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“High Mobility Group A1 (HMGA1) protein inhibits p53-mediated intrinsic apoptosis interacting with Bcl-2 at mitochondria”

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ABSTRACT

The High mobility group A (HMGA) non-histone chromosomal proteins play key roles in chromatin architecture and orchestrate the assembly of nucleoprotein complexes involved in gene transcription, replication, and chromatin structure. HMGA overexpression and gene rearrangement are frequent events in human cancer. Their importance in cancer progression and their role in apoptosis has been defined by a new physical and functional interaction between HMGA1 and p53 proteins. HMGA1 inhibits p53-mediated apoptosis modulating the transcription of p53 target genes as *Mdm2, p21*^{Waf1}, *Bax, Bcl-2* and promoting Hipk2 relocalization from the nucleus to the cytoplasm. Even though HMGA1 proteins have been identified as nuclear proteins, abundant HMGA1 expression has been frequently detected in the cytoplasm of cancer cells but it has been often considered as an artefact likely do a very abundant HMGA1 expression, and no deep investigation has been undertaken to clearly demonstrate the presence of these proteins in the cytoplasm, and to unveil the functional role of this localization. Preliminary studies obtained by a screening of an Antibody Array^{TM} strongly suggest possible cytoplasmic interactors of HMGA1 proteins in tumoral cell lines.

My thesis project is focalized on the interaction between HMGA1 and anti-apoptotic factor Bcl-2. I have identified and characterized a new subcellular localization of HMGA1 proteins by analysis of total, nuclear and cytoplasmic cell lysates from several normal, and tumor-derived cell lines. Then, I confirmed the interaction HMGA1-Bcl-2 in cytoplasm *in vivo* and *in vitro*. Moreover, since p53 interacts with Bcl-2 blocking its anti-apoptotic function, I identified a new mechanism that allows HMGA1 proteins to inhibit the p53-mediated intrinsic-apoptotic pathway. Indeed, HMGA1 localizes at mitochondria and displaces it from the binding to Bcl-2 enhancing its anti-apoptotic function.

Finally, I reported the correlation between the HMGA1 cytoplasmic localization and a more aggressive phenotype in thyroid, breast and colon cancer.

1. BACKGROUND

1.1 HMGA protein family

Cell proliferation and differentiation are highly coordinated processes during development and require the precise regulation of gene expression. One process that facilitates the orchestration of these changes in gene expression patterns is remodelling of chromatin structure, which in turn modulates the interaction of transcription regulatory proteins with DNA. These changes in chromatine structures are effected by so-called “architectural transcription factors” (Muller *et al*. 2001).

The high mobility group (HMG) proteins are abundant heterogeneous, non-histone components of chromatine that act as such architectural factors contributing to transcriptional regulation. The HMGA proteins, including HMGA1 (isoforms HMGA1a, HMGA1b, HMGA1c) and HMGA2, are the key of assembly of multiprotein complexes of transcriptional factors and co-factors, which constitute the so called “transcriptome” or “enhanceosome” of several genes. The HMGA proteins have a similar structure and are well conserved during evolution. Each protein contains three DNA binding domains containing short basic repeats, the so-called “AT-hooks”, with which they bind AT-rich sequences in the minor groove of DNA, and an acidic carboxy-terminal tail that is believed to be important for protein-protein interaction and for recruitment of specific proteins to enhanceosome (Reeves *et al*. 2001).

The human *HMGA1* gene, located at the chromosome band 6p21, spans 10 kb and consists of 8 exons, among which only exons from 5 to 8 are transcribed in mRNA and codes for the HMGA1 proteins that have a molecular weight of 19-20 kDa (Friedmann *et al.* 1993). 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 spliced variants in mammalian cells (Figure 1.1) (Johnson *et al.* 1989; Friedmann *et al.* 1993).

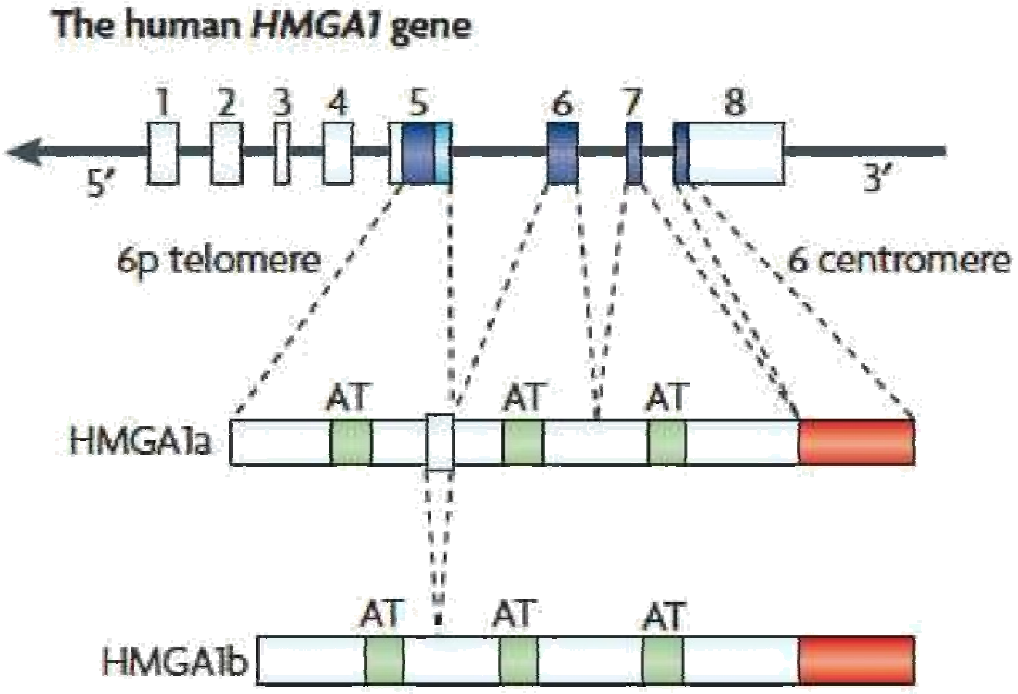


Figure 1.1. Schematic representation of HMGA1a and HMGA1b proteins.
Each protein contains three basic domain, named AT hook (green box), with which they bind DNA, and an acidic carboxy-terminal region (red box).

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).

Then, the human *HMGA2* gene, located at chromosom band 12q13-15, spans more than 140 kb and consists of 5 exons, all of them coding for HMGA2 protein (Figure 1.2). Alternative spliced mRNAs from this gene have also been detected in leiomyoma tumors (Hauke *et al.* 2001; Kurose *et al.* 2001).

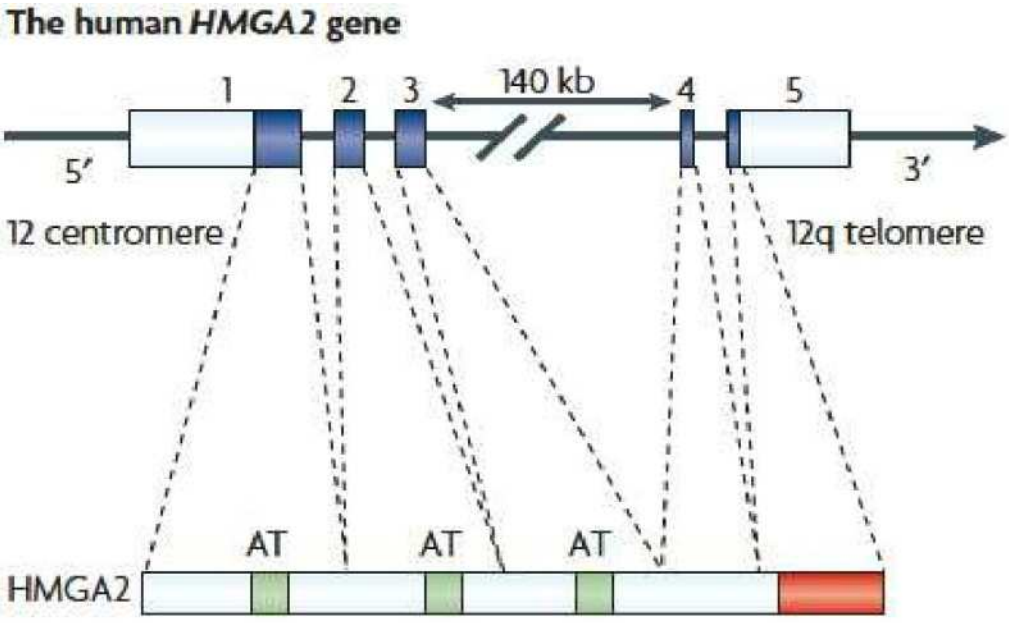


Figure 1.2. Schematic representation of HMGA2 protein. HMGA2 protein contains three basic domain, named AT hook (green box), with which they bind DNA, and an acidic carboxy-terminal region (red box).

Both *HMGA* genes are widely expressed during embryogenesis, whereas their expression is absent or low in adult tissues (Zhou *et al.* 1995, Chiappetta *et al.* 1996). In particular, expression of HMGA2 has not been detected in any of the several adult mouse and human tissues tested (Rogalla *et al.* 1996).

Low expression has been observed in CD34-positive haematopoietic stem cells (Rommel *et al.* 1997), in mouse preadipocytic proliferating cells (Anand and Chada 2000) and in meiotic and post-meiotic cells secondary spermatocytes and spermatidis (Chieffi *et al.* 2002, Di Agostino *et al.* 2004). Conversely, HMGA1 is ubiquitary expressed albeit at low levels, in adult murine and human tissues (Chiappetta *et al.* 1996).

1.2 Multiple functions of HMGA proteins

Many reports illustrate how the HMGA proteins participate in processes such as the regulation of gene expression, virus integration and expression, embryogenesis, differentiation and neoplastic transformation.

In normal cells, the expression of HMGA proteins is restricted to embryogenesis, it decreases with organogenesis and in normal adult cells is very low or almost absent. At later stages, the expression pattern becomes more restricted; in particular, HMGA1 expression is confined to specific body organs of ectodermal, mesodermal and endodermal origin, while HMGA2 expression is restricted to those of mesenchimal origin.

After embryogenesis, the *HMGA* genes are re-expressed at high levels in transformed cells and in tumors. This elevated expression, detected in a variety of tumors having different origins (Giancotti *et al.* 1989, Fedele *et al.* 1996), suggested that these proteins could be used as diagnostic markers of neoplastic transformation/progression. Indeed, it has been well established that many human neoplasias, including thyroid, prostatic, cervical, colorectal, pancreatic and ovarian carcinoma, show a strong increase of both HMGA1a and HMGA1b proteins (Chiappetta *et al.* 1998; Bandiera *et al*.1998; Abe *et al*. 2000; Kim *et al.* 1995; Chiappetta *et al.* 2001; Abe *et al.* 2002; Abe *et al*. 2003; Masciullo *et al.* 2003; Fusco and Fedele 2007). The first evidence of a direct role played by these factors in tumorigenesis came from transfection in normal rat thyroid cells of an antisense construct for HMGA2 that prevented retrovirally induced neoplastic transformation (Berlingieri *et al.* 1995). The increased expression of HMGA proteins was later shown to promote tumor progression in different cell lines.

In addition of overexpression, HMGA proteins was later shown to promote tumour progression in different cell lines.

In addition of overexpression in malignant tumors, HMGA de-regulation, as a result of specific chromosomal rearrangements, has also been reported in a variety of common benign tumours. Structure alterations for both HMGA genes

have been reported, but rearrangements of *HMGA2* gene at 12q15 are particulary frequent especially in lipomas and leiomyomas, making this gene probably the most commonly rearranged one in human neoplasia.

1.3 Mechanisms of action of HMGA proteins

The HMGA proteins have roles in assembling or modulating macromolecular complexes that are involved in various biological processes: HMGA proteins directly bind to the DNA, modifying its conformation and consequently facilitating the binding of a group of transcriptional factors (TF). They interact with both DNA and TFs to generate a multiprotein stereospecific complex bound to DNA (Fig. 1.3 a).

HMGA proteins have been shown to participate in this way in the regulation of many genes, the best studied being the interferon (IFN)-β gene (Thanos D, Maniatis T, 1992).

The activation of IFN-β expression is due to a multifactor complex that assembles in the nucleosome-free enhancer region of the gene, including the factors NFkB, interferon regulatory factor, activating transcriptional factor (ATF2)/JUN and the HMGA1a protein.

The HMGA proteins can also influence gene transcription through protein-protein interactions with transcription factors (Figure 1.3 b) by modifying its conformation and enhancing the affinity of its binding to DNA.

The enhancement of transcriptional activity of the TF serum-response factor by HMGA1a is an example of this mechanisms (Chin *et al.* 1998).

Finally, the HMGA proteins have the ability to alter chromatin structure (Figure 1.3 c). Indeed, they have been shown to be important elements that are associated with matrix and scaffold-associated regions. They are specific segments of genomic DNA that have a high affinity for the nuclear matrix and that are enriched in AT sequences. These sequences anchor chromatin to the nuclear scaffold and organize topologically independent DNA domains which

have functional roles both in DNA replication and transcription (Galande S.*et al*. 2002). The binding of HMGA proteins to these regions de-represses transcription by displacement of histone H1 by DNA.

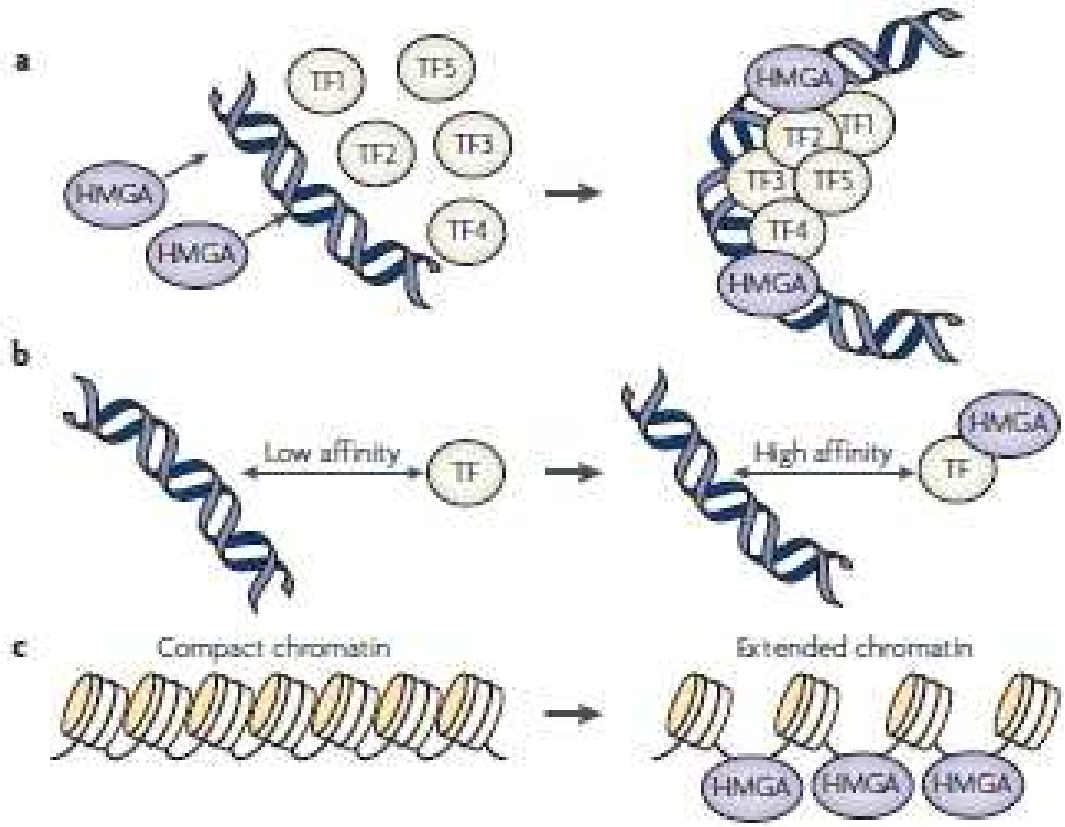


Figure 1.3 a) HMGA proteins bind Dna; **b)** HMGA are involved in protein-protein interaction; **c)** HMGA proteins alter the chromatine structure.

1.4 Regulation of gene expression by HMGA proteins

The regulation of gene transcription in eukaryotic cells depends by chromatine structure. HMGA proteins do not have transcriptional activity *per se*; however, by interacting with the transcription machinery they alter the structure and thereby regulate, negatively or positively, the transcriptional activity of several genes (Thanos,D. *et al*. 1992; Thanos D. *et al*. 1993);(Table 1.1).

HMGA proteins might participate in the regulation of gene transcription, influencing chromatin structure, by two mechanisms: one model suggests that HMGA proteins act to influence the structure of large regions or domains of chromatin, whereas a second model assumed a restricted or localized effect on chromatin and nucleosome structure. The promoter regions of many of these regulated genes contain multiple AT-rich sequence that 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.

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)	***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)
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)	***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)
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)	**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 Role of HMGA proteins in development

The high expression of HMGA proteins during embryogenesis suggests that they have an important role in development and are involved in the control of cell growth and differentiation. Infact, it has been demonstrate that HMGA proteins induce 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). Then, *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 (Friedmann *et al.* 1993; Johnson *et al.* 1990; Ogram and Reeves 1995; Holth *et al.* 1997).

Also the phenotypic characterization of knockout mice for each of the *HMGA* genes confirms crucial roles for these proteins in different aspects of development. Indeed, 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.

1.6 Role of HMGA proteins in cancer

In contrast to normal tissues, high levels of HMGA1 and HMGA2 are a common feature of human benign and malignant tumors. The correlation between the levels of HMGA proteins and the malignant phenotype made it important to determinate their role in the process of cell transformation and, in particular, to investigate whether their increase is a phenomenon associated with cell transformation or whether it has a causative relationship with the malignant phenotype.

Benign tumors of mesenchymal origin (lipomas, leiomyomas, fibroadenomas, pulmonary hamartomas and endometrial polyps) often contain chromosomal rearrangements involving *HMGA2* gene. These rearrangements result in the formation of new hybrid genes that code for chimeric proteins in which the AT-hooks of HMGA2 are fused to ectopic peptidic sequences (Hess 1998), with loss of the C-terminus and of the 3'UTR of the gene.

It is probably that the truncation of *HMGA2* gene and loss of its 3'UTR rather than the formation of a new chimeric gene is crucial for cell trasformation (Kazmierczak *et al*.1995, Kools and Van de Ven *et al*. 1996).

Rearrangements and overexpression of the HMGA2 gene have also been described in non-mesenchymal benign human tumors, such as pituitary adenomas (Finelli *et al*. 2002, Pierantoni *et al*. 2005).

Consistently, transgenic mice overexpressing HMGA1 or HMGA2 develop pituitary adenomas, demostrating the casual role of HMGA proteins in the transformation of pituitary gland (Fedele *et al*. 2002, Fedele *et a.l* 2005).

In addiction, 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).

Overexpression of HMGA proteins is a costant feature of several human malignant neoplasias, including thyroid, prostate, uterus, breast, lung, uterine cervix, colonrectum, ovary and pancreas carcinomas (Fedele *et al*. 2001). Moreover, HMGA protein expression is associated with a highly malignant phenotype (Tallini and Dal Cin 1999) and is a poor prognostic index as their overexpression often correlates with the presence of metastasis and with reduced survival (Fusco and Fedele 2007); (Table 1.2).

Table 1.2 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)

The mechanisms that lead to transformation 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 (p21Waf1/Cip1), as well as cooperating with p53 to activate transcription of the p53 inhibitor MDM2. This results in a reduction of p53-dependent apoptosis in cells expressing p53 and exogenous or endogenous HMGA1 after activation by UV light (Pierantoni *et al*. 2006), (Figure 1.4).

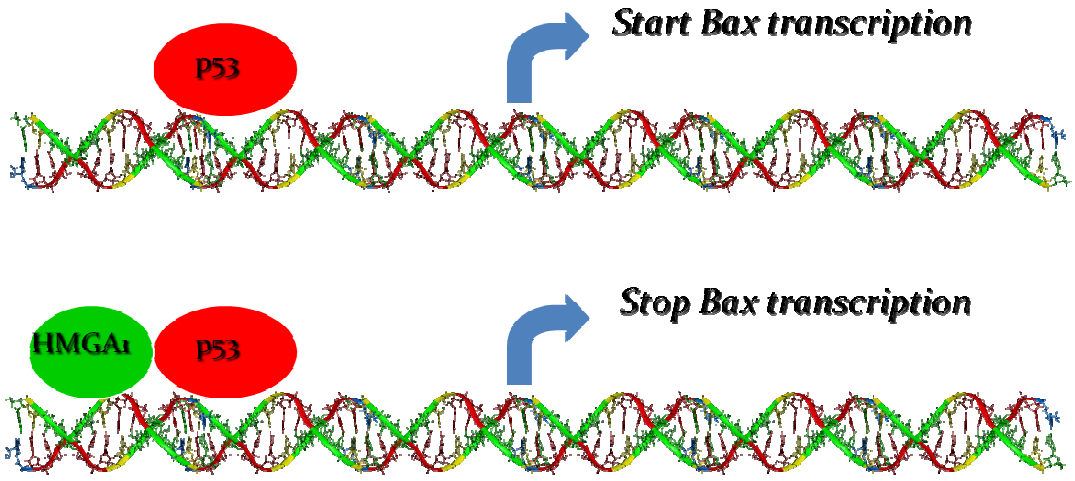


Figure 1.4 HMGA1 regulates Bax transcription.
p53 up-regulate the transcription of pro-apoptotic factor Bax, the co-expression of HMGA1 reduce the activity of p53 on Bax promoter. HMGA1 expression alone did not affect the activity of any promoter, it acts in the regulation of these promoters only cooperating with p53 protein.

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), a serin threonine kinase (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 1.5).

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.

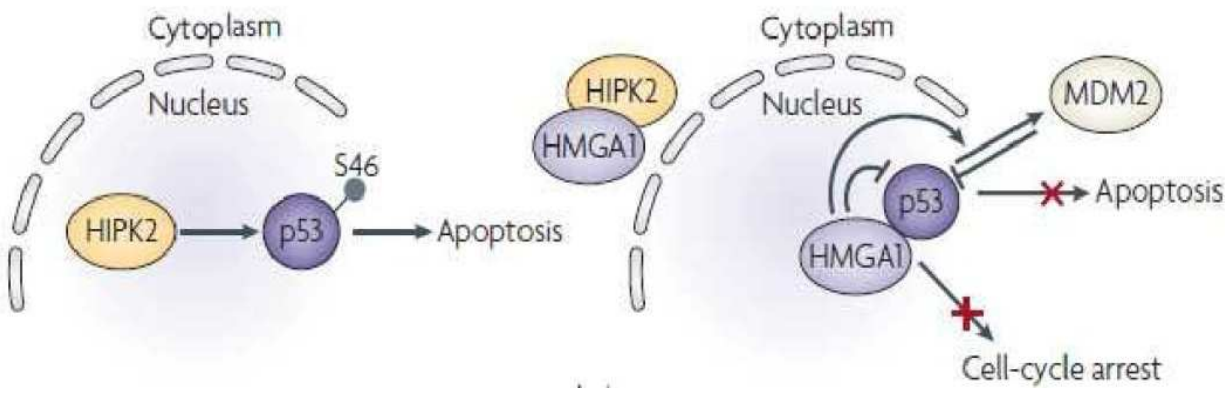


Figure 1.5. Schematic representation of p53 inhibition by high mobility group A (HMGA)1. In response to DNA damage, p53 induces either cell-cycle arrest or apoptosis. p53 phosphorylation at S46 by homeodomain-interacting protein kinase 2 (HIPK2) is one determinant of the outcome because it occurs only after severe, non repairable 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.

We have identified also another mechanism by which HMGA1 overexpression contributes to escape from apoptosis leading to neoplastic transformation based on Blc-2 induction. In fact, HMGA1 overexpression promotes the reduction of Brn-3a binding to the *Bcl-2* promoter, thereby blocking the Brn-3a corepressor function on Bcl-2 expression following p53 activation. In particular HMGA1 overexpression promote Hipk2 relocalization in the cytoplasm and inhibition of p53 transcriptional repression exerted on the *Bcl-2* promoter, while HIPK2 over-expression reestablished HIPK2 nuclear localization and sensivity to apoptosis (Figure 1.6A).

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 (Figure 1.6 B).

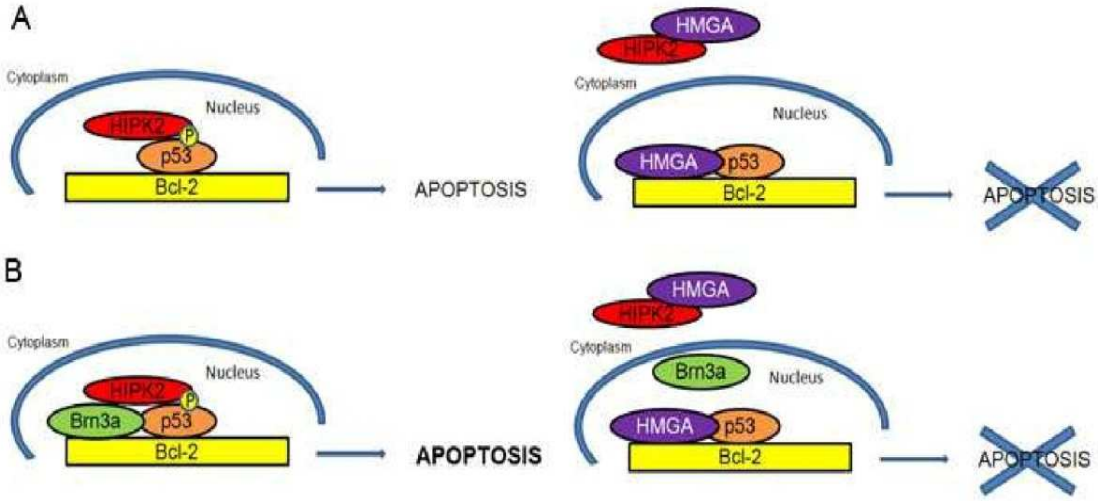


Figure 1.6 Induction of Bcl-2 expression By HMGA proteins
A) Hipk2 phosphorylates p53 that represses Bcl-2 promoter.HMGA1 overexpression promove Hipk2 relocalization in the cytoplasm and inhibits the repression exerted by p53 on *Bcl-2* promoter. **B)** Brn3a acts as corepressor of *Bcl-2* transcription. The overexpression of HMGA1 promotes Hipk2 delocalization from nucleus to the cytoplasm and the reduction of Brn3a binding on Bcl-2 promoter.

These mechanisms described might have important implications in tumorigenicity as well as in the development of the tumor resistance to antineoplastic treatments.

1.7 Interactors of HMGA proteins

Another important charcteristic of HMGA proteins is the capability to interact directly with many transcriptional factors. As reported in Table 1.3, 21 different transcription factors have been identified by coimmunoprecipitation assay and pull down assay.

These interactors have two points in common: the sites of interaction include part, or all, of more AT-hook peptide motifs plus some flaking regions and these sites include amino acid residues that are known to be extensively modified *in vivo* by post-translation modifications like phosphorylation, acetylation or methylation. In particular, post-translation modifications are important because 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 1.3 Transcription factors that physically interact with HMGA proteins

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

1.8 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 (Figure1.7): 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 three nuclear export signal sequences (Vousden and Lu 2002).

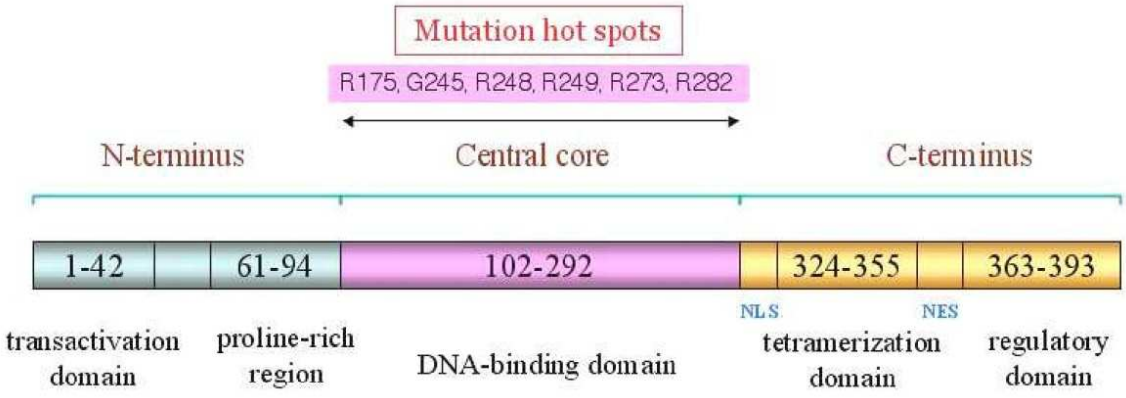


Figure 1.7. Schematic representation of the p53 structure. p53 contains 393 amino acids, consisting of three functional domains, i.e. an N-terminal activation domain, DNA binding domain and C-terminal tetramerization domain. The N-terminal domain includes transactivation subdomain and a PXXP region that 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 post-translational 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 TP53 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. 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).

1.9 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 different 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 modifications, thus resulting in activation of p53-targeted genes (Fritsche *et al.* 1993).

The induction of the transcription dependent cell death program requires p53 phosphorylation at Ser 46, which is necessary for the 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 different and constitute downstream

effectors of signaling pathways that elicit different 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-damage inducible 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.

The products of these gene may induce apoptosis through either an extrinsic or an intrinsic pathway, namely the death receptor pathway and the mitochondrial pathway respectively (Figure 1.8).

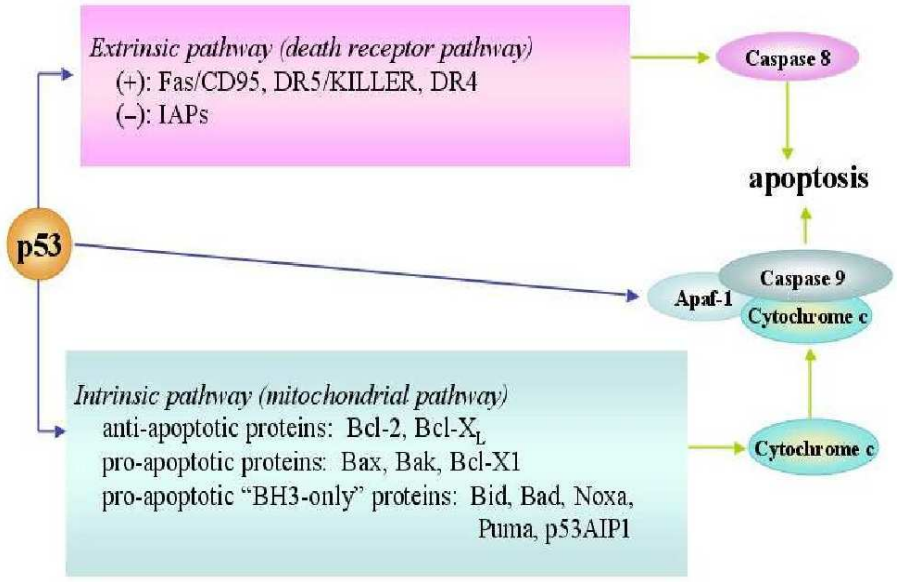


Figure 1.8. p53-associated genes and pathways involved in apoptotic cell death.

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.

Recently, an emerging area of research unravels additional activities of p53 in cytoplasm, where it triggers apoptosis and inhibits autophagy (Douglas *et al.* 2009). Activation of cytosolic p53 can directly induce mitochondrial outer membrane permeabilization (MOMP) by forming inhibitory complexes with the protective Bcl-XL and Bcl-2 proteins, resulting in cytocrome c release.

MOMP is usually inhibited by anti-apoptotic multidomain proteins of Bcl-2 family (such as Bcl-2, Bcl-XL and Mcl-1), and is conditional on pro-apoptotic multidomain proteins from the same family (in particular Bax and Bak) that can homo-oligomerize within the outer mitochondrial membrane to form MOMP-mediating supramolecular structures. A set of distinct pro-apoptotic "BH3-only" proteins can directly interact with Bax or Bak to trigger their homo-oligomerization and hence MOMP and/or neutralize one or more anti-apoptotic

multidomain proteins. p53 has been suggested to act like a BH3-only protein, either as a direct activator of Bax and/or Bak or as a de-repressor (Motohiro *et al*. 2003). The pro-apoptotic effects of cytoplasmic p53 are not dependent on transcription (Figure 1.9). However the control of transcription by nuclear p53 decisively contributes to the function of cytoplasmic p53. 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). These results reveal that regulation of apoptosis is an important and evolutionarily conserved tumor suppressor function of p53.

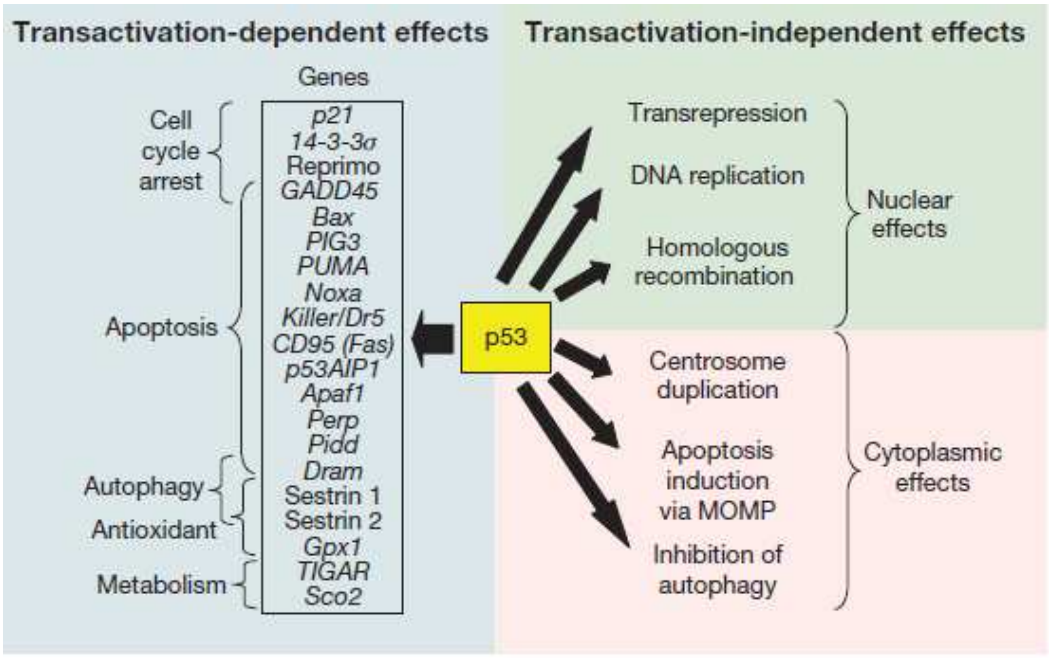


Figure 1.9 Classification of p53 activities. On the left side, some genes that are transactivated by p53 are exemplified, together with a few of the functional consequences of p53 activation. On the right side, transactivation –indipendent effects of p53 are listed. These can be divided into nuclear and extra-nuclear (cytoplasmic) p53 activies.

1.10 Bcl-2 and apoptosis

Bcl-2 family proteins serve as critical regulators of pathways involved in apoptosis, acting to either inhibit or promote cell death. Altered expression of these proteins occurs commonly in human cancers, contributing to neoplastic cell expansion by suppressing programmed cells death and extending tumor cell life span. Moreover, because chemotherapeutic drugs typically exert their cytotoxic actions by inducing apoptosis, the ultimate efficacy of most anticancer drugs can be heavily influenced by the relative levels and state of activation of members of the Bcl-2 family (John C Reed Oncogene 1998).

The apoptosis-suppressing *Bcl-2* gene was discovered as a proto-oncogene found at the breakpoints of t(14;18) chromosomal traslocations in low-grade B-cell lymphomas. Initial gene transfection studies of Bcl-2 demonstrated that over-production of the protein significantly prolongs cell survival in the face of classical apoptotic stimuli, including lymphokine deprivation from factor-dependent hematopoietic cells, glucocorticoid treatment of thymocytes and lymphoid leukemia cells, ϒ-irradiation of thymocytes, and NGF-deprivation from fetal sympathetic neurons (reviewed in Reed, 1994). Conversely, antisense-mediated suppression of Bcl-2 expression was demonstrated to induce or accelerate cell death (Reed *et al*., 1990). It was thus that Bcl-2 emerged as the first example of an intracellular apoptosis-suppressor and the first identified proto-oncogene which contributed to neoplasia through effects on cell life span regulation rather than cell division.

Bcl-2 is a multifunctional protein. Three general functions for Bcl-2 and some of its anti-apoptotic homologous such as Bcl-XL have been identified: a) dimerization with other Bcl-2 family proteins; b) binding to non-homologous proteins; and (c) formation of ion-channels/pores (Reed 1997; Schendel *et al.* 1998; Vaux and Strasser, 1996; Zamzami *et al.*, 1998).

The Bcl-2 family proteins are composed of three classes: anti-apoptotic 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). Sequence comparisons of the Bcl-2 family members have revealed up to four conserved domains, called Bcl-2 Homology (BH) domains: BH1, BH2, BH3 and BH4 (Reed *et al.* 1998). Mutagenesis studies identified a conserved domain, BH3, within several of the pro-apoptotic Bcl-2 family members which was show to be critical for both dimerization with anti-apoptotic proteins such as Bcl-2 or Bcl-XL and for induction of apoptosis (reviewed in Kelekar and Thompson, 1998). Of note, several pro-apoptotic members of Bcl-2 family protein contain the BH3 domain as their only apparent similarity with other members of the family, constituting the so-called "BH3-only" branch of the Bcl-2 family (Figure 1.10).

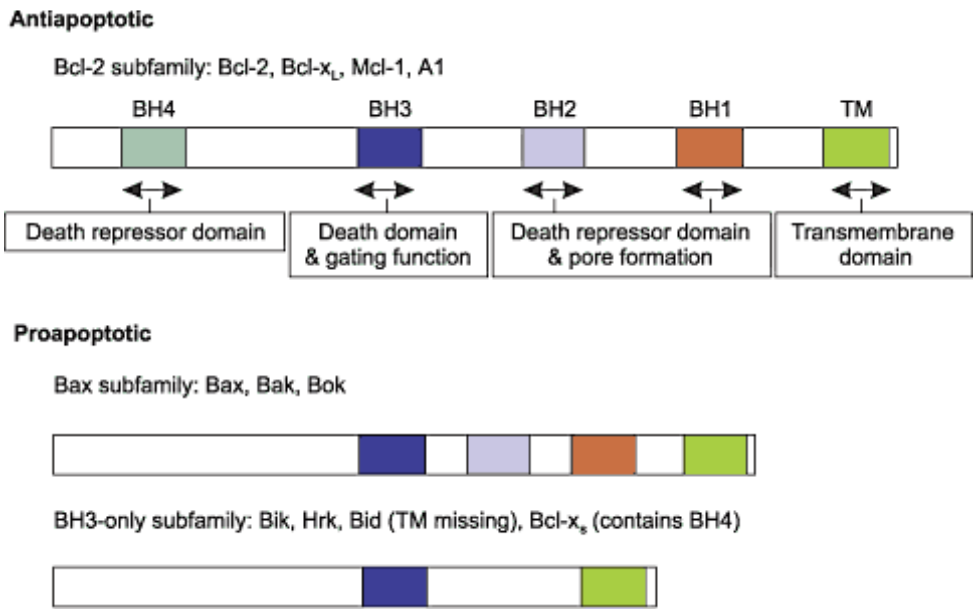


Figure 1.10. The Bcl-2 family. The topologies of several of the Bcl-2 family proteins are depicted, illustrating the BH1, BH2,BH3 and BH4 domains, as well as the transmembrane (TM) domains. The Bcl-X_L andBcl-X_S protein arise through alternative mRNA splicing mechanisms from the same gene.

Most Bcl-2 family members contain a C-terminal hydrophobic stretch of amino acids that anchors them in membranes, predominantly the outer mitochondrial membrane, endoplasmic reticulum and nuclear envelope. However, at least two pro-apoptotic Bh3-only proteins, Bad and Bid, lack membrane-anchoring domains *(*Wang *et al.* 1996; Yang *et al.* 1995*).* The locations of these proteins in cells is dynamically controlled by their association/dissociation with other Bcl-2 family proteins, resulting in a regulated translocation between the cytosol and the surface of membranous organelles where other Bcl-2 family proteins reside. In the regulation of the intrinsic pathway, pro-apoptotic gene products such as Bax, Bid, Puma, Noxa, and p53AIP1 localize at 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). Activation of death receptors can licence the mitochondrial apoptosis pathway by caspase-8 mediated cleveage of Bid into tBid, connecting the extrinsic with the intrinsic apoptosis pathway in an amplification loop (Figure 1.11).

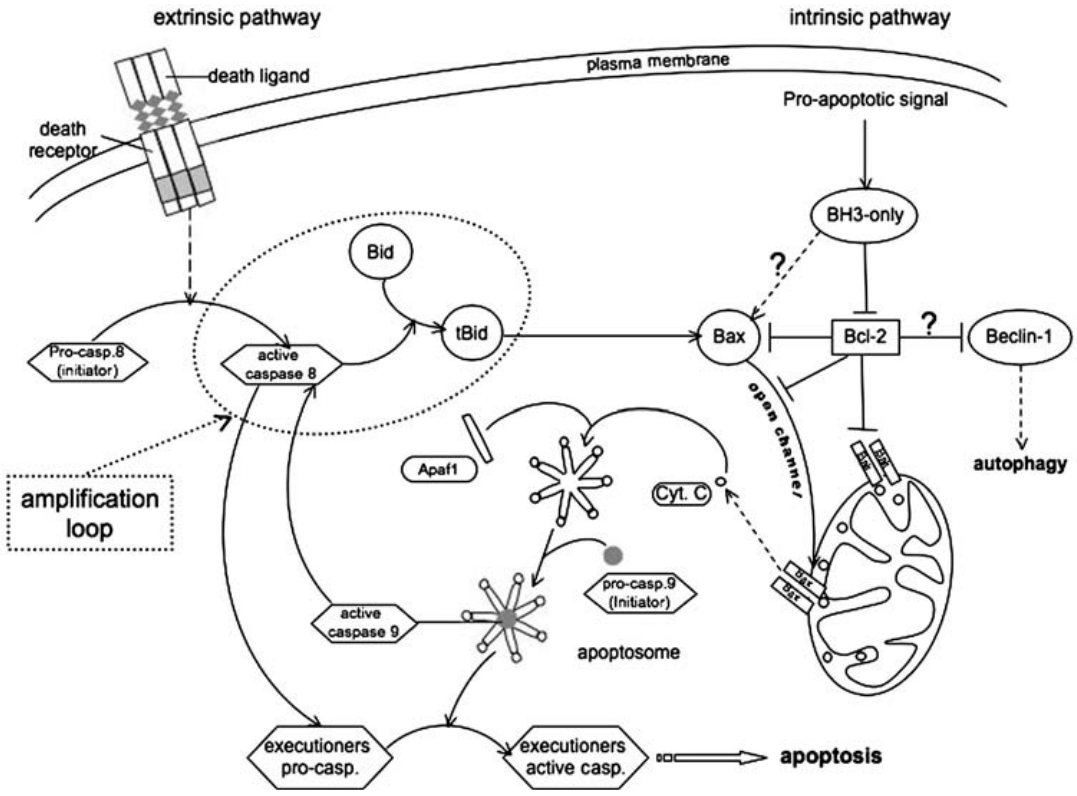


Figure 1.11. Cell death signalling regulated by the Bcl-2 family. Prosurvival-members of the Bcl2 family preserve mitochondrial integrity by preventing activation of Bax and/or Bak until neutralised by BH3-only proteins. This leads to mitochondrial outer membrane permabilization, release of apoptogenic factors, including cytochrome c, required for apoptosome formation and activation of caspase cascade. Activation of death receptors can licence the mitochondrial apoptosis pathway by caspase-8 mediated cleavage of Bid into tBid, connecting the extrinsic with the intrinsic apoptosis pathway in an amplification loop.

The quantitatively most important of these various mechanisms for controlling cell death most likely varies, depending on particular type of cell and the cell death stimulus involved. Moreover Bcl-2's properties include transcriptional and post-transcriptional control of gene expression, protein turnover, alterations of protein conformations, protein phosphorylation, proteolytic processing, and protein translocation.

1.11 HMGA1 levels influence mitochondrial function

Unique physical and biochemical properties give HMGA1 proteins the ability to function in the array of nuclear processes. These properties do not, however, exclude the possibility of a "non-nuclear" role for the proteins. Any region of DNA known to be high in AT content is thus a potential candidate for containing HMGA1 binding sites, regardless of specific sequence. This allows the proteins to directly interact with a number of platforms including satellite repeats (Strauss *et al*., Cell 1984), SARs/MARs (Zhao *et al*. 1993) and multiple gene promoter elements (Reeves *et al*. 2001). Functional versatility and mobility is increased by the relative small size (12-19 kDa) and inherent flexibility (Lehn *et al.* 1988) of the HMGA1 proteins. As free molecules, the proteins have little if any secondary structure (Evans *et al.*1992; Evans *et al.* 1995).

This characteristic is thought to play a significant role in the ability of HMGA1 to interact with multiple proteins and DNA elements as well as to induce conformational changes in these substrates (Reeves *et al.* 2001).

Multiple studies have shown that HMGA1 function is regulated by *in vivo* post-translational phosphorylation, acetylation and methylation (Reeves *et al.* 2001; Banks *et al*. 2000; Edberg *et al.* 2004). These modifications results from both internal and external signaling events that affect important biological events such as cellular activation and proliferation, apoptosis and cell cycle progression. Moreover, secondary biochemical modifications contribute to the ability of the cell to control both the function and distribution of these highly mobile and dynamic proteins. In particular, the specific phosphorylation of two HMGA1 threonine residues by cdc2 kinase during the G2/M phase of the cell cycle results in a 20-fold decrease in the DNA binding affinity of the modified protein, indicating the cell stage-specific mobilization of HMGA1 for an as yet undetermined function

(Nissen *et al.* 1991). Initial synchronization studies revealed a dynamic, cell cycle-dependent translocation of HMGA1 proteins from the nucleus into the cytoplasm and mitochondria of NIH3T3 cells (Dement *et al.* 2005). HMGA1

retains its DNA binding capabilities within the mitochondria and associates with the regulatory D-loop region in vivo.

Another work reveals that HMGA proteins are located in the nuclei of normal cell except during the late S/G(2) phases of the cell cycle, when HMGA1 proteins reversibly migrate to the mitochondria, where it binds to mitochondrial DNA (mtDNA). In many cancer cells, this controlled shuttling is lost and HMGA1 is found in mitochondria throughout the cell cycle (Mao L. *et al.*, Mol.Cell.Biol.2009). These evidence indicate that this nucleocytoplasmic movement is very dynamic, is cell-cycle dependent and is both directional and reversible, with the protein moving from nucleus to the cytoplasm and then back again. The mitochondrial localization of the HMGA1 proteins opened new areas of research regarding possible organelle specific functions for the proteins respect to normal and abnormal cellular functions, including cancer.

2. AIMS OF STUDY

The aim of the my study is to define the subcellular endogenous localization of HMGA1 proteins evaluating the presence of these proteins not only in the nuclei of cancer cell lines but also in the cytoplasm in order to identify the possible interactors in this cellular compartiment.

In fact, even thought the HMGA1 proteins have been frequently detected in the cytoplasm of cancer cells, no deep investigation has been undertaken to clearly demonstrate the presence and the functions of these proteins in cytoplasm.

On the basis of data that I have reported in the background, I focalized my attention on B-cell lymphoma gene 2 (Bcl-2) as possible cytoplasmic interactor.

Bcl-2 is an anti-apoptotic protein anchored to external membrane of mitochondria that inhibits the release of cytocrome C into the cytosol wich, in turn, activates caspase -9 and caspase-3 leading to apoptosis.

Moreover, *Bcl-2* gene is altered in many tumours including melanoma, breast, prostate, and lung carcinomas, and its transcription is regulated by HMGA1.

Therefore, my work aims to verify the localization of the HMGA1 protein at cytoplasmic level and its binding to Bcl-2 including the biological consequences of this interaction.

3. MATERIALS AND METHODS

3.1 Cell culture and transfections

HEK293, GC1, HeLa, HBL100, BT549, MDA-MB-231, MDA-MB-468, MCF7, T47D, ND7, and MEFs cells were maintained in DMEM with 10% fetal calf serum (GIBCO), glutamine, and antibiotics. H1299 cells were maintained in RPMI-1640 Medium with 10% fetal calf serum (GIBCO), glutamine, and antibiotics. Cells were transfected with plasmids by lipofectamine-plus reagent (Invitrogen) as suggested by the manufacturer.

3.2 Expression constructs

The pCMV/*Hmga1b* is described elsewhere (Pierantoni et al, 2001). pHaemagglutinin (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: pHA-A2 (amino acids 1-109) is constituted by the entire coding sequence of the *HMGA2;* pHA-A1b (amino acids 1-96) is constituted by the entire coding sequence of the *HMGA1b* isoform; pHA-A1b (1-79) 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 (Δ42-52) contains the *Hmga1b* coding sequence deprived of the second AT-hook domain; 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. To construct the EGFP-*Hmga1b* expression vector, the entire *Hmga1b* coding sequence was amplified by PCR with pairs of primers linked to

restriction sites (*EcoR*I and *Kpn*I) and cloned in the pEGFP-C2 plasmid (Clontech).

3.3 Western blotting and immunoprecipitation assay

TCE were prepared with lysis buffer (50mM Tris Hcl pH 7.5, 5mM EDTA, 300mM NaCl, 150mM KCl, 1mM dithiothreitol, 1% Nonidet P40, and a mix of protease inhibitors). For co-immunoprecipitation experiments, antigens and Abs were incubated for 3 h and then supplemented with protein A-sepharose or G-sepharose beads (Millipore). 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). 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 (Amersham Pharmacia). The Abs used for immunoprecipitation and Western blotting purchased from Santa Cruz Biotechnology were: anti-HA (Y11), anti-Sp1 (H225), anti-p53 (DO1), anti-Bcl-2 (100), anti-Cytochrome c (7H8) and anti-γ-tubulin (C11); anti-COX IV (3E11), anti-Phospho-(ser15) p53 (9284) and anti-Caspase-7 (9492) from Cell Signaling; anti-Caspase-9 (MAB8301) purchased from R&D Systems; anti-HMGA1 are polyclonal Ab raised against a synthetic peptide located in the NH2-terminal region (Pierantoni et al, 2006).

3.4 *In vitro* translation and protein-protein binding

The pET2c-*HMGA1b* construct was previously described (Baldassarre et al, 2003). His recombinant protein was produced in Escherichia coli BL21 cells. Stationary phase cultures of E. coli cells transformed with the plasmid of interest were diluted in LB with ampicillin (100 mg/ml), grown at 30°C to an OD_{600} of

0.6 and induced with 0.1mM IPTG. After an additional 2 h at 30°C, the cultures were harvested and resuspended in 10 ml of cold PBS (140mM NaCl, 20mM sodium phosphate pH 7.4), 1mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors (Boehringer). The cells were broken by French Press.

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 500mM imidazol and dialysed in PBS. The purified Bcl-2 and p53 proteins were respectively purchased from GenWay and BD Pharmingen. The recombinant proteins were subjected to protein-protein binding *in vitro* in NETN buffer (20mM Tris-HCl pH 8.0, 100mM NaCl, 1mM EDTA, and 0.5% Nonidet P-40) for 1 h at 4°C. Recombinant proteins and Abs were incubated for 3 h and then supplemented with protein A-sepharose or G-sepharose beads (Millipore). The resins were then extensively washed in the same buffer. The bound proteins were separated by SDS-PAGE, and analyzed by Western blotting.

3.5 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 moAb (Roche) and the FITC-conjugated goat anti mouse IgG (Jackson). Cells were stained simultaneously for DNA with Hoechst 33342 before observation with a fluorescent microscope (Zeiss). For mitochondrial staining, cells transfected with EGFP-HMGA1b were stained with Mito-ID^{TM} Red Detection Kit as suggested by the manufacturer.

3.6 Viability, apoptosis and caspase detection

Apoptosis was monitored by FACS, measuring the mitochondrial membrane potential (DF), determining the activation of Caspase-9 and TUNEL assay. Cells were harvested, pooled with the supernatant, washed once in PBS with Ca^{2+}/Mg^{2+} and processed for the different assays. For FACS analysis, cells were trypsinized, fixed in 70% ethanol and stored at 4 °C for a few days. Then, cells

were washed with PBS w/o Ca^{2+} and Mg^{2+}, stained with 50μg/ml propidium iodide containing RNase (20μg/ml) and analyzed with a FACS Calibur cytofluorimeter. For the measurement of the DF, the JC-1 (cationic dye that signal the loss of mitochondrial membrane) staining was used. After washing in PBS with Ca^{2+}/Mg^{2+}, cells were resuspended in complete medium and incubated with 2.5 mg/ml JC-1 (Molecular Probes) for 20 min at room temperature in the dark. After two washes in PBS with Ca/Mg, samples were placed on ice and immediately analyzed by a BD FACScan cytofluorimeter by using the BD CellQuest software package. For TUNEL assay, cells were fixed in paraformaldehyde solution (4% in PBS, pH 7.4) for 30 min at room temperature and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. Apoptotic nuclei were detected using TUNEL labelling reaction according to the manufacturer's instructions (Roche Biochemicals). TUNEL labelling and phase contrast images were analyzed by AXIO VISION 3.0 program. Caspase-9 colorimetric assay (PromoKine) was analyzed according to the manufacturer's instructions.

3.7 Isolation of nuclear/cytoplasmic fractions

Nuclear and cytoplasmic fractions were prepared as follows: 1-2 x 10^6 cells, scraped off the plate with PBS, were resuspended in hypotonic lysis buffer (10mM HEPES pH 7.9, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA) added with protease inhibitors (Roche). After resuspension, NP-40 was added to a final concentration of 0.6% and the nuclei were isolated by centrifugation at 10000 r.p.m. for 30 s at 4°C. After removal of the supernatant (i.e. the cytoplasmic extract), nuclei were resuspended in nuclear extract buffer (20mM HEPES pH 7.9, 25% glycerol, 0.4M NaCl, 0.1mM EDTA, 0.1mM EGTA), rocked for 15 min at 4°C and then recovered by centrifugation at 14000 r.p.m. for 5 min at 4°C.

3.8 Isolation of mitochondria and treatment with proteinase K in vitro

Mitochondria were isolated by differential centrifugation, using Mitochondria Fractionation Kit (BioVision). For enzymatic digestion, isolated mitochondria were resuspended in suspension buffer and treated with proteinase K 0.2 mg/ml for 20 min at 30°C in the presence or absence of Triton X-100 (1% final concentration). PMSF was then added to a final concentration of 2mM to stop the reaction.

3.9 Cytochrome C release from isolated and purified mitochondria

Intact mitochondria were isolated as above described and incubated at 37°C for 1 h with His-HMGA1b and recombinant p53 in MSB buffer (400mM mannitol, 50mM Tris, pH 7.2, 10mM KH_2PO_4, 5 mg/ml BSA) containing a cocktail of protease inhibitors. Samples were centrifuged at 5500 r.p.m. for 15 min at 4°C. The resulting supernatant and pelleted mitochondrial fractions were analyzed by Western blot. Pelleted mitochondria were washed two times with MSB buffer before analysis.

3.10 Immunohistochemistry

For immunohistochemistry, 6µm paraffin sections were deparaffinised and then placed in a solution of absolute methanol and 0.3% hydrogen peroxide for 30 min and then washed in PBS before immunoperoxidase staining. The slides were then incubated overnight at 4ºC in a humidified chamber with the antibodies diluted 1:100 in PBS. The slides were subsequently incubated with biotinylated goat anti-rabbit IgG for 20 min (Vectostain ABC kits, Vector Laboratories) and then with premixed reagent ABC (Vector) for 20 min. The immunostaining was performed by incubating the slides in diaminobenzidine (DAB-DAKO) solution containing 0.06 mM DAB and 2 mM hydrogen peroxide in 0.05% PBS, pH 7.6, for 5 min, and, after chromogen development, the slides were washed, dehydrated with alcohol and xylene and mounted with cover slips using a

permanent mounting medium (Permount). The antibodies used in this study were raised against the synthetic peptide SSSKQQPLASKQ specific for the HMGA protein. They were affinity purified against the synthetic peptide (Pierantoni et al, 2006). Tissue samples were scored as positive for immunohistochemistry when tissue immunoreactivity was detected in at least 10% of the cells. Negative controls were performed by omitting the first antibody. The specificity of the reaction was confirmed by the lack of tissue immunoreactivity after pre-incubation of the antibody with molar excess of the HMGA1 synthetic peptide.

4. RESULTS

4.1 HMGA1 is present at cytoplamic level other than in the nucleus

To determine whether HMGA1 proteins localize also at cytoplasmic level, we analyzed differential cell fractions from human embryonic kidney 293 cells (HEK293) transiently transfected with vectors encoding the full length haemagglutinin (HA)-tagged HMGA1b protein and a series of NH2- and COOH-terminal deletion mutants (Figure 4.1A). Western Blot analysis showed that the protein coded for the full-length HA-*HMGA1b* and almost all the HA-*HMGA1b* deletion mutant constructs was present in both the nuclear and cytoplasmic extracts (Figure 1B). One exception was represented by the HA-*HMGA1b* (1-43) construct that is deprivated of the second AT-hook domain that contains the nuclear signal localization (NSL): indeed, *HMGA1b* (1-43) showed only a cytoplasmic localization (Figure 4.1B).

Sp1 and γ-tubulin were used as markers of nuclear/cytoplasmic separation as well as loading controls (Figure 4.1B); Sp1 is a nuclear protein and the levels of γ-tubulin in cytoplasm are high.

To confirm these data, we performed immunofluorescence studies, using HA antibodies and markers that selectively stain nucleus (Hoechst) in HEK293 cells transiently transfected with HA-HMGA1b deletion mutants constructs.

The results, shown in Figure 4.1C, confirm that the HMGA1 full-length protein was detected in both the nuclear and cytoplasmic compartments after transfection with almost all the constructs, whereas the protein coded for the HA-HMGA1b (1-43) construct was located only in the cytoplasm.

To better characterize the cellular localization of endogenous HMGA1 proteins, we analyzed total, nuclear, and cytoplasmic cell lysates from several normal and tumor-derived cell lines. Western Blotting analyses demonstrated that HMGA1 localized at nuclear level only in normal cell lines, such as HEK293, neuronal

cell line (ND7), spermatogonia cell line (GC1) (Figure 4.1 D) and in two breast carcinoma cell lines (T47D and MCF7). Conversely, HMGA1 was present at cytoplasmic level other than in the nucleus in several breast (HBL100, BT549, MDA-MB-231, MDA-MB-468), lung (H1299), cervical (HeLa) cancer cell lines (Figure 4.1E).

Interestingly, mouse embryonic fibroblasts (MEFs) behaved as most of the cancer cell lines showing HMGA1 expression in both the compartments (Figure 4.1 E). Furthermore, HMGA1 was revealed at nuclear and cytoplasmic level also in human seminomas, whereas it was only nuclear in the normal testis (Figure 4.1 E).The same experiments performed to analyze the cellular localization of the other member of the HMGA protein family, HMGA2, revealed that this protein was not detected at cytoplasmic level, but only in the nucleus (Figures 4.1B and 4.1C).

This result suggests that the cytoplasmic localization is restricted to only the HMGA1 members of the HMGA protein family and not for HMGA2 protein.

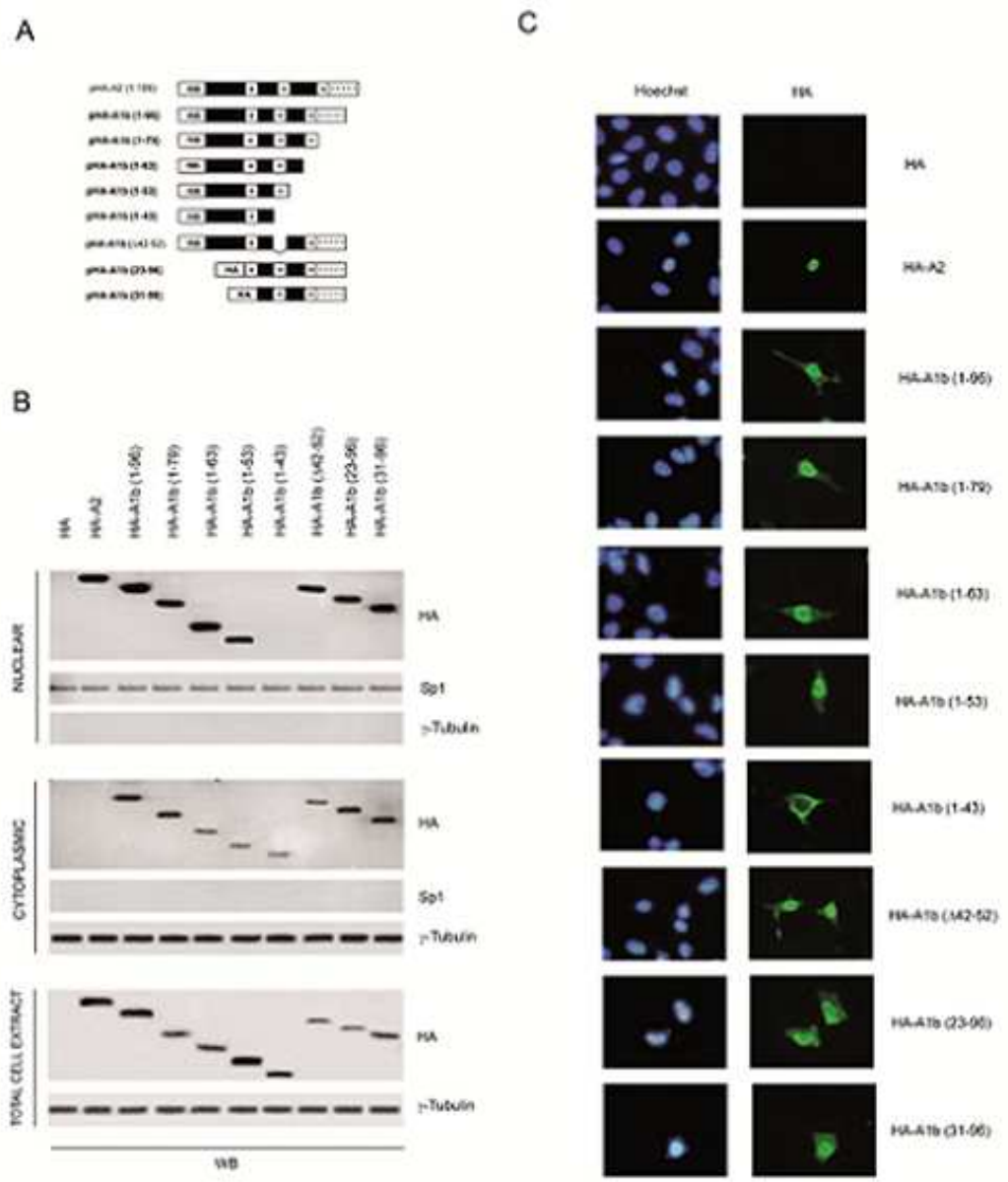


Figure 4.1 HMGA1b localize also in the cytoplasm. A) Diagram of the HA-tagged *Hmga2* and *Hmga1* wilde-type and deletion mutants used in western blotting and immunofluorescence analysis. The AT-hook domains (+) and the acidic tail (- - - - -) are indicated. **B)** Nuclear, cytoplasmic and total cell extracts from HEK293 cells were analyzed by Western blotting for the HA-HMGA2 and HA-HMGA1 wilde-type and mutant proteins. Sp1 and γ-tubulin were used as markers of nuclear/cytoplasmic separation as well as loading controls. **C)** Subcellular localization of HA-HMGA2 and HA-HMGA1 mutant proteins in HEK293 cells transfected with the indicated vectors. Nuclei were stained with Hoechst.

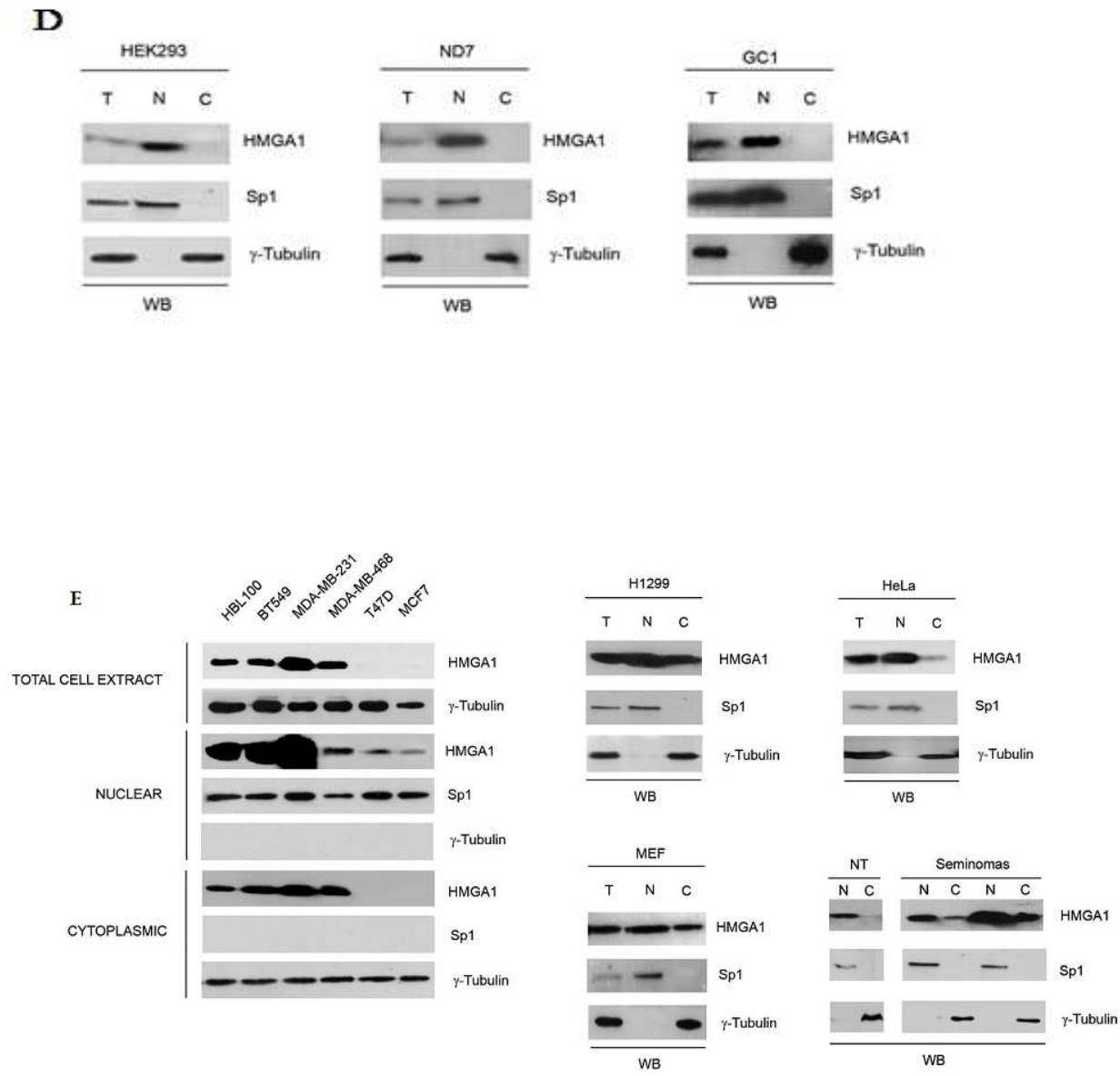


Figure 4.1. HMGA1 is located into the cytoplasm of cancer-derived cell lines and mouse embryonic fibroblasts. D) Total (T), nuclear (N) and cytoplasmic (C) cell extracts from HEK293, ND7, and GC1 cells were analyzed by Western blotting for the HMGA1 protein. **E)** Immunoblot analysis of HMGA1 expression in total (T), nuclear (N) and cytoplasmic (C) cell extracts from several breast tumor cell lines (HBL100, BT549, MDA-MB-231, MDA-MB-468, T47D, MCF7) (left), lung cancer cell line (H1299), cervical cancer cell line (HeLa), mouse embryonic fibroblasts (MEFs), human normal testis and seminomas (right). Sp1 and γ-tubulin were used as markers of nuclear/cytoplasmic separation as well as loading controls.

4.2 HMGA1 binds Bcl-2 *in vitro* and *in vivo*

In order to characterize the role of HMGA1 proteins into the cytoplasm, we have thought to identify potential interactors of HMGA1 looked for new molecular partners. Since Bcl-2 localizes to mitochondrial outer and, to a lesser degree, inner membrane (Akao *et al.* 1994), and Bcl-2 has been recently identified by us as possible HMGA1-interacting partner by an Antibody Array TM (Hypromatrix Incorporation), we asked whether HMGA1 is able to bind Bcl-2.

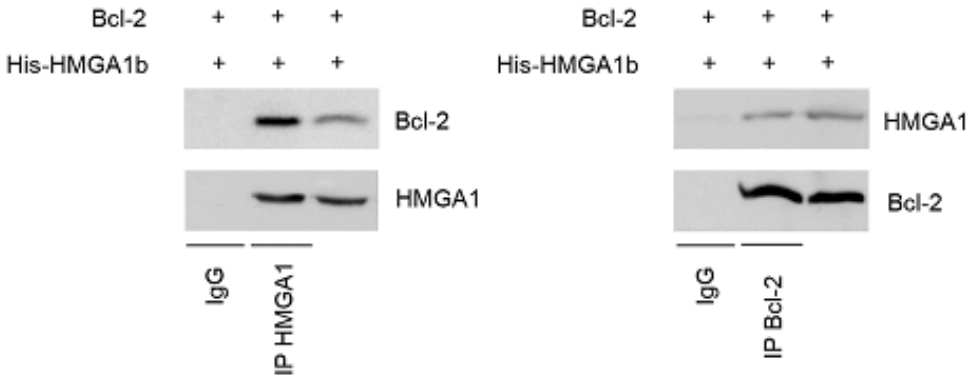
To this aim, we first evaluated the ability of HMGA1 and Bcl-2 purified proteins to interact *in vitro*. Indeed, Bcl-2 recombinant protein was incubated with the His-HMGA1b protein, that is the full length protein conjugated with tag of histidine, and the complexes were immunoprecipitated with anti-HMGA1 or anti-Bcl-2 antibodies and analyzed by Western blotting with the reciprocal antibodies.

As shown in the Figure 4.2 A, HMGA1 and Bcl-2 proteins can directly interact.

To verify this interaction *in vivo*, total cell extracts (TCE) from HEK293 cells transiently transfected with pCEFL-HA and pCEFL-HA/*HMGA1b* constructs were immunoprecipitated with anti-HA monoclonal antibodies and analyzed by Western blotting with anti-Bcl-2 polyclonal antibodies. Bcl-2 protein was present in the immunocomplexes from HA-*HMGA1b* transfected cells (Figure 4.2 B, upper panel). The reciprocal experiment performed immunoprecipitating with anti-Bcl-2 antibodies and revealing with anti-HMGA1 antibodies confirmed this interaction (Figure 4.2 B, lower panel).

Moreover, we have detected this association also between the endogenous proteins into cytoplasmic cell extract (CCE) from mammalian cancer cell lines MDA-MB-231 (Figure 4.2 C).

A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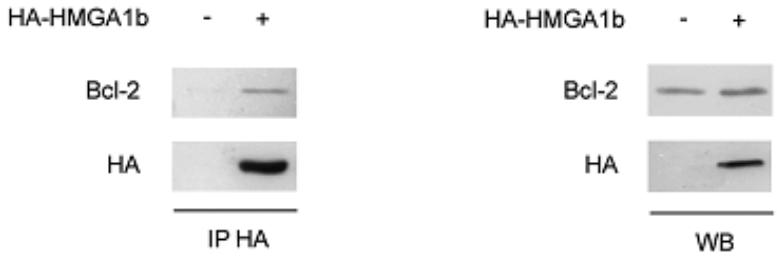
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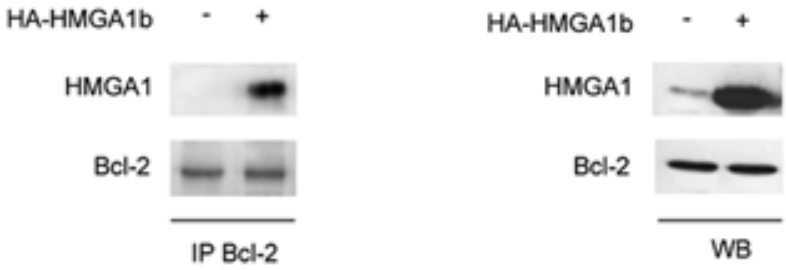
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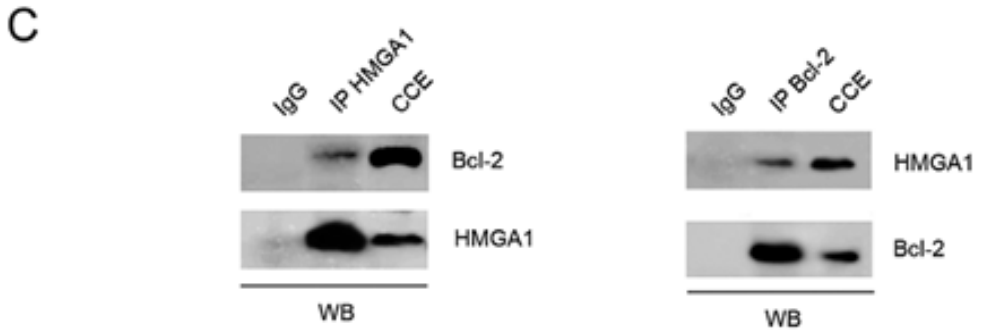
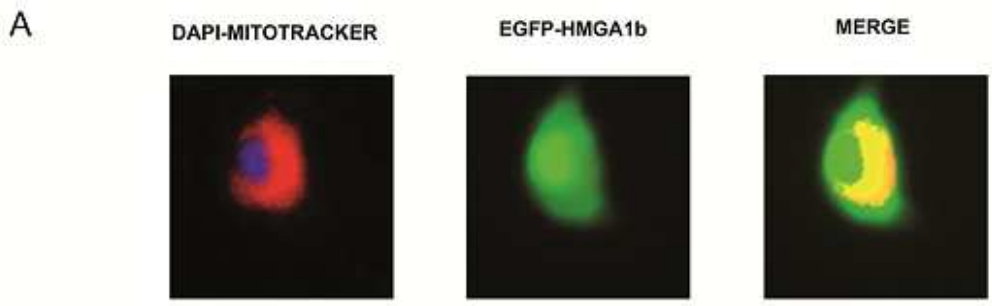


Figure 4.2. *In vitro* and *in vivo* characterization of the HMGA1/Bcl-2 interaction. A) The *in vitro* interaction between HMGA1b and Bcl-2 was assessed by immunoprecipitation assay with anti-HMGA1 (left panel) or anti-Bcl-2 (right panel) antibodies and blotting with the reciprocal antibodies after preincubation of the two proteins. The relative inputs are the His-HMGA1b and Bcl-2 recombinant proteins (loaded as controls). **B)** HEK293 cells were transfected with pCEFL-HA and pCEFL-HA/*Hmga1b* vectors. After 48 h, total cell extracts were prepared, and equal amounts of proteins were immunoprecipitated with anti-HA (upper panel) or anti-Bcl-2 (lower panel) antibodies, and the immunocomplexes were analyzed by Western blotting using the reciprocal antibodies. Cellular extracts used for immunoprecipitation experiments were analyzed by Western blotting (right panels). **C)** Co-immunoprecipitation with the anti-HMGA1 or anti-Bcl-2 antibodies with the cytoplasmic endogenous HMGA1 and Bcl-2 proteins (CCE from parental MDA-MB-231 cells). IgG indicates the negative control of immunoprecipitation using unrelated antibodies.

4.3 HMGA1 localizes in the internal compartments and on the outer membrane of mitochondria

It has been previously published that members of the HMGA1 protein subfamily are localized, almost exclusively, in the nuclei of normal cells except during the late S and G_2 phases of the cell cycle (Mao *et al*. 2009), when a minor protein fraction reversibly migrates out of the nucleus and into the mitochondria (Dement *et al*. 2005). However, this highly regulated shuttling is frequently disrupted in malignant cells that overexpress HMGA1 and these proteins are found in the mitochondria at all stages of the cell cycle (Dement *et al.*, 2005, 2007). To further characterize the role of HMGA1 into the cytoplasm and its association with mitochondria, we performed an immunofluorescence assay in HEK293 cells transfected with a vector encoding EGFP-HMGA1b protein, and incubated with a marker that selectively stains mitochondria, MitoTracker Red

dye. This red-fluorescent dye is able to stain mitochondria in live cells and its accumulation is dependent upon membrane potential. Merging of the signals by microscopy supported the partial co-localization of the cytoplasmic EGFP-HMGA1b protein fraction at the mitochondria (Figure 4.3 A). Therefore, to determine the mitochondrial compartment in which HMGA1 localizes, we performed an enzymatic digestion of mitochondrial fractions from HMGA1b- and empty vector-transfected HEK293 (Figure 4.3 B) and MDA-MB-231 cells, that show the presence of HMGA1 at cytoplasmic level (Figure 4.3 C). After digestion with proteinase K, a non-specific protease unable to cross the outer mitochondrial membrane (Reef *et al*. 2006), HMGA1 was partially recoverable in the pellet, whereas Bcl-2, a tail-anchored protein to the mitochondrial membrane, was degraded. HMGA1 disappeared, as it occurs also for p53 that is able to binds HMGA1 and is present in cytoplasm, only after solubilisation of inner mitochondrial membranes by Triton X, a detergent capable to destroy internal and external mitochondrial membrane (Figure 4.3 B). These findings indicated that HMGA1 is located in the internal compartments and on the outer membrane of mitochondria (Figures 4.3 B and 4.3 C). As control of experiment, we used Cox-IV that is a protein located on the mitochondrial inner membrane. The degradation of CoxIV protein after treatment with Triton-X but not with proteinase K, confirmed the results.



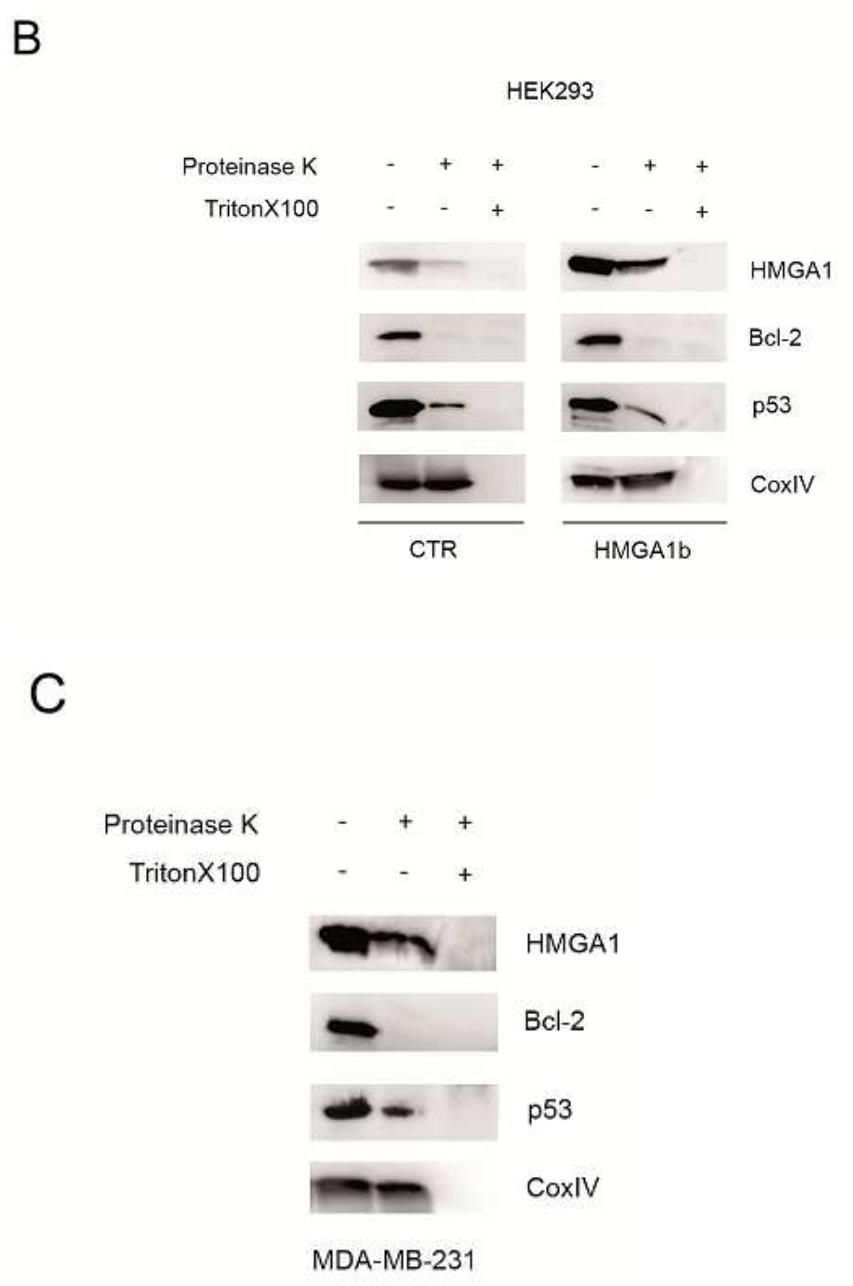


Figure 4.4. HMGA1 localizes at the mitochondria in the internal compartments and on the outer membrane. A) EGFP-HMGA1b subcellular localization in HEK293 cells. Nuclei and mitochondria were respectively stained with Hoechst and MitoTracker Red dye. **B)** Mitochondrial fractions from control and HMGA1b-transfected HEK293 cells were enzymatically digested by Proteinase K in the presence or absence of Triton X-100 (1%) and subsequently analyzed by Western blotting for the indicated proteins. **C)** Mitochondrial fractions from MDA-MB-231 cells were treated as in B and analyzed by Western blotting for the indicated proteins. Cyclooxygenase IV (CoxIV), localized to the inner mitochondrial membrane, was used as loading control.

4.4 HMGA1 displaces Bcl-2 from the binding to p53

We have previously demonstrated physical and functional interaction between HMGA1 and p53 (Pierantoni *et al*. 2006). This interaction modulates the transcription of p53 target genes such as *Mdm2, p21^{waf1}, Bax, Bcl-2*, and inhibits p53-mediated apoptosis (Pierantoni *et al*. 2006, 2007; Esposito *et al*. 2010).

As suggested by the data previously described, we asked whether HMGA1 counteracts p53-mediated apoptosis acting also on the p53 interactors in the cytoplasm, in particular at mitochondrial level. It is known that the cytochrome C release mediated by p53 depends on its ability to bind members of the anti-apoptotic Bcl-2 family (i.e. Bcl-2 and Bcl-xL) and to inactivate their inhibitory effect exerted on the pro-apoptotic proteins Bax and Bak (Mihara *et al*. 2003; Deng *et al*. 2006).

Therefore, we investigated whether HMGA1 affects p53 binding to Bcl-2 by performing a co-immunoprecipitation assay in the MDA-MB-231 cells incubated with doxorubicin, that is able to activate the endogenous p53, in the presence or absence of HMGA1b protein overexpression.

As expected, doxorubicin increases the formation of the Bcl-2/p53 complex leading to cell death, whereas HMGA1b overexpression significantly reduces the Bcl-2/p53 binding (Figure 4.4 A).To confirm this result, we used a cell-free system in which p53 and Bcl-2 recombinant proteins were incubated in presence or not of a recombinant wild-type HMGA1b protein. As shown in Figure 4.4 B, HMGA1b recombinant protein displaced Bcl-2 from the binding to p53.

This result demonstrates that HMGA1b directly interferes with the p53/Bcl-2 binding.

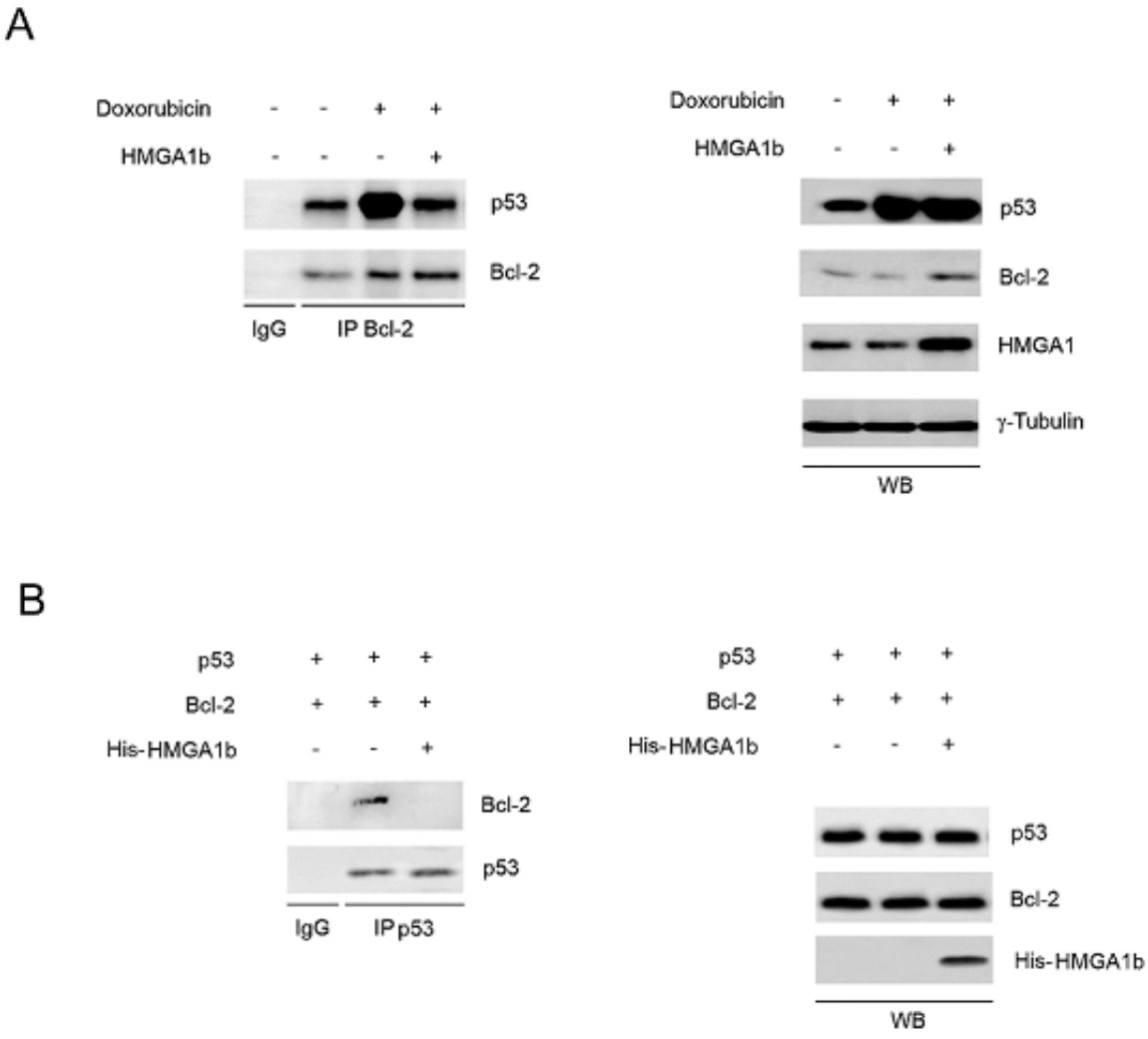


Figure 4.4. HMGA1 displaces Bcl-2 from the binding to p53. A) Lysates from MDA-MB-231 cells transfected with control or HMGA1b expressing vectors treated or not with doxorubicin were subjected to immunoprecipitation with anti-Bcl-2 antibodies, then immunocomplexes were analyzed by Western blotting using p53 antibodies. Western blot with Bcl-2 antibodies was used as a control of the equal loading of the immunoprecipitated Bcl-2 protein (left panel). Cellular extracts used for immunoprecipitation experiments were analyzed by Western blot with specific antibodies to verify protein expression levels (right panel). Y-Tubulin expression was used as a control for equal protein loading. **B**) p53 and Bcl-2 recombinant proteins were co-immunoprecipitated in the presence or absence of His-HMGA1b recombinant protein. The samples were separated by SDS-PAGE and transferred on Immobilon-P membranes. The filters were probed with either p53 or Bcl-2 antibodies (left panel). Western blotting analysis showed the amount of recombinant proteins used (right panel).

4.5 HMGA1 counteracts p53-intrinsic apoptosis

The data shown above strongly suggest that the presence of HMGA1 at mitochondria may affect p53-mediated mitochondrial apoptosis (also called p53-intrinsic apoptosis) through the reduction of p53/Bcl-2 binding. Indeed, p53 activates the apoptosis through two distinct pathways: transcriptional activation of proapoptotic genes and facilitation of mitochondrial outer membrane permeabilization (MOMP) with release of cytochrome C (Chipuk & Green, 2006). Earlier studies on p53-intrinsic apoptosis have pointed to p53 ability to permeabilize membranes of isolated mitochondria and permit cytochrome C release (Mihara et al, 2003; Chipuk et al, 2004; Deng et al, 2006).

Therefore, we investigated whether HMGA1 counteracts p53-mediated MOMP in this cell-free system by testing the ability of recombinant p53 to permeabilize mitochondria isolated from HEK293 or MCF7 in presence or absence of HMGA1b recombinant protein. As expected, p53 caused release of cytochrome C from HEK293 and MCF7 mitochondria (Figure 4.5 A), whereas the presence of HMGA1 greatly reduced such release resulting in depletion of cytochrome C from the supernatant fraction.

These data strongly support the existence of a negative role of mitochondrial-localized HMGA1 on the p53 permeabilization function. To verify whether the mitochondrial HMGA1 activity has functional effects, HEK293 and MCF7 cells were transfected with control or *HMGA1b* expressing vectors and treated with a lethal dose of UV (40 J/m^2) to induce cytochrome C release mediated by p53 activity. As expected, UV treatment induces cytochrome C release into the cytosolic fraction of control cells at the indicated time (Figures 4.5B and 6C).

No such effect could be observed in HEK293 and MCF7 cells transfected with *HMGA1b* expression vector (Figures 4.5 B and 6C).

The inhibitory effect of HMGA1 on p53-induced cytochrome C release was further confirmed analyzing mitochondrial and cytosolic fractions isolated from wild-type and null MEFs for the *hmga1* gene after UV irradiation.

Figure 4.5 D shows that UV light was less efficient in triggering the release of cytochrome C from the mitochondria of wild-type MEFs compared with mitochondria from *hmga1* minus MEFs. Consistently, HEK293 cells overexpressing HMGA1b protein showed a reduction of caspase 9 cleavage and a decreased number of apoptotic cells after UV irradiation in comparison with the control cells (Figure 4.6 E, left panel and right panel, respectively).

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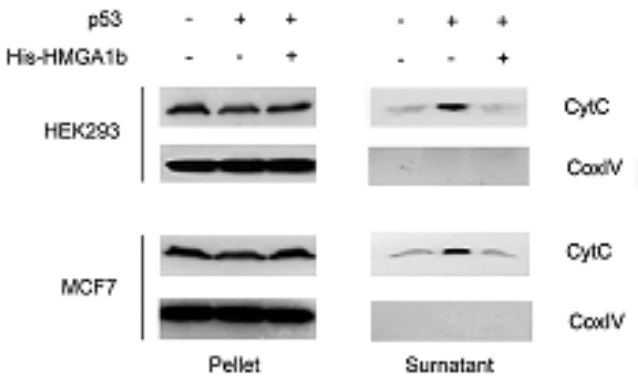

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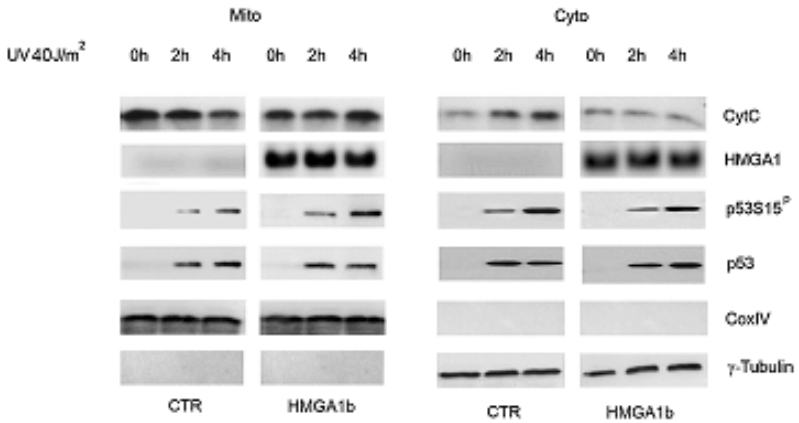


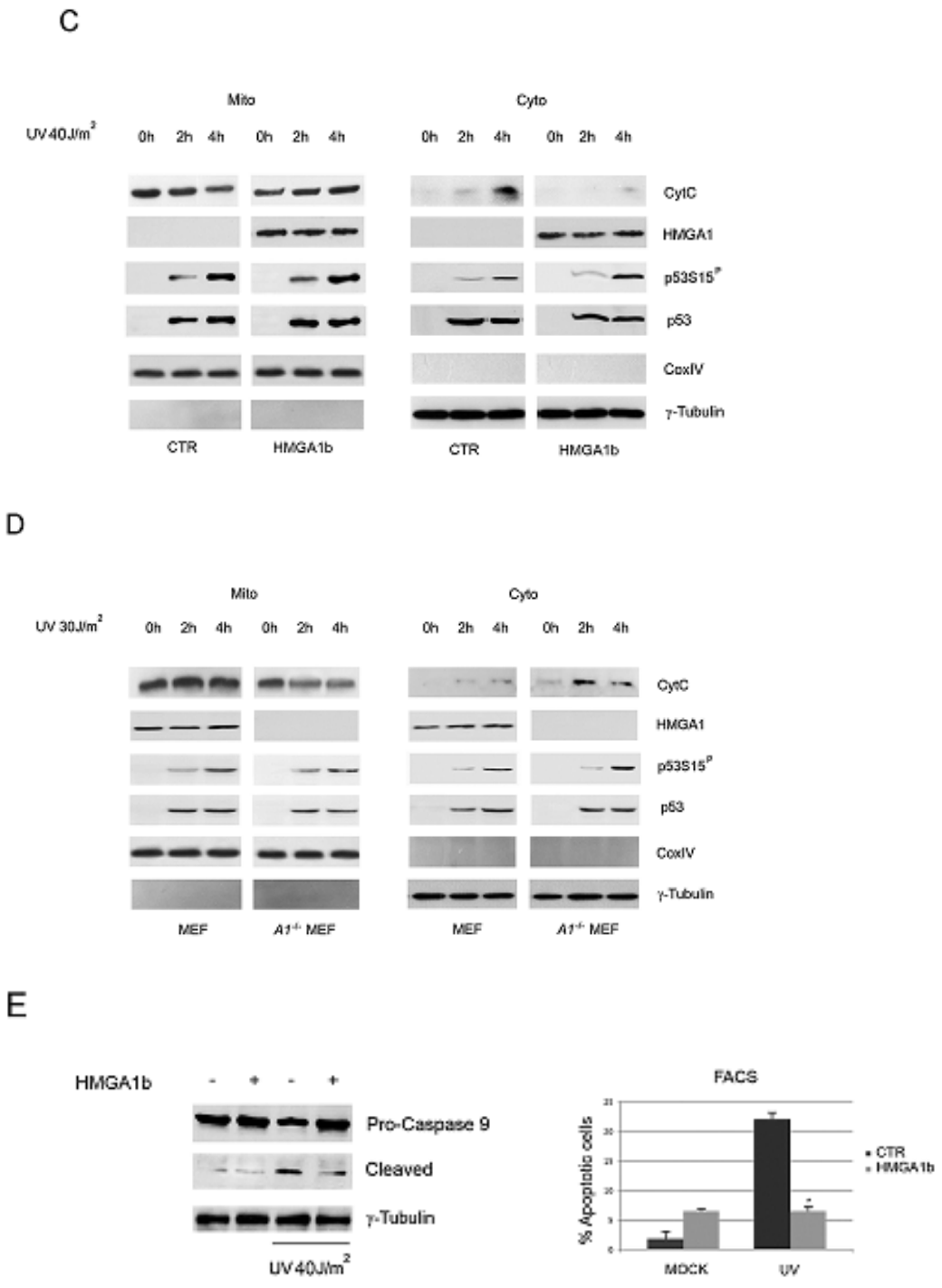


Figure 6. HMGA1 counteracts p53-mediated release of cytochrome C. A) Mitochondria isolated from HEK293 and MCF7 cells transfected with HMGA1 or the backbone vectors were incubated with p53 recombinant protein. After 60 min incubation at 37℃, reactions were centrifuged, and pellet and supernatant fractions analyzed by Western blot. **B-C)** HEK293 and MCF7 cells were transfected with HMGA1 expressing vector and, after 24 h, cells were treated or

not with 40 J/m^2 of UV irradiation. At the indicated time points, mitochondrial and cytosolic fractions were collected and analyzed by Western blotting with the indicated antibodies. **D)** Wild-type and *hmga1* null MEFs were either untreated or treated with 40 J/m^2 of UV irradiation. At the indicated time points, mitochondrial and cytosolic fractions were collected and analyzed by Western blotting. Cytosolic and mitochondrial contamination was verified by CoxIV and γ-Tubulin used also as loading control. **E)** HEK293 cells were treated as in **B,** and apoptosis was assessed by Western blotting analysis of Caspase-9 cleavage (left panel) and counting the number of apoptotic cells by FACS (right panel).

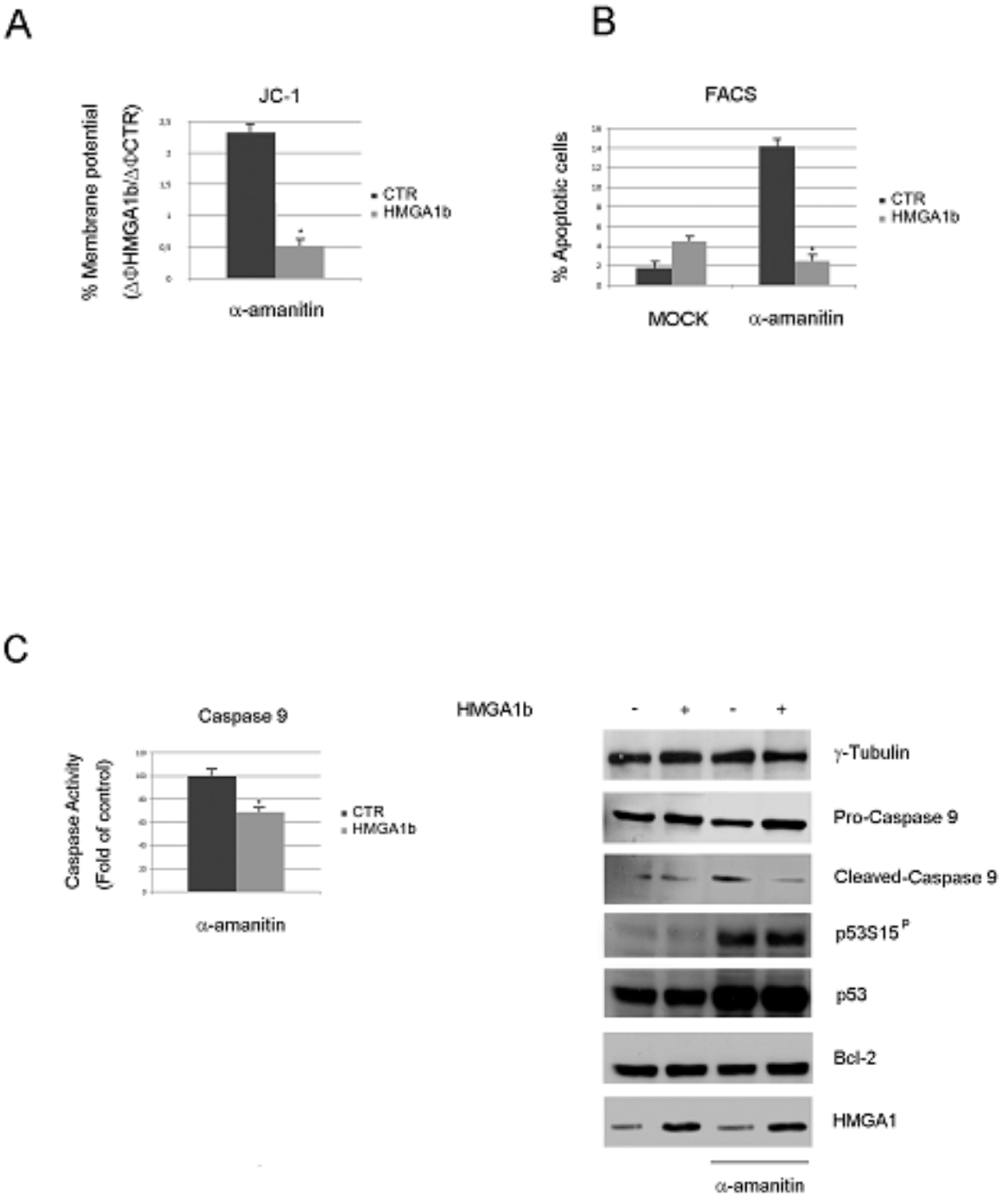
4.6 HMGA1 inhibits p53-mediated apoptosis by a transcriptional-independent mechanism

It has been already demonstrated that HMGA1 binds to p53 and inhibits its transcriptional activity. Therefore, to exclude that HMGA1 might regulate mitochondrial function and inhibit apoptosis through mechanisms independent from its mitochondrial localization but just altering p53 transcriptional activity, HEK293 cells were treated with α-amanitin, a specific inhibitor of RNA polymerase II-dependent transcription and an activator of p53-mitochondrial apoptosis (Arima *et al.*, 2005). In the presence of α-amanitin, overexpression of HMGA1 was still able to reduce the number of HEK293 cells with depolarized mitochondria (Figure 4.6 A). To depolarized HEK293 cells, we have used a JC-1 Assay Kit using JC1, a cationic dye that signal the loss of mitochondrial membrane. As expected, HMGA1 was also able to reduce the percentage of apoptotic cells (Figure 4.6 B) and caspase 9 activation (Figure 4.6 C) following treatment with α-amanitin in comparison with the control cells.

The same results were obtained in MCF7 cells, where HMGA1 overexpression counteracts caspase 7 and caspase 9 cleavages after α-amanitin treatment (Figure 4.6 D). Furthermore, the HA-HMGA1b (1-43) deletion mutant protein, which localizes only into the cytoplasm, was still able to inhibit caspase 9 activation following UV exposure. Indeed, it only partially counteracts cell apoptosis in comparison with the HA-HMGA1b full length protein (Figure 4.6 E).

Therefore, these results confirm the transcription-independent activity of HMGA1 in the repression of apoptosis and further support its negative

regulation towards p53-intrinsic apoptosis leading to the conclusion that HMGA1 can inhibit apoptosis through transcriptional and non-transcriptional pathways.



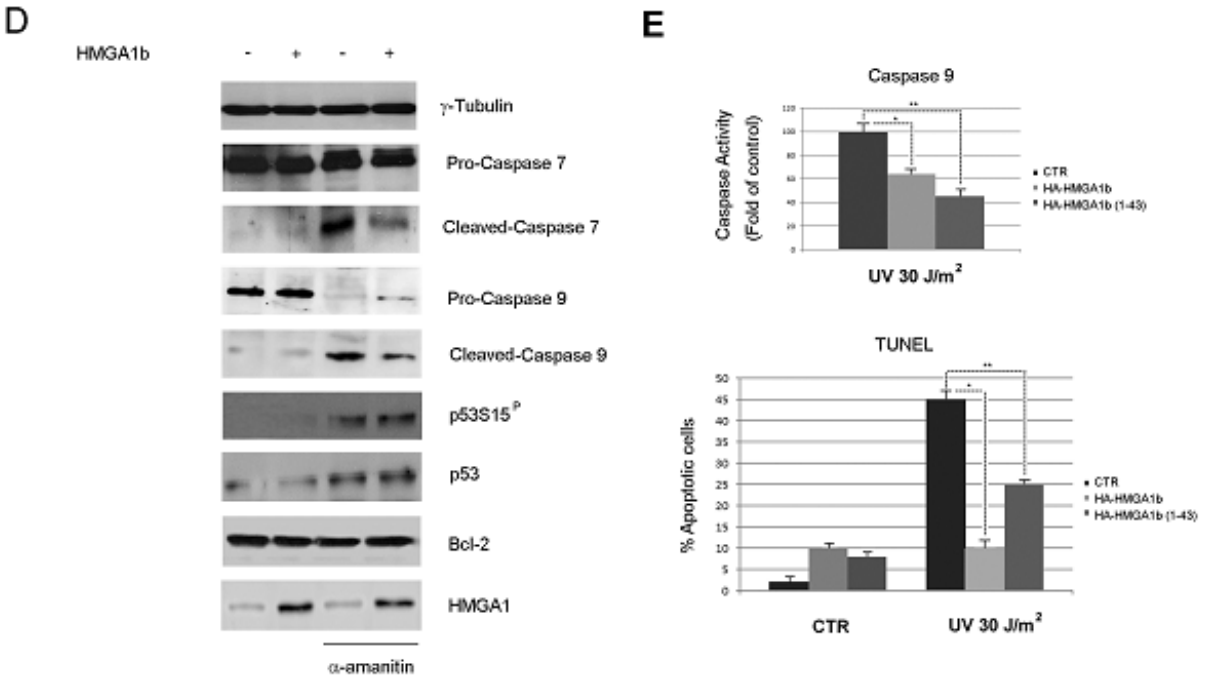


Figure 4.6. Transcription-independent activity of HMGA1 on inhibition of apoptosis. A-C) HEK293 cells were transfected with HMGA1 or the backbone vectors and after 24 h either untreated or treated with 10 μg/ml α-amanitin. After 16 h, cell death was assessed by JC-1 assay **(A)**, by counting the number of apoptotic cells by FACS **(B)**, by Caspase-9 colorimetric assay and Western blotting analysis of Caspase-9 cleavage **(C)**. The data are representative of three independent experiments. Each bar represents the mean and lines indicate standard deviation (s.d.) (*t*-test, **P*<0.05). **D)** MCF7 cells were treated as in **A** and apoptosis was assessed by Western blotting analysis of Caspase-7 and Caspase-9 cleavage. **E)** HEK293 cells were transfected with control, HA-HMGA1b or HA-HMGA1b (1-43) expressing vectors and after 24 h either untreated or treated with 30 J/m^2 of UV irradiation. After 16 h, cell death was assessed by Caspase-9 colorimetric assay (upper panel) and Tunel assay (lower panel). Each bar represents the mean of three independent experiments, and lines indicate standard deviation (s.d.) (*t*-test, **P*<0.05, ***P*<0.05).

4.7 Cytoplasmatic localization of HMGA1 correlates with higher aggressive tumor histotype

The interaction of HMGA1 protein with Bcl-2 and a consequent possible impairment of the p53 apoptotic activity would suggest another oncogenic effect of HMGA1, other those due to its regulation of the transcriptional activity, and thereby an higher aggressivity of the cells where HMGA1 is located at the cytoplasmic level.

Then, we proceeded to the analysis of thyroid, breast and colon carcinoma samples at different degree of malignancy to correlate the HMGA1 cytoplasmic location with the tumour malignancy.

Therefore, we analyzed the expression and localization of the HMGA1 protein in differentiated and undifferentiated thyroid carcinomas by immunohistochemistry. As shown in Figure 4.7, representative papillary and follicular thyroid carcinomas, that are well-differentiated and poorly aggressive carcinomas, showed a specific nuclear staining for HMGA1 (Figures 4.7 A and B, respectively). Conversely, anaplastic thyroid carcinomas, that represent one of the most aggressive tumor in the mankind, evidenced a strong cytoplasmic staining (a representative case is shown in Figure 4.7 C). Equally, a strong HMGA1 cytoplasmic staining was also found in aggressive breast and colon carcinomas (representative cases are shown in Figures 4.7 E and 4.7 G, respectively), whereas I*n Situ* breast ductal carcinomas and colon adenomas showed nuclear specific staining for HMGA1 (representative cases are shown in Figures 4.7 D and 4.7 F, respectively).

These data have confirmed by the analysis of data reported in Table 4.7. Analyzing some number of tumoral samples by immunohistochemistry, we have noted that the percentage of samples that present a cytoplasmic localization of HMGA1 are undifferentiated tumours or carcinomas.

Taken together, these data indicate a correlation between HMGA1 positivity at cytoplasmic level and a higher aggressive tumour histotype.

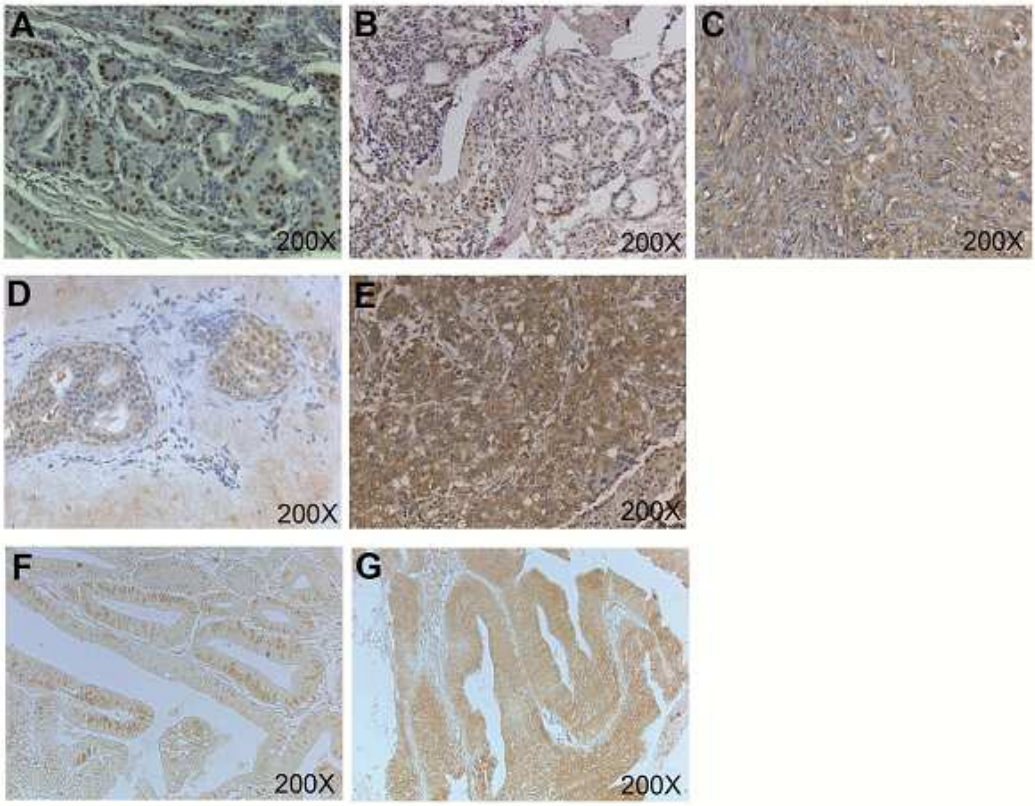


Figure 4.7. Immunohistochemical analysis of HMGA1 protein expression in human tumors. Paraffin sections were analyzed by immunohistochemistry using home made anti-HMGA1 rabbit polyclonal antibodies. **A**) Immunostaining of a papillary thyroid carcinoma (200 x). Specific nuclear immunoreactivity was observed. **B**) Immunostaining of a follicular thyroid carcinoma (200 x). Specific nuclear immunoreactivity was observed in follicular cells. **C**) Immunostaining of a anaplastic thyroid carcinoma (200 x). A strong cytoplasmic staining was observed in undifferentiated cancer cells. **D**) Immunostaining of an *In Situ* breast ductal carcinoma showing nuclear immunoreactivity and a very weak cytoplasmic staining (200 x). **E**) Immunostaining of an aggressive ductal breast carcinoma (G3) showing a strong cytoplasmic staining (200 x). **F)** Immunostaining of a colon adenoma with severe dysplasia showing nuclear immunoreactivity. **G)** Immunostaining of a colon carcinoma showing a strong cytoplasmic staining in the malignant cells.

Table 4.7

Tumor Histotype	Nuclear HMGA1 staining	Nuclear and cytoplasmic HMGA1 staining	Number of samples
Follicular thyroid carcinomas	10	0	10
Papillary thyroid carcinomas	16	2	18
Anaplastic thyroid carcinomas	1	9	10
In *Situ* breast ductal carcinomas	8	0	8
Breast carcinomas	2	9	11
Colon adenomas	12	0	12
Colon carcinomas	4	10	14

5. DISCUSSION AND CONCLUSIONS

The High mobility Group A proteins (HMGAs) are a family of small non-histone chromatin factors that do not have transcriptional activity *per se* but act like architectural factors (Muller *et al*. 2001).

They are able to bind directly the DNA, modifying its conformation and facilitating the binding of a group of transcriptional factors. They can also influence gene transcription through direct protein-protein interactions with transcription factors leading nuclear multiprotein complexes formation and promoters and enhancers of several genes (Reeves *et al*. 2001), (Fusco and Fedele 2007) .

HMGA proteins overexpression is a feature of several human malignancies and it is clearly demonstrated that they play a critical role in this process since the block of their expression inhibits cell transformation. They represent a good prognostic marker of neoplasia. These proteins have been identified as nuclear proteins; they exert their oncogenic activity acting at transcriptional level. Indeed, it has been reported that they are able to:

a) induce the activity of the transcription factor E2F1 and AP1 ;

b) induce Cyclin A expression;

c) inactivate p53-induced apoptosis;

d) impair DNA repair;

e) enhance the expression of proteins involved in inflammation;

f) modulate the expression of microRNAs and gene involved in epithelial-mesenchymal transition.

In this study, I demonstrated that HMGA1 localizes not only in the nuclei but also at cytoplasmic level where interacts with Bcl-2 at mitochondria and inhibits the p53-intrinsic-apoptotic pathway. I have reported that HMGA1 is located also in the cytoplasm of cancer cell lines and mouse embryonic fibroblasts.

This localization is restricted only for HMGA1 family members and not for HMGA2, that is exclusively in the nucleus. In the cytoplasm HMGA1 has a

diffuse localization at mitochondria in which it is present in the internal compartments as well as on the outer membrane.

HMGA1 is able to bind Bcl-2 that is anchored to the external mitochondrial membrane interfering with the binding between Bcl-2 and p53, thus counteracting the cytochrome C release. This results evident after lethal dose of UV light because the levels of cytochrome C in cytosol decreased in presence of HMGA1 overexpressed. Previously, our group demonstrated that HMGA1 sustains Bcl-2 transcription and at the same time promotes Bcl-2 activity also at cytoplasmic level. Then, to exclude the transcriptional activity of HMGA1 proteins in inhibiting the mitochondria p53-mediated apoptosis, I have performed experiments using α-amanitin. These experiments have demonstrated that HMGA1 is able to inhibit the intrinsic p53-mediated apoptosis by transcriptional independent mechanisms. The interaction of HMGA1 protein with Bcl-2 and the possible impairment of the p53 apoptotic activity would suggest another oncogenic effect of HMGA1 other those due to its regulation at the transcriptional level, and thereby a higher aggressivity of the cells where HMGA1 is located at the cytoplasmic level . Therefore, HMGA1 overexpression contributes to cancer progression impairing p53 activity, notwithstanding the absence of p53 gene mutations and/or deletions, through the already studied transcriptional activity (Pierantoni et al, 2006, 2007; Esposito et al, 2010) and, in addition, through this new cytoplasmic mechanism.

Consistently, the HMGA1 cytoplasmic localization correlates with a more aggressive histotype of cancer. Indeed, HMGA1 is localized in the cytoplasm of anaplastic thyroid carcinomas, colon and breast carcinomas, whereas it is located only at nuclear level in the differentiated thyroid carcinomas, *in situ* breast carcinomas and in colon adenomas.

Therefore, the data reported here demonstrate the ability of the HMGA1 proteins to localize in the cytoplasm, in particular at mitochondria, where they inhibit apoptosis by displacing p53 from Bcl-2, thus sustaining the Bcl-2 oncogenic activity.

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