et al*. 1995, Schoenmakers *et al*. 1995). Conversely, malignant neoplasias show an abundant expression of the *HMGA2* gene that is required for malignant cell transformation (Berlingieri *et al*. 1995, Giancotti *et al*. 1985, 1987, Abe *et al*. 2003). Consistently with the development of prolactin adenomas in *HMGA2* transgenic mice, induction of HMGA2 expression was observed in human prolactinomas in association with amplification and/or rearrangement in most of the tumor samples analyzed (Finelli *et al*. 2002), whereas the *HMGA2* gene was not expressed at all in normal pituitary gland.

The aim of the present work has been to assess the putative involvement of the *HMGA2* gene in another pituitary adenoma subtype, such as the NFPA. NFPAs are benign neoplasias that differ from prolactinomas in showing more frequently a normal karyotype and a lower frequency of trisomy 12 when the karyotype is aberrant (Finelli *et al*. 2000). Therefore, we analyzed *HMGA2* gene expression and possible cytogenetic alterations in a representative panel of 18 NFPAs. Results obtained by fluorescence *in situ* hybridization (FISH) analysis and reverse transcriptase (RT)-PCR expression show that the majority of NFPAs express HMGA2, which, at odds with prolactinomas, is not associated with over-representation of the *HMGA2* region, and only in a few cases is driven by rearrangement of the gene.

Materials and methods

Patients and tumor specimens

The NFPA tissue samples were obtained at transsphenoidal surgery from 18 patients, 11 of whom had undergone surgery for visual defects (pituitary adenomas (PAs) 80, 84, 86, 92, 100, 105, 107, 109, 114, 116 and 120), five for prevention (PAs 82, 93, 99, 103 and 120) and two for an increase in tumor size (PAs 110 and 112). The non-functioning secreting pituitary adenomas were clinically and hormonally characterized on the basis of standard endocrinological criteria; the tumor subtype was confirmed by routine immunohistochemistry analysis (Table 1). Seven of 18 tumors (PAs 80, 84, 103, 110, 114, 115, 112) presented with invasion of cavernous sinus. Most of the patients had not received any chemotherapy or radiation therapy

Table 1 Summary of the results obtained from immunohistochemical, cytogenetic interphase FISH (I-FISH) and RT-PCR analyses on a panel of NFPAs

NFPA	Immuno-histochemistry	Karyotype	I-FISH of chromosomes αX, 5, 8, 12	HMGA2 I-FISH (%nuclei)			HMGA2 RT-PCR	
				Disomy	tri/tetrasomy	sp[1]	1-3 ex	1-5 ex
80	negative	46, XY[8]		~90		7[2]	+	−
82[3]	30%FSH, 2% GH	46, XY[4]		~94			−	−
84	60% FSH	nd	Tris X	~97			+	+
86[3]	negative	46, XY[31]		>98			−	−
92	5% LH, 5% TSH	44~46, XY, der(1)ins(11q1p)[3][c p5]		>94			+	nd
93	30% FSH	45~47, XY, +X[12], +11[2], +17[2] −17[3], −19[3][cp19]	Tris X	>96			nd	nd
99[5]	60% LH, 40% FSH	53~60<3n>, XXY, −1[5], −2[6], −5[4], −7[6], −8[5], −13[5], −17[7], −19[5], −22[5], 2mar[cp8]/46, XY[3]	Tris 12	12	74[4]/0	13[6]	+	−
100[7]	90% ACTH, 1% PRL	46~50, XY, +9[3], +12[3], +14[2] [cp 4]	Tris 12		>96/0		−	−
103[7]	70% TSH, 1% PRL	nd	Tetra 12		0/>70		−	−
105	80% FSH, 2% LH	47, XX, +8	Tris 8	>90			+	nd
107[7]	20% FSH	nd	Tris 12 and X		~90/0		+	+
109	negative	46, XX[3]		>97			−	−
110	10% LH	46, XY[3]		>97			+	+
112	negative	46, XY[2]		>98			+	+
114[7]	40% FSH	nd	Tris 12	~80	~20/0		+	+
115	2% FSH, 2% LH	46, XY[5]		99			+	+
116	5% FSH, 2% LH	46, XX[5]		>94			+	+
120		46, XX[6]		>98			+	+
C1		46, XX		>96	2/1	0	nd	
C2		46, XY		>97	1/2	0.3	nd	
C3		46, XX		>98	1/2	0	nd	
C4		46, XY		>97	1/2	0	nd	

[1]sp, split red/green signals.
[2]Nuclei with additional signal(s) given by BAC 698i6 targeting the HMGA2 3′ region.
[3]Tumors previously studied in Finelli *et al.* 2002, and studied in this work with centromeric/pericentromeric probes in I-FISH.
[4]In PA 99 we observed a heterogeneous pattern of FISH signals with a major trisomic clone (53%) characterized by three signals of the same intensity of control cells and a trisomic subclone (21%) with one/two *HMGA2*-specific signals of reduced intensity.
[5]Over-representation of the HMGA2 region as compared to the copy number of chromosome 12 assessed by dual-color FISH of D12Z3 and HMGA2 BACs in 36% of nuclei controlled.
[6]We observed uncoupled signals in 13% of the nuclei in particular 8% of nuclei with one/two signal/s given by the probe covering the HMGA2 5′ gene (BAC 669g18) and 5% of nuclei with one/two signal/s given by the probe targeting the HMGA2 3′ region (BAC 698i6).
[7]No over-representation of the HMGA2 region as compared to the copy number of chromosome 12 as specified above.
nd, not determined; FSH, follicle-stimulating hormone; GH, growth hormone; LH, luteinizing hormone; PRL, prolactin; TSH, thyroid-stimulating hormone; ACTH, adrenocorticotrophin.

before surgery. Histological analysis was performed as described previously (Finelli *et al*. 2000). PAs 82 and 86 were described previously (Finelli *et al*. 2002).

Cell cultures and cytogenetic analysis

The primary pituitary cell cultures were set up as described elsewhere (Bettio *et al*. 1997). The phytohemagglutinin (PHA)-stimulated peripheral blood cultures were set up according to standard procedure. The Q-banding of fluorescence using Quimacrine (QFQ) banding technique was used for cytogenetic analysis, and the International System for Human Cytogenetic Nomenclature was adopted (Mitelman 1995).

FISH studies

FISH analysis on nuclei was performed by using the following alphoid probes: pZ8.4 (D8Z8) and pDMX1 (DXZ1; Archidiacono *et al*. 1995), and pBR12 (D12Z3; Baldini *et al*. 1990). YAC 882a10, which maps on chromosome 5p13, was from the CEPH YAC library, as described by Finelli *et al*. (2000), while *HMGA2* BAC clones (698i6 and 669g18), encompassing the 5′ (5′ untranslated region and exons 1–3) and the 3′ (exons 3–5 and the 3′ untranslated region) portions of *HMGA2* gene are described in previous works (Finelli *et al*. 2002, Pierantoni *et al*. 2003).

The procedure described by Lichter *et al*. (1990) and Lichter & Cremer (1992), with some modifications, was used for dual-color FISH experiments on interphase nuclei from direct tumor preparations or short-term culture tumor preparations. Briefly, the probes were labeled by nick-end translation with biotin or digoxygenin (Roche Molecular Biochemicals, Germany). For each *in situ* hybridization experiment, 200 ng labeled alphoid probe and/or 500 ng labeled YAC/BAC probes were used in a 10 µl volume of hybridization solution. The FISH procedure, detection of biotin- and digoxygenin-labeled probes, nuclei/chromosome counterstaining and digital-image analysis are described elsewhere (Finelli *et al*. 2000). The images were edited using Adobe Photoshop 7 (Adobe System, Mountain View, CA, USA). As described previously, scoring was based on >200 nuclei per each tumor sample and for reference purposes the background percentage of nuclei with more or less than two signals and the percentage of nuclei with a split hybridization signal were calculated. PHA-stimulated lymphocytes from healthy individuals were hybridized in parallel.

RNA extraction and RT-PCR analysis

Pituitary adenomas were dissected rapidly, frozen on dry ice and stored at −80 °C. Total RNA was extracted using TRI reagent solution (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's protocol. 5 µg total RNA, digested with RNase-free DNase, were reverse-transcribed using random hexanucleotides as primers (100 mM) and 12 units avian myeloblastosis virus RT (Promega). The cDNA was amplified in a 25 µl reaction mixture containing 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.4 mM of each primer and 1 unit *Taq* DNA polymerase (Perkin-Elmer). After a denaturing step (95 °C for 2 min) the cDNA was further amplified in 20 PCR cycles (95 °C for 1 min, 58 °C for 30 s and 72 °C for 30 s). The following primers were used to amplify the *HMGA2* transcript: forward primer, 5′-CGAAAGGTGCTGGGCAGCTCCGG-3′, which maps onto the first exon; reverse primer, 5′-CCATTTCCTAGGTCTGCCTCTTG-3′, which maps onto the third exon; reverse primer II, 5′-CTAGTCCTCTTCGGCAGACTC-3′, which maps onto the fifth exon.

Expression of the *GAPDH* gene was used as an internal control for the amount of cDNA tested. The specific primers were: forward, 5′-ACATGTTCCAATATGATTCC-3′; reverse, 5′-TGGACTCCACGACGTACTCA-3′ (corresponding to nucleotides 195–215 and 355–335, respectively). The reaction products were analyzed on a 2% agarose gel, and transferred to GeneScreen plus nylon membranes (Dupont, Boston, MA, USA). The membranes were hybridized with a *HMGA2* cDNA probe. cDNA probes obtained by PCR were labeled with [^{32}P]dCTP using random oligonucleotide primers (Ready-To-Go; Pharmacia) at a specific activity equal to or higher than 7×10^8 c.p.m./µg.

Results

Chromosome analysis

Table 1 shows a list of the 18 NFPA samples analyzed in our study. Their immunohistochemical patterns are also specified. All the tumors were examined using conventional cytogenetics, either on direct or short-term culture chromosome preparations, with successful karyotyping of 14 tumors. An abnormal karyotype was found in five adenomas (PAs 92, 93, 99, 100 and 105), while an apparently normal karyotype was observed in the remaining nine tumors (Table 1, third

column). Peripheral blood cells from healthy individuals were also analysed (C1–C4 in Table 1).

Interphase FISH

To assess the normal/abnormal chromosomal set of tumors where cytogenetic analysis failed (PAs 84, 103, 107 and 114) or only a few metaphases could be analyzed, we performed FISH of centromeric/pericentromeric probes specific to chromosomes found at increased dosages in previous studies (Finelli *et al*. 2000), i.e. chromosomes 5, 8, 12 and X. By this approach, we observed the presence of trisomy X in PA 84, trisomy 12 in PA 114, tetrasomy 12 in PA 103 and combined trisomy 12 and X in PA 107, accounting for the four tumors where conventional cytogenetics failed. In addition, we could confirm trisomy 8 in PA 105, trisomy X in PA 93, trisomy 12 in PA 100 and trisomy 12 and an extra X-specific signal in PA 99 (Table 1, fourth column). Based on previous findings in prolactin-secreting adenomas (Finelli *et al*. 2002), we conducted FISH experiments on 16 non-functioning secreting pituitary adenomas to establish the dosage and putative rearrangements of *HMGA2*. Interphase dual-color FISH was performed on nuclei from direct/short-term tumor preparations using *HMGA2*-specific probes and different combinations of *HMGA2*-specific BAC probes with a D12Z3-specific alphoid probe. As reported in Table 1, dual-color FISH of *HMGA2*-specific BACs showed on 12 out of 18 NFPAs two pairs of red/green overlapping spots. This pattern corresponds to that of peripheral blood cells from healthy individuals (C1–C4 in Table 1) in the great majority of nuclei (90–99%). Conversely, an increased dosage of the target region was detected in five tumors, namely PAs 99, 100, 107 and 114, showing *HMGA2* trisomy, and PA 103, which showed *HMGA2* tetrasomy in 20–96% of nuclei. A heterogeneous pattern of FISH signals was given by PA 99: in fact, this sample displayed a major trisomic clone (53%), characterized by three signals of the same intensity of control cells, a trisomic subclone (21%), with one/two *HMGA2*-specific signals of reduced intensity, and minor disomic subclones (summing up to 13%) with one regular (red/green) *HMGA2* signal and one split (either red or green) signal (Fig. 1A and Table 1). Signals of decreased intensity as well as split signals are highly suggestive of intra-*HMGA2* rearrangements. PA 80 was disomic for *HMGA2* in most cells but contained a small subclone (7%) with an additional signal given by the 3′ *HMGA2* BAC (Fig. 1B). FISH results with *HMGA2* BACs on both NFPAs showing subclones with an atypical pattern were confirmed in independent experiments. Co-hybridization of *HMGA2* BACs with chromosome 12 alphoid-specific probe was then performed on the two above NFPAs to detect selective overrepresentation of the *HMGA2* region, in addition to the trisomy of chromosome 12, when present. These FISH experiments revealed in PA 99 a number of spots higher than that given by the alphoid probe in about 36% of the nuclei. This percentage derived from the sum of the trisomic subclone with signals of reduced intensity (21%) and that of disomic subclones with split signals (13%).

HMGA2 gene overexpression in non-functioning secreting pituitary adenomas

RT-PCR analysis, using primers specific for exons 1 and 3 of *HMGA2* gene, was performed in parallel to check the *HMGA2* expression in the NFPAs evaluated by cytogenetics and interphase FISH. 15 tumors could be investigated together with PAs 82 and 86, used as negative controls in our previous study (Finelli *et al*. 2002). Insufficient material has been obtained to evaluate the *HMGA2* expression in PA 93. As shown in Fig. 1C, most tumors (12/15) showed *HMGA2*-specific mRNA, whereas only PAs 100, 103 and 109 were negative. Notable differences in the levels of *HMGA2* mRNA could be appreciated among tumors, with a group that expresses high levels of *HMGA2* mRNA (PAs 112, 114, 115 and 120) and one that expresses *HMGA2* at low levels (PAs 84, 92, 105, 110 and 116). As expected, *HMGA2* was not expressed in normal pituitary gland (Fig. 1C, lane NP; Zhou *et al*. 1995). To verify the presence of truncated transcripts of the *HMGA2* gene, we evaluated the expression of the entire *HMGA2* transcript in 15 of the NFPA tumours. To this end, we have utilized primers specific for exons 1 and 5 of the *HMGA2* gene, which amplify the entire coding sequence. Eight adenoma samples, PAs 84, 107, 110, 112, 114, 115, 116 and 120, showed *HMGA2* gene expression, indicating the presence of a standard-sized transcript, whereas in the other samples, PAs 80, 82, 86, 99, 100, 103 and 109, no amplification was observed (Fig. 1D). The results from PAs 82, 86, 100, 103 and 109 confirm those obtained by using the other *HMGA2* primer pair. The results obtained for PAs 80 and 99 were consistent with the FISH results, where we observed a hybridization pattern suggestive of intra-*HMGA2* rearrangements. For all PCR assays the amplification was also performed with non-reverse-transcribed RNAs to exclude DNA contamination (data not shown).

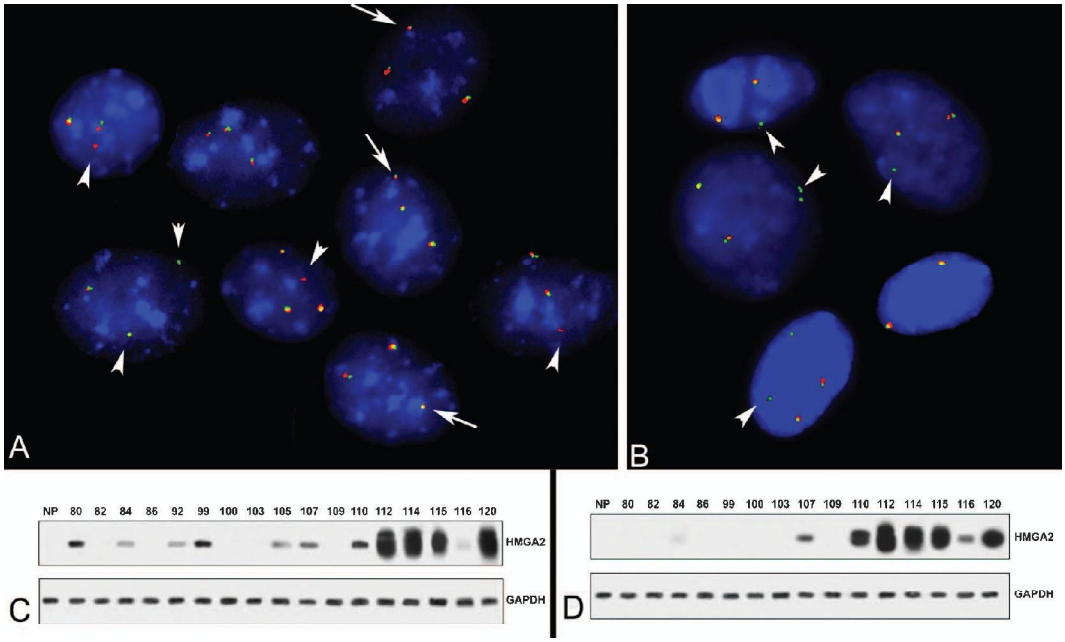

Figure 1 Interphase FISH analysis and *HMGA2* gene expression in human non-functioning secreting pituitary adenomas. (A) Dual-color FISH of probes 669g18 (green)/698i6 (red) shows a heterogeneous hybridization pattern on PA 99 that displays nuclei with three signals of the same intensity, nuclei with one/two *HMGA2*-specific signals of reduced intensity (arrows) and nuclei with red/green *HMGA2* signals and split (either red or green) signals (arrowheads). (B) The same two probes show in most cells of PA 80 a disomic hybridization pattern for *HMGA2* and only a small fraction of nuclei with an additional signal given by the 3′ *HMGA2* BAC. (C) Total RNA was extracted from the indicated tumor samples, and the *HMGA2* expression was analyzed by RT-PCR with a pair of primers located on exons 1 and 3. NP, normal pituitary gland; lane numbers indicate PA numbers (see Table 1). (D) RNA from normal pituitary gland (NP) and from indicated adenoma samples were amplified with two primers that map to exons 1 and 5. All cDNAs were co-amplified with *GAPDH* as an internal control.

Discussion

Previous data from our group suggest an involvement of the *HMGA2* gene in human prolactinomas. In fact, overrepresentation of the genomic region where *HMGA2* resides (12q14) and/or rearrangement of the gene were demonstrated by FISH experiments in association with an increased expression of *HMGA2* gene (Finelli *et al*. 2002). A causal role of HMGA2 in prolactinomas was supported by the appearance of prolactin/growth hormone adenomas in transgenic *HMGA2* mice (Fedele *et al*. 2002), and by data indicating an oncogenic role for this protein (Fedele *et al*. 1998). Here, we extended the analysis of the *HMGA2* gene to a sample of 18 NFPAs to assess its putative involvement by increased dosage and rearrangement in this common pituitary adenoma subtype. Results obtained by FISH analysis and RT-PCR show that most NFPAs express HMGA2. However, differently from prolactinomas, HMGA2 expression is not commonly associated with overrepresentation of the *HMGA2* region, and is associated with *HMGA2* rearrangement only in two out of 17 NFPAs. In fact, in both the PA 80 and 99 samples, *HMGA2* rearrangement could be observed, by interphase FISH scoring, in low-represented tumor clones, showing uncoupling of the signals given by BACs monitoring the contiguous 5′ and 3′ portions of *HMGA2* with loss of either signal in a fraction of cells. Moreover, in PA 99 different rearrangements were triggered by the initial *HMGA2* break, as assessed by the heterogeneity in the intensity of the uncoupled FISH signals and the alternative loss of one of them in different tumor subclones (Fig. 1A).

Despite the fact that *HMGA2* rearrangement was monitored only in a fraction of cells in tumors PAs 80 and 99, RT-PCR analysis showed only aberrant transcripts. This suggests that microrearrangements of the gene or sequence alterations, not detectable by FISH analysis, also affected the majority of tumor cells with apparent integrity of the *HMGA2* region by FISH analysis. Interestingly, one of the two rearranged NFPAs, PA 99, showed a consistent fraction of cells

trisomic for chromosome 12, which represents likely a primary genetic event that might facilitate the occurrence of further rearrangements. Indeed, evidence has been provided that polysomy promotes structural instability in tumor-cell chromosomes through asynchronous replication and breaks within late-replicating regions (Kost-Alimova *et al*. 2004). The *HMGA2* region falls within a G-dark band, which likely corresponds to a late-replicating region that may become a preferential site of structural rearrangements in the unstable polysomic chromosome 12. As already demonstrated in a high number of benign tumors of mesenchymal origin (Schoenmakers *et al*. 1995) and for two cases of prolactinomas (Finelli *et al*. 2002), the rearrangement of the *HMGA2* gene in PAs 80 and 99 results in a break in the large intervening sequence (IVS3) that separates the third from the fourth exon. This would induce the oncogenic potential of the HMGA2 protein because of the loss of its C-terminal tail, as demonstrated previously (Fedele *et al*. 1998, Battista *et al*. 1999).

As demonstrated previously for prolactinomas, the sole trisomy 12 is not sufficient for HMGA2 expression, which is associated with overrepresentation of the *HMGA2* region and/or rearrangement. In fact, PAs 100 and 103 were trisomic and tetrasomic for chromosome 12, but they did not have overrepresentation of the 12q14 region (data not shown) and did not express HMGA2. Since overrepresentation of *HMGA2* region, via trisomy or tetrasomy, is quite common in prolactinomas (Finelli *et al*. 2002), and rare in NFPAs, we retain that the polysomy rearrangement is a major contributor to *HMGA2* activation in prolactinomas, while it is implicated less in NFPAs, most of which have a diploid karyotype. Since FISH experiments have shown the common lack of an extra chromosome 12 and rearrangements only in a small percentage of cells that would trigger new rearrangement in the polysomic chromosome, and most NFPAs express high levels of *HMGA2* transcript, other mechanisms able to activate HMGA2 expression should occur in human NFPAs. Alternative HMGA2-activating mechanisms, among which sequence alterations or dysregulation by cryptic rearrangements, need further study. In fact, it cannot be excluded that the *HMGA2* overexpression may be due to the same, still mainly unknown, mechanisms responsible for *HMGA2* overexpression in malignant neoplasias.

In conclusion, the findings reported here extend those obtained in prolactinomas by confirming the involvement of HMGA2 in pituitary oncogenesis. Since *HMGA2* transgenic mice never develop NFPAs, and since rearrangements of *HMGA2* are rare in this subtype, we can also hypothesize that whereas in most human prolactinomas *HMGA2* overexpression would represent one of the initial and causal events, in most NFPA subtypes *HMGA2* overexpression might represent a secondary event that occurs independently of the specific initializing event and might be responsible for tumor progression. However, this hypothesis needs to be validated by future work.

Acknowledgements

This work was supported by grant from the Ministero della Sanità to Istituto Auxologico Italiano, and from AIRC, the Progetto Finalizzato 'Biotecnologie' of the CNR, the MURST projects 'Terapie antineoplastiche innovative' and 'Piani di Potenziamento della Rete Scientifica e Tecnologica'. COST ACTION B19 'Molecular Cytogenetics of Solid Tumors' is acknowledged for stimulating discussion. We thank the Associazione Partenopea per le Ricerche Oncologiche (APRO) for its support. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Cell Death and Differentiation (2006) 13, 1554–1563
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High Mobility Group A1 (HMGA1) proteins interact with p53 and inhibit its apoptotic activity

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Received 01.8.05; revised 12.10.05; accepted 15.11.05; published online 09.12.05
Edited by M Oren

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.**

Cell Death and Differentiation (2006) **13,** 1554–1563.
doi:10.1038/sj.cdd.4401839; published online 9 December 2005

Keywords: apoptosis; HMGA1; p53; antibody array; UV

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] ovary[18] and breast[19] 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 NH_2- 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

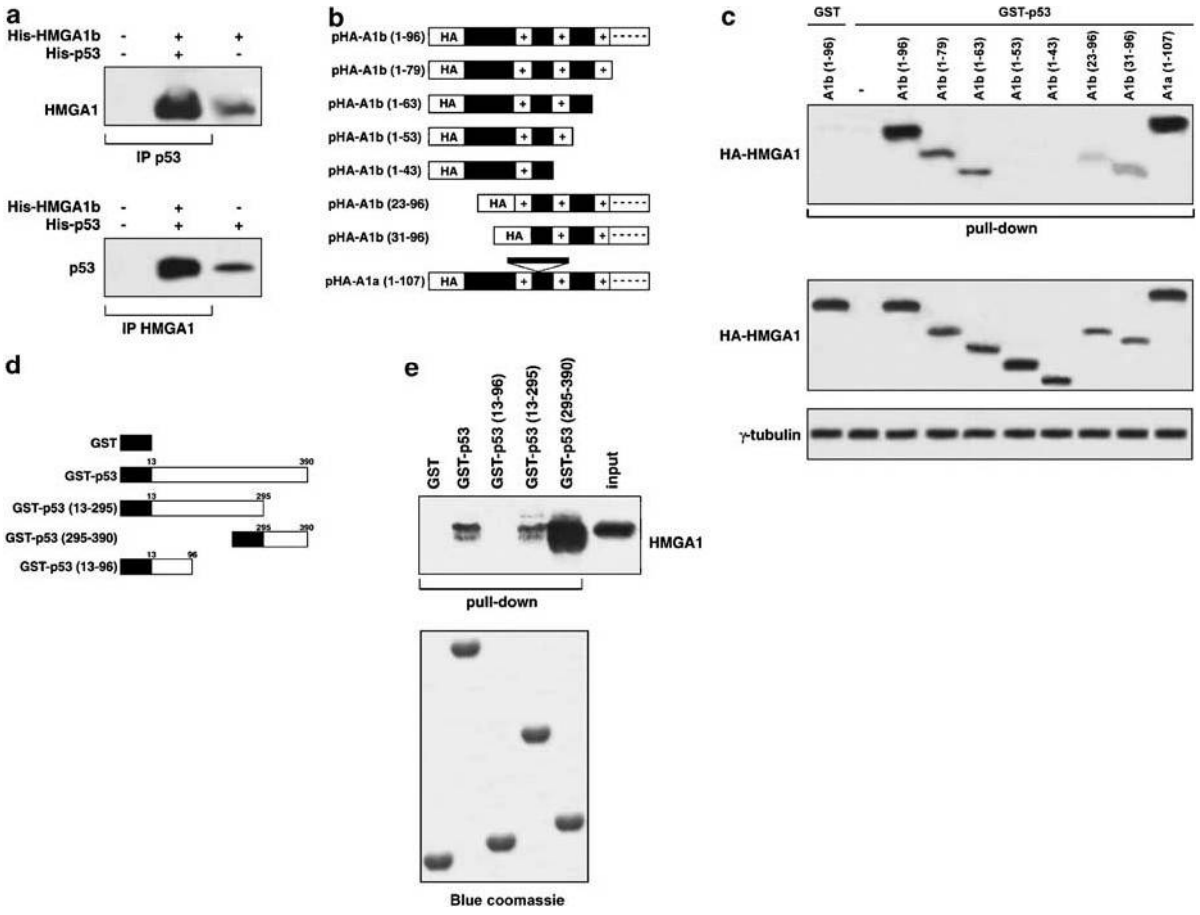

Figure 1 *In vitro* interaction between HMGA1 and p53. (**a**): The *in vitro* interaction between p53 and HMGA1b was assessed by immunoprecipitation assay with anti-p53 (upper panel) or anti-HMGA1 (lower panel) antibodies and blotting with the reciprocal antibodies after preincubation of the two proteins. 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 NH_2-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 and 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

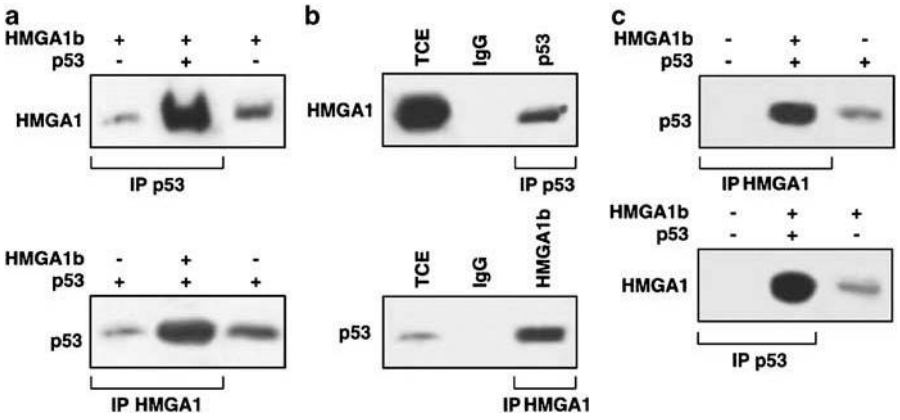

Figure 2 *In vivo* characterization of the HMGA1/p53 interaction. (**a**) 293 cells were transfected with pCMV/*Hmga1b* vector alone or together with pCMV-*p53*. After 48 h, total cell extracts were prepared and equal amounts of proteins were immunoprecipitated with anti-p53 (upper panel) or anti-HMGA1 (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 ^{32}P-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

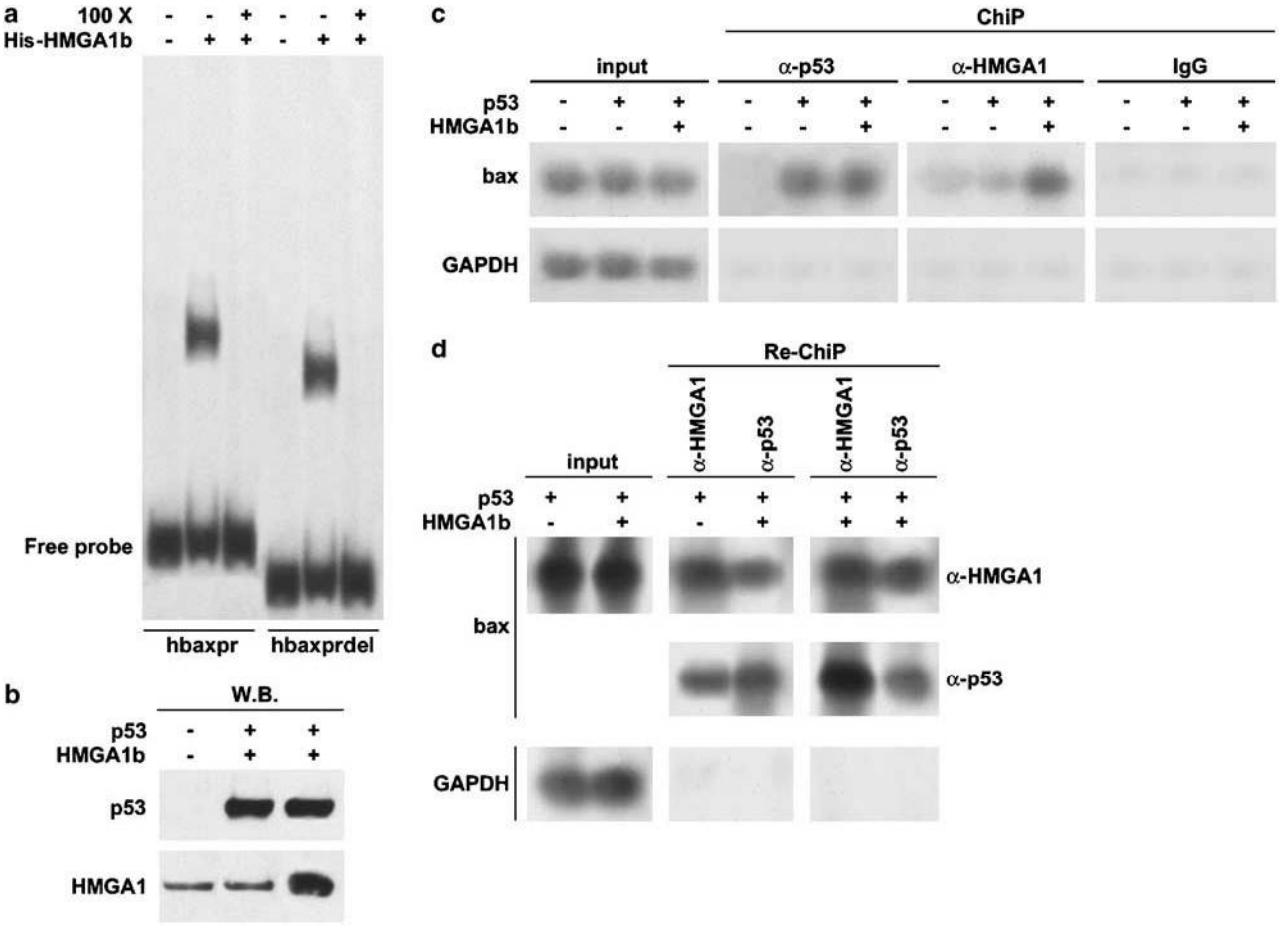

Figure 3 HMGA1 binds the human *Bax* promoter *in vitro* and *in vivo*. (**a**) EMSA performed with a radiolabelled oligonucleotide spanning from nt −441 to −500 of the human *Bax* promoter (hbaxpr) and the same region deprived of p53-binding site (hbaxprdel) incubated with 50 ng of recombinant HMGA1b 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

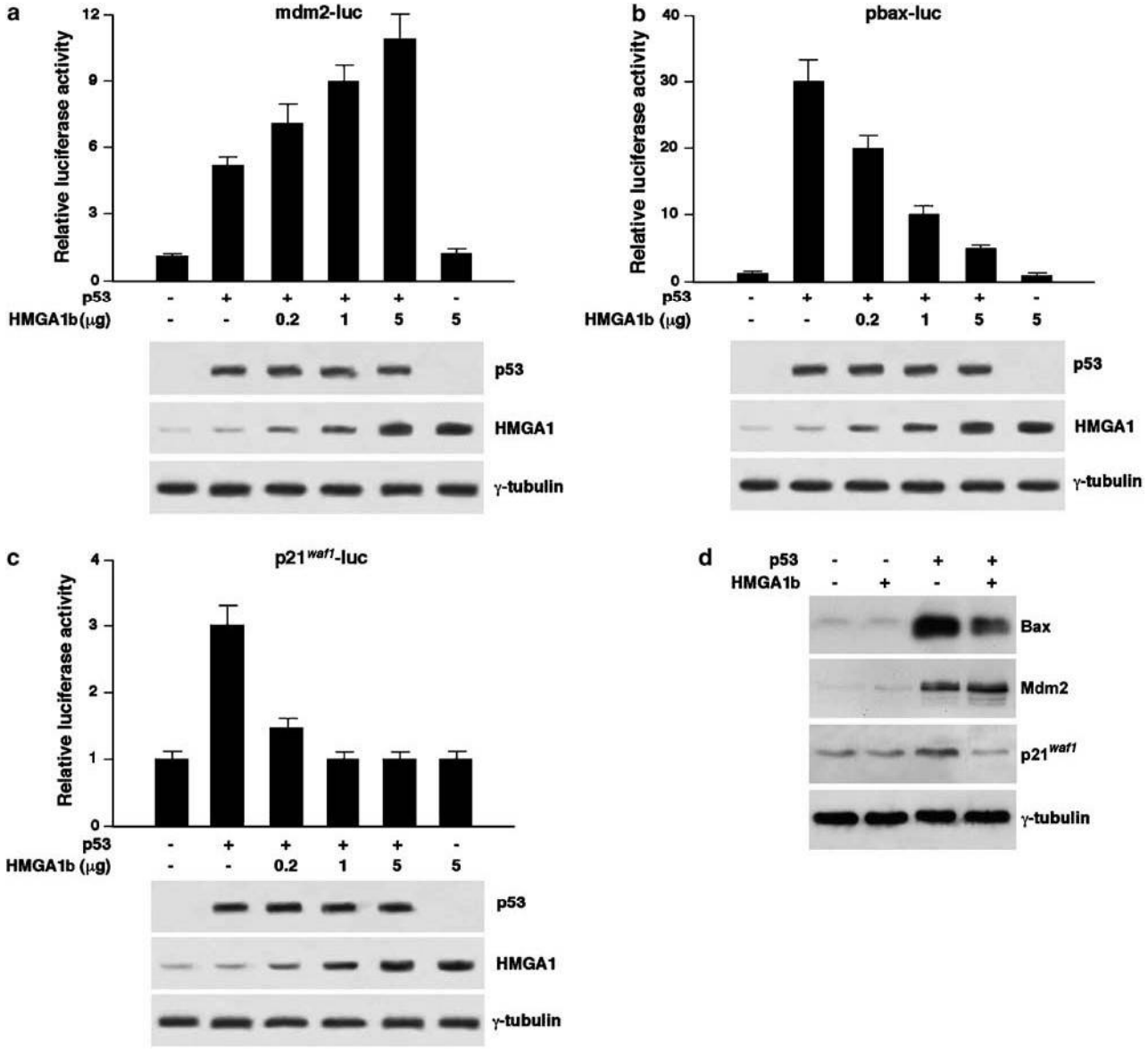

Figure 4 HMGA1b modulates p53 transcriptional activity. Dose–response analysis of increasing amounts of HMGA1b on p53 activity on the *mdm2* (**a**), *Bax* (**b**), and *p21*^{waf1} (**c**) luciferase-reporter vectors transiently transfected in p53-null H1299 cells. All transfections were performed in 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

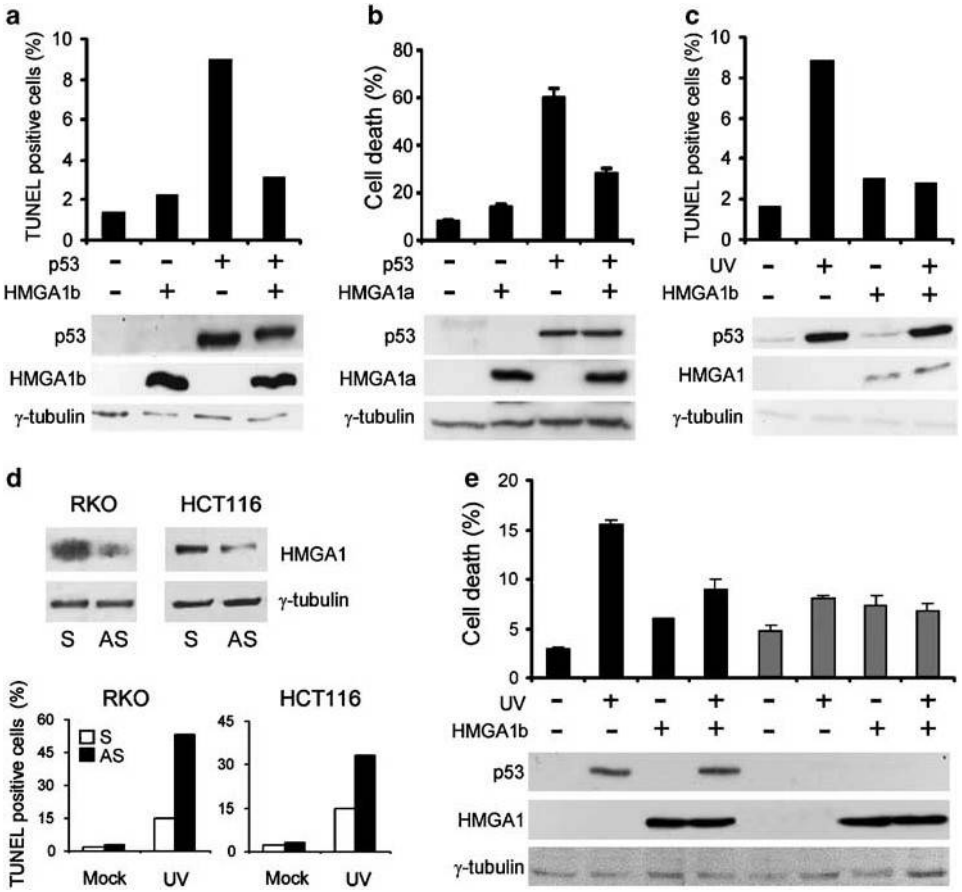

Figure 5 HMGA1 interferes with the apoptotic activity of p53. (**a**) p53 null H1299 cells were infected with the control vector dl70.3 (— in the p53 row) or with the wtp53-carrying Adp53 (+ in the p53 row). At 1 h postinfection, cells were transfected with the pCMV control vector (— in the HMGA1b row) 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 μM.

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 μg/ml), grown at 30°C to an OD_{600} 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 μl 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 (Hypromatrix 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 Fl393 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

NH_2-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 μg poly(dC-dG), 2 μg BSA and 10% glycerol, to a final volume of 20 μl, for 10 min at room temperature. The samples were incubated for 15 min after addition of 2.5 fmol of a ^{32}P-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 μl 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 μl 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 μl of 1% SDS, 0.1 M $NaHCO_3$. This step was repeated twice.

In Re-Chip experiments, complexes were eluted by incubation for 30 min at 37°C in 250 μl 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 μl of 5 M NaCl. Then were added 10 μl 0.5 mM EDTA, 20 μl 1 M Tris-HCl pH 6.5 and 20 μg of Proteinase K, and incubated for 1 h at 45°C. DNA was purified by Phenol/$CHCl_3$, 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′-GTATTCCCCCAGGTTTACATG-3′; h-*GAPDH*-pr-dw-5′-TTCTCCATGGTGGTGAAGAC-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 μg/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.

Acknowledgements

We are grateful to Jean Ann Gilder (Scientific Communication) for editing the text, and to Barbara Cecchinelli for GST-p53 vectors. We are grateful to Giorgia Bracaglia for providing her data about the effects exerted from HMGA1a isoform on p53 apoptotic activity. We thank the Associazione Italiana Ricerca sul Cancro (AIRC), the Progetto Finalizzato 'Biotecnologie' of the CNR, the MURST projects 'Terapie antineoplastiche innovative' and 'Piani di Potenziamento della Rete Scientifica e Tecnologica', the Associazione Partenopea per le Ricerche Oncologiche (APRO) and the Ministero della Salute for their support. CR is recipient of a fellowship from Fondazione Italiana per la Ricerca sul Cancro (FIRC).

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High-mobility group A1 inhibits p53 by cytoplasmic relocalization of its proapoptotic activator HIPK2

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High-mobility group A1 (HMGA1) overexpression and gene rearrangement are frequent events in human cancer, but the molecular basis of HMGA1 oncogenic activity remains unclear. Here we describe a mechanism through which HMGA1 inhibits p53-mediated apoptosis by counteracting the p53 proapoptotic activator homeodomain-interacting protein kinase 2 (HIPK2). We found that HMGA1 overexpression promoted HIPK2 relocalization in the cytoplasm and inhibition of p53 apoptotic function, while HIPK2 overexpression reestablished HIPK2 nuclear localization and sensitivity to apoptosis. HIPK2 depletion by RNA interference suppressed the antiapoptotic effect of HMGA1, which indicates that HIPK2 is the target required for HMGA1 to repress the apoptotic activity of p53. Consistent with this process, a strong correlation among HMGA1 overexpression, HIPK2 cytoplasmic localization, and low spontaneous apoptosis index (comparable to that observed in mutant p53–carrying tumors) was observed in WT p53–expressing human breast carcinomas. Hence, cytoplasmic relocalization of HIPK2 induced by HMGA1 overexpression is a mechanism of inactivation of p53 apoptotic function that we believe to be novel.

Introduction

The high-mobility group (HMG) proteins are low–molecular weight nuclear factors with nonhistone chromosomal accessory functions (1). The A subgroup of HMG (i.e., HMGA1a, HMGA1b, HMGA1c, and HMGA2) interacts with the minor groove of many AT-rich promoters and enhancers (2) and plays key roles in chromatin architecture and gene transcription control (2, 3). HMGA proteins do not directly exert transcriptional activity and are considered architectural transcription factors. By a complex network of protein-DNA and protein-protein interactions, they organize chromatin into the structure required by the basal transcription machinery to execute gene transcription (4). In physiological conditions, HMGA proteins are expressed at a high level during embryogenesis (5, 6), while their expression becomes low to undetectable in adult tissues. High HMGA expression in adult life is associated only with pathological conditions such as human carcinomas of thyroid (7, 8), colon (9–11), prostate (12), pancreas (13), cervix (14), ovary (15), and breast (16) tissues. A functional role of this aberrant HMGA overexpression in cancers has been previously demonstrated. We previously showed that in rat thyroid cells, blockage of HMGA synthesis prevents tumor transformation by murine transforming retroviruses (17, 18). Furthermore, infection of different carcinoma cells by a recombinant adenovirus carrying the HMGA1b cDNA in antisense orientation induces apoptosis and interferes with tumor growth in vivo (19). To get clues on the mechanism(s) through which HMGA proteins exert their tumorigenic effect, we recently searched for HMGA interactors by analysis of Ab arrays and found that HMGA1 binds the p53 oncosuppressor and decreases its apoptotic activity, strongly supporting the idea that HMGA1 contributes to tumor transformation by interfering with the apoptotic function of p53 (20). HMGA1 exerts this effect by repressing the p53 transcriptional activity on target genes such as *BAX* and $p21^{Waf}$, and by chromatin reimmunoprecipitation experiments we showed that HMGA1 and p53 are present in the same complexes bound to the *BAX* gene promoter. However, despite the negative action of HMGA1 on p53 transcriptional activity on this promoter, HMGA1 overexpression did not decrease the presence of p53 in this region, suggesting a mechanism of p53 inactivation independent of its DNA-binding activity, at least on this promoter.

The WT p53 protein, which is mutated or inactivated in most human cancers, is a master regulator of the apoptotic program (21, 22). p53 is a sequence-specific transcription factor that regulates the expression of genes involved in cell cycle arrest or apoptosis in response to genotoxic damage or cell stress, including tumor cell response to many antineoplastic treatments (23). It has been proposed that the posttranslational modifications that activate p53 are responsible for p53-mediated biological outcomes (24, 25). Among the p53 posttranslational modifications, phosphorylation at serine residue 46 (Ser46) was identified as a specific modification involved in apoptosis, at least in part through transcriptional regulation of specific target genes (26–28). We and others have previously shown that homeodomain-interacting protein kinase 2 (HIPK2) binds to and activates p53 by phosphorylating it at Ser46 (29, 30). Furthermore, HIPK2 was also shown to promote apopto-

Nonstandard abbreviations used: Ad, adenovirus; EGFP, enhanced GFP; HIPK2, homeodomain-interacting protein kinase 2; HMG, high-mobility group; LMB, leptomycin B; MDM2, mouse double minute 2; NF-YB, nuclear factor YB; TCE, total cell extract.

Conflict of interest: The authors have declared that no conflict of interest exists.
Citation for this article: *J. Clin. Invest.* **117**:693–702 (2007). doi:10.1172/JCI29852.

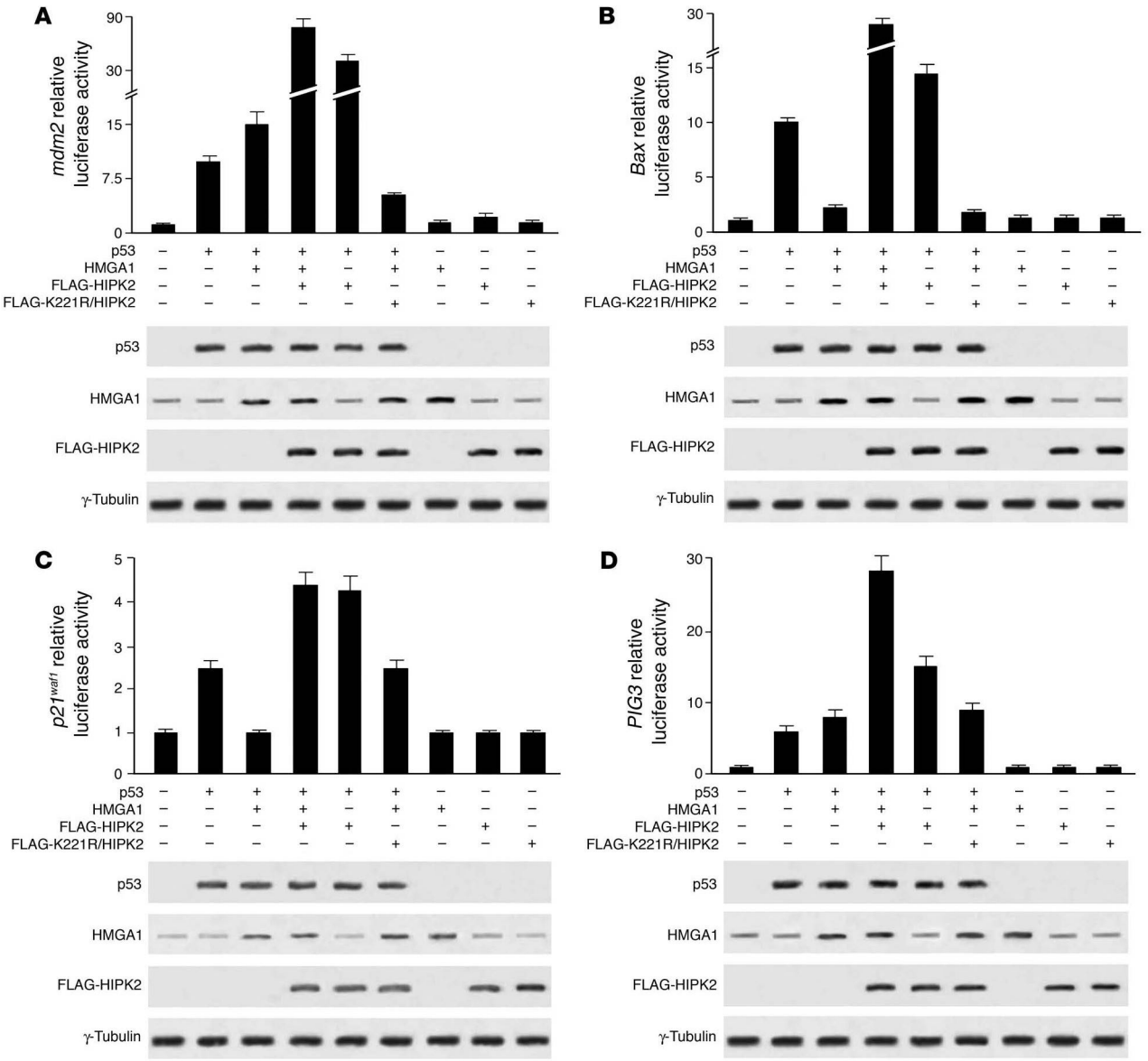

Figure 1

HMGA1, p53, and HIPK2 coexpression activates proapoptotic genes. Effect of FLAG-tagged WT HIPK2 or the kinase-defective HIPK2 mutant (FLAG-K221R/HIPK2) on the activity exerted by HMGA1 on reporter vectors *mdm2* (**A**), *bax* (**B**), $p21^{waf1}$ (**C**), and *PIG3* (**D**). p53-null H1299 cells were used as recipient. The pCMV-p53 vector encoding WT p53 protein was transfected alone or with the indicated plasmids. All transfections were performed in duplicate; data are mean ± SD of 5 independent experiments. Empty vectors were used as a control. Western blot analyses of p53, HMGA1, and FLAG-HIPK2 proteins expression from 1 indicative experiment are shown in the lower panels.

sis by acting on targets different from p53, such as the p53 family members p73 and p63 (31), the transcriptional corepressor c-terminal binding protein (32), the p53 inhibitor mouse double minute 2 (MDM2) (33), and the scaffold protein Axin (34), supporting a major role for HIPK2 in the regulation of apoptosis. In addition, we showed that HIPK2 physically interacts with HMGA1b in vitro and in vivo, but the HIPK2-mediated function apparently occurs regardless of this interaction (35).

Here we report that HMGA1 interferes with p53-induced apoptosis by counteracting its proapoptotic activator HIPK2. In particular, HMGA1 overexpression prevented the nuclear localization of HIPK2 and the phosphorylation of p53Ser46, while these events were restored by HIPK2 overexpression or HMGA1 downregulation by tumor cell treatment with HMGA1-specific antisense oligonucleotides. Consistent with a causal role for HIPK2 relocalization in the HMGA1-induced inhibition of p53 apoptotic activity, HIPK2 depletion by RNA interference suppressed the antiapoptotic effect of HMGA1 expression. In agreement with these data, immunohistochemical analyses of biopsy tissue from 69 human breast carcinomas showed significant association among HMGA1 overexpression, HIPK2 cytoplasmic localization, and low spontaneous apoptosis index in the presence of WT p53. Our data support the hypothesis that HMGA1 exerts its tumorigenic activity by forcing a cytoplasmic localiza-

tion of the p53 proapoptotic activator HIPK2, thus inhibiting p53 apoptotic function.

Results

HIPK2 is involved in the HMGA1/p53-mediated transcriptional regulation of p53 target genes. Results of our recent studies indicate that HMGA1 interferes with the p53-mediated transcription of the p53 effectors $p21^{waf1}$ and Bax while cooperating with p53 in the transcription of its inhibitor MDM2. We did not observe any detectable modification in the amount of p53 present on the *BAX* promoter analyzed by chromatin reimmunoprecipitation experiments upon HMGA1 overexpression (20). Thus, we asked whether other nuclear factors are responsible for the HMGA1 effects on p53 transcription. We concentrated our attention on HIPK2 for 2 main reasons: (a) HIPK2 contributes to p53 promoter selection by direct p53 phosphorylation at Ser46 and indirect p53 acetylation (28–30, 36, 37) and (b) HIPK2 binds HMGA1, and the functional role of this interaction is unknown (35). To evaluate whether HIPK2 expression affects HMGA1/p53-mediated transcription, we measured the activity of p53-dependent promoters in the presence of various combinations of p53, HMGA1, and HIPK2 proteins. p53-null H1299 cells were cotransfected with expression vectors encoding HMGA1 and p53 in combination or not with HIPK2 and with reporter vectors carrying the luciferase gene under the control of p53-responsive promoters *mdm2*, *BAX*, $p21^{WAF1}$, and *PIG3*. As expected from our recent observations (20), HMGA1 cooperated with p53 in the transcription of its inhibitor MDM2 (Figure 1A), while it repressed the p53-mediated transcription of the p53 effectors *BAX* and $p21^{WAF1}$ (Figure 1, B and C). No effect was observed on the *PIG3* promoter (Figure 1D). This HMGA1 activity was dependent on the presence of p53, because the sole expression of HMGA1 did not affect promoter activities (Figure 1). To test whether HIPK2 contributes to this transcriptional regulation, we coexpressed HIPK2 with p53 and HMGA1. HIPK2 dramatically increased the activation of the *mdm2* and *PIG3* promoters, but reverted the repressive effect exerted by HMGA1 on the *BAX* and $p21^{WAF1}$ promoters (Figure 1). Indeed, except for the $p21^{WAF1}$ promoter, the activation induced by coexpression of the 3 factors exceeded that induced by HIPK2 and p53 without HMGA1. This effect can be attributed to HIPK2 activity, because the kinase-dead HIPK2-K221R mutant did not affect or even rescue the HMGA1/p53 transcriptional activity. Taken together, these results indicate that HIPK2 reverts the inhibitory activity of HMGA1 on the p53 effector promoters and points to a complex interplay among p53, HIPK2, and HMGA1 in the regulation of p53–target gene expression.

HIPK2 reverts the effects of HMGA1 on p53 apoptotic function. We previously showed that the variation of p53–target gene transcription induced by HMGA1 overexpression was associated with reduction of p53 apoptotic activity (20). Because HIPK2 is a strong proapoptotic activator of p53 and its coexpression with HMGA1 modifies the HMGA1 activity on p53-mediated transcription, we examined whether HIPK2 counteracts the antiapoptotic effect of HMGA1. p53-null H1299 cells were transiently transfected with expression vectors encoding for HMGA1 or HIPK2, alone or in combination, and infected with a WT p53 recombinant adenovirus (Adp53) or an empty control virus (dl70.3). Expressions of the transduced proteins were determined by Western blot analyses (Figure 2A, lower panel). Cell death was measured by trypan blue exclusion (Figure 2A, upper panel) and TUNEL assay (Figure 2B), which revealed that HIPK2 overexpression inhibited the antiapoptotic activity of HMGA1 and induced a level of cell death comparable to that promoted by p53/HIPK2 coexpression. In order to evaluate whether these results can be reproduced in more physiological conditions such as in WT p53–carrying cells during cell stress, human colon carcinoma RKO cells were induced to either over- or underexpress HMGA1 by transfection of HA-HMGA1–encoding vector or HMGA1-specific antisense oligonucleotide.

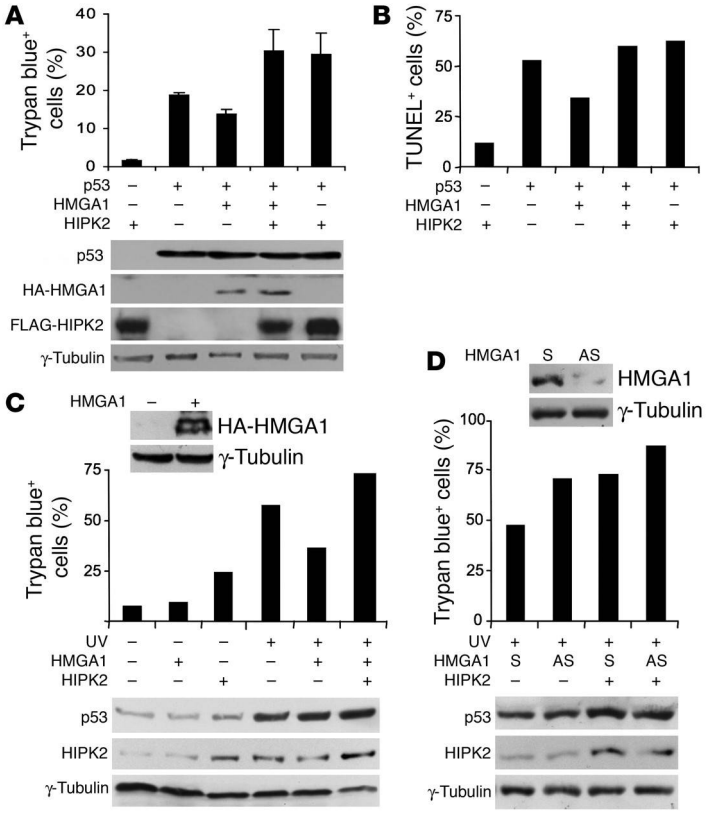

Figure 2

HIPK2 rescues the HMGA1 antiapoptotic effect on p53. (**A**) H1299 cells were infected with dl70.3 (– in the p53 row) or Adp53 (+ in the p53 row) viruses at 60 MOI. One hour after infection, cells were transfected with pCEFL-Hmga1 vector or pCEFL control vector and pEGFP-HIPK2 vector or pEGFP empty vector. After 48 hours, floating and adherent cells were collected and analyzed by trypan blue exclusion. Mean ± SD of 3 independent experiments are shown. Expression of the indicated proteins was analyzed by Western blot; the results of 1 indicative experiment of the 3 performed are reported. Expression of γ-tubulin shows equal loading of samples. (**B**) The same cells reported in **A** were analyzed by TUNEL assay. One indicative experiment is reported. (**C**) WT p53–carrying RKO cells overexpressing HA-HMGA1 protein or not, as detected by Western blot (upper panel), were transiently transfected with HIPK2 expression vector as indicated and subjected or not to UV light irradiation (50 J/m^2), and the percentage of cell death was measured by trypan blue exclusion 36 hours after irradiation. Expression of the indicated proteins was analyzed by Western blot; shown is 1 indicative experiment of the 3 performed. (**D**) RKO cells were transiently transfected with HMGA1-specific sense (S) or antisense (AS) oligonucleotides to reduce HMGA1 expression, as detected by Western blot (upper panel). Upon transfection of HIPK2 expression vector, cells were irradiated with UV light and analyzed as in **C**.

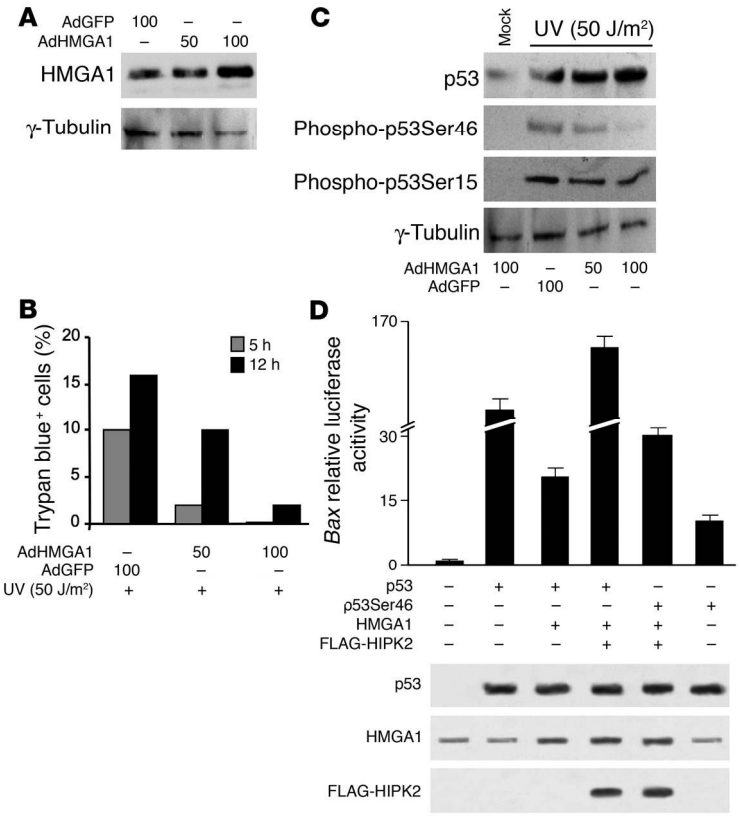

Figure 3
Contribution of p53 phosphorylation at Ser46 to HMGA1/HIPK2-induced *BAX* promoter activity. (**A**) Western blot analysis of the indicated protein in WT p53–carrying HCT116 cells 48 hours after infection at the indicted MOI. (**B**) The same cells analyzed in **A** were treated with UV light at 50 J/m². At 5 and 12 hours after UV light irradiation, the rate of cell death was measured by trypan blue exclusion. (**C**) The same cells treated and analyzed in **B** were harvested at 5 hours after UV light irradiation to perform Western blot of the indicated proteins. (**D**) p53-null H1299 cells were transfected with the indicated expression vectors, and luciferase activity was determined. All transfections were performed in duplicate; data are mean ± SD of 3 independent experiments. Empty vectors served as control. The expression of p53, HMGA1, and HIPK2 proteins was determined by Western blot.

Activation of endogenous p53 by UV light was associated with induction of cell death that, as expected from our previous results (20), was partially inhibited by HMGA1 overexpression. Consistent with the results obtained above by exogenous p53 expression, HMGA1-induced inhibition of cell death was completely overcome by HIPK2 (Figure 2C). In addition, in RKO cells transfected with HMGA1-specific sense or antisense oligonucleotides, HIPK2 overexpression increased cell susceptibility to UV light as well as HMGA1 depletion, and both HMGA1 depletion and HIPK2 overexpression further induced cell death (Figure 2D). Taken together, these results suggest that HIPK2 might be at least one of the targets for the antiapoptotic activity of HMGA1.

Phosphorylation of p53 at Ser46 is involved in the antiapoptotic activity of HMGA1 overexpression. Phosphorylation of human p53 at Ser46 determines promoter selection and induction of apoptosis (27, 28). Because HIPK2 phosphorylates p53 at Ser46 and the kinase-dead HIPK2-K221R mutant did not modify the p53-mediated transcriptional activity, we asked whether p53Ser46 phosphorylation is involved in the HMGA1-mediated inhibition of p53 activity. The WT p53–carrying HCT116 cells were infected with WT HMGA1 recombinant adenovirus (AdHMGA1) or control virus (AdGFP) at the same MOI (Figure 3A) and treated or not with an apoptotic dose of UV light. As expected from our previous data (Figure 2C and ref. 20), HMGA1 overexpression protected cells from apoptosis in a dose-dependent manner (Figure 3B). Interestingly, this protection was associated with reduced phosphorylation of p53Ser46 (Figure 3C). Comparable results were obtained in RKO cells (data not shown). No difference was observed in the total amount of p53 or in its phosphorylation at Ser15, a HIPK2-independent post-translational modification involved in p53 activation for either growth arrest or apoptotic functions (24, 25), which supported a direct role for the inhibition of the p53Ser46 phosphorylation pathway in apoptosis resistance induced by HMGA1 overexpression.

Next, we measured HMGA1/HIPK2-mediated transcription of the *BAX* promoter in the presence of the p53Ser46A mutant, which cannot be phosphorylated by HIPK2. The p53Ser46A mutant was much less efficient than WT p53 in activating the *BAX* promoter; however, HMGA1 coexpression still suppressed promoter activity while HIPK2 restored it (Figure 3D), suggesting that the HIPK2 kinase activity required to revert the effect of HMGA1 is not confined to the Ser46 phosphorylation of p53.

HIPK2 is required for the HMGA1-mediated repression of p53-induced apoptosis. To verify the requirement for HIPK2 in HMGA1-mediated repression of p53 apoptotic activity, expression of the endogenous HIPK2 protein was depleted by stable transfection of H1299 cells with a pSUPER vector carrying HIPK2- or β-gal–specific RNA-interfering sequences (see Methods). As shown in Figure 4A, HIPK2 expression was decreased in the H1299 HIPK2-interfered (HIPK2i) cells compared with that of control vector–transfected H1299 cells (β-Gali). The 2 populations were infected with Adp53 in the presence or absence of overexpressed HMGA1. As expected from previous data (37), HIPK2-depleted cells were more resistant to p53-mediated apoptosis (Figure 4B). However, when HMGA1 was overexpressed together with p53, the cells were protected from apoptosis only in the presence of endogenous HIPK2, while no protection was observed in the HIPK2-depleted cells (Figure 4B, upper panel). This protection was not caused by differences in the expression levels of the transduced proteins, since the amounts of p53 and HMGA1 were comparable in the 2 populations (Figure 4B, lower panel). To verify whether activation of the endogenous p53 protein can induce a similar outcome, WT p53–carrying RKO cells were stable transfected with the same pSUPER vectors described above, and HIPK2 depletion was verified by Western blotting (Figure 4C). Consistent with the results obtained by exogenous p53 expression, HMGA1 overexpression protected from UV light–induced apoptosis only in HIPK2-expressing cells (Figure 4D). These data indicate that HMGA1 represses the apoptotic activity of p53 by counteracting HIPK2.

HMGA1 exerts its antiapoptotic function by interfering with the nuclear localization of HIPK2. We previously showed that HMGA1 physically interacts with HIPK2 (35) and that HIPK2 colocalizes with p53 in the nuclear bodies and activates p53 apoptotic function

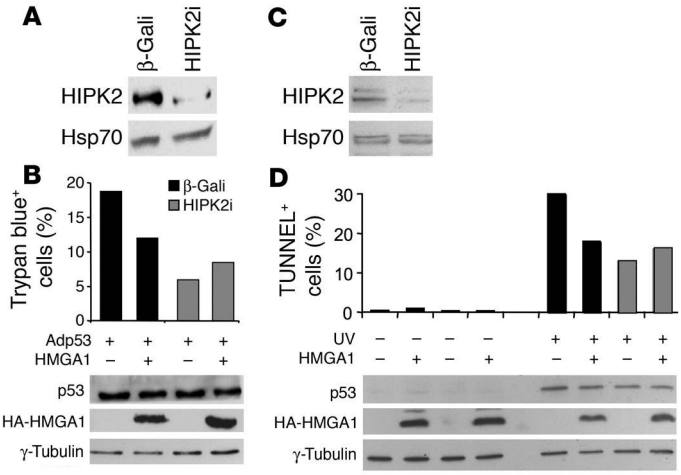

Figure 4
HIPK2 is required for HMGA-mediated repression of apoptosis. (**A**) Western blot analysis of endogenous HIPK2 in p53-null H1299 cells stably transfected with pSUPER-lacZ (β-Gali) or pSUPER-HIPK2 (HIPK2i) vectors. Heat shock protein 70 (Hsp70) was used as loading control. (**B**) The same cells used in **A** were infected with Adp53 and transfected with the pCEFL-Hmga1 vector or pCEFL empty vector. Cell death was measured as in Figure 2B, while TCEs from the same cells used in **B** were analyzed by Western blot as in Figure 2B for the indicated proteins. (**C**) Western blot analysis of endogenous HIPK2 in WT p53–expressing RKO cells stably transfected as in **A**. (**D**) The same cells used in **C** were transfected with HMGA1 and irradiated with UV light (50 J/m^2) to activate the endogenous p53. The apoptotic index was calculated by TUNEL assay.

(29, 30). Because HMGA1 inhibits p53-induced apoptosis by acting on HIPK2, we evaluated the interaction and the localization of HIPK2 in the presence or absence of overexpressed HMGA1. We confirmed the physical interaction between the 2 proteins (Figure 5A) and observed a dose-dependent cytoplasmic localization of HMGA1 (Figure 5, B and C) that was strongly associated with cytoplasmic relocalization of HIPK2 (Figure 5D). This subcellular distribution of HIPK2 depended on the relative ratio between HMGA1 and HIPK2, because HIPK2 overexpression restored HIPK2 in the nuclei (Figure 5, E and F). Consistently, specific antisense oligonucleotide depletion of HMGA1 on RKO cells, which endogenously express HIPK2, WT p53, and high levels of HMGA1 (Figure 6A), resulted in increased nuclear localization of endogenous HIPK2 protein, as assessed by Western blot analysis on separated nuclear/cytoplasmic cell extracts, while p53 remained in the nucleus in each condition (Figure 6B). Together with our observation that this specific antisense oligonucleotide treatment was also associated with increased UV light–induced apoptosis (Figure 2D and ref. 20), these results strongly suggest that HMGA1 inhibits p53 apoptotic function by interfering with the nuclear localization of the p53 activator HIPK2.

To confirm this hypothesis, enhanced GFP–HIPK2 (EGFP-HIPK2) or its kinase-dead EGFP-K221R mutant were expressed in RKO cells in the presence or absence of the nuclear export inhibitor leptomycin B (LMB) (38). LMB treatment promoted more robust nuclear compartmentalization of HIPK2 (Figure 6C) and stronger induction of apoptosis than did mock treatment (Figure 6D), supporting the notion that HIPK2 nuclear localization is necessary for p53 activation and function (39).

HMGA1 overexpression strongly associates with HIPK2 cytoplasmic localization in breast carcinomas. To evaluate whether the relationship between HMGA1 overexpression and HIPK2 subcellular localization defined in tumor cell lines can occur in vivo, we analyzed both HMGA1 expression and HIPK2 nuclear and cytoplasmic compartmentalization in 69 stage I and stage II breast cancer patients whose clinical characteristics have been described previously (40). As shown in Table 1, HMGA1 was overexpressed in 35 of 69 cases (51%) while HIPK2 was positive in 44 tumors (64%), with a cytoplasmic pattern of reactivity in 27 patients (39%) and a nuclear pattern in 17 (25%). Of interest, HMGA1 overexpression significantly correlated with the cytoplasmic compartmentalization of HIPK2 (i.e., in the HMGA1-positive samples, HIPK2 showed a cytoplasmic pattern in 57% of the cases and a nuclear one in 26%; $P < 0.0001$, χ^2 test). In addition, in the HMGA1-positive samples, the percentage of p53-positive (surrogate of mutant p53) breast cancers was 55% in patients presenting nuclear localization of HIPK2 and 37% in those presenting a cytoplasmic one (Figure 7A). To evaluate whether this correlation was associated with resistance to apoptosis, as would be predicted by our in vitro model, we performed a TUNEL assay on 4 selected groups of HMGA1-positive breast carcinomas (Figure 7, B and C) according to their cytoplasmic (Figure 7D) or nuclear (Figure 7E) positivity for HIPK2 and their p53 status. As summarized in Figure 7F, when HIPK2 was relocalized in the cytoplasm, we observed a low apoptotic index in both p53-negative (surrogate of WT p53) and p53-positive (surrogate of mutant p53) tumors (p53-negative, 2.79 ± 0.253; p53-positive, 2.32 ± 0.244). In contrast, when HIPK2 was localized in the nucleus, the apoptotic index was low only in the mutant p53-positive tumors (p53-positive, 2.76 ± 0.500; p53-negative, 9.00 ± 2.546), as expected. These results indicate that an association among HMGA1 overexpression, HIPK2 cytoplasmic localization, and resistance to apoptosis even in the presence of WT p53 can occur in vivo, at least in breast carcinoma.

Discussion

Defects in apoptosis are thought to play a major role in tumorigenesis as well as in tumor response to anticancer treatments (23). Recently, we reported that HMGA1, whose aberrant expression is implicated in the process of carcinogenesis, binds to the p53 oncosuppressor and inhibits its apoptotic activity (20). At the molecular level, this inhibition is associated with increased transcription of the p53 inhibitor MDM2 and repression of the p53 effectors *BAX* and *p21^{WAF1}* (20). These observations were subsequently confirmed and extended to members of the p53 family by Frasca and coauthors (41). In the present study, we investigated the mechanism through which HMGA1 modifies p53 transcriptional activity and inhibits apoptosis. We found that HMGA1 repressed p53 apoptotic activity by promoting the cytoplasmic relocalization of the p53 proapoptotic activator HIPK2.

HIPK2 physically and functionally interacts with p53 and activates its apoptotic function by specifically phosphorylating human p53Ser46 and mouse p53Ser58 (29, 30, 42). In particular, it was shown that p53Ser46 phosphorylation changes the affinity of p53 for different promoters with a shift from growth arrest–related genes to apoptosis-related ones (27, 28). HIPK2 promotes

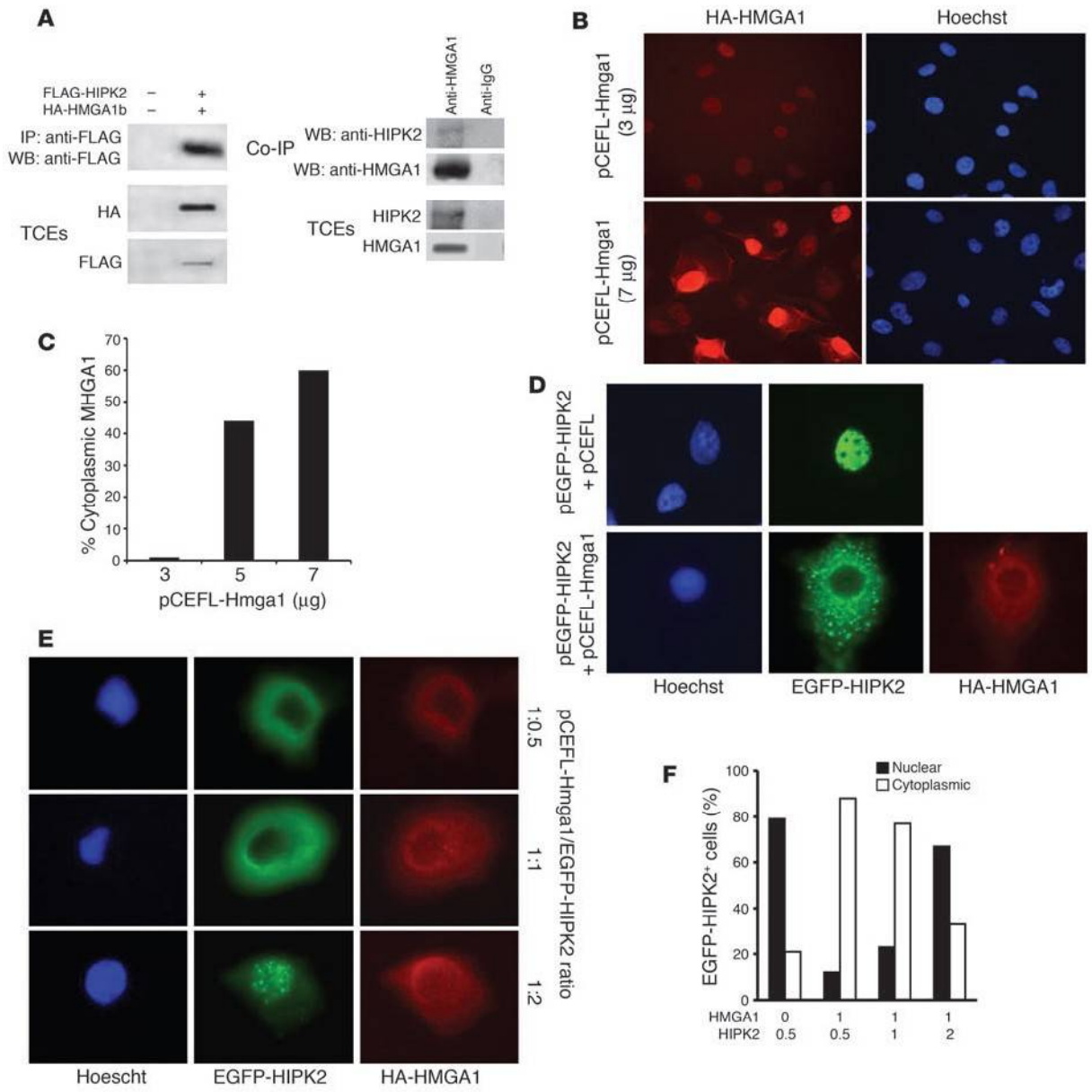

Figure 5

HMGA1 inhibits p53-induced apoptosis by interfering with HIPK2 nuclear localization. (**A**) For coimmunoprecipitation of exogenous proteins, H1299 cells were transfected as indicated and TCEs were immunoprecipitated with anti-HA Ab. Immunocomplexes were analyzed by Western blot (WB) as indicated. For coimmunoprecipitation of endogenous proteins, TCEs from H1299 cells were immunoprecipitated with anti-HMGA1 or anti-IgG as negative control and analyzed by Western blot. (**B**) H1299 cells were transfected with increasing doses of pCEFL-Hmga1. HA-HMGA1 expression was analyzed by immunofluorescence with anti-HA and tetramethylrhodamine isothiocyanate–conjugated Abs. Nuclei were stained with Hoechst. Images are from 1 representative experiment of the 3 performed. (**C**) H1299 cells were transfected and immunostained as in **B**; the percentage of HMGA1-positive cells with cytoplasmic staining was counted. (**D**) EGFP-HIPK2 subcellular localization in H1299 cells transfected with the indicated vectors. EGFP-HIPK2 is visible by its intrinsic green fluorescence. HA-HMGA1 expression was analyzed as in **B**. (**E**) H1299 cells were transfected with pCEFL-Hmga1 and pEGFP-HIPK2 expression vectors at the indicated molar ratios. EGFP-HIPK2 and HA-HMGA1 expression and their subcellular localization were analyzed by immunofluorescence as in **D**. (**F**) H1299 cells were transfected and immunostained as in **E**. The percentages of HMGA1-positive cells with nuclear or cytoplasmic staining of EGFP-HIPK2 were counted. Cells with a pCEFL-Hmga1 dose of 0 were cotransfected with pCEFL empty vector.

p53-mediated transcriptional activation of proapoptotic factors such as *BAX*, PIG3, Noxa, and p53-regulated apoptosis-inducing protein and repression of antiapoptotic factor such as Galectin-3 through phosphorylation of human p53Ser46 and mouse p53Ser58 (27–30, 42, 43). HIPK2 and p53 colocalize in the nuclear bodies together with their typical components promyelocytic leukemia protein (PML) and SP100 nuclear antigen (29, 30, 39, 44). A PML-independent, nuclear, dotted distribution of EGFP-HIPK2 has also been reported; however, HIPK2-dependent p53 phosphorylation and the subsequent p53-medi-

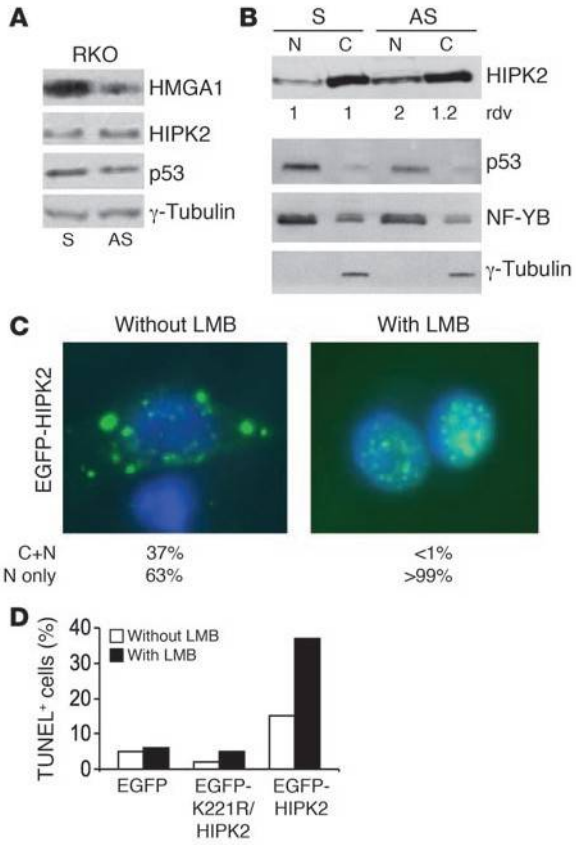

Figure 6
HIPK2 nuclear localization strongly increases HIPK2-induced apoptosis. (**A**) Western blot analyses of endogenous HMGA1, HIPK2, and p53 in WT p53–carrying RKO cells transduced with HMGA1-specific sense or antisense oligonucleotides. γ-Tubulin was used as loading control. (**B**) From the same cells as in **A**, nuclear (N) and cytoplasmic (C) extracts were prepared 36 hours after transduction and analyzed by Western blotting for the indicated proteins. NF-YB (48) and γ-tubulin were used as markers of nuclear/cytoplasmic separation as well as loading controls. Quantitative analysis was performed, and the relative density value (rdv) of HIPK2 was calculated as a ratio between HIPK2 and NF-YB in the nuclear extracts and between HIPK2 and γ-tubulin in the cytoplasmic extracts. (**C**) RKO cells were transfected with EGFP-HIPK2 vector and cultured in the presence or absence of 10 nM LMB for 16 hours. Subcellular localization of the EGFP construct was examined by fluorescence microscopy. Cells were simultaneously stained for DNA using Hoechst. The staining phenotype was categorized in 2 groups, 1 with nuclear HIPK2 staining only, and 1 with both nuclear and cytoplasmic staining. (**D**) RKO cells were transfected with the indicated EGFP vectors and treated with LMB as in **C**. At 20 hours after transfection, both floating and adherent cells were harvested to measure cell death by TUNEL assay.

ated activity requires the presence of PML (39), indicating that HIPK2 nuclear localization is necessary for p53 activation. Here we show that the nuclear localization of HIPK2 was inhibited by HMGA1 overexpression and restored by HMGA1 depletion or HIPK2 overexpression. In addition, forced nuclear localization of HIPK2 induced by the nuclear export inhibitor LMB strongly increased HIPK2-induced apoptosis.

We previously showed that HMGA1 interacts with HIPK2. However, the functional relevance of this interaction was unclear (35). The data we describe here show that an imbalance in the ratio between HMGA1 and HIPK2 resulting in HMGA1 overexpression elicited an antiapoptotic effect by inducing HIPK2 accumulation in the cytoplasm and impairing p53Ser46 phosphorylation. Conversely, overexpression of HIPK2 compared with that of HMGA1 reestablished HIPK2 nuclear localization and rescued the rate of p53-mediated apoptosis in the presence of HMGA1. This rescue was associated with recovery of p53 transcriptional activity on the *BAX*, *PIG3*, and *p21*^{WAF1} promoters as well as further activation of the *mdm2* promoter.

Besides *TP53* gene mutations, several mechanisms of p53 protein inactivation have been shown to contribute to tumor development, including p53 maintenance in the cytoplasm (45, 46). The finding that cytoplasmic localization of HIPK2 is determined by greater expression of HMGA1 than of HIPK2 strongly supports the idea that HMGA1 contributes to tumorigenicity by interfering with the tumor-suppressing activity of p53 through cytoplasmic localization of its activator HIPK2. This mechanism might account for the progression of neoplasms in which p53 apoptotic activity is impaired, notwithstanding the absence of p53 gene mutations and/or deletions. It is noteworthy that an analysis of 69 breast carcinoma patients showed a significant association among HMGA1 overexpression, cytoplasmic localization of HIPK2, and low spontaneous apoptosis index (comparable to that observed and described in tumors carrying mutant p53), supporting the hypothesis that in these tumors there is an inactivation of p53 apoptotic function mediated by HMGA1-dependent cytoplasmic localization of HIPK2.

In conclusion, our data strongly support the existence of what we believe to be a new mechanism of p53 inactivation through HMGA1-mediated cytoplasmic localization of the p53 activator HIPK2. This mechanism might have important implications in tumorigenicity as well as in the development of tumor resistance to antineoplastic treatments.

Methods

Cell culture, transfections, and transactivation assays. HCT116, RKO, and H1299 cells were maintained in DMEM supplemented with 10% fetal calf serum (GIBCO; Invitrogen), glutamine, and antibiotics. 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 previously described reporter vectors (20, 29) and normalized with the use of a cotransfected β-gal construct. Luciferase activity was analyzed by Dual-Light System (Applied Biosystems).

For inhibition of HMGA1 expression, antisense and corresponding sense oligonucleotides were designed and synthesized specifically by BIOGNOSTIK. Oligonucleotides were added once at a concentration of 2 mM. For inhibition of nuclear exports, cells were treated with 1 nM LMB for 18 hours.

Expression constructs. The pCAG-p53, pCAG-p53Ser46, pCMV-Hmga1, pCEFL–HA-HMGA1, pFLAG-HIPK2, pFLAG-K221R/HIPK2, pEGFP-HIPK2, and pEGFP-K221R constructs have been described previously (29, 35, 42).

Table 1
Relationship between HIPK2 and HMGA1 in samples from 69 breast cancer patients

HIPK2 expression	HMGA1-positive	HMGA1-negative	Total cases
Nuclear	11 (26%)	6 (22%)	17 (25%)
Cytoplasmic	24 (57%)	3 (11%)	27 (39%)
Negative	7 (17%)	18 (67%)	25 (36%)
Total cases	42	27	69

$P < 0.0001$, χ^2 test; $P < 0.005$, χ^2 test for trend. The latter test indicates how much of the association between HMGA1 and HIPK2 nuclear or cytoplasmic localization is accounted for by linear trend.

Western blotting and coimmunoprecipitation. Total cell extracts (TCEs) 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) or in nondenaturing buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.5% Triton; and 5 mM EDTA] for coimmunoprecipitation. Immunoprecipitation was carried out by incubating 1–3 mg of TCEs with Abs preadsorbed to protein G-agarose (Pierce Biotechnology). Immunocomplexes were collected by centrifugation, separated by SDS-PAGE, and blotted onto nitrocellulose membrane (Bio-Rad). Differential nuclear and cytoplasmic cell lysates were obtained as reported previously (47). Protein extracts were separated by SDS-PAGE and transferred to Immobilon-P Transfer membranes (Millipore). Membranes were blocked with 5% nonfat 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 (Amersham). The Abs used for Western blotting were as follows: anti-FLAG M5 mAb (Sigma-Aldrich); anti-HA 12CA5 mAb (Roche); anti-p53 DO1 mAb and anti–heat shock protein 70 polyclonal Ab (Santa Cruz Biotechnology Inc.); sheep anti-p53 polyclonal Ab (Ab-7; Calbiochem); rabbit anti–phosphorylated p53Ser46 polyclonal and rabbit anti–phosphorylated p53Ser15 polyclonal Abs (Cell Signaling Technology); anti–nuclear factor YB (anti–NF-YB) (48); anti-HMGA1 polyclonal Ab (raised against a synthetic peptide located in the NH2-terminal region; ref. 20); and anti-HIPK2 polyclonal Ab (30). Anti-γ-tubulin (Santa Cruz Biotechnology Inc.) was used for loading control.

Recombinant adenoviruses and adenoviral infection. The early genes 1 and 3-defective recombinant adenovirus dl70.3, Adp53 (49), and AdGFP and AdHMGA1 (19) were amplified and titrated on HEK293 cells as described previously (49).

Cell viability and TUNEL assays. Both floating and adherent cells were collected and counted in a hemocytometer after the addition of trypan blue. The percentage of dead cells (e.g., number of blue cells per total number of cells) was determined by scoring 100 cells per chamber 3 times. Cell numbers were determined in duplicate.

For TUNEL assay on in vitro cell lines, 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 mg/ml Hoechst 33258 for 2 minutes, and mounted with coverslip in 25% glycerol in PBS. At least 400 cells were counted in each plate.

For TUNEL assay on human breast cancer tissue, the fluorescent in situ detection of apoptosis in formalin-fixed, paraffin-embedded breast cancer tissues was performed using the MEBSTAIN Apoptosis kit II (IMMUNOTECH) according to the manufacturer's instructions. After deparaffinization and rehydration, 50 μl of terminal deoxynucleotidyl transferase–mediated nick end-labeling reaction mixture was applied to slides, which were then incubated at 37°C for 60 minutes. Sections were examined using a fluorescence microscope, and the number of apoptotic cells was counted in 8 high-power fields (original magnification, ×400) per section. Counts were averaged to determine the number of apoptotic cells.

Indirect immunofluorescence. Cells plated in 35-mm dishes were fixed in 2% formaldehyde in PBS and permeabilized in a solution of 0.25% Triton X-100 in PBS. Immunofluorescence was obtained with the anti-HA 12CA5 mAb (Roche) and the tetramethylrhodamine isothiocyanate–conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.). Cells were stained simultaneously for DNA with Hoechst 33342 before observation with a fluorescent microscope (Zeiss).

Plasmid construction for RNA interference and cell transfection. The pSUPER-β-gal and pSUPER-HIPK2 plasmids were constructed as reported previously (43), and Western blot analysis for STAT1 protein was used to exclude stimulation of IFN production in the stably transfected cell lines. The pSUPER-β-gal vector carrying an interfering sequence for the *LacZ* gene was used as a control.

Patients and tissue specimens. The 69 stage I, stage IIa, and stage IIb breast cancer patients (median age, 50 years; range, 30–76 years) included in this

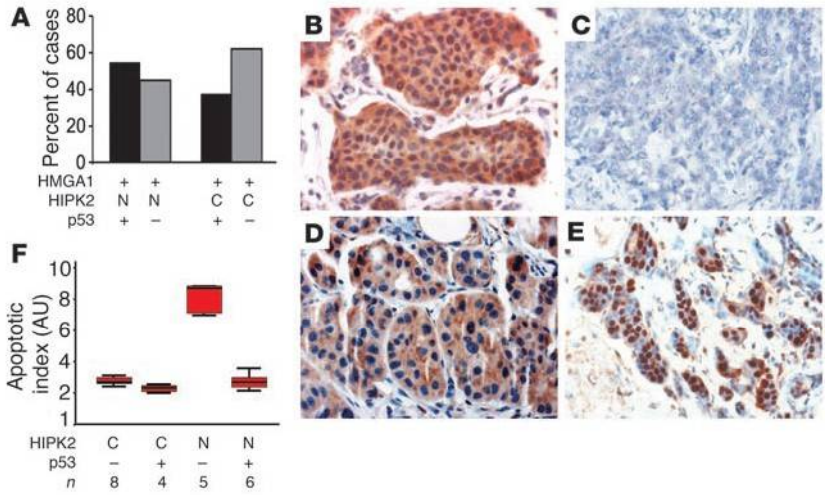

Figure 7
HMGA1 and HIPK2 immunostaining in breast cancer. (**A**) Histogram showing the distribution of p53 immunostaining in 44 tumor samples that expressed both HMGA1 and HIPK2. (**B–E**) Streptavidin-biotin immunoperoxidase staining of invasive breast ductal carcinomas displaying HMGA1 overexpression (**B**), HMGA1 absence (**C**), and distinct cytoplasmic (**D**) or nuclear (**E**) localization of HIPK2. Original magnification, ×40. (**F**) Quantification of apoptotic index in 4 groups of HMGA1 positive breast carcinomas according to their HIPK2/p53 phenotypes (8 cases: HIPK2 positive in the cytoplasm/p53 negative; 4 cases: HIPK2 positive in the cytoplasm/p53 positive; 5 cases: HIPK2 positive in the nucleus/p53 negative, and 6 cases: HIPK2 positive in the nucleus/p53 positive). The apoptotic index was counted in 10 fields per tumor evaluating the apoptotic index mean value in each group. The mean apoptotic index in HIPK2 nuclear-positive, p53-negative tumors was significantly higher than those of the other 3 groups ($P < 0.0001$; Bonferroni test). Lines represent median values, shaded boxes represent twenty-fifth and seventy-fifth percentiles, and whiskers represent minimum and maximum values. *Y* axis values indicate the mean value of the number of cells. Data are mean ± SD.

study were surgically treated at the Regina Elena Cancer Institute (40). This series included 58 invasive ductal carcinomas, 7 invasive lobular carcinomas, 2 tubular carcinomas, and 2 medullary carcinomas, of which 51 were T1 and 18 T2 and 50 were node-negative and 19 node-positive. Tumors were staged according to the Unione Internationale Contre le Cancer TNM system 2002, and graded according to Bloom and Richardson (50). The study was reviewed and approved by the ethics committee of the Regina Elena National Cancer Institute, and written informed consent was obtained from all patients.

Immunohistochemistry. Breast cancer specimens were fixed for 18–24 hours in 4% buffered formaldehyde and then processed through to paraffin wax. HMGA1, HIPK2, and p53 were evaluated by immunohistochemistry on 5-μm-thick paraffin-embedded tissues. Sections harvested on SuperFrost Plus slides (Menzel-Glaser) were deparaffinized, rehydrated, and pretreated in a thermostatic bath at 96°C for 40 minutes in 10 mM citrate buffer (pH 6). Polyclonal Abs directed against HMGA1 (diluted 1:50) and HIPK2 (1 μg/ml) and anti-p53 mAb purchased from DakoCytomation (clone DO7) were incubated for 60 minutes at room temperature. The reactions were revealed using Super Sensitive Link-Label Detection System purchased from Biogenex (Space), using 3-amino-9-ethylcarbazole (AEC substrate chromogen; DakoCytomation) as chromogenic substrate. All sections were slightly counterstained with Mayer hematoxylin and mounted in aqueous mounting medium (Glycergel; DakoCytomation). HMGA1 and HIPK2 proteins were considered overexpressed when more than 10% of the neoplastic cells presented a strong immunoreaction in the cytoplasm and/or in the nucleus. p53 was considered positive only when a distinct nuclear stain was observed in more than 10% of cancer cells. Evaluation of the immunohistochemical data was performed independently and in blinded manner by 2 investigators (M. Mottolese and A.D. Benedetto).

Statistics. The χ^2 test was used to test the relationship between HMGA1 overexpression and HIPK2 nuclear or cytoplasmic compartmentalization. A *P* value of less than 0.05 was considered statistically significant for both tests. The tests indicate how much of the association between HMGA1 and HIPK2 nuclear or cytoplasmic localization is accounted for by linear trend. To determine differences in apoptotic index among tumors according to HMGA1, HIPK2, and p53 immunohistochemical phenotypes, the Bonferroni test was used.

Acknowledgments

We are grateful to Laura Ciuffini and Francesca Puglisi for sharing their experiments on subcellular fractionations and to M. Lienhard Schmitz for the kind gift of the anti-HIPK2 polyclonal Ab. This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro, the Progetto Finalizzato "Biotecnologie" of the CNR, and MURST projects "Terapie antineoplastiche innovative" and "Piani di Potenziamento della Rete Scientifica e Tecnologica" from the Ministero della Salute as well as by EC FP6 funding (contract 503576). This publication reflects the authors' views and not necessarily those of the European Union. The EU is not liable for any use that may be made of the information contained herein. We thank the Associazione Partenopea per le Ricerche Oncologiche (APRO) for its support. Cinzia Renaldo is recipient of a FIRC fellowship.

Received for publication July 25, 2006, and accepted in revised form December 5, 2006.

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RESEARCH ARTICLE

Identification of new high mobility group A1 associated proteins

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High mobility group A (HMGA) proteins (HMGA1a, HMGA1b, HMGA1c and HMGA2) are nonhistone chromosomal proteins that do not have transcriptional activity *per se*, but they orchestrate the assembly of multiprotein complexes involved in gene transcription, replication and chromatin structure through a complex network of protein–DNA and protein–protein interactions. To better understand their mechanisms of action, we have used a combination of coimmunoprecipitation, 1-D gel SDS-PAGE and MS to identify new potential molecular interactors. We have found 11 proteins that associate with HMGA1. These proteins belong to three different classes: mRNA processing proteins, RNA helicases and protein chaperones. Some interactions were confirmed by coimmunoprecipitation and pull-down experiments in human embryonal kidney 293 cells. These experimental data suggest that HMGA1 proteins can associate with proteins that are strictly involved in chromatin structure and in several important mRNA processing steps, supporting the idea that HMGA1 proteins can also participate in these events.

Received: January 16, 2007
Revised: June 5, 2007
Accepted: July 25, 2007

Keywords:
High mobility group A1 proteins / New interactors / Tandem mass spectrometry

1 Introduction

The mammalian high mobility group A (HMGA) nuclear proteins are a family of nonhistone chromatin-associated proteins. This family consists of four known members: HMGA1a, HMGA1b and HMGA1c, derived from an alternative splicing of the same gene [1, 2], and HMGA2, encoded by a different gene [3]. The HMGA proteins are characterized by the presence of three 'AT hook' motifs that bind to the minor groove of stretches of AT-rich region of DNA [4]. They participate in a wide variety of cellular processes, including regulation of chromatin architecture and gene transcription control, by serving as generalized chromatin effectors, either enhancing or suppressing the ability of more usual transcriptional activators and repressors to act within the confines of chromatinized DNA [5]. In fact, HMGA1 proteins are involved in specific protein–DNA and protein–protein interactions that induce structural changes in chromatin, and are implicated in the formation of complexes called 'enhanceosomes' on promoters and enhancers of several

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Abbreviations: FA, formic acid; **HA,** hemagglutinin; **HMGA,** high mobility group A; **TCE,** total cellular extract

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genes whose transcription they regulate [6]. Enhanceosome formation serves as a stereospecific platform to recruit RNA polymerase II and other cofactors necessary for transcription initiation. HMGA proteins could also regulate the transcription by binding the AT-rich sequences called 'scaffold attachment regions' (SARs) and promoting an 'open' chromatin domain structure that is permissive for transcription, or by direct interaction with nucleosomes [7].

HMGA proteins seem to play an important role during embryonic development [8]. In fact, their expression is high during embryogenesis and low or absent during the adult life [9]. HMGA proteins are also expressed at high level in many human malignant neoplasias [10–17], and their expression is correlated to the acquisition of the neoplastic phenotype [18–20].

Despite the wealth of data associating aberrant or overexpression of HMGA proteins with neoplastic transformation, still little is known about the molecular mechanisms by which these proteins exert their effect. The identification of new HMGA associated proteins could somehow help to clarify their function. Proteomic represents a valuable approach to identify new potential interactors. Therefore, to gain insights into the functions fulfilled by HMGA1 proteins, we have developed a proteomic analysis to identify new HMGA1 interactors. Coimmunoprecipitation of HMGA1 associated proteins followed by MS analysis allowed us to identify 11 potential HMGA1 interactors. These proteins are mainly involved in several aspects of RNA secondary structure and metabolism, including transcription and pre-mRNA splicing, revealing an important role of HMGA1 proteins also in chromatin remodelling finalized to mRNA processing.

2 Materials and methods

2.1 Cell culture, expression constructs and transfection

Human embryonal kidney (HEK) 293 cells were maintained in DMEM, supplemented with 10% FCS (GIBCO-BRL, Life Technologies, Gaithersburg, MD), 2 mM L-glutamine, 10 U/mL penicillin and 10 ng/mL streptomycin at 37°C under 5% CO_2 atmosphere.

pHemagglutinin (pHA)-tagged *Hmga1b* expression plasmid was obtained amplifying and inserting the murine *Hmga1b* cDNA into the pCEFL-HA expression vector (pCEFL-HA/*HMGA1b*) [21].

To construct the GST fusion genes, the entire and/or portions of *Hmga1a* and *Hmga1b* coding sequences were amplified by PCR with pairs of primers linked to restriction sites (*EcoR*I and *BamH*I), and cloned in the pGEX-2T plasmid (Promega): pGST-HMGA1a (1–107) is constituited by the entire coding sequence; pGST-HMGA1b (1–96) is constituited by the entire coding sequence; pGST-HMGA1b (1–54) is constituted by the first 54 amino acids including the first two AT-hook domains; pGST-HMGA1b (55–96) is constituted by the last 42 amino acids including the last AT-hook domain. The GST-RFG fusion protein was generated by cloning the entire RFG cDNA in the pGEX-2T plasmid (Promega) [22].

HEK293 cells were transiently transfected with 5 μg of plasmid by lipofectamine-plus reagent as suggested by the manufacturer (Invitrogen).

2.2 Samples preparation, immunoprecipitation and Western blotting

For preparative gels, 8×10^7 cells were transfected with 50 μg of empty vector or with pHA-tagged *Hmga1b* expression plasmid. For total cellular extract (TCE) preparation, cells were harvested 48 h after the transfection. Cells were then lysed in 7 mL of RIPA buffer (50 mM Tris HCl pH 7.5, 5 mM EDTA, 300 mM NaCl, 150 mM KCl, 1 mM DTT, 1% NP-40 and a mix of protease inhibitors), and clarified by ultracentrifugation at $200\,000 \times g$ at 4°C for 30 min. For immunoprecipitation experiments, 10 mg of TCE was incubated with 14 μg of anti-HA antibodies for 3 h, and then supplemented with protein A-Sepharose beads (Pharmacia Biotech). One hour later, the beads were collected and washed five times with lysis buffer, boiled in 150 μL of Laemmli sample buffer, and 9/10 of this sample were then treated as follows in Section 2.5. Conversely, for analytical gels, 1 mg of TCE was incubated with 1,4 μg of anti-HA antibodies for 3 h, and then supplemented with protein A-Sepharose beads (Pharmacia Biotech). One hour later, the beads were collected, washed five times with lysis buffer, and boiled in 15 μL of Laemmli sample buffer. For immunoblotting analysis, 50 μg of TCE or immunoprecipitated proteins were separated by SDS-PAGE and transferred to Immobilon-P Transfer membranes (Millipore). Membranes were blocked with 5% nonfat milk proteins and incubated with the appropriate antibodies at the appropriate dilutions. The filters were then incubated with horseradish peroxidase-conjugated secondary antibodies, and the revelations were performed by ECL method (Amersham Biosciences).

2.3 Pull-down assay

The pGST-HMGA1a (1–107), pGST-HMGA1b (1–96), pGST-HMGA1b (1–54), pGST-HMGA1b (55–96) and pGST-RFG constructs are described above. GST fusion proteins were produced in *E. coli* BL21 cells. Stationary phase cultures of *E. coli* cells transformed with the plasmid of interest were diluted ten times in LB with ampicillin (100 mg/mL), grown at 30°C to an OD_{600} of 0.6, and induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After 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 PMSF and protease inhibitors (Boehringer). The cells were broken by French Press and centrifuged at $5000 \times g$ at 4°C for 30 min. The supernatants were

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then incubated at 4°C for 1 h with 250 μL of glutathione-Sepharose beads (Amersham Pharmacia Biotech). The resins were washed with PBS and protease inhibitors. The GST fusion proteins were incubated with 200 μg of TCEs from parental 293 cells in NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 0.5% NP-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.

2.4 Antibodies

The antibodies used for immunoprecipitation and Western blotting were: anti-p68 monoclonal Ab (Pab 204), gently provided from F. V. Fuller-Pace; anti-HA 12CA5 mAb from Roche; antihuman FUS/TLS from Euromedex (Mundolsheim, France); anti- β-1 tubulin (G-8), anti-Hsc71 and anti-γ-tubulin (D10) from Santa Cruz (California, USA); anti-DDX1 from BD Biosciences (New Jersey, USA); anti-DDX17 and anti-TAFII68 from Bethyl (Texas, USA); anti-HMGA1b polyclonal Ab was raised against a synthetic peptide located in the NH_2 terminal region [23].

2.5 Proteomic analysis

All reagents for the proteomic analysis were of the highest purity available. Water was purified using a MilliQ system (Millipore, Bedford, MA); acetic acid (AcOH), iodoacetamide and formic acid (FA) were from Fluka (Buchs, Switzerland); methanol was from Merck (Darmstadt, Germany); ACN was from Biosolve (Valkenswaard, The Netherlands); Tris® and SDS were from Invitrogen (Carlsbad, CA) and Biosafe, respectively; Blue coomassie was from BioRad (Hercules, CA); trypsin proteomic grade and DTT were from Sigma–Aldrich. Proteins used as molecular mass references (high molecular weights) were from Amersham Biosciences (Piscataway, NJ). The proteins immunoprecipitated as mentioned above were separated by SDS-PAGE on a 8% polyacrylamide gel under well defined conditions with 550 V·h [24], and then revealed by staining with Blue coomassie R250 Biosafe (BioRad). The coomassie dye bonds to arginine and aromatic amino acids, and any dye, that is not bound to proteins, diffuses out of the gel during the destaining step. The proteins were fixed by incubating the gel in a solution containing 30% methanol and 7.5% AcOH for 1 h, and washed three times in water. Gel was then incubated in Blue coomassie overnight, and background staining was removed by five washes in water. Gel was stored at 4°C in 1% AcOH. From the 1-DE polyacrylamide gel, the bands were excised and subjected to in-gel tryptic digestion. Protein digestion was conducted as previously described [25], modified as described below. Briefly, the gel pieces were destained in 100 μL of 50 mM ammonium bicarbonate, and 50% ACN at room temperature. The gel slices were dried in neat ACN before performing cysteines reduction with DTT and alkylation with iodoacetamide. Pieces of gel were reswollen in 20 μL of 20 mM ammonium bicarbonate and 6.25 ng/μL of trypsin from pig (Sigma, St. Louis, MO, USA; type IX-S, ref T-0303). After overnight incubation at room temperature, the supernatant was removed before the addition of 1% FA solution in water. This solution was pooled with the previous supernatant. A second extraction using 50% ACN was performed to improve peptide material recovery and then pooled with the previous supernatant. The extracted solution was partially dried by Speed-Vac for a final 2–5 μL volume.

2.6 LC-MS/MS

Peptide separations were carried out on an Ultimate HPLC pump coupled to a Famos autosampler (both from Dionex/LC Packings, Amsterdam, Holland). MS data were acquired on a QTRAP 2000 triple quadrupole-linear IT mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Ontario, Canada). The Analyst 1.4 software controlled both the mass spectrometer and the HPLC system. Peptides separation was carried out on a 75 μm id × 15 cm commercial column packed with C18 PepMap material (100 Å, 3 μm particles size, Dionex). Separations were conducted using a 45 min HPLC cycle corresponding to a 25 min Gradient from 1% A (1% ACN, 0.1% FA, 98.9% water) up to 80% B (80% ACN, 0.1% FA, 19.9% water). Solvents were continuously degassed using helium bubbling. A calibrator cartridge (NAN-75, Dionex) was used to split the micropump flow to obtain a final flow rate of 200 nL/min on the RP column. The Q-Trap instrument was tuned for peptide and fragment mass accuracy before its utilization for this study using the methods and consumables defined by the manufacturer. The collision energy is dynamically calculated as a linear function of the precursor ion defined by a slope and an intercept for each ion charge state. Each MS cycle was including an enhance MS (EMS) survey scan in 400–1400 m/z range, an enhancement resolution (ER) scan range of 10 m/z and two enhanced product ion (EPI) scans ranging between 100 and 1800 m/z. After the two cycles of fragmentation, each precursor ion was automatically excluded for 60 s from the successive MS cycles.

2.7 Data processing and database searches

For the export of raw CID spectra in a format compatible with database search by MASCOT, a script provided by the manufacturer was used (Mascot.dll, ver. 1.6b5, Matrix Science, London). CID spectra for precursors less than 1.0 Da apart were grouped if they occurred within the ten following cycles. Spectra were rejected if they contained less than ten peaks with a precursor intensity lower than 50 counts. An on-site biprocessor version of the MASCOT protein identification software (version 1.9) was used for this data analysis. The peptides are not redundant matched peptides and manually verified. All data treatments were conducted with the optimized parameters including the enzyme specificity, *i.e.* trypsin, with cleavage when K or R is followed by P, fixed

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cysteine modification (carbamidomethylation) and variable modifications of methionine (monooxidized methionine) and asparagine/glutamine (formally 'deamidation', but due to deisotoping artefact during data extraction). The Human subset of the merged Swiss-Prot/TrEMBL database version 41.0 and 24.0, respectively was used for searches. No protein mass restriction was used, and all protein identifications were manually validated.

3 Results and discussion

3.1 Proteomic analysis of HMGA1 interactors

In order to isolate and identify new HMGA1 interacting proteins, we employed proteomic analysis. To this aim, an expression vector coding for the full-length *Hmga1b* cDNA tagged with the influenza virus HA epitope was transiently transfected in human embryonic kidney 293 cells. The expression of HA-HMGA1b protein was verified by Western blotting analysis using both anti-HA and anti-HMGA1b antibodies (Fig. 1A). About 10 mg of TCE was then utilized for the proteomic analysis. In particular, proteins associated with HA-HMGA1 were purified by coimmunoprecipitation using anti-HA antibodies. Preliminary SDS-PAGE experiments showed a large number of protein bands gathered in the high molecular region of the gel. Therefore, the immunoprecipitated material was fractionated on an 8% 1-D gel to enhance the resolution at high molecular weight. Proteins were stained with Blue coomassie as shown in the Fig. 1B. Two different standard molecular weights were loaded on the gel (lanes 1 and 2 of the Fig. 1B). The comparison between the third lane, corresponding to the immunoprecipitated lysate of empty-vector overexpressing cells, and the fourth lane, which corresponds to that of HA-HMGA1b overexpressing cells, allowed us to identify several bands which were present exclusively in the immunoprecipitated sample of HA-HMGA1b overexpressing cells. Then, the gel was sequentially cut in several fragments, indicated by square brackets from A to F in Fig. 1B. Proteins contained in each fragment were digested inside the gel with trypsin, and the extracted material was analysed by RP-HPLC ESI-MS/MS. Acquired data were converted for protein identification using the MASCOT software against the human subset of Swiss-Prot and TrEMBL databases. All data treatments were conducted with optimized parameters, and all protein identifications were manually validated. Among the gel slices digested, only six led to the identification of 11 different

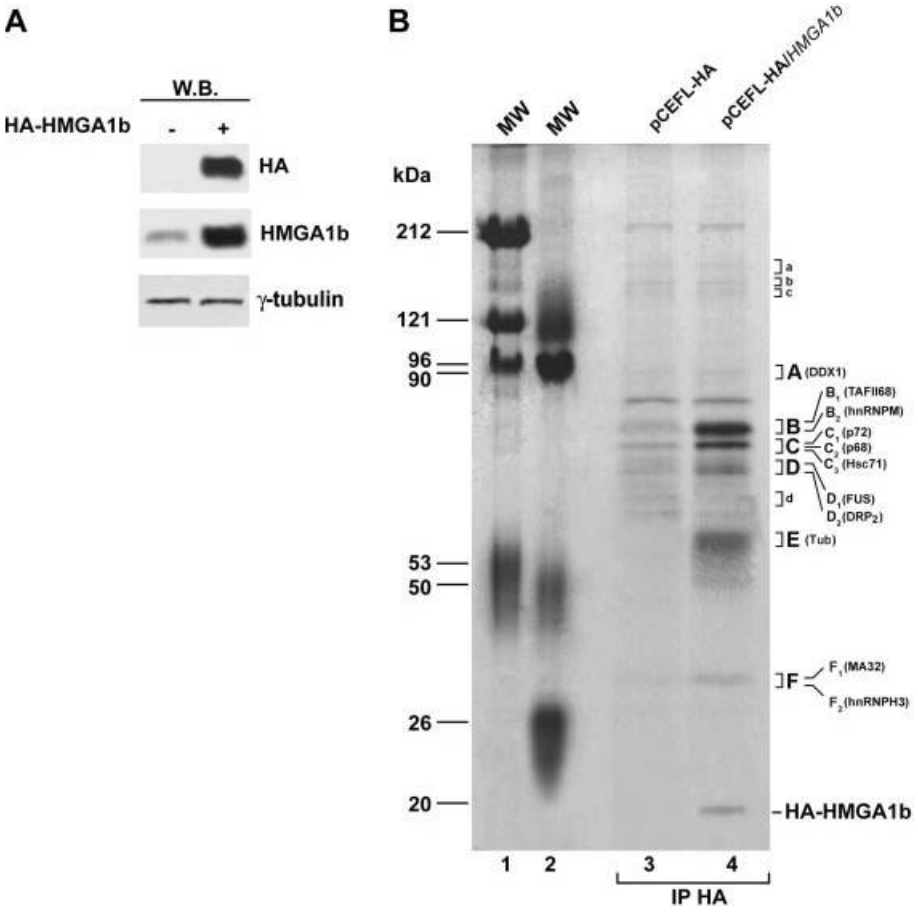

Figure 1. Analysis of HMGA1 interactors separated by SDS-PAGE. (A) TCEs derived from pCEFL-HA empty vector or pCEFL-HA/*HMGA1b* overexpressing cells were analysed by Western blotting with anti-HA and anti-HMGA1b antibodies. γ-Tubulin was used to equalize protein loading. (B) Equal amount of total cellular proteins derived from control cells (lane 3) and from HA-HMGA1 overexpressing cells (lane 4) were immunoprecipitated with anti-HA antibodies, separated by SDS-PAGE on an 8% polyacrylamide gel, and stained with Bio-Safe Blue coomassie. The gel was sequentially cut, and each cut, that had MS successful result, was labelled. The position of the fragments that have had success in spectrometric analysis are indicated by square brackets on the right side of the picture from A to F. The other gel slices, indicated on the right side of the picture from a to d, did not lead to a significant protein identification. Two different molecular masses of known proteins are loaded in the lanes 1 and 2.

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proteins corresponding to 50 polypeptides (Fig. 1B). The final results are summarized in Table 1, where the names and Swiss-Prot/TrEMBL accession numbers of the identified proteins and their function are reported. It was not surprising that each excised band from 1-DE gel separation contained more than one protein. The other gel slices, indicated from 'a to d' on the picture did not lead to significant protein identification (Fig. 1B). In several cases, other peptide sequences were not sufficiently discriminant to identify a single protein but they gave only information about protein family. Moreover, peptides corresponding to heterogeneous nuclear ribonucleoproteins M and H3 (hnRNP M and hnRNP H3), tubulin beta-1 chain and heat shock cognate 71 (hsc71), demonstrated to be HMGA1 molecular partners by other groups [26], were also found in our study (Table 1).

3.2 *In vivo* and *in vitro* characterization of HMGA1 interactors

To validate the interactions between HMGA1 proteins and some of the new identified associated proteins *in vivo*, TCEs from 293 cells transfected with the pCEFL-HA empty vector or with the full-length *Hmga1b* cDNA tagged with the influenza virus HA epitope were immunoprecipitated with the antibodies against p68 helicase, FUS and DDX1. The following filters were then immunoblotted with anti-HA antibodies. As shown in Fig. 2A, left panel, a great amount of HMGA1b protein was present in all of the immunocomplexes from 293 cells overexpressing HA-HMGA1b protein, confirming that HMGA1 and the indicated proteins really interact each other. Coimmunoprecipitations with isotype-matched IgG were also performed as control of the specificity of the interactions.

To examine the effective amount of the molecular interactors identified in 293 cells, we have evaluated their protein levels in empty vector- or HA-HMGA1b- expressing cells by Western blotting analysis performed with the appropriate antibodies. As shown in the Fig. 2A, right panel, the amount of each interactor is independent from HMGA1 expression. Moreover, we have confirmed the HA-HMGA1b expression level in transfected 293 cells by Western blotting using anti-HA antibodies. These experiments have demonstrated that the interactions, evaluated through proteomic analysis, are real, and that HMGA1 proteins interact, at least in this cellular system, with the new identified proteins.

To further examine the specificity of these interactions, and to map the regions of HMGA1b protein required for the binding to its molecular interactors, pull-down assays were performed incubating a total lysate deriving from 293 cells with the HMGA1b recombinant protein fused to GST and with two HMGA1b-deletion mutants: GST-HMGA1b (1–54) and GST-HMGA1b (55–96) (scheme in Fig. 2B). As shown in the Fig. 2C, upper panel, all the interactors, with the exception of Tubulin β-1, bind the C-terminal end of the HMGA1b protein. TAFII68, DDX1 and, in a smaller amount, DDX17, are able to also bind the NH_2-terminal region of HMGA1b protein. Tubulin β-1 chain, conversely, is able to only bind the HMGA1b NH_2- terminal region (Fig. 2C, upper panel). Finally, we have also analysed the interactions between the long HMGA1a isoform, which differs from the short isoform

Table 1. HMGA1 interactors identified by MS analysis

Gel fragment	Protein description	Swiss-Prot Acc. No.	Function	Score (MASCOT)	# Matched peptides	% of recovery
A	ATP-dependent helicase (DDX1)	Q92499	ATP-dependent helicase	286	8	13
B_1	Heterogeneous nuclear ribonucleoprotein M (hnRNP M)	P52272	Processing events of pre-mRNAs	133	4	8
B_2	TATA-binding protein associated factor 2N (RB56) (TAFII68)	Q92804	Transcription initiation	115	5	10
C_1	Probable RNA-dependent helicase p72 (DDX17)	Q92841	RNA-dependent ATP-ase helicase	357	7	13
C_2	Probable RNA-dependent helicase p68 (DDX5)	P17844	RNA-dependent ATP-ase helicase	266	6	11
C_3	Heat shock cognate 71 kDa Protein	P11142	Chaperone	129	3	6
D_1	RNA-binding protein FUS	P35637	Annealing of ss DNAs and D-loop formation in superhelical ds	130	3	8
D_2	Dihydropyrimidinase related protein-2 (DRP-2)	Q16555	DNA Hidrolase activity	57	2	5
E	Tubulin beta-1 chain	P07437	Constituent of micro-tubules	107	3	7
F_1	Complement component 1 (MA32)	Q07021	Splicing	223	3	10
F_2	Heterogeneous nuclear ribonucleoprotein H3 (hnRNP H3)	P31942	Processing events of pre-mRNAs	131	2	9

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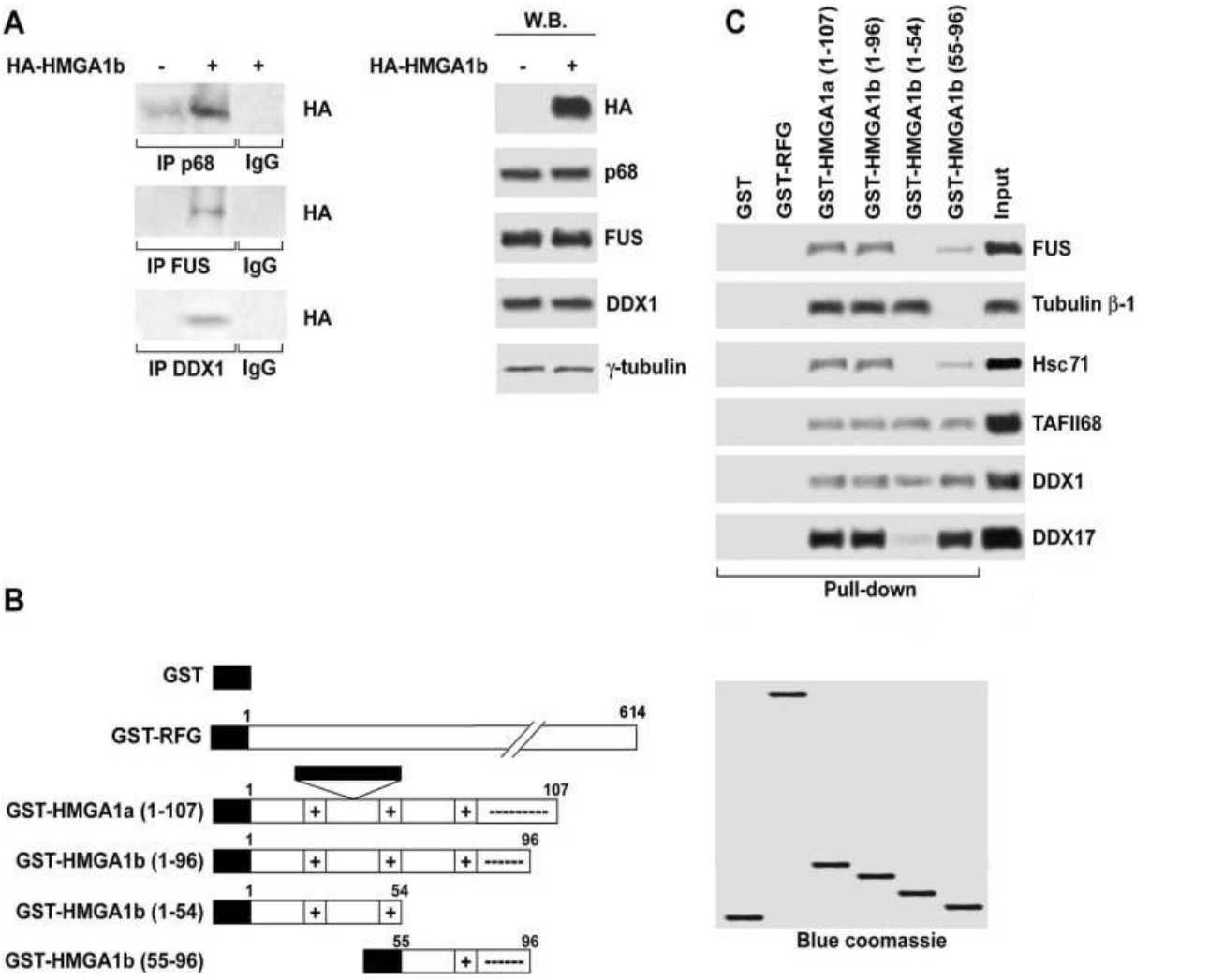

Figure 2. *In vivo* and *in vitro* interactions between HMGA1 proteins and MS identified interactors. (A) *In vivo* interactions by coimmunoprecipitation assay between HMGA1b protein and some identified interactors. Two hundred and ninety three cells were transfected with empty vector or with pCEFL-HA/*Hmga1b* expression vector. Forty-eight hours post-transfection, TCEs were prepared and equal amount of proteins were immunoprecipitated with anti-p68, anti-Fus, anti-DDX1. The immunocomplexes were then analysed by Western blotting using anti-HA antibodies (left panel). Coimmunoprecipitations with isotype-matched IgG were also performed as negative control for the specificity of the interactions. The expression level of the HA-HMGA1b protein and its interactors was detected by Western blotting using specific antibodies as indicated. γ-Tubulin was used to equalize protein loading (right panel). (B) Schematic representation of GST fusion proteins used in the pull-down assay. (C) GST pull-down assays of the indicated GST-fusion proteins with the endogenous cellular extracts from 293 cells. The relative inputs are TCEs derived from 293 cells. The bound complexes were separated by SDS-PAGE, and the filter was incubated with the relative antibodies (upper panel). Gel was stained with Blue coomassie to show that equal amount of GST-fusion proteins were used in the assay (lower panel).

HMGA1b only for the deletion of 11 amino acids between the first and the second AT-hook domains. To this aim, TCE from 293 cells was incubated with the GST-HMGA1a fusion protein. As shown in Fig. 2C, upper panel, the HMGA1a isoform is also able to bind all the identified interactors of the short HMGA1b isoform. No interacting proteins were detectable in the complexes obtained incubating TCE with the GST protein alone or with an unrelated GST fusion protein such as GST-RFG (Fig. 2C, upper panel).

3.3 Functional classification of the HMGA1 interactors

3.3.1 DEAD-box proteins

Among the RNA chaperones, that ensure the correct folding of RNA molecules, there are the RNA helicases of the DEAD-box family and related families. The term RNA helicase refers to enzymes that locally unwind complex RNA struc-

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tures, an activity that is associated with the hydrolysis of a nucleoside triphosphate (NTP), preferentially ATP [27]. DEAD represents the one letter code for the tetrapeptide Asp-Glu-Ala-Asp. DEAD box proteins modulate RNA secondary structure in all cellular processes involving RNA, including transcription, pre-mRNA processing, ribosome biogenesis, RNA export, translation initiation and RNA degradation [28, 29]. Several DEAD box proteins have been shown to bind HMGA1 proteins in this study, such as DDX1, p68 and p72. DDX1 is a human DEAD box protein that also contains a region with homology to heterogeneous nuclear ribonucleoprotein U (hnRNP U) [30], which mediates the binding of TFIIH to the RNA polymerase II holoenzyme [31].

p68 (DDX5) [32] and p72 (DDX17) [33] are highly related members of the DEAD box family, and are 'established' RNA helicases. They have been implicated in growth regulation and in both pre-mRNA and pre-rRNA processing. These proteins interact with p300/CBP coactivator, with the RNA polymerase II holoenzyme [34], and also with the transcriptional repressor histone deacetylase I (HDACI) [35]. Moreover, p68 potently synergizes with the p53 tumour suppressor protein to stimulate transcription of p53-dependent promoters, acting as coactivator of p53-dependent apoptosis [36]. All these data support the role of p68 and p72 helicases as transcriptional regulators [35]. The recent reports that HMGA1 proteins interact with p53 [23] and modulate HDAC1 activity [37], and the fact that CBP/p300 is able to interact and to acetylate HMGA1 proteins [38], strongly support the validity of the interactions between HMGA1 proteins and p68 or p72 helicases, found by our proteomic approach. These findings suggest that HMGA1 proteins could be involved not only in basal transcriptional activity, but also in post-transcriptional mechanisms, supporting the idea, also deriving from other studies [26], that implicates HMGA proteins in the participation of the spliceosome complex.

3.3.2 FUS/TLS and TAFII68

TLS, also known as FUS, is the product of a gene commonly translocated in liposarcomas (*TLS*), and it is prototypical of a class of nuclear proteins that contains a C-terminal domain with a distinct RNA recognition motif (RRM) surrounded by Arg-Gly-Gly (RGG) repeats [38]. TLS and the related protein TAFII68/RB56 [39, 40], also identified in our study as potential interactor of HMGA1 proteins, have other features that distinguish them from the most heterogeneous ribonuclear proteins (hnRNPs). In particular, the NH2-terminal region is different from other RNP-carrying proteins; this region could serve as a transcriptional activation domain, and it is able to directly interact with components of the transcriptional machinery, such as TFIID and RNA polymerase II [41].

3.3.3 Other molecular interactors

Among the identified interactors in this work, four of them, different from the above described, have recently been identified as molecular partners of HMGA1 proteins by other group [26]. They are: the heat shock cognate 71 kDa protein (hsc71), the heterogeneous nuclear ribonucleoproteins H3 and M (hnRNP H3 and hnRNP M), and tubulin β-1 protein. Hsc71 is mainly involved in chaperoning and protein folding [42]; hnRNP H3 and hnRNP M are involved in splicing process, and, in particular, in early heat shock-induced splicing arrest [43]; tubulin β-1 is one of the most common member of the tubulin family, which makes up microtubules [44].

We have also identified as interactors DRP-2 and MA32 proteins. The dihydropyrimidinase related protein 2 (DRP-2) presents a hydrolase activity, and it is an important molecule in guiding neuronal development [45]; the complement component 1 (MA32) is so named for its characteristic to bind the globular heads of C1Q inhibiting C1 activation and is a subunit of human pre-mRNA splicing factor SF2 [46], which prevents exon skipping, ensures the accuracy of splicing and regulates alternative splicing. Therefore, these other HMGA1 interactors confirm a role of HMGA1 proteins in post-transcriptional processes.

4 Concluding remarks

HMGA proteins are chromatinic proteins which act as architectural factors, and play a general role modulating gene expression in different cellular events, altering the chromatin structure, and facilitating the formation of high-order nucleoprotein structures in which multiple protein–protein and protein–DNA contacts enhance the specificity and the stability of the regulatory complexes. Therefore, specific sets of genes are activated or repressed by assembling complex nucleoprotein structures, also termed enhanceosomes, containing the components of basal transcription and various transcriptional factors bound to their cognate promoter/enhancer elements. In this work, we have identified new several proteins associated with HMGA1 proteins, which are involved in DNA- and RNA-dependent essential nuclear processes. In particular, our data implicate HMGA1 proteins in post-transcriptional processes, above all in mRNA processing.

We are grateful to Dr. ssa M. Monti for critical reading of the manuscript. This work was supported by grants from AIRC, the Progetto Finalizzato 'Biotecnologie' of the CNR, the MURST projects 'Terapie antineoplastiche innovative' and 'Piani di Potenziamento della Rete Scientifica e Tecnologica'. We thank the Associazione Partenopea per le Ricerche Oncologiche (APRO) for its support.

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Journal of Pathology
J Pathol 2008; **214**: 58–64
Published online 12 October 2007 in Wiley InterScience
(www.interscience.wiley.com) **DOI:** 10.1002/path.2249

Original Paper

Detection of high-mobility group proteins A1 and A2 represents a valid diagnostic marker in post-pubertal testicular germ cell tumours

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No conflicts of interest were declared.

Received: 10 May 2007
Revised: 10 August 2007
Accepted: 26 August 2007

Abstract

The high-mobility group A (HMGA) non-histone chromosomal proteins HMGA1 and HMGA2 are architectural factors. They are abundantly expressed during embryogenesis and in most malignant neoplasias, whereas their expression is low or absent in normal adult tissues. Their over-expression is known to have a causal role in cellular neoplastic transformation. Previous studies from our group have shown that their expression is restricted to specific germinal cells. In this study we have evaluated, by immunohistochemistry, the expression of HMGA1 and HMGA2 in a series of post-pubertal testicular tumours of different histological types, including 30 seminomas, 15 teratomas, 15 embryonal carcinomas and 10 mixed germinal tumours with a prominent yolk sac tumour component. HMGA1 protein expression was detected in all seminomas and embryonal carcinomas analysed, but not in teratomas or yolk sac carcinomas. Conversely, HMGA2 was present only in embryonal carcinomas and yolk sac carcinomas, but not in seminomas or teratomas. The immunohistochemical data were further confirmed by Western blot and, at the mRNA level, by RT–PCR analyses. These findings indicate that HMGA1 and HMGA2 are differently expressed with respect to the state of differentiation of testicular germ cell tumours (TGCTs), with over-expression of both proteins in pluripotential embryonal carcinoma cells and loss of expression of HMGA1 in yolk sac tumours and of both proteins in the mature adult tissue of teratoma areas. Therefore, the different profiles of HMGA1 and HMGA2 protein expression could represent a valuable diagnostic tool in some cases in which the histological differential diagnosis is problematic.
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Keywords: **HMGA proteins; testis; spermatogenesis; fertility; testicular tumours**

Introduction

Testicular germ cell tumours represent the most common malignancy in males aged 15–34 years and represent a major cause of death attributable to cancer in this age group [1]. Testicular germ cell tumours (TGCTs) can be subdivided into seminoma and non-seminoma germ cell tumours (NSGCTs), which consist of embryonal cell carcinoma, choriocarcinoma, yolk sac tumour and teratoma. Neoplasms containing more than one tumour cell component, e.g. seminoma and embryonal cell carcinoma, are referred to as mixed germ cell tumours. Seminomas and NSGCTs not only present distinctive clinical features but also show significant differences as far as therapy and prognosis are concerned [2].

The mammalian high mobility group A (HMGA) family of chromosomal proteins includes HMGA1a and HMGA1b, which are encoded by the same gene, *HMGA1*, through alternative splicing [3], and the closely related HMGA2 [4]. They are small, non-histone, chromatin-associated proteins that bind DNA in AT-rich regions through three basic domains called 'AT-hooks'. Members of the HMGA protein family have no intrinsic transcriptional activity, but they can regulate transcription by altering the architecture of chromatin, so furthering the assembly of multiprotein complexes of transcriptional factors [5]. Both genes are widely expressed during embryogenesis [6], whereas their expression is low or absent in normal adult tissues. Functional null for the *Hmga2* gene results in a pygmy phenotype with a drastic reduction

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in fat tissue, and is associated with a longer cell cycle of embryonic fibroblasts [6], whereas transgenic mice carrying an activated *Hmga2* gene show a giant phenotype associated with pelvic/abdominal lipomatosis [7], suggesting HMGA2 as a key protein in embryonic development and adipocytic cell growth. Conversely, both heterozygous and homozygous mice for the *Hmga1*-null allele show cardiac hypertrophy due to a direct role of HMGA1 on cardiomyocytic cell growth regulation, indicating a critical role of HMGA1 in heart development and growth. Both HMGA1 and HMGA2 proteins are over-expressed in several experimental and human malignancies [4]. Several studies have shown that their over-expression has a causal role in malignant cell transformation. In fact, the block of their expression has been shown to prevent thyroid cell transformation and lead malignant cells to death [8,9]. Moreover, *in vitro* and *in vivo* studies demonstrated an oncogenic activity of the over-expressed HMGA proteins, since either HMGA1 or HMGA2 over-expression is able to transform mouse and rat fibroblasts [10], and transgenic mice over-expressing either HMGA1 or HMGA2 develop NK-T cell lymphomas and pituitary adenomas [11–13]. We have previously shown that HMGA1 is present in mitotic cells (spermatogonia and primary spermatocytes), whereas HMGA2 is highly expressed in meiotic and post-meiotic cells (secondary spermatocytes and spermatids) [14,15]; in addition, we have demonstrated a specific function for HMGA2 in the regulation of spermatogenesis, because this differentiation programme is dramatically hampered in *Hmga2* $^{-/-}$ mice in spite of the presence of *Hmga1* [15]. The aim of the present study was to evaluate HMGA1 and HMGA2 expression in TGCTs to assess correlation with differential histological diagnosis.

Materials and methods

Tissue samples

The tissue bank of the National Cancer Institute 'G. Pascale' provided 70 cases of cryopreserved tissue from 30 seminomas, 15 teratomas, 15 pure embryonal carcinomas and 10 mixed tumours with a prevalent component of yolk sac tumour (in particular, eight with a pattern of microcystic and solid, one with a pattern of microcystic and papillary, and one with a pattern of microcystic, papillary and hepatoid). Ethics Committee approval was given in all instances.

Antibodies

Antibodies were obtained from the following sources: (a) polyclonal rabbit antibody anti-HMGA1 raised against a synthetic peptide located in the NH_2-terminal region (SKSSQPLASKQEKDGT) [16]; (b) polyclonal rabbit antibody anti-HMGA1 (No. 4078, Abcam, Cambridge, UK); (c) polyclonal rabbit antibody anti-HMGA2 raised against a synthetic peptide located in the NH_2-terminal region (SARGEGAGQPSTSAQG) [15]; (d) polyclonal rabbit antibody anti-HMGA2 (No. 41878, Abcam); (e) polyclonal rabbit antibody anti-SP1 (No. sc-14027, Santa Cruz Biotechnology, CA, USA).

Histological analysis and immunohistochemistry

For light microscopy, tissues were fixed by immersion in 10% formalin and embedded in paraffin wax by standard procedures. Sections (5 μm) were stained with haematoxylin and eosin or processed for immunohistochemistry. For each paraffin-embedded sample, 4 μm serial sections mounted on slides and pretreated for immunohistochemistry were dewaxed in xylene and brought through graded ethanols to deionized distilled water. Before staining for immunohistochemistry, the sections were incubated in a 750 W microwave oven for 15 min in 10 mM buffered citrate, pH 6.0, to complete antigen unmasking. The classical avidin–biotin peroxidase complex (ABC) procedure was used for immunohistochemistry. In the ABC system, endogenous peroxidase was quenched by incubation of the sections in 0.1% sodium azide with 0.3% hydrogen peroxide for 30 min at room temperature. Non-specific binding was blocked by incubation with non-immune serum (1% TRIS–bovine albumin for 15 min at room temperature). The sections were incubated overnight with antibodies against HMGA1 at a dilution of 1 : 200 and with antibodies against HMGA2 (diluted 1 : 200) [14–16]. For HMGA1 and HMGA2 detection, two additional antibodies were used which gave similar results (not shown). The following controls were performed: (a) omission of the primary antibody; (b) substitution of the primary antiserum with non-immune serum diluted 1 : 500 in blocking buffer; (c) addition of the target peptide used to produce the antibody (10^{-6} M). No immunostaining was observed after any of the control procedures. Peroxidase activity was developed with the use of a filtered solution of 5 mg 3,3′-diaminobenzideine tetrahydrochloride (dissolved in 10 ml 0.05 M Tris buffer, pH 7.6) and 0.03% H_2O_2. Mayer's haematoxylin was used for nuclear counterstaining. The sections were mounted with a synthetic medium. Antibodies against the HMGA1 and HMGA2 proteins are described elsewhere [14–16].

Protein extraction and western blot analysis

Total cell extracts (TCEs) 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). Protein concentration was estimated by a modified Bradford assay (Bio-Rad). The protein extracts were boiled in Laemmli sample buffer, separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to Immobilon-P transfer membranes (Immobilon Millipore Corporation, Bedford, MA, USA). The membranes were blocked

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with 5% non-fat milk proteins and incubated with the antibody at the appropriate dilution. Antibodies for the detection of HMGA1 and HMGA2 protein are described elsewhere [14–16]. For Western blot analyses, two additional antibodies were used for HMGA1 and HMGA2, which gave the same results (not shown). Bound antibodies were detected using horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence (Amersham Life Sciences, Buckinghamshire, UK). As a control for equal loading of protein lysates, the blotted proteins were probed with antibodies against specific protein 1 (SP1).

RNA extraction and RT–PCR analysis

TGCTs were rapidly dissected, frozen on dry ice and stored at −80 °C. Total RNA was extracted using TRI-reagent solution (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's protocol. Total RNA (5 μg) digested with RNAse-free DNAse, were reverse-transcribed, using random hexanucleotides as primers (100 mM) and 12 units AMV reverse transcriptase (Promega). The cDNA was amplified in a 25 μl reaction mixture containing 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.4 mM each primer and 1 U Taq DNA polymerase (Perkin-Elmer). After a denaturing step (95 °C for 2 min) the cDNA was further amplified in 20 PCR cycles (95 °C for 1 min, 58 °C for 30 s and 72 °C for 30 s). The following primers were used to amplify the HMGA1 transcript: forward primer, 5′-AGAGACCTCGGGGCCGACCA-3′; reverse primer, 5′-GATGCCCTCCTCTTCCTCC-TT-3′ (corresponding to nucleotides 140–160 and 272–251, respectively). The following primers were used to amplify the HMGA2 transcript: forward primer, 5′-CGAAAGGTGCTGGGCAGCTCCGG-3′, which maps onto the first exon; reverse primer, 5′-CCATTTCCTAGGTCTGCCTCTTG-3′, which maps onto the third exon. Expression of the *GAPDH* gene was used as an internal control for the amount of cDNA tested. The specific primers were: forward, 5′-ACATGTTCCAATATGATTCC-3′; reverse, 5′-TGGACTCCACGACGTACTCA-3′ (corresponding to nucleotides 195–215 and 355–335, respectively). The reaction products were analysed on a 2% agarose gel and transferred to GeneScreen$^+$ nylon membranes (Dupont, Boston, MA, USA). The membranes were hybridized with *HMGA1* and *HMGA2* cDNA probes. cDNA probes obtained by PCR were labelled with [^{32}P]dCTP, using the random oligonucleotide primers (Ready-to-Go DNA labelling kit; Pharmacia Biotech, Piscataway, NJ, USA) at a specific activity ≥7 × 108 cpm/μg.

Results and discussion

We evaluated the expression of HMGA1 and HMGA2 proteins in normal and TGCTs by immunohistochemistry, using polyclonal antibodies raised against the different NH_2-terminal regions of the HMGA1 and HMGA2 proteins and polyclonal antibodies raised against the different COOH-terminal regions of the HMGA1 and HMGA2 proteins. These antibodies are specific for HMGA1 or HMGA2 protein without cross-reactivity. Immunohistochemical assays were first performed on sections of normal human testis (Figures 1A, 2A). In accordance with our previous results obtained on mouse testis [14], both the spermatogonia and primary spermatocytes showed specific nuclear positivity for HMGA1 (Figure 1A), whereas only secondary spermatocytes showed specific nuclear positivity for HMGA2 (Figure 2A).

Subsequently, HMGA1 and HMGA2 expression was examined in a series of post-pubertal TGCTs, including 30 seminomas, 15 pure embryonal carcinomas, 10 mixed tumours with relevant yolk sac tumours, and 15 mixed tumours with relevant teratoma component. Moreover, HMGA1 and HMGA2 were evaluated in 15 intratubular germ cell tumours (ITGCTs) areas in 15 of 30 examined seminomas. The results of this analysis, summarized in Table 1, showed an intense HMGA1 immunoreactivity in ITGCTs, seminomas and embryonal carcinomas, but neither in epithelial and mesenchymal areas of teratomas or in yolk sac carcinomas, whereas HMGA2 expression was observed in embryonal carcinomas and yolk sac carcinomas (with no differences between the variants observed), but not in ITGCTs, seminomas and teratomas. Some representative immunohistochemical data are shown in Figures 1B–E and 2B–E for HMGA1 and HMGA2, respectively. No staining was observed when the same samples were stained with the antibodies preincubated with the peptides against which antibodies were raised or in the absence of the primary antibodies (data not shown).

Western blot analysis confirmed HMGA1 protein expression in seminomas and embryonal carcinomas, and HMGA2 protein expression in embryonal carcinomas, and yolk sac tumours (Figure 3A). To verify whether the altered HMGA1 and HMGA2 protein

Table 1. HMGA1 and HMGA2 immunohistochemical data in post-pubertal TGCTs

Tumour	Number of cases ($n = 70$)	Mean age [years (range)]	HMGA1 expression (no. of cases)	HMGA2 expression (no. of cases)
Seminoma	30	30.3 (20–47)	+++ (23) ++ (7)	− (30)
EC	15	30.8 (22–46)	+++ (12) ++ (3)	+++ (14) ++ (1)
YST	10	28.7 (22–44)	− (10)	+++ (7) ++ (3)
Teratoma	15	26 (20–30)	− (15)	− (15)
ITGCT*	15	27.3 (20–36)	+++ (13) ++ (2)	− (15)

* Associated areas in 15 of 30 seminomas examined.
EC, embryonal carcinoma; YST, yolk sac tumour; ITGCT, intratubular germ cell tumour; −, not expressed; +++, high expression; ++, moderate expression.

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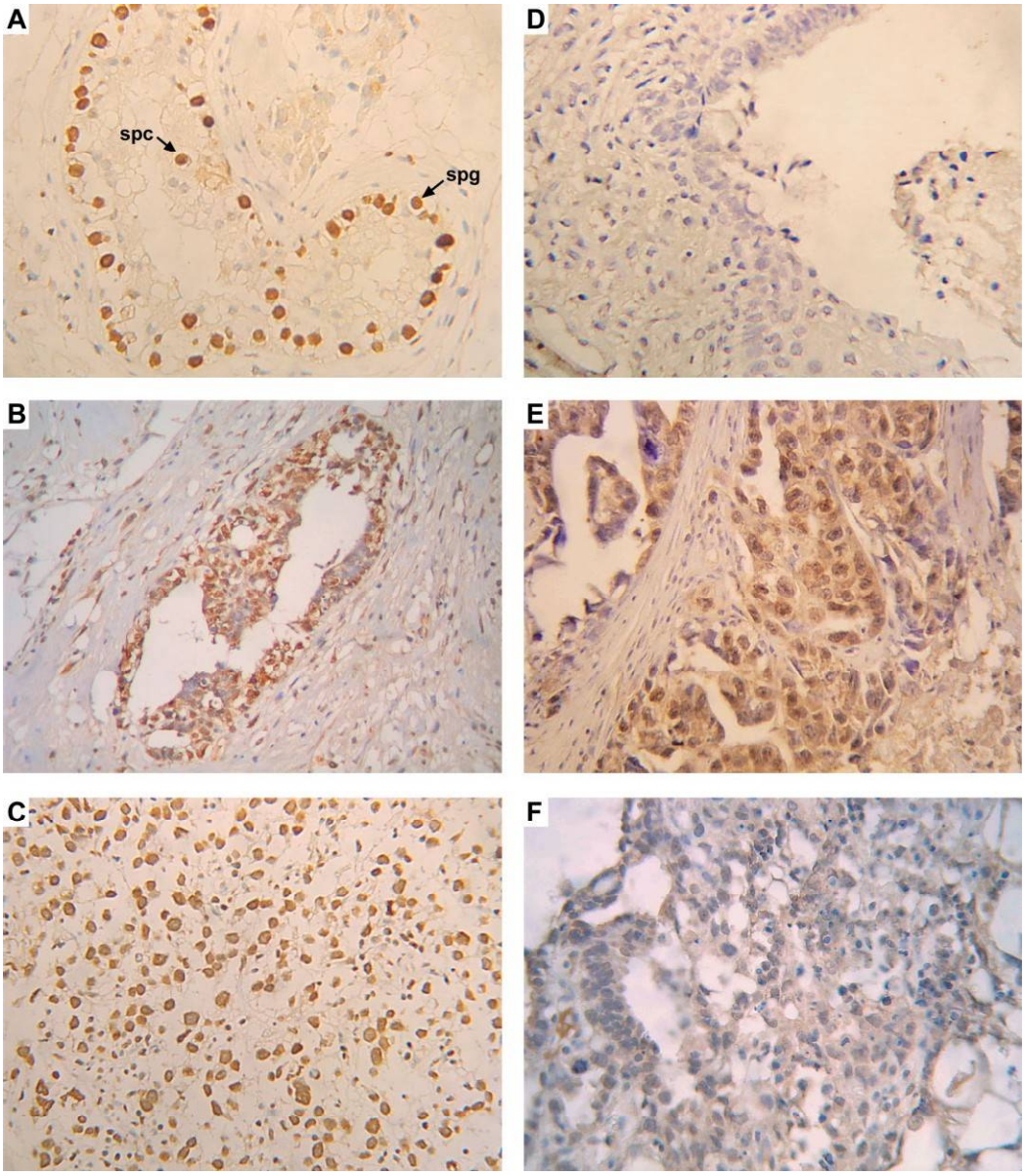

Figure 1. Immunohistochemical analysis of HMGA1 expression in normal testis and testicular germ cell tumours. (A) HMGA1 expression in human normal testis, in which nuclear positivity was observed in spermatogonia (spg) and spermatocytes (spc). (B) HMGA1 expression in intratubular germ cell tumour, in which intense nuclear positivity was observed. (C) Classic seminoma with intense and diffuse nuclear HMGA1 positivity. (D) Teratoma with mesenchymal component, in which no HMGA1 positivity was observed. (E) Embryonal carcinoma with intense nuclear HMGA1 positivity. (F) Absent expression of HMGA1 in a yolk sac tumour. All magnifications ×40

expression observed in TGCTs is associated with an alteration of mRNA expression levels, we analysed the expression of *HMGA1* and *HMGA2* mRNA by reverse transcription–PCR on the same cases utilized for immunohistochemistry and western blot analyses. As shown in Figure 3B, an abundant HMGA1 mRNA amplification was observed in seminomas and embryonal carcinoma, but not in teratomas and yolk sac tumours. Conversely, *HMGA2* mRNA was abundantly amplified from embryonal carcinoma and yolk sac tumours, but not from seminomas and teratomas (Figure 3B).

Post-pubertal TGCTs show significant clinical and biological differences with respect to TGCTs of prepubertal age [17]. In fact, the usual post-pubertal TGCTs are derived from an ITGCT that is never observed in pre-pubertal TGCTs [18]. Generally, ITGCTs are considered direct precursors of seminomas, leading to the hypothesis that non-seminomas (NS-TGCTs) progress from ITGCTs through an intermediate seminoma stage [19]. In particular, embryonal carcinoma cells could further differentiate into somatic tissue, giving rise to teratomas [18–20]. The different HMGA1 and HMGA2 profiles fit this histological typing scheme (Figure 4). In fact, HMGA1 and HMGA2 coexpression was observed only in embryonal carcinoma cells that correspond to developmentally pluripotent embryonic cells in the preimplantation stage of spermatogenesis, while spermatogonia–spermatocytic-derived tumours, e.g.

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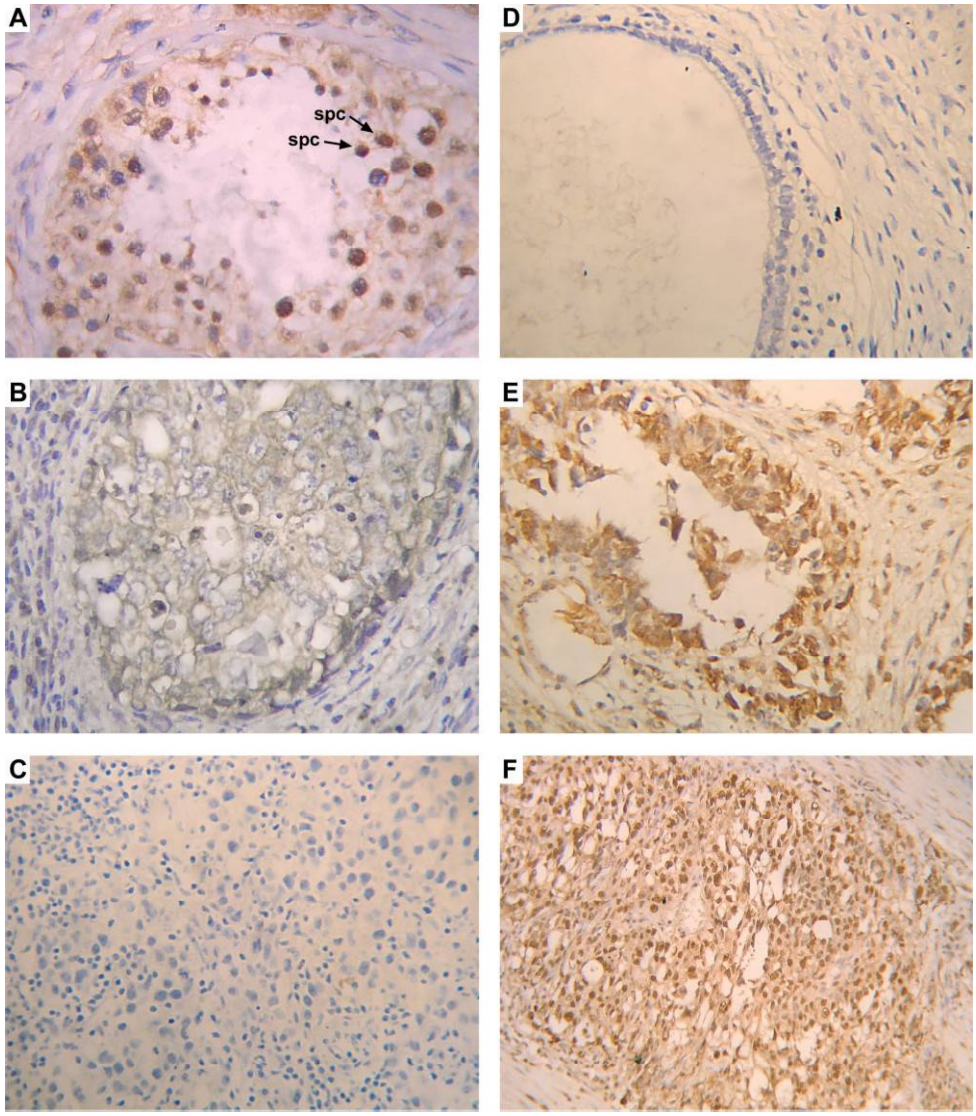

Figure 2. Immunohistochemistry analysis of HMGA2 expression in normal testis and testicular germ cell tumours. (A) HMGA2 expression in human normal testis in which nuclear HMGA2 positivity was observed in spermatocytes (spc). (B) Intratubular germ cell tumour. (C) Classic seminoma. (D) Teratoma with mesenchymal component, in which no HMGA2 positivity was observed. (E) Embryonal carcinoma with intense nuclear HMGA2 positivity. (F) Nuclear expression of HMGA2 in a yolk sac tumour. All magnifications ×40

seminomas, show only HMGA1 over-expression. Conversely, no expression of both HMGA1 and HMGA2 proteins was detected in teratomas. In addition, extra-embryonic differention of yolk sac tumours shows only expression of HMGA2 protein.

This work enhances the current view that HMGA1 and HMGA2, previously believed to play the same role in carcinogenesis, may play different roles in different tissues. The difficulties of pathological diagnosis of TGCTs are multiple, with a rate of 6% of diagnostic error [21]. It is noteworthy that the different expression profile of HMGA1 and HMGA2 proteins could be a useful tool for diagnosis of TGCTs. In fact, classical morphological criteria allow us to easily distinguish seminomas from NS-TGCTs, the former being classically constituted by solid sheets of monomorphic large cells in a lymphoid background. However, the presence of microcystic or pseudotubular areas, because of excessive tissue oedema, could create a problematic differential diagnosis with yolk sac tumours and embryonal carcinoma [18], emphasizing the role of the pathologist in histological classification and staging of TGCTs. Correct histological classification, particularly in distinction between seminoma and NS-TGCTs, appear important for prognosis and therapeutic management. However, incorrect histological definition could be particularly critical in the choice of therapeutic strategies, with a significant impact on the morbidity and mortality of patients. Currently, testicular cancers are curable in the majority of patients. In fact, particularly for low stages, radiosensitivity demonstrated in seminomas leads to irradiation of retroperitoneal lymph nodes, whilst radiation insensitivity

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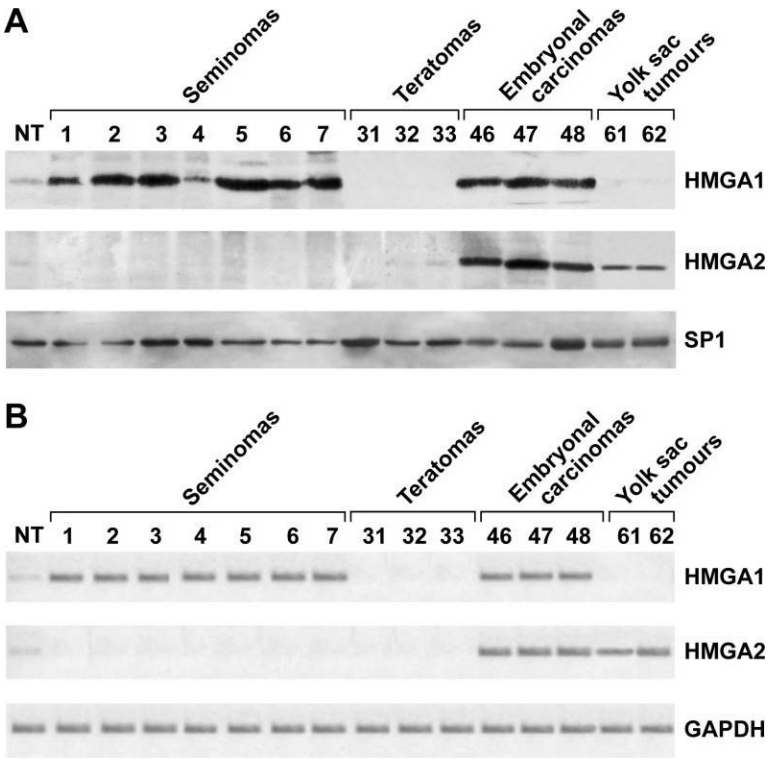

Figure 3. (A) Western blot analysis of HMGA1 and HMGA2 expression in normal testis and and testicular germ cell tumours. 40 μg total tissue lysates were resolved on 14% SDS–PAGE, transferred onto nitrocellulose filters and western-blotted with anti-HMGA1 and anti-HMGA2 polyclonal antibodies. SP1 was analysed as a loading control. (B) Expression of *HMGA1* and *HMGA2* mRNA in normal testis and testicular germ cell tumours by RT–PCR analysis. Total mRNA was extracted, reverse-transcribed and subjected to PCR analysis for *HMGA1* and *HMGA2*. The integrity of mRNA samples was determined using *GAPDH* as a control. NT, normal testis

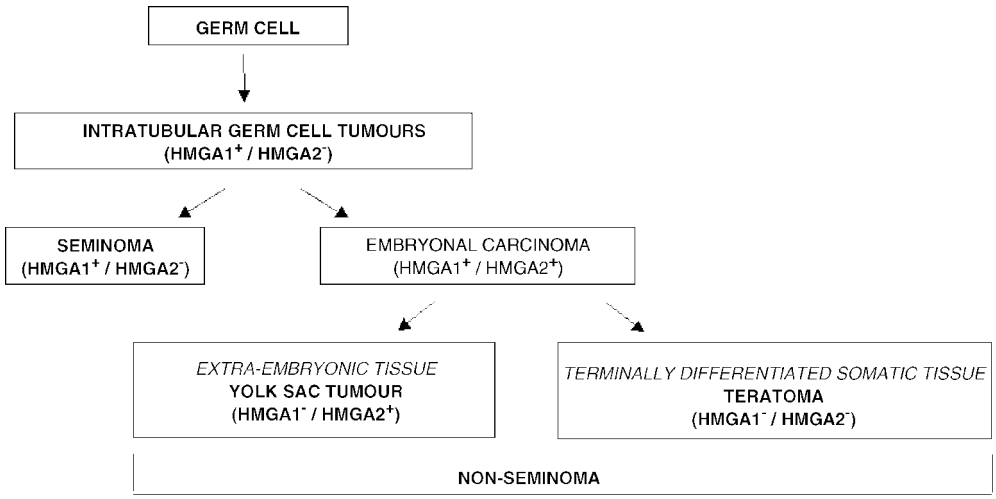

Figure 4. Schematic representation of the histological types of testicular germ cell tumours in relation to HMGA1 and HMGA2 expression

of non-seminomatous tumours leads to consideration of lymph nodes removal or platin-based therapy [21].

Clearly, the coexpression of HMGA1 and HMGA2 proteins, together with other well-known molecular markers (e.g. CD30, α-fetoprotein, SOX2 and cytokeratin) in neoplastic cells is suggestive of an embryonal carcinoma diagnosis, while only HMGA1 expression is indicative of seminomas and only HMGA2 expression is specific of yolk sac tumours. Indeed, the use of HMGA1 and HMGA2 together with the above-mentioned markers could improve diagnostic specificity in the classification of TGCTs to provide adequate therapeutic strategies.

Acknowledgements

This study was supported by the Associazione Italiana per la Ricerca sul Cancro (AIRC). We thank Fabrizio Fiorbianco

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(www.studiociotola.it) for skilful technical assistance with the artwork.

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