Evidence of functional specificity within the MAGE-I family of tumor expressed proteins

Thesis submitted for the Degree of Doctor Philosophiae

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1. Summary

The Melanoma Antigen Genes (MAGE) belong to a large family of highly conserved genes, sharing an elevated degree of sequence homology. The characteristic feature of MAGE proteins is a C-terminal domain present in all the members of the family, termed the MAGE homology domain (MHD). Based on their expression pattern MAGE genes are classified in MAGE-I and MAGE-II genes. MAGE-I genes expression is restricted to tumor and male germ cells, and for this reason they form part of a growing group of genes named Cancer Testis Antigens (CTA). Expression of MAGE-I genes seems to be an early event during gametogenesis and tumorigenesis, and correlates with genomewide hypomethylation, an important event frequently observed in carcinogenesis.

Since their discovery in 1991, MAGE-I genes were mostly studied for their potential use in immunotherapy against cancer or as prognostic markers in tumors. The biological roles that these proteins play in tumor development and progression were poorly investigated. Moreover, due to their sequence homology, MAGE-I proteins are still considered functionally redundant proteins. In the present work, we functionally characterized different MAGE-I genes, in particular MageA2 and MageB2 genes, demonstrating their functional specificity.

We show that MageA2 protein confers wild-type p53 tumor suppressor-sensitive resistance to chemotherapeutic drugs, such as etoposide, by recruitment of HDAC3 to p53/MageA2 complex, thus repressing p53 transactivation function. The mechanism responsible for the repressive effect of MageA2, relies on an impaired acetylation of both p53 and histones surrounding p53 binding sites by MageA2/HDAC3 complexes. The correlation between MAGE-A expression and resistance to apoptosis has been analyzed in short-term melanoma cell lines, where combined treatment with etoposide and trichostatin A (an inhibitor of histone deacetylases) restores the p53 response and reverts chemoresistance in cells expressing high levels of MAGE-A.

We also present evidence that MageA2 is able to repress PML3-induced p53 activity in a specific manner, by affecting PML3 mediated p53 acetylation at the PML3 nuclear bodies (PML3-NBs). The relevance of MageA2 expression on PML3 activity has been analyzed in a normal cellular context, in which PML3 induces premature senescence, an important barrier against cell transformation. In this regard, we demonstrate that MageA2 impairs the

senescence response associated to PML3 expression in normal human fibroblast. A possible mechanism for the inhibitory effect of MageA2 on PML3 is that MageA2 could interfere with PML3 sumoylation. The specificity of MageA2 functions is demonstrated by the fact that, despite high level of homology, MageA4 is not recruited to the NBs, it does not affect p53 activity nor is able to interfere with PML3 induced senescence.

Finally, we have preliminarily characterized the MageB2 protein, showing that it specifically localizes to the nucleolus where it is able to interact with many nucleolar proteins. Nucleolar stress induces MageB2 relocalization to the nucleoplasm, a characteristic behavior of nucleolar proteins that regulate processes such as rRNA metabolism or RNA processing. Moreover, since we observed that MageB2 induces pRb relocalization to the nucleoli and increases E2F1 transactivation function, including E2F1-induced rRNA transcription, we hypothesize that it could play a positive role in the regulation of cell proliferation.

Altogether the work presented here consistently supports the notion that, despite the high level of sequence homology, there is a clear degree of functional specificity within members of the MAGE-I family. Hence, we can hypothesize that different MAGE-I proteins, for instance MageA2 and MageB2, could act within different pathways in the regulation of complex processes such as apoptosis, proliferation, and senescence. By targeting different signal transduction pathways their final outcome could be related to the establishment and progression of the tumors where they are expressed.

In this Thesis, we give a comprehensive view on the functional differences among MAGE-I members, focusing on Mage-A and Mage-B members. Implementation of our investigation could be the first step leading to understanding of how expression of specific MAGE-I members could impact cancer cell behaviour thus prompting the use of MAGE-I genes as novel cancer specific targets for the development of new drug-based therapies.

2. Introduction

The Melanoma Antigen Genes (MAGE)

MAGE genes belong to a large family of closely related genes characterized by a high degree of sequence homology (Fig. A) (Chomez, De Backer et al. 2001). The first human members of the MAGE gene family that have been described (now referred as MAGE-I subfamily) are expressed in tumor cells but silent in normal adult tissues except in male germ cells and placenta. Consequently, MAGE-I genes are considered tumor-specific antigens belonging to the Cancer/Testis Antigens (CTA) superfamily (van der Bruggen, Traversari et al. 1991; Rogner, Wilke et al. 1995; Chomez, De Backer et al. 2001; Barker and Salehi 2002). Based on this findings, MAGE-I proteins have become important targets for cancer immunotherapy (Marchand, van Baren et al. 1999; Thurner, Haendle et al. 1999; Kruit, van Ojik et al. 2005). However, other members of the family were recently found to be expressed in normal cells (now referred as MAGE-II subfamily), indicating that the family is larger and more disparate than initially expected (Lucas, Brasseur et al. 1999; Pold, Zhou et al. 1999; Chomez, De Backer et al. 2001). Sequence homology analysis has led to the recognition of other members and to date more than 60 MAGE genes have been identified and classified in different groups and subfamilies (Chomez, De Backer et al. 2001; Xiao and Chen 2004) (Table A).

a) The discovery of tumor specific MAGE proteins

In the last decades great efforts have been devoted to the search for tumor antigens, which could be used to direct the cytolytic capacities of the human immune system against cancer (Old 1981; Boon and Old 1997). An ideal cancer antigen for immunotherapy should be specifically and stably expressed by the tumor, absent from normal tissues, and crucial for survival of cancer cells. An important turning point in this search was the adoption of an approach called autologous typing, in which cultured tumor and normal cells from a patient were used as targets for specific immune recognition by the patient's own antibodies and T cells (Old 1981; Knuth, Danowski et al. 1984).

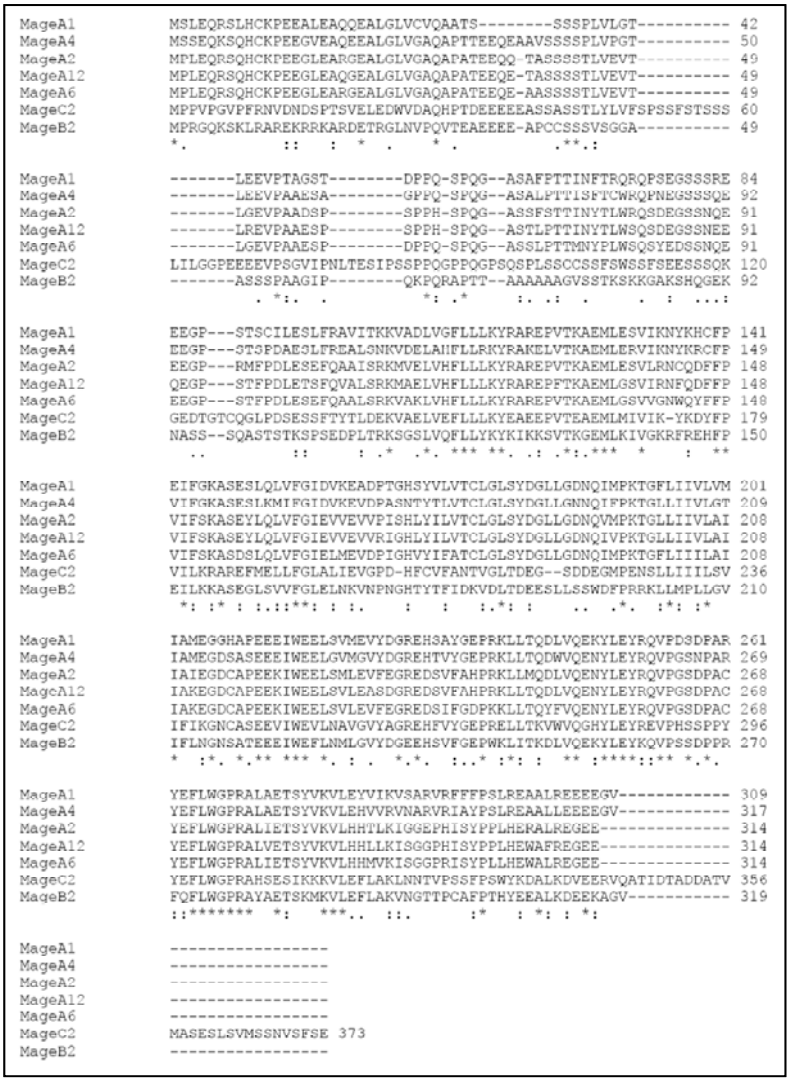

Fig. A: Multiple sequence alignment of selected MAGE type I members, showing the high degree of sequence homology shared by these proteins.

The first CTA was identified in 1991 through autologous typing and the application of a newly developed DNA-cloning methodology for defining the targets of T-cell recognition (Van Pel, van der Bruggen et al. 1995). A patient with melanoma who had an unusually favorable clinical course was identified as having cytotoxic T cells that recognized autologous tumor cells (Knuth, Wolfel et al. 1989). Using this system, the gene encoding the tumor antigen MZ2-E was cloned (van der Bruggen, Traversari et al. 1991; Traversari, van der Bruggen et al. 1992). The gene was termed Mage1 (then renamed MageA1) and closely related genes Mage2 (MageA2) and Mage3 (MageA3) were subsequently identified in the same cell line (Gaugler, Van den Eynde et al. 1994; Chomez, De Backer et al. 2001). Expression of MageA1 was detected in melanomas, some breast carcinomas and other tumor

types, but not in any normal tissues except testis (Chomez, De Backer et al. 2001). In the following years, 12 new closely related MAGE-A genes were identified and showed to be clustered at the Xq28 (De Plaen, Arden et al. 1994; Chen, Scanlan et al. 1997; Chomez, De Backer et al. 2001). A search for the gene responsible for the sex-reversal phenotype revealed a second cluster of MAGE genes, which encoded the MAGE-B genes and was located at Xp21.3 (Muscatelli, Walker et al. 1995). Subsequently, a third cluster, encoding the MAGE-C genes was identified at Xq26-27 (Chen, Gure et al. 1998; Lucas, De Smet et al. 1998; Gure, Stockert et al. 2000). MAGE-A, MAGE-B and MAGE-C genes form the MAGE-I subfamily of tumor-specific MAGE genes.

Type	Subtype	Gene name	Length of amino acids	Expression status
I	MAGE-A	MAGE-A1	280	T
		MAGE-A2	314	T
		MAGE-A3	314	T
		MAGE-A4	317	T
		MAGE-A5	320	T
		MAGE-A6	314	T
		MAGE-A7	80	P
		MAGE-A8	318	T
		MAGE-A9	315	T
		MAGE-A10	369	T
		MAGE-A11	319	T
		MAGE-A12	314	T
		MAGE-A13	-	P
		MAGE-A14	-	P
		MAGE-A15	-	P
I	MAGE-B	MAGE-B1	347	T
		MAGE-B2	319	T
		MAGE-B3	346	T
		MAGE-B4	346	T
		MAGE-B5	111	T
		MAGE-B6	407	T
		MAGE-B7	-	P
		MAGE-B8	-	P
		MAGE-B9	-	P
		MAGE-B10	348	T
		MAGE-B11	-	P
		MAGE-B12	-	P
		MAGE-B13	-	P
		MAGE-B14	-	P
		MAGE-B15	-	P
		MAGE-B16	320	T
		MAGE-B17	203	T
I	MAGE-C	MAGE-C1	1142	T
		MAGE-C2	373	T
		MAGE-C3	346	T
		MAGE-C4	115	T
		MAGE-C5	-	P
		MAGE-C6	-	P
		MAGE-C7	-	P
II	MAGE-D	Necdina	321	N
		MAGE-D1	778	N
		MAGE-D2	606	N
		MAGE-D3	1387	N
		MAGE-D4	741	N
II	MAGE-E	MAGE-E1	741	N
		MAGE-E2	-	N
		MAGE-E3	424	N
II	MAGE-F	MAGE-F1	307	N
II	MAGE-G	MAGE-G1	100	N
II	MAGE-H	MAGE-H1	219	N
II	MAGE-I	MAGE-I1	-	P
		MAGE-I2	-	P
II	MAGE-J	MAGE-J1	-	P
II	MAGE-K	MAGE-K1	-	P
II	MAGE-L2	MAGE-L2	529	N

Table A: The MAGE family genes
T: expressed in tumor or germinal cells. P: pseudogene. N: expressed in normal cells. -: unknown.

b) Structure, classification and organization of MAGE genes

The principal feature of MAGE proteins, is a large central region termed MAGE homology domain (MHD). The MHD is a sequence of 165-171 amino acids, generally found near the C-terminus of the protein. This domain corresponds to a region of high homology and it is shared by all the members of the MAGE family (Fig. B). The MHD is present in proteins of many multicellular organisms, including *Drosophila*, *Aspergillus* and *Arabidopsis thaliana* but is apparently lacking in *Caenorhabditis elegans* and in unicellular organism such as protozoa (Chomez, De Backer et al. 2001; Barker and Salehi 2002). However, it has recently been reported that the yeast protein Nse3 shows significant level of homology with human MAGE proteins (Sergeant, Taylor et al. 2005). In humans the MHDs can be subdivided into five distinct regions that represent distinct areas of conservation (Fig. C). Subdomains 1, 3, and 5 are very similar, while the linker sequences are the less conserved portions of the MHD (Fig. C). Structural predictions based on the aligned MHD sequences suggest that the MHD contains at least four potential α–helical domains and five regions of β-sheet structure, but the true structure of this domains remains unresolved (Fig. C) (Barker and Salehi 2002). Despite its intra- and inter-species conservation, the MHD shows no significant homology to other proteins that would provide clues to its cellular function.

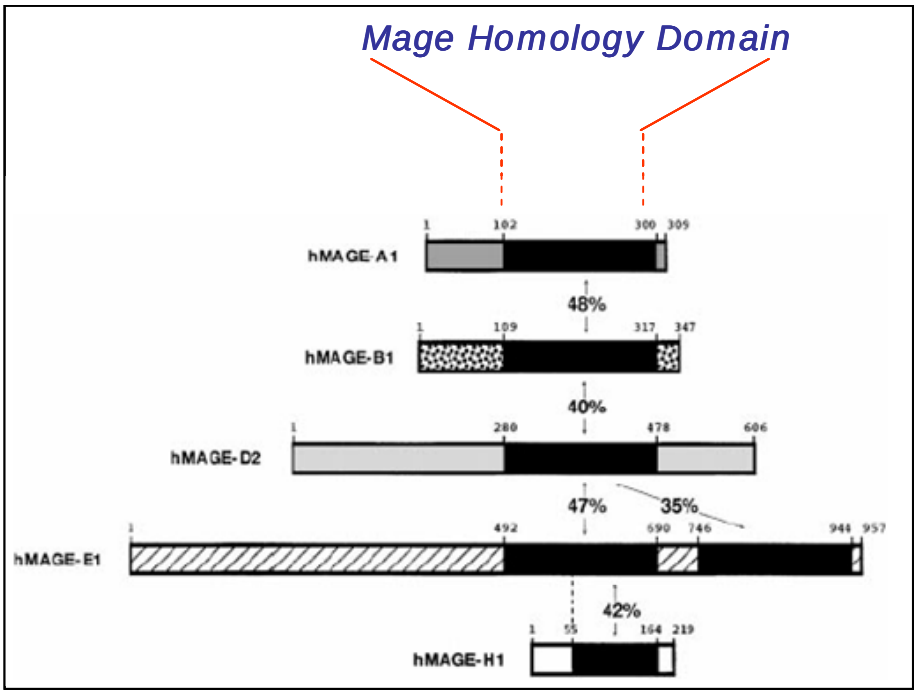

Fig. B: Schematic representation of MAGE proteins. The percentage of identical amino acids between the MAGE conserved domains are indicated. Different fillings of the N- and C- terminal regions of the proteins represent sequences that share no homology.

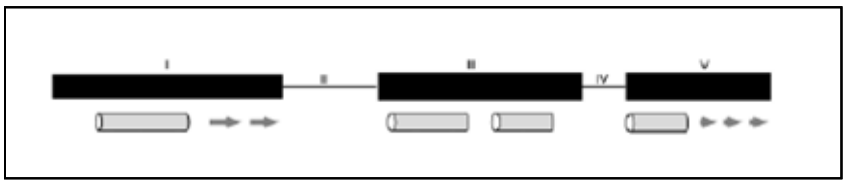

Fig. C: Schematic representation of the MHD structure. Black boxes represent highly conserved regions, while lines represent less conserved regione. Cylinders and arrows represent predicted α-helices and β-sheets, respectively.

Based on expression profiles, MAGE genes were classified as belonging to either subgroup I (MAGE-I genes) or II (MAGE-II genes) (Barker and Salehi 2002). MAGE-I genes include three subfamilies that cluster at the X-chromosome, MAGE-A at Xq28, MAGE-B at Xp21 and MAGE-C at Xp26-27 (Fig. D). The expression of these genes is restricted to some malignant tumors, testis and placenta, but is not found in other normal tissues (Chomez, De Backer et al. 2001). For this reason, these genes belong to the expanding group of genes termed cancer/testis antigens (CTA). Most of MAGE-I proteins consist of little more than a single highly conserved MHD flanked by a small amount of poorly conserved N- and C-terminal sequence (Barker and Salehi 2002). MAGE-II genes include the subfamilies MAGE-D, -E, -F, -G, -H, -I, -J, -K and L2, that localize in chromosome 3, 15 and X (Fig. D). These genes are expressed in various normal adult human tissues. Some MAGE-II proteins (Necdin, MAGE-F and -G) show a structure similar to that of MAGE-A and –B, however others (MAGE-D, -E and –L) are larger proteins with extended N- or C-termini and in some cases contain a second, less conserved MHD (MHD2) (Fig. E). The ORFs of several MAGE-II genes are encoded by multiple exons and some give rise to alternative spliced mRNAs (Barker and Salehi 2002).

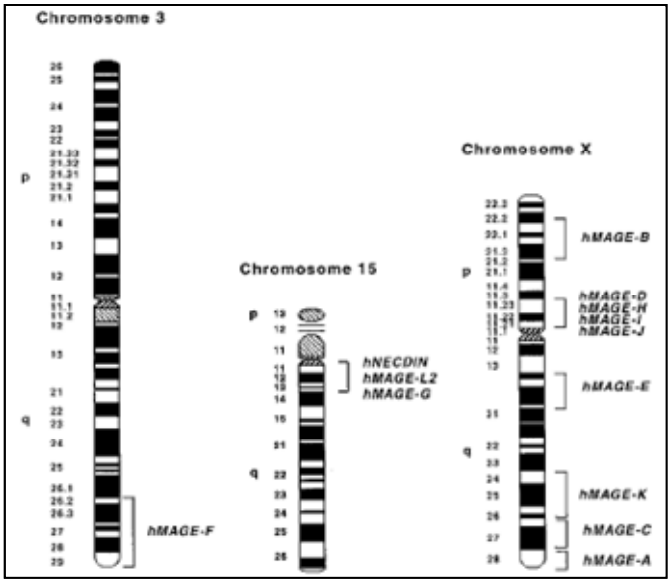

Fig. D: Chromosomal locations of the human MAGE subfamilies.

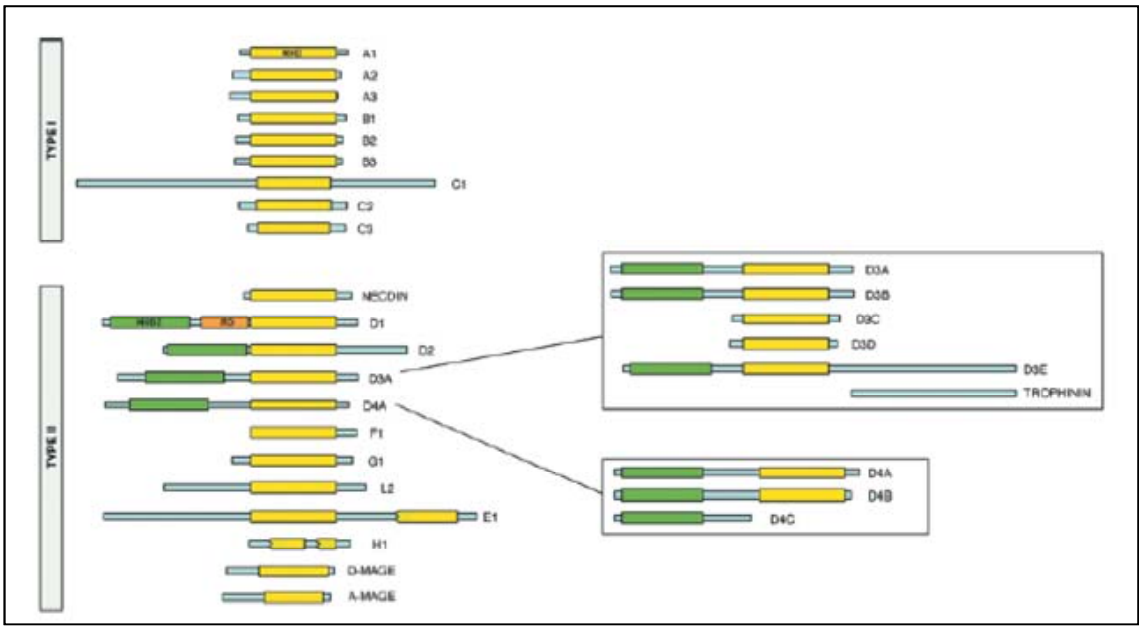

Fig. E: Domain structures of selected MAGE family members, with the MHD indicated in yellow and MHD2 in green. Alternatively spliced isoforms of MageD3 and D4 are indicated in boxes. The jagged lines in the MHDs in MageE1and H1 indicate the presence of truncated MHDs.

Phylogenetic analysis of the MHD sequences showed that type I and type II genes define distinct branches within the MAGE family (Fig. F) (Barker and Salehi 2002).

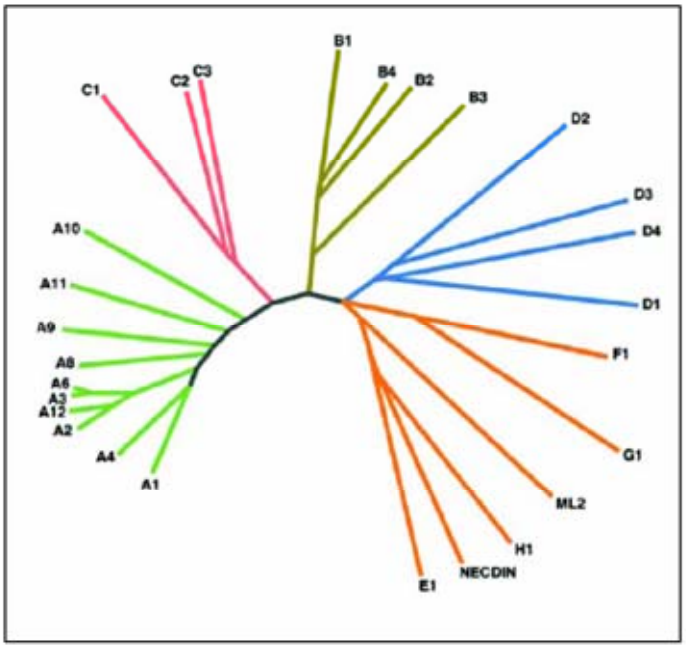

Fig. F: Phylogenetic tree of aligned MHDs.

Structural analysis of MAGE genes suggest that they derived from an ancestral gene, probably belonging to MAGE-D, due to the high level of complexity of this subfamily, by processes of retroposition and further duplication, events that probably occurred more recently, thus indicating an unusually rapid evolution of this gene family (Chomez, De Backer et al. 2001).

c) Regulation of MAGE genes expression

MAGE-I genes are ideal targets for cancer vaccines because of their expression in a wide range of human tumors. Indeed, recent clinical studies showed promising results in anticancer immunotherapy against MAGE-I antigens (Marchand, van Baren et al. 1999; Hersey, Halliday et al. 2008; Zhang, Zhang et al. 2008). Nevertheless, heterogeneous intratumor expression of MAGE-I genes may hamper the effectiveness of these therapies and this has increased the interest in the analysis of the regulation of the expression of these genes.

The expression of the various MAGE-I genes varies greatly among tumor types and they are frequently co-expressed in the same tumor, suggesting a possible cooperative effect in favoring the malignant phenotype through different functions that may act in synergy. These

observations are also consistent with MAGE-I expression being the result of the activation of a coordinated gene-expression program, rather than being independent events.

A critical element in the induction of CTA genes expression (including MAGE-I) appears to be promoter demethylation. Methylation of CpG islands within gene promoters is responsible for gene silencing, due to both its effect on chromatin structure and to transcription factor binding (Baylin and Herman 2000). Alterations in DNA methylation and chromatin re-structuring, occur during two phases in the human life cycle: gametogenesis and early embryogenesis (Kimmins and Sassone-Corsi 2005). So far, all CTA genes studied have methylated CpG islands in normal somatic tissues and are activated by demethylation during spermatogenesis (Weber, Salgaller et al. 1994; De Smet, De Backer et al. 1996; De Smet, Lurquin et al. 1999). Global DNA hypomethylation and gene-specific hypomethylation are processes which often accompany tumorigenesis and global hypomethylation in tumors has been correlated with CTA gene expression.

Recent studies have demonstrated the relevance of DNA methylation on the regulation of MAGE-I expression. Demethylating agents such as 5-Aza-2'-Deoxycytidine (5-aza-CdR), and more recently valproate, have shown to induce the expression of MageA1 and MageB2, respectively, in tumor cells that do not express these genes and in several normal cell lines (Weber, Salgaller et al. 1994; De Smet, De Backer et al. 1996; Karpf, Lasek et al. 2004; Milutinovic, D'Alessio et al. 2007). Further studies revealed that cells that do not express MageA1 nevertheless contain transcription factors capable of inducing significant MageA1 promoter activity, Ets transcription factor being responsible for its expression (De Smet, De Backer et al. 1996). This implied that lack of MageA1 transcription in somatic cells requires a local repression mechanism to prevent activation by these ubiquitous transcriptions factors. A CpG-rich promoter was defined in the promoter of MageA1, which unlike classical CpG-rich promoters, is methylated in all normal somatic tissues (De Smet, Lurquin et al. 1999). Moreover, although MZ2-MEL melanoma cells contain an active unmethylated MageA1 gene, they lack the ability to induce demethylation of newly integrated MageA1 transgenes previously methylated *in vitro* before transfection. In the same cells unmethylated MageA1 transgenes were protected against remethylation, and this appeared to depend on the level of transcriptional activity. Therefore, it was proposed that hypomethylation of MageA1 in tumors relies on a past demethylation event and on the presence of appropriate transcription factors that maintain the promoter unmethylated (De Smet, Loriot et al. 2004). Moreover, by using this cellular system it was shown that transient down-regulation of the DNA methyltransferase DNMT1 leads to the activation and stable hypomethylation of MageA1

(Loriot, De Plaen et al. 2006). Similar results were obtained by double knockout of DNMT1 and DNMT3b (James, Link et al. 2006) or in knockout mice for the Histone H3 Lys 9 (H3-K9) methyltrasferase, GLP/Eu-HMTase1 (Tachibana, Ueda et al. 2005). Altogether these data evidence the importance of epigenetic regulatory mechanisms for the control of MAGE-I expression.

In addition, other reports have suggested that chromatin remodeling mechanisms, like histone acetylation, could contribute to MAGE-A expression. It was demonstrated that treatment with the histone deacetylase (HDAC) inhibitor Trichostatin A up-regulates the expression of MAGE-A genes as induced by demethylating agents (Wischnewski, Pantel et al. 2006).

BORIS (Brother of the regulator of imprinted sites) is a recently identified CTA (Loukinov, Pugacheva et al. 2002), which encodes a paralogous of CTCF (CCCTC-binding factor), an ubiquitously expressed nuclear factor involved in genetic regulatory processes, such as context-dependent transcription silencing or activation, and organization of epigenetically controlled chromatin insulators that regulates imprinted genes in somatic cells. Like CTCF, BORIS contains an 11-zinc finger domain that mediate interaction with specific cis-regulatory sequences. These proteins are expressed in a mutually exclusive fashion during the development of male germ cells. In particular, demethylation events are associated with the expression of BORIS and with CTFC silencing, suggesting a complementary function of the two proteins during development and differentiation of the male germ line. BORIS is expressed also in many tumor types, and maps in a region (20q13) highly amplified in some cancer cells, its expression favors proliferation and neoplastic transformation (Klenova, Morse et al. 2002). Recent studies have demonstrated that BORIS is implicated in the expression of some CTAs, including MAGE-I genes, like MageA1, A2, B1 and B4 (Vatolin, Abdullaev et al. 2005). Conditional expression of BORIS in normal cells leads to induction of MageA1 through binding and consequent demethylation of MageA1 promoter. Moreover, BORIS seems to be important for the expression of CTAs after addition of demethylating agents. Nevertheless, a more recent work revealed that many melanoma cells express MageA1 and other CTAs in the absence of BORIS activation, suggesting that BORIS is neither necessary nor sufficient for the activation of other CTAs (Kholmanskikh, Loriot et al. 2008).

d) Functions of MAGE genes

Because many MAGE genes are highly homologous and are co-regulated and often co-expressed during gametogenesis and tumorigenesis, it has been suggested that many of them could have redundant functions. Moreover, since the discovery of MAGE proteins, most of the studies were focused on targeting these proteins for antitumor immunotherapies; and analysis of their expression in different tumors being mostly used as a prognostic tool. For these reasons their biological functions have remained poorly characterized. A central question is whether MAGE expression contributes directly to tumorigenesis or is a functional irrelevant by-product of the process of cellular transformation, possibly associated with global-chromatin changes. Clues have emerged, however, to indicate that expression of MAGE genes could have a role in human tumorigenesis (Simpson, Caballero et al. 2005). In fact MAGE-I genes expression could be detected in early phases of lung and breast carcinogenesis and, in addition, it has been demonstrated that cells expressing MAGE-I members acquire chemoresistance (Jang, Soria et al. 2001; Otte, Zafrakas et al. 2001; Seth, Krop et al. 2002).

Within MAGE-I subfamily, MAGE-A proteins are the best characterized. By yeast two hybrid assays it was identified the transcriptional regulator SKI-interacting protein (SKIP) as a binding partner for **MageA1**. Binding of MageA1 to SKIP depends on the extreme carboxyl terminus of the MAGE protein. SKIP connects DNA-binding proteins to other proteins that either activate or repress transcription, and participates in a wide range of signaling pathways, including those involving vitamin D, retinoic acid, estrogens, glucocorticoids, NOTCH1 and transforming growth factor-β. In the NOTCH1 pathway, MageA1 was found to disrupt the SKIP-mediated NOTCH1 signal transduction by binding to SKIP and recruiting histone deacetylase. Therefore, at least in this signaling pathway MageA1 can act as transcriptional repressor (Laduron, Deplus et al. 2004). The function of MageA1 in the germ line has not been elucidated. It is likely that MageA1 represses the expression of genes required for differentiation, as it is expressed in spermatogonia but not during later developmental stages.

Recent data indicate that expression of certain MAGE-A genes in cancer cells contributes directly to the malignant phenotype and response to therapy. In particular, the relationship between sensitivity to tumor necrosis factor (TNF) and **MageA1**, **MageA2** or **MageA3** expression was examined in a range of human cell lines, where it was found that cell lines expressing at least one of the three MAGE genes were more resistant to TNF-mediated cytotoxicity (Park, Kong et al. 2002). It has also been shown that MageA2 or **MageA6** genes

lead to the acquisition of resistance to chemotherapeutic drugs such as paclitaxel and doxorubicin in human cell lines (Bertram, Palfner et al. 1998; Duan, Duan et al. 2003; Glynn, Gammell et al. 2004; Suzuki, Yoshida et al. 2007).

MageA4 was identified as a partner for the oncoprotein gankyrin. Both MageA4 and gankyrin are frequently overexpressed in hepatocellular carcinomas (Fu, Wang et al. 2002; Nagao, Higashitsuji et al. 2003). Gankyrin destabilizes the retinoblastoma tumor suppressor, contributing to entry into the cell cycle and escape from cell-cycle arrest and/or apoptosis; in addition it binds the cyclin-dependent kinase 4 (Cdk4) inhibiting subsequent interaction with $p16^{INK4A}$ and $p18^{INK4C}$ (Li and Tsai 2002). Finally, gankyrin binds Mdm2 to facilitate the binding and consequent ubiquitination and degradation of p53 (Higashitsuji, Higashitsuji et al. 2005). MageA4 suppresses these activities through its binding to gankyrin, consequently inhibiting both anchorage-independent growth *in vitro* and tumor formation in athymic mice of gankyrin-overexpressing cells (Higashitsuji, Itoh et al. 2000). This repressive effect of MageA4 occurs through the action of a peptide that is naturally cleaved from the carboxyl terminus of MageA4, which induces p53-independent apoptosis (Sakurai, Itoh et al. 2004). MageA4 negatively regulates the expression of $p21^{Cip1}$ through the binding of the transcription factor Miz-1, found to be an interactor of MageA4 by yeast two hybrid analysis. More recently it has been demonstrated, that in non-small cell lung carcinomas MageA4 can induce apoptosis and that treatment of these tumors with the chemotherapeutic agent etoposide seems to increase the apoptotic effect (Peikert, Specks et al. 2006).

MageA11 was found to have a role in the regulation of androgen-receptor function by modulating its internal domain interactions (Bai, He et al. 2005). MageA11 stabilizes ligand-free androgen receptor in the cytoplasm, leading to its accumulation. In the presence of agonist, the exposure of the amino-terminal ligand-binding domain of the receptor increases the recruitment of SRC/p160 co-activators, consequently amplifying the transcriptional activity of the pathway. Binding between MageA11 and the androgen-receptor is regulated by the epidermal growth factor (EGF)-dependent phosphorylation and ubiquitination of MageA11 (Bai and Wilson 2008).

Recent studies have demonstrated that the small interfering RNA (siRNA) mediated suppression of **MAGE-A**, **-B** and **–C** gene expression slows proliferation and induces caspase independent apoptosis in human and murine mast cell lines, and suppresses growth of malignant cells in an *in vivo* murine model of mastocytosis (Yang, O'Herrin et al. 2007).

Altogether, these studies formly point to the functional roles of MAGE-I proteins expression in the regulation of proliferation/survival of tumor cells.

Among MAGE-II proteins, **Necdin** is the better characterized one. Necdin is a neural differentiation-associated gene product isolated from murine embrional carcinoma P19 cells (Maruyama, Usami et al. 1991). The Necdin gene is expressed in most of the postmitotic neurons through the central nervous system and peripheral nervous system (Maruyama, Usami et al. 1991; Aizawa, Maruyama et al. 1992; Niinobe, Koyama et al. 2000). Necdin strongly suppresses cell proliferation, promotes neuronal differentiation, and inhibits death of several cell lines and primary neurons (Hayashi, Matsuyama et al. 1995; Taniura, Taniguchi et al. 1998; Kobayashi, Taniura et al. 2002; Takazaki, Nishimura et al. 2002; Kuwako, Hosokawa et al. 2005). Necdin binds to many regulatory proteins such as the transcription factors E2F1, E2F4, and p53 leading to the repression of their transcriptional activity and consequently inhibiting cell growth and apoptosis (Taniura, Taniguchi et al. 1998; Taniura, Matsumoto et al. 1999; Kuwako, Taniura et al. 2004; Kurita, Kuwajima et al. 2006; Hasegawa and Yoshikawa 2008). Necdin binds also to the neurotrophin receptors p75, TrkA (Tcherpakov, Bronfman et al. 2002; Kuwako, Taniura et al. 2004; Kuwako, Hosokawa et al. 2005) and to the Dlx (distal-less homeobox) homeodomain proteins through its binding to MageD1 (another MAGE-II family member), promoting differentiation of GABAergic neurons (Kuwajima, Nishimura et al. 2006). Since all these interactors are involved in cell cycle regulation, differentiation, and apoptosis, necdin may play an integral role in mitotic arrest, differentiation, and survival of postmitotic neurons (Yoshikawa 2000; Andrieu, Meziane et al. 2006). More recently it has been reported that Necdin expressed in skeletal muscle cells mediates muscle regeneration by promoting myoblast survival and differentiation (Deponti, Francois et al. 2007; Bush and Wevrick 2008). The human Necdin gene is located in chromosome 15q11-q12 (Nakada, Taniura et al. 1998), a region deleted in the genomic imprinting-associated neurobehavioral disorder Prader-Willi syndrome (PWS). The Necdin gene is maternally imprinted and expressed only from the paternal allele in human and mouse (Jay, Rougeulle et al. 1997; MacDonald and Wevrick 1997). Furthermore, Necdin-deficient mice display several phenotypes such as postnatal lethality, impaired neuronal development, and abnormal behaviors, which are consistent with some of the PWS symptoms (Gerard, Hernandez et al. 1999; Muscatelli, Abrous et al. 2000; Ren, Lee et al. 2003; Kuwako, Hosokawa et al. 2005; Zanella, Watrin et al. 2008). These findings suggest that deletion of the paternal Necdin allele leads to the abnormal development of specific neurons in PWS.

MageL2 is also an imprinted gene that, similarly to Necdin, maps to the same chromosomal region associated with PWS. It is expressed predominantly in the developing central nervous system, particularly within hypothalamus, cerebral cortex, and spinal cord (Boccaccio, Glatt-

Deeley et al. 1999; Lee, Kozlov et al. 2000). Highest MageL2 expression levels are observed during neurogenesis. The tissue specific and developmental expression pattern of MageL2 is consistent with a role in the PWS (Kozlov, Bogenpohl et al. 2007).

MageD1, also named NRAGE or Dlxin, plays important roles in regulation of apoptosis and transcription. NRAGE interacts with p75 neurotrophin receptor ($p75^{NTR}$), a TNF receptor family member responsible for binding the tyrosine kinase receptor (Trk) and forming a complex that facilitates binding of neurotrophin, which in turn activates the Trk receptor. $p75^{NTR}$ is also capable of mediating cell death (Barker 1998; Barrett 2000; Kaplan and Miller 2000). NRAGE- $p75^{NTR}$ binding occurs through the intracellular segment of $p75^{NTR}$ located near the plasma membrane. Overexpression of NRAGE inhibits the interaction between $p75^{NTR}$ and the Trk receptor and induces cell apoptosis (Salehi, Roux et al. 2000). Another NRAGE-binding protein, XIAP, a member of the inhibitors of apoptosis family (IAP), can inhibit apoptosis by binding to activated caspases. The NRAGE-XIAP complex facilitates the degradation of XIAP, suggesting that NRAGE accelerates cell death by degrading XIAP and activating caspases (Jordan, Dinev et al. 2001). Other works suggested that NRAGE can induce cell death by other mechanisms. NRAGE interacts and inhibits Che-1, (also known as apoptosis-antagonizing transcription factor (AATF), a protein involved in cell cycle control and gene transcription), by sequestering Che-1 to the cytoplasmic compartment and targeting it for proteasome-dependent degradation (Di Certo, Corbi et al. 2007).

MageD1 interacts also with homeodomain proteins Msx2, Dlx7 and Dlx5, that participate in the development of the craniofacial, limbic and nervous systems (Bendall and Abate-Shen 2000). Msx genes are expressed in dividing or in apoptotic cells, and they behave as transcriptional repressors controlling cell cycle. Dlx genes are expressed in developing cells and behave as transcriptional activators. The effect of MageD1 binding to these genes remains unknown, but it was demonstrated that MageD1 is required for Dlx5 activity (Masuda, Sasaki et al. 2001). The activity of MageD1 on Msx2 is also regulated by Ror2, a membrane tyrosine kinase receptor that control morphogenetic development (Schlessinger 2000). Ror2 binds the c-terminus of MageD1, sequestering it to the plasmatic membrane and inhibiting its interaction with nuclear Msx2 (Matsuda, Suzuki et al. 2003). In addition, MageD1 interacts with Praja1, an ubiquitin ligase protein that regulates the transcriptional function of Dlx5 by inducing MageD1 degradation (Sasaki, Masuda et al. 2002).

It was also reported that MageD1 binds BRCA-2, an important factor in the double strand break repair process and many other cellular functions such as inhibition of cell proliferation. Mutations of BRCA-2 at the germinal level favor the predisposition to breast and ovarian

carcinomas. Binding between MageD1 and BRCA-2, induces MageD1 stabilization and seems to be required for the p53-dependent anti-proliferative function of BRCA-2 (Tian, Rai et al. 2005).

Moreover, more recently it was suggested that MageD1 can suppress the motility and adhesion response of tumor cells by interfering with actin cytoskeleton reorganization and hypoxia inducible factor 1-dependent gene expression (Shen, Xue et al. 2007).

The p53 tumor suppressor protein

The tumor suppressor p53 was first identified in 1979 (DeLeo, Jay et al. 1979; Lane and Crawford 1979; Linzer and Levine 1979), and since then it becomes one of the most studied protein in human diseases, primarily because the majority of human tumors have defects in the p53 pathway. Through nearly 30 years of intensive studies, extensive knowledge has been achieved on the p53 pathway and a great extent of complexity has been unveiled.

The importance of p53 in cancer development is illustrated by the fact that cancer cells can escape the tumour suppression function of p53 through missense mutations of the p53 gene. Actually, 50% of human cancers carry mutations in the p53 gene (Olivier, Hussain et al. 2004). In addition, other components of the p53 pathway are frequently altered in tumours bearing wild type p53. Is thus possible to suggest that the p53 pathway is compromised to some degree in all human cancers.

p53 has a short-half life and is normally maintained at low levels in unstressed cells by continuous ubiquitylation and subsequent degradation by the 26S proteasome (Haupt, Maya et al. 1997; Kubbutat, Jones et al. 1997). However, in response to a large number of stress signals, including DNA damage, oncogene activation, viral infection, telomere erosion, hypoxia, temperature change, nucleolar disruption and nucleotide depletion, p53 is rapidly stabilized and accumulates in the nucleus, where it forms a homotetrameric complex (Friedman, Chen et al. 1993). Only tetrameric p53 seems to be fully active as transcriptional activator or repressor of distinct target genes that contain p53 sequence-specific binding sites (Davison, Yin et al. 1998). Furthermore, p53 interacts with more than 100 cellular proteins and influences the cellular response to stress signals through transcription-dependent and – independent pathways (Fuster, Sanz-Gonzalez et al. 2007). Thus, p53 functions as a key integrator that translates diverse stress signals into different cellular outcomes, ranging from cell-cycle arrest, DNA repair/genome stability, apoptosis, autophagy, cell migration and

senescence, to differentiation, embryo implantation, regulation of metabolism, and angiogenesis (Murray-Zmijewski, Slee et al. 2008).

The activity of p53 is tightly regulated, and depending on the stimulus and the cellular environment, the protein undergoes a series of post-translational modifications, that affect its stability, sub-cellular localization and its ability to interact with different co-factors and to bind to its target promoters. The most commonly reported post-translational modifications of p53 include phosphorylation of serines and/or threonines and acetylation, ubiquitylation, sumoylation, neddylation and methylation (Bode and Dong 2004; Murray-Zmijewski, Slee et al. 2008).

a) Structure of the p53 protein

The p53 protein consists of 393 amino acids and is commonly divided into three functional domains, the N-terminal, the central core and the C-terminal domains (Fig. G).

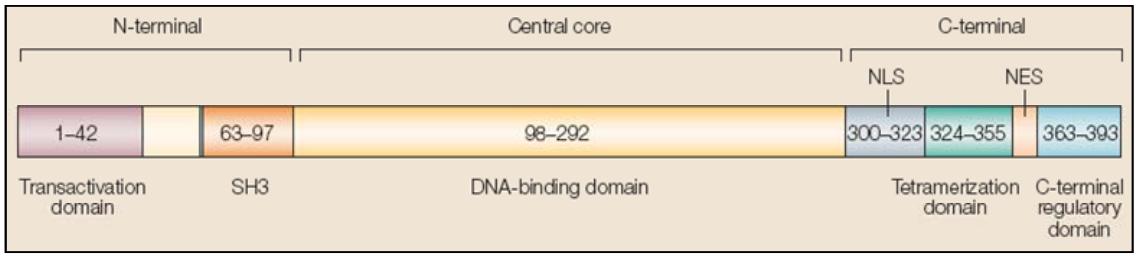

Fig. G: Schematic representation of the p53 functional domains

The acidic amino-terminal domain, or the **transactivation domain** (TA, amino acids 1-62), is required for transcriptional activation (Fields and Jang 1990). This portion is sufficient to activate transcription by interaction with components of the basal transcriptional machinery like the TATA-binding protein (TBP) and TBP associated proteins (TAFs) as well as with p300/CBP (Liu, Miller et al. 1993). The transcriptional activation domain is finely regulated both by post-translational modifications and by interaction with protein partners such as p300/CBP, Mdm2 and Pin1. Mdm2, the most critical negative regulator of p53, interacts with residues 17–27 and mediates the ubiquitination of p53 C-terminal lysine residues. Moreover Mdm2, together with MdmX, prevents the binding of transcriptional co-activators such as p300, interfering with the transactivation function of p53.

Adjacent to the transactivation domain there is a **proline-rich domain** (PRD, amino acids 63-97) containing five repeats of the amino acid motif PXXP (where P designates proline and X any amino acid) in humans while only two in mice (Walker and Levine 1996). This region was thought to be significant for p53 regulation since PXXP motifs create binding sites for Src homology 3 (SH3) domain-containing proteins and can modulate signal transduction (Kay, Williamson et al. 2000). Indeed, the p53 PXXP motifs may contribute to interactions with the transcription co-activator p300 (Dornan, Shimizu et al. 2003) thus influencing p53 acetylation. Furthermore, the prolyl isomerase Pin1 binds to Thr81-Pro82 site upon Thr81 phosphorylation and induces a conformational change on Pro82. This may reduce Mdm2 binding thus influencing p53 stability (Wulf, Liou et al. 2002; Zacchi, Gostissa et al. 2002), consistently with other evidence indicating that the PRD modulates Mdm2 binding (Berger, Vogt Sionov et al. 2001; Dumaz, Milne et al. 2001). Furthermore, the functional importance of the PRD is suggested by studies showing that this domain is dispensable for cell cycle arrest but is essential for apoptosis (Sakamuro, Sabbatini et al. 1997; Venot, Maratrat et al. 1998) being required both for transcriptional activation of pro-apoptotic genes (Bergamaschi, Samuels et al. 2006) and for direct apoptotic function of p53 at mitochondria (Chipuk, Kuwana et al. 2004). Yet, a recent study conducted in mice indicated that, although the PRD may play some role in the regulation of p53, as mice $p53^{\Delta P}$ (that bear p53 lacking the PRD) display increased Mdm2-mediated degradation and decreased transactivation capacity (Toledo, Lee et al. 2007), the PXXP motifs are not essential for p53 tumor suppressor functions, as their depletion does not significantly affect p53 accumulation and transactivation, and exhibits only little effect on cell cycle control or apoptosis.

The **core domain** of p53 (amino acids 98-292) contains the sequence-specific DNA binding domain (DBD) required for p53 to function as a transcriptional activator (el-Deiry, Kern et al. 1992). The canonical p53-responsive element contains two decamers or half sites, PuPuPuC(A/T)(A/T)GPyPyPy, which are separated by a spacer of 0–13 base pairs. Several studies indicate that a p53 monomer binds the pentameric sequence and that a tetramer binds the full consensus site (Cho, Gorina et al. 1994; Ma, Pan et al. 2005). The crystal structure of the p53-DBD bound to DNA has revealed that the conserved regions are crucial for the p53–DNA interaction (Cho, Gorina et al. 1994). The larger part of the DBD forms an antiparallel β-sandwich. This β-sandwich serves as a scaffold that supports the structures important for the interaction with DNA, specifically two large loops and a loop-sheet-helix motif. Most of the interactions between p53 and its target proteins take place in the DBD of p53. The importance of sequence-specific DNA binding for p53 to function as a tumor suppressor is highlighted by

the fact that 97% of tumor-associated mutations cluster in this domain (Sigal and Rotter 2000). The mutations situated in the DBD can disrupt specific DNA binding by several possible ways. Mutations at some positions (e.g. Arg273His) lose direct contacts with DNA and are therefore named "contact mutants" (Cho, Gorina et al. 1994; Bullock, Henckel et al. 1997). Some other mutants, defined "conformational mutants" (e.g. Arg175His, Gly245Ser, Arg249Ser, Arg282Trp) show reduced binding due to destabilization of the tertiary structure of p53 DBD (Wong, DeDecker et al. 1999; Bullock, Henckel et al. 2000). Mutation at position 248 (Arg248Trp), in addition to breaking DNA-protein contacts, also introduces extensive structural changes into the DBD (Wong, DeDecker et al. 1999).

The **tetramerization domain** (amino acids 300-355) of p53, is linked to the DBD by a flexible linker. This region consists of a β-sheet structure that determines the dimeric association and an α-helical structure that mediates the successive association between two dimmers (Lee, Harvey et al. 1994; Jeffrey, Gorina et al. 1995). Tetramerization is required for efficient transactivation by p53 (Pietenpol, Tokino et al. 1994). This domain contains also the nuclear localization signal (NLS) (amino acids 316-325) and the nuclear export signal (NES) (amino acids 340-351).

The last 30 amino acids of p53 constitute a basic C-terminal domain (CT, amino acids 363-393) that has been regarded as a **regulatory domain** due to its ability to influence p53 activity. A great number of post-translational modifications occur in this domain upon stress-signalling. Nearly every residue within this domain is in fact subjected to at least one post-translational modification. This domain does not form a regular secondary structure and is able to interact directly with DNA and RNA (Lee, Elenbaas et al. 1995; Ayed, Mulder et al. 2001). In particular CT is able to bind ssDNA ends, insertion/deletion mismatches, recombination intermediates and γ-irradiated DNA *in vitro* thus implying its ability to recognize damaged DNA and DNA repair intermediates *in vivo* (Bakalkin, Selivanova et al. 1995; Lee, Elenbaas et al. 1995; Zotchev, Protopopova et al. 2000). Moreover some recent works demonstrated that the CT is important for binding to non-linear DNAs (McKinney and Prives 2002; Fojta, Pivonkova et al. 2004; Palecek, Brazda et al. 2004) and is involved in the ability of p53 to diffuse linearly on DNA (McKinney, Mattia et al. 2004). These studies also revealed that the CT is required for efficient promoter activation by p53.

The family of p53 related transcription factors includes its homologues p63 and p73 (Kaghad, Bonnet et al. 1997; Yang, Kaghad et al. 1998). p53 family members have high structural similarity (Fig. H). They all contain the central DNA binding domain (DBD), the N-terminal

transactivation domain (TA) and the oligomerization domain (OD). p63 and p73 are transcribed from two major promoters thus can be expressed with (TA forms) or without (ΔN forms) the amino terminal TA domain (Stiewe, Zimmermann et al. 2002). Moreover, p63 and p73 splicing variants, designated by greek letters, differ in the regions at the carboxy terminal end. Here also resides the most obvious structural difference between p53 and its siblings consisting in the fact that the α-forms of p63 and p73 contain a sterile α motif (SAM) that is absent in p53 (Kim and Bowie 2003). The homology shown by p53, p63 and p73 had first suggested that the products of this gene family share similar or even redundant functions. However, difference in regulation and activity among the p53 members emerged quite rapidly. Many reports highlighted that the three members of p53 family might be critical regulators of different processes. In particular, p53 is mainly involved in DNA-damage response while p73 and p63 play key roles in development. This is strikingly highlighted by the phenotypes of mice with targeted deletions for each of the three family members. While p53-null mice are tumor-prone, underlining the key function of p53 in tumor suppression, they lack a clear developmental phenotype that is instead evident upon disruption of p63 and p73 (Yang, Schweitzer et al. 1999; Yang, Walker et al. 2000; Pellegrini, Dellambra et al. 2001; Brunner, Hamel et al. 2002). Interestingly, phylogenetic analysis demonstrated that the primordial ancestor of all three genes is actually much more related to p63 and p73, while p53 appears as the most recently evolved member of its family (Yang, Kaghad et al. 2002). It might be hypothesized that p53 lost some of its ancestral functions that are instead carried out by its homologues, yet it has gained the ability to protect cells from tumorigenesis. This seems also to be confirmed by the evidence that p53 is mutated in almost 50% of all human malignancies, while mutations in the p63 and p73 genes are rare (Nomoto, Haruki et al. 1998; Irwin and Kaelin 2001). However, there are reports showing that also p63 and p73 have tumor suppressive activities in human tumors. On the one hand they contribute to p53 tumor suppression, as it has been shown that mice heterozygous for mutations in both p53 and p63 or p53 and p73, lead to a more aggressive tumor phenotype (Flores, Sengupta et al. 2005) and that p53 requires at least one of its homologues to function properly as an inducer of pro-apoptotic genes upon DNA damage (Flores, Tsai et al. 2002). On the other hand they might have a specific role in tumor suppression as their loss or down-regulation leads to tumorigenesis at specific tissues (Ahomadegbe, Tourpin et al. 2000; Park, Lee et al. 2000; Puig, Capodieci et al. 2003; Park, Kim et al. 2004) with a tumor spectrum that differs from that of p53 and that reflects their pattern of expression.

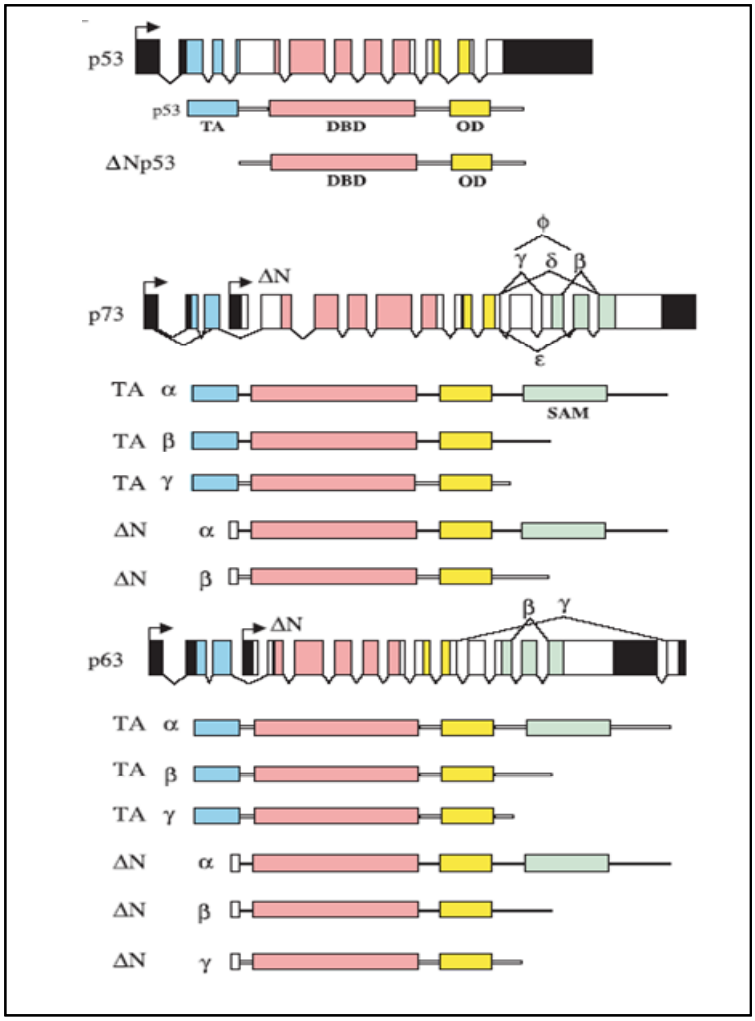

Fig. H: Gene architecture of the p53 family. The p53 family includes the three genes p53, p63, and p73. They have a modular structure consisting of the TA, the DBD, and the oligomerization domain. All genes are expressed as two major types: full-length proteins containing the TA domain and DN proteins missing the TA domain. The products of p73 and p63 are more complex than p53 and contain a COOH-terminal SAM domain and a transactivation inhibitory domain in their a isoforms. p63 and p73 also contain two promoters. The P1 promoter in the 5V untranslated region produces TA proteins that are transcriptionally active, whereas the P2 promoter produces DN proteins with dominant-negative functions toward themselves and toward wild-type p53. In addition, extensive COOH-terminal splicing and, in TP73, additional NH2-terminal splice variants of the P1 transcript further modulate the p53-like functions of the TA proteins.

b) p53 protein regulation

When cells respond adequately to p53-activating signals, the consequent biological outcome may vary considerably. As it appears from a large number of studies, much of the choice does not depend on p53 itself. Rather it is the cellular context, as defined by the balance of intracellular and extracellular signalling events which dictates the direction undertaken by p53 response. Multiple proteins contribute to the activation of p53 in response to diverse stress stimuli. These include proteins that modify p53 for both stabilization and transcriptional activation, that reverse these modifications, that enhance the translation of p53 mRNA or that alter its subcellular localization.

b.1) Regulation of p53 stability

In the absence of stress, p53 abundance is maintained at very low levels. Multiple lines of evidence indicate that MDM2 and MDMX play a major role in regulating p53 stability.
MDM2 was first isolated from a mouse double minute chromosome that was present at a high copy number in a spontaneously transformed derivative of mouse 3T3 cells (Cahilly-Snyder, Yang-Feng et al. 1987). Its role as an oncogene was assessed after the observation that its over-expression lead to immortalization of rodent primary fibroblasts and induced a fully transformed phenotype in cultured cells. Early studies ascribed the transforming capabilities of MDM2 to its ability to form a complex with p53 (Momand, Zambetti et al. 1992) and to the observation that its overexpression leads to abrogation of both p53-mediated G_1 phase cell cycle arrest and induction of apoptosis in cultured cells (Chen, Wu et al. 1996; Haupt, Barak et al. 1996). The MDMX oncogene was identified because of its ability to interact with p53 (Shvarts, Steegenga et al. 1996) and later as a MDM2 partner (Tanimura, Ohtsuka et al. 1999). Like MDM2, MDMX acts as an oncogene and both are found amplified in many cancers including soft tissue, breast, lung and small intestine cancer (Momand, Jung et al. 1998; Danovi, Meulmeester et al. 2004). The two protein share a great structural similarity and are able to homo- and heterodimerize through their RING-finger domain (Tanimura, Ohtsuka et al. 1999). Importantly, the highest similarity among the two proteins resides in the region encompassing the p53 binding domain where the amino acid residues necessary for the interaction with p53 are strictly conserved (Freedman, Epstein et al. 1997). Conversely, the same amino acids on p53 are required for both the interaction with MDM2 and with MDMX (Bottger, Bottger et al. 1997). Yet, an important difference between MDM2 and MDMX is

that the RING domain of MDM2 is essential for its action as an E3-ubiquitin ligase, whereas MDMX apparently has no intrinsic ubiquitin-ligase activity.

Genetic studies revealed that germ line inactivation of either MDM2 or MDMX leads to early embryonic lethal phenotypes that are completely overcome by concomitant inactivation of the p53 gene (Jones, Roe et al. 1995; Montes de Oca Luna, Wagner et al. 1995; Parant, Chavez-Reyes et al. 2001; Finch, Donoviel et al. 2002; Migliorini, Lazzerini Denchi et al. 2002). These studies also indicated that MDM2 and MDMX are non-redundant p53 inhibitors, as normal levels of either regulator cannot compensate for the loss of the other. These evidences clearly indicate that p53 is a key downstream target of MDM2/MDMX and that MDM2/MDMX are essential negative regulators of p53.

MDM2 and MDMX mediate the inactivation of p53 through different molecular mechanisms. Both MDM2 and MDMX interfere with the transcriptional activity of p53. This was initially thought to occur by virtue of their binding to the N-terminal domain of p53, masking the transactivation domain of p53 (Oliner, Pietenpol et al. 1993), and disabling its interactions with the basal transcriptional machinery and essential co-factors (Chen, Lin et al. 1995). Indeed, MDM2/MDMX bind to the N-terminus of p53 within a domain that serves to recruit the histone acetyltransferases p300 and CBP (Lambert, Kashanchi et al. 1998; Dumaz and Meek 1999) thus interfering with p53 acetylation by p300 (Kobet, Zeng et al. 2000; Sabbatini and McCormick 2002). MDM2 also associates with the histone deacetylase HDAC1, targeting it toward p53 deacetylation (Ito, Kawaguchi et al. 2002); MDM2 has also been shown to localize with p53 at its responsive elements on the p21 and MDM2 genes under normal growth conditions and to dissociate upon DNA damage (White, Talbott et al. 2006) thus suggesting that the recruitment of a MDM2-HDAC1 complex to chromatin might potentially promote histone deacetylation and transcriptional silencing. In addition, it has been reported that MDM2 is capable of binding directly to histones promoting their monoubiquitylation and leading to transcriptional repression (Minsky and Oren 2004). Moreover, MDM2 further inhibits p53 transcriptional functions by catalyzing the conjugation of NEDD8, a small ubiquitin-like protein, to p53 Lys-370, 372 and 373 (Xirodimas, Saville et al. 2004).

The most important role played by MDM2 is however the regulation of p53 turn-over. MDM2 is in fact an E3-ubiquitin ligase that upon binding to p53 can catalyze either mono- and poly-ubiquitylation of p53, leading to nuclear export or proteasomal degradation, respectively (Li, Brooks et al. 2003). In contrast to MDM2, MDMX does not act as an E3 ubiquitin ligase and by itself cannot stimulate p53 ubiquitylation and proteasome-dependent degradation (Jackson

and Berberich 2000; Stad, Little et al. 2001). However, MDMX can stabilize MDM2 preventing its auto-ubiquitylation (Sharp, Kratowicz et al. 1999; Stad, Little et al. 2001), while MDM2 mediates MDMX poly-ubiquitylation (Linares, Hengstermann et al. 2003; Pan and Chen 2003).

It has been shown that MDM2 mediates both monoubiquitylation or polyubiquitylation of p53 depending on its relative amount with respect to p53 (Li, Brooks et al. 2003): when MDM2 is abundant, it promotes effective p53 polyubiquitylation, leading to subsequent p53 degradation by the 26S proteasome. Several proteins have been demonstrated to assist the ubiquitylation of p53 by MDM2. Gankyrin increases the ubiquitin-ligase activity of MDM2 and p53 degradation by favouring the association of the MDM2-p53 complex to the 26S proteasome (Higashitsuji, Higashitsuji et al. 2005). Moreover, transcription factor Yin Yang 1 (YY1), a protein that plays a key role in development, increases p53 degradation by stabilizing the complex p53-MDM2 (Sui, Affar el et al. 2004). Intriguingly, it has been shown that p300 cooperates with MDM2 to promote p53 poly-ubiquitylation (Grossman 2001) by acting as an E4 ubiquitin ligase (Grossman, Deato et al. 2003). This reveals a complex interplay between the two proteins that needs further elucidation.

Conversely, when MDM2 levels are scarce, p53 becomes monoubiquitylated and rather than being degraded, p53 is exported into the cytoplasm. Notably, MDM2 and p53 do not leave the nucleus together, it seems rather that mono-ubiquitylation of p53 by MDM2 leads to an unmasking of the NES within the C-terminus of p53 (Gu, Nie et al. 2001; Li, Brooks et al. 2003). Movement of p53 into the cytoplasm has been thought for long time to have an inhibitory effect on p53 activity. Clearly, this translocation prevents p53 from transcriptional activation of its target genes, however it might be important for transcription-independent functions of p53 such as interactions with mitochondrial proteins in the apoptotic response (Mihara, Erster et al. 2003; Chipuk and Green 2004).

On the other hand phosphorylation of MDM2 and MDMX enhances their degradation by auto-ubiquitylation and by reduction of their association with the de-ubiquitylating enzyme HAUSP, further releasing p53 from its negative regulation (Li, Chen et al. 2002; Li, Brooks et al. 2004; Brooks, Li et al. 2007).

The p53-MDM2 interplay is tightly regulated in a negative feedback loop wherein p53 stimulates MDM2 synthesis through transactivation of its promoter (which contains two adjacent p53 binding sites) while MDM2 inhibits p53 activity. Interestingly, MDMX is not a p53 transcriptional target and its abundance is mainly regulated through the interaction with HAUSP.

p53 stabilization and activation is furthermore regulated by other pathways that crosstalk with the p53-MDM2 axis. A feedback loop governing p53 activity involves the AKT kinase and the PIP-3 phosphatase PTEN. In detail, AKT phosphorylates MDM2 and induces its translocation into the nucleus, where it down-regulates p53 (Mayo, Dixon et al. 2002; Zhou, Gu et al. 2003). By inhibiting AKT and by blocking the nuclear entry of MDM2, PTEN sustains p53 activity (Mayo and Donner 2002). On the other hand, in damaged cells, p53 activates the transcription of PTEN (Stambolic, MacPherson et al. 2001) thus facilitating formation of a cycle in which p53 induces PTEN and PTEN stabilizes p53.

Upon increased oncogenic signalling, p53 activation and stability is regulated also by the tumor suppressor ARF. ARF increases p53 stability as it down-modulates MDM2 ubiquitin ligase activity both by sequestering it to the nucleolus (Honda and Yasuda 1999; Lowe 1999; Damalas, Kahan et al. 2001) and by interfering with its interaction with YY1 (Sui, Affar el et al. 2004). Notably p53 itself has been shown to down-regulate ARF expression (Robertson and Jones 1998). In this way increased levels of p53 act in a negative feedback, resulting in down-regulation of ARF transcription and concomitant decreases in ARF levels. This will then allow MDM2 to bind to p53 and reduce its stability by targeting it for degradation.

Other proteins that act as E3 ubiquitin ligase have been shown to target p53 and promote its proteasome-mediated degradation. Pirh2, a RING-H2 domain-containing protein, interacts with p53 and promotes MDM2-independent p53 ubiquitylation and degradation (Leng, Lin et al. 2003). Similar to MDM2, Pirh2 is a p53 responsive gene and participates in a comparable auto regulatory negative feedback loop. Another E3 ligase, COP1, has also been described recently as a direct ubiquitin ligase for p53 (Dornan, Wertz et al. 2004). COP1 is also a p53-inducible gene and can ubiquitinate and degrade p53.

ARF-BP1/Mule/HectH9 was recently identified as a HECT domain E3 ligase that can ubiquitinate and degrade p53 (Chen, Kon et al. 2005). ARF interacts with ARF-BP1/Mule/HectH9 and inhibits its activity thus leading to p53 stabilization.

Another route to degradation for p53 is mediated by the ubiquitin ligase CHIP (chaperone-associated ubiquitin ligase), that has been shown to poly-ubiquitinate p53 upon transient binding to HSP90 and HSP70. This mechanism might contribute to maintaining low levels of p53 under physiological conditions as CHIP depletion augment p53 basal levels (Esser, Scheffner et al. 2005).

Recently, a family of ubiquitin ligases called caspase 8/10-associated RING proteins (CARPs) were described. CARPs target either unmodified p53 or p53 that has been phosphorylated on serine 20 for degradation, independently of MDM2 (Yang, Rozan et al. 2007).

b.2) Regulation of p53 by posttranslational modifications

p53 undergoes a great variety of post-translational modifications that influence its stability and its transcriptional activity. These include phosphorylation of serines and threonines, acetylation, mono- and poly-ubiquitylation, sumoylation, neddylation and methylation of lysines. The actual pattern of post-translational modifications is complicated since the same residue might be modified in different ways by different enzymes. Notably nearly all of these modifications occur in the N-terminal or in the C-terminal domains.

- Phosphorylation

Many kinases, including ATM, ATR, Chk1, Chk2, casein kinase1 and 2 (CK1, CK2), c-JUN NH2-terminal kinase (JNK), Erk, p38, Aurora Kinase A, glycogen synthase kinase-3β (GSK3β), HIPK2 and DYRK2, have been shown to phosphorylate p53 after DNA damage. So far, 17 phosphorylation/dephosphorylation sites have been detected following DNA damage induced by ionizing radiation or ultraviolet (UV)-light irradiation. In humans, these sites include Ser6, 9, 15, 20, 33, 37 and 46, and Thr18 and 81 in the amino-terminal region; Ser315 and Ser392 in the C-terminal domain; and Thr150, Thr155 and Ser215 in the central core. In addition, Thr55, Ser376 and Ser378 seem to be constitutively phosphorylated in unstressed cells (Gatti, Li et al. 2000) (Fig. I). Only a few p53 sites are reported to be phosphorylated by one specific protein kinase. Clearly, significant redundancies are observed, yet this multiplicity may provide a failsafe mechanism and a distinctive combination of phosphorylated residues could be required for further modifications, leading to maximal activation.

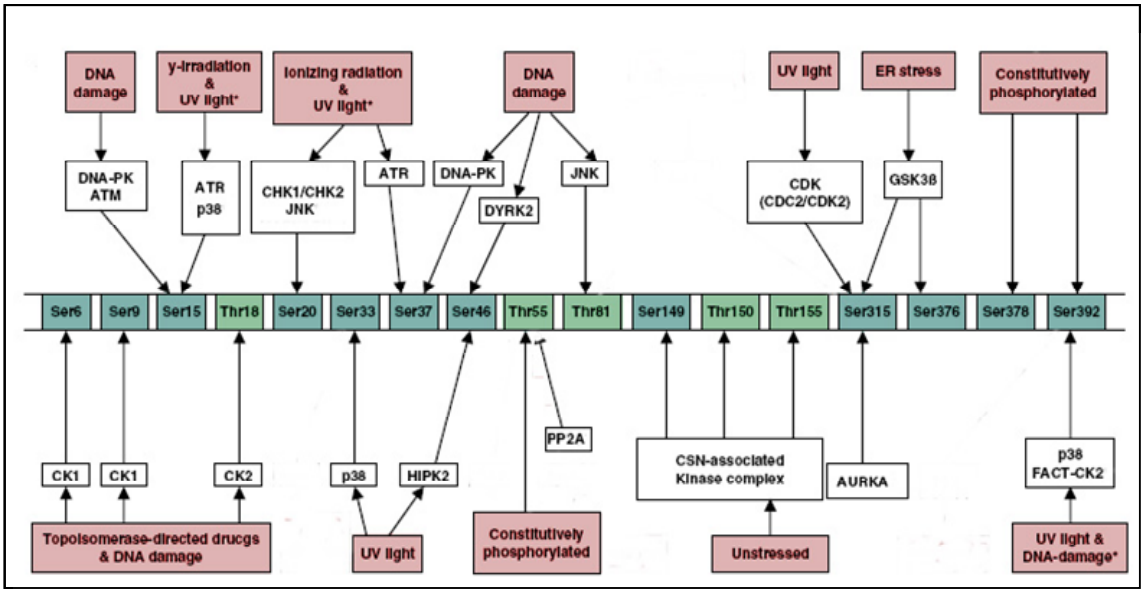

Fig. I: Schematic representation of p53 phosphorylation sites and the enzymes involved in p53 phosphorylation upon different stimuli

The phosphorylation of the amino-acid residues Ser15, Thr18 and Ser20 in the N-terminal domain of human p53 are the most extensively studied. These residues are located in, or close to, the region of p53 that also binds to MDM2/MDMX (Appella and Anderson 2001; Ito, Lai et al. 2001; Sabbatini and McCormick 2002; Bode and Dong 2004). Ser15 is phosphorylated in an ATM-dependent manner early in response to γ-irradiation (Banin, Moyal et al. 1998; Waterman, Stavridi et al. 1998), being ATR the kinase responsible for Ser15 phosphorylation upon UV radiation (Tibbetts, Brumbaugh et al. 1999). Also p38 phosphorylates p53 at this residue in response to UV radiation (Bulavin, Saito et al. 1999). Data from *in vitro* or overexpression studies indicate that phosphorylation at Ser15 stimulates p53-dependent transactivation, growth arrest and apoptosis in response to DNA damage (Fiscella, Ullrich et al. 1993; Shieh, Ikeda et al. 1997). However, there exist conflicting data on whether Ser15 phosphorylation affects MDM2 binding or not (Shieh, Ikeda et al. 1997; Dumaz and Meek 1999), but it is known that it increases the interaction with the acetyltransferase CBP (Lambert, Kashanchi et al. 1998). Phosphorylation of other residues, including Thr18 and Ser20, which occurs later in the response to DNA damage, was shown to depend on initial phosphorylation at Ser15 and seems to interfere with the interaction of p53 with MDM2, thus promoting p53 stabilization (Chehab, Malikzay et al. 1999; Dumaz, Milne et al. 1999; Unger, Juven-Gershon et al. 1999).

Phosphorylation of Ser46 by the kinases HIPK2, p38 and DYRK2 was reported to mediate selectivity in promoter binding by p53 and to specifically promote the induction of apoptosis inducing genes, such as p53-regulated apoptosis-inducing protein1 (p53AIP1) (Oda, Arakawa et al. 2000; D'Orazi, Cecchinelli et al. 2002; Taira, Nihira et al. 2007). Moreover, HIPK2-mediated phosphorylation of p53 at Ser46 is required for the acetylation of p53 at Lys382 by CBP (Hofmann, Moller et al. 2002). Phosphorylation at Ser46 is sufficient for p53 to induce the PTEN tumor suppressor protein relative to MDM2 (Mayo, Seo et al. 2005). The shift of p53 gene targeting from MDM2 to PTEN results in sustained p53 activation and diminished survival as p53 and PTEN coordinate cell death by facilitating one another's expression and functions.

Moreover, under normal conditions p53 represses its own phosphorylation at Ser46 due to MDM2-mediated HIPK2 degradation (Rinaldo, Prodosmo et al. 2007). This creates another positive feedback loop in which MDM2 degrades HIPK2 thus decreasing p53 phosphorylation at Ser46. Under these conditions p53 will activate MDM2 transcription rather than PTEN. These findings support the notion that the cell-cycle-arresting functions of p53 include active inhibition of the apoptotic ones, and furthermore indicates that phosphorylation at Ser46 is an important determinant of p53 choice as it acts like a switch between two opposite feedback loops.

Within the DNA binding domain, Ser215 phosphorylation by Aurora Kinase A abolishes the DNA binding ability of p53 (Liu, Kaneko et al. 2004); Thr150 and Thr155 are phosphorylated by two COP9 signalosome-associated kinases, CK2 and protein kinase D, leading to ubiquitin-dependent degradation of p53 (Uhle, Medalia et al. 2003). Interestingly, a recent report demonstrated that Ser149 of p53 is O-GlcNAcylated and that this modification is associated with decreased phosphorylation of p53 at Thr155, resulting in decreased p53 ubiquitylation (Yang, Kim et al. 2006). This offers another demonstration of the dynamic interplay between different post-translational modifications in p53.

With respect to the CT-domain, phosphorylation of p53 at serine 315 and serine 376, by GSK-3β upon ER stress, has been shown to increase its cytoplasmic localization and reduce its stability (Qu, Huang et al. 2004; Qu and Koromilas 2004; Pluquet, Qu et al. 2005). Phosphorylation of Ser315 by the cyclinB-dependent kinase p34(Cdc2), was reported to increase the transactivation potential of human p53 in response to radiation damage, possibly by promoting nuclear retention, mediated via interaction of p53 with E2F1 (Blaydes, Luciani et al. 2001; Fogal, Hsieh et al. 2005). Phosphorylation at the same site by aurora kinase A, however, was suggested to promote MDM2-dependent ubiquitylation and proteolysis of p53,

arguing for an inhibitory role of this modification (Katayama, Sasai et al. 2004). Ser392 is phosphorylated by p38 or CK2 in response to UV-irradiation, but only very inefficiently after γ-irradiation. An UV-activated protein kinase complex that phosphorylates Ser-392 of p53 *in vitro* has been identified. This kinase complex contains CK2 and the chromatin transcriptional elongation factor FACT (a heterodimer of hSpt16 and SSRP1). FACT seems to alter the specificity of CK2 in the complex such that it selectively phosphorylates p53 over other substrates including casein. Phosphorylation by the kinase complex enhances p53 activity (Keller, Zeng et al. 2001). It has been demonstrated that damage-stimulated phosphorylation of p53 at Ser392 by CK2 has an important impact on p53 structure as it can increase intrinsic thermostability of the core DNA-binding domain thus enhancing p53 affinity for its consensus DNA binding site.

In addition to stress-induced phosphorylation there are also a few sites in p53 which are dephosphorylated in response to stress signals, such as Ser376 and Thr55. The loss of phosphorylation from Ser376 creates a consensus binding site for 14-3-3σ and in turn increases the affinity of p53 for sequence-specific binding sites on DNA (Waterman, Stavridi et al. 1998). Dephosphorylation by PP2A at Thr55 instead has been shown to stabilize p53 (Li, Cai et al. 2007). It is likely that an ordered pattern and interdependence of stress-induced modifications to p53 exist in cells. However, the relevance and the precise role of phosphorylation on p53 in its stabilization and activation remain controversial. While it seems likely that the complex network of stress-induced modifications to p53 is important in generating a functional molecule, there is also evidence that p53 can be activated without these changes. It was demonstrated that p53 stabilization occurs as well upon mutation of a series of known stress-induced phosphorylation sites (Fuchs, O'Connor et al. 1995; Blattner, Tobiasch et al. 1999). Further evidence pointing to a lack of requirement for post-translational modification in p53 stabilization comes from the use of small molecule inhibitors of the p53–MDM2 interaction. In fact, p53 accumulates in cells treated with Nutlin-1 (a cis-imidazoline analogue that displaces p53 from its complex with MDM2) and this is followed by an increase in the levels of both p21 and MDM2 consistent with activation of the p53 pathway. Yet, unlike DNA damage, nutlins did not induce phosphorylation of p53 but stabilized, unphosphorylated form of p53 which was equally efficient at sequence-specific DNA binding and induction of apoptosis (Thompson, Tovar et al. 2004).

These controversial results does not mean, however, that phosphorylations on p53 are irrelevant or do not occur *in vivo*; nature appears to be able to compensate quite easily for the loss of one or even two or more of these modifications in p53.

- **Acetylation**

p53 can be acetylated at several lysines by different HAT (Fig. J). Acetylation of p53 by p300/CBP and PCAF occurs in response to DNA damaging agents, such as UV- and γ-irradiation. CBP and p300 acetylate p53 at lysines within the C-terminal domain (Lys370, 372, 373, 381, 382) (Avantaggiati, Ogryzko et al. 1997; Gu and Roeder 1997; Lill, Grossman et al. 1997). Moreover, Lys320 and Lys305 in the nuclear localization domain are acetylated by PCAF (p300/CBP-associated factor) and p300, respectively (Sakaguchi, Herrera et al. 1998; Liu, Scolnick et al. 1999). Finally, the MYST family acetyl transferases, hMOF and TIP60, were recently shown to acetylate p53 at Lys120 in the DBD (Legube, Linares et al. 2002; Berns, Hijmans et al. 2004; Doyon, Selleck et al. 2004).

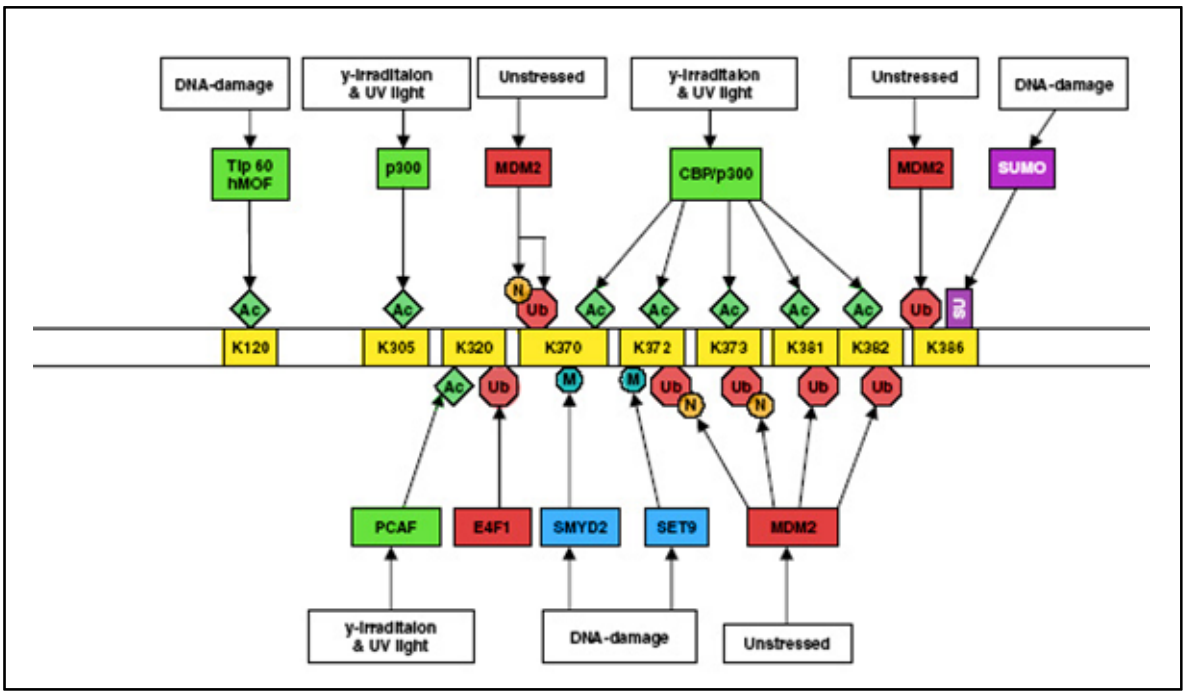

Fig. J: Schematic representation of post-translational modifications occurring on p53 at Lysine residues. Ac, acetylation; M, methylation; Ub, ubiquitylation; N, NEDDylation; SU, sumoylation. The enzymes mediating the modification are indicated.

The role of acetylation in mediating p53 binding to DNA has been largely debated and is still controversial (Gu and Roeder 1997) yet, what is commonly accepted is that it potentiates the recruitment of co-factors thus favoring p53 transcriptional activation.

Consistently, reports showing that acetylated p53 is enriched at target promoters in a stress dependent manner (Liu, Xia et al. 2003; Mantovani, Tocco et al. 2007) suggest a role for p53 acetylation in affecting its transcriptional activation. While some studies could show that acetylation favours the recruitment of co-factors and histone acetyltransferases (TTRAP and CBP), thus increasing p53 transcriptional activity (Barlev, Liu et al. 2001), other suggested that the enhancing effect of p300/CBP on p53 transactivation function was independent of p53 acetylation (Espinosa and Emerson 2001).

Nevertheless, a number of recent studies indicate that acetylation of specific lysine residues on p53 has differential effects on the choice of the target genes induced by p53. Using acetylation-mimicking lysine to glutamine mutations, functional differences between acetylation of Lys320 versus Lys373 were reported. Acetylation of Lys320 in p53 was shown to favour interaction with high-affinity p53-binding sites in target genes, promoting cell survival and cell cycle arrest. In contrast, acetylation of Lys373 led to a stronger interaction of p53 with low-affinity binding sites, which are found in pro-apoptotic target genes and therefore promoted cell death (Knights, Catania et al. 2006).

Acetylation on Lys120 by the MYST family acetyl transferase Tip60 has also been shown to modulate p53 transcriptional activity and to be necessary for apoptotic response. Notably, Lys120 is specifically acetylated by Tip60 (Legube, Linares et al. 2004). Lys120-acetylated p53 was reported to accumulate upon DNA damage preferentially on the promoters of pro-apoptotic target genes, including Bax and PUMA.

Acetylation might also enhance p53 transcriptional activity through promoting the assembly of an active transcriptional apparatus. It is known that TFIID binding to the core promoter element is required for assembly of a functional transcriptional initiation complex (Thomas and Chiang 2006). TAF1, the larger subunit of TFIID has been shown to interact with the C-terminal domain of p53 on p21 promoter (Espinosa, Verdun et al. 2003; Li and Wu 2004). In a recent study it has been demonstrated that the acetylation of two lysine residues in p53, Lys373 and Lys382, leads to a direct interaction with the Double Bromodomain (DBrD), a structural protein module that recognizes acetylated lysines of TAF1 to induce the transcription of p21. Notably, also the recruitment of CBP is mediated through the binding of its single bromodomain to p53 acetylated on Lys382 (Mujtaba, He et al. 2004). Recruitment of HATs has been shown both to enhance p53 acetylation and to favour the acetylation of histones on p53 responsive promoters thus favouring transcriptional activation.

Interestingly, it has been proposed that DNA-binding is required to allow p53 acetylation by p300; in its absence the C-terminal domain of p53 is not accessible for acetylation by p300

and this provides an intrinsic negative regulatory mechanism to prevent acetylation until the tetramer is promoter bound. After DNA binding, allosteric effects mediate an exposure of the acetylation motif to allow DNA-dependent acetylation of the tetramer. Notably, the conformational change allows p300 to contact p53 on its PRD and this interaction is essential for DNA-dependent acetylation of p53 (Dornan, Shimizu et al. 2003). Indeed, upon PRD deletion, p300 can still bind to p53 on its N-terminal LXXLL motif encompassing Thr18-Ser20, but cannot acetylate DNA-bound p53. It has been suggested that the prolyl-isomerase Pin1 may assist structural rearrangements that occur upon DNA binding thus promoting the DNA-dependent acetylation of p53 by p300. This may occur at least in part through phosphorylation induced isomerization of Thr81-Pro82 within the PRD. Indeed, it has been demonstrated that mutations affecting this site, result in impaired acetylation of p53 at Lys 373 and Lys382 (Mantovani, Tocco et al. 2007). Moreover, Pin1-mediated Pro82 isomerization within the PRD has been shown to be required for Ser20 phosphorylation by Chk2 (Berger, Stahl et al. 2005), which promotes p53 binding to p300 (Dornan and Hupp 2001). DNA-dependent acetylation of p53 stabilizes p300 binding (Dornan, Shimizu et al. 2003) and directs the organization of transcription complexes on the p53 bound promoters thus promoting the transcriptional functions of p53.

The acetylation status of p53 can be regulated via deacetylation by an HDAC1 (histone deacetylase 1)-containing complex (Murphy, Ahn et al. 1999) or by the NAD-dependent histone deacetylase SIRT1 (silent information regulator 2a, Sir2a) (Vaziri, Dessain et al. 2001). Moreover, overexpression of HDAC1, 2 and 3 repress p53 transcription (Juan, Shia et al. 2000). Deacetylation was shown to repress p53-dependent transcriptional activation, apoptosis and growth arrest (Luo, Su et al. 2000; Luo, Nikolaev et al. 2001). Moreover, as the lysine residues within the CTD of p53 are also targets for ubiquitylation, it was proposed that acetylation of these residues may promote the stabilization of p53 by interfering with proteasomal degradation (Brooks and Gu 2003). In addition to mediating p53 ubiquitylation, MDM2 also seems to interfere with the acetylation of p53. Indeed, it was shown to inhibit p53 acetylation by p300 and PCAF (Ito, Lai et al. 2001; Jin, Zeng et al. 2002) and to promote the HDAC1-mediated deacetylation of p53 (Ito, Kawaguchi et al. 2002). It has been observed that many tumor-associated proteins can mediate p53 deacetylation; the oncogenic form of PML, PML-RAR (Insinga, Monestiroli et al. 2004), and the metastasis-associated MTA2/PID (Luo, Su et al. 2000), have been shown to recruit HDACs to exert their negative control on p53 function.

- Other modifications

Lysine residues are also targeted by methyl transferases (Fig. J). Methylation of p53 can occur at least at two different sites, leading to opposite effects on p53 function. Methylation at Lys372 by the methyl-transferase Set9, increases the stability of p53, restricts it to the nucleus and enhances p53 dependent transcription, whereas methylation of Lys370, mediated by another methyl transferase, Smyd2, leads to repression of transcriptional activity (Chuikov, Kurash et al. 2004; Huang, Perez-Burgos et al. 2006). In human cells, the histone lysine-specific demethylase LSD1 interacts with p53 to repress p53-mediated transcriptional activation and to inhibit the role of p53 in promoting apoptosis (Huang, Sengupta et al. 2007). While *in vitro* LSD1 removes both monomethylation (K370me1) and dimethylation (K370me2) at K370, *in vivo*, LSD1 shows a strong preference to reverse K370me2, which is performed by an unknown methyltransferase, distinct from Smyd2. Interestingly, methylation at K370me2 has a different role in regulating p53 from that of K370me1: K370me1 represses p53 function, whereas K370me2 promotes association with the co-activator 53BP1 (p53-binding protein 1) through tandem Tudor domains in 53BP1. Thus, LSD1 represses p53 function through the inhibition of interaction of p53 with 53BP1. Taken together, these observations show that p53 is dynamically regulated by lysine methylation and demethylation and that the methylation status at a single lysine residue confers distinct regulatory outputs.

Lysine residues are also subjected to other post-translational modifications such as ubiquitylation, sumoylation and NEDDylation (Fig. J). p53 is ubiquitinated by several E3 ubiquitin-ligases among which MDM2 is the best characterized (see above). MDM2 can induce both monoubiquitylation of p53, leading to nuclear export (Gu, Nie et al. 2001) and mitochondrial localization (Marchenko, Wolff et al. 2007), and polyubiquitylation that leads to degradation by the 26S proteasome (Li, Brooks et al. 2003).

Three lysines targeted for ubiquitination (Lys 370, Lys372 and Lys373) are also subjected to NEDDylation. The C-terminal glycine residue of the ubiquitin-like protein NEDD8 is covalently linked to p53 by MDM2 thus inducing transcriptional repression (Xirodimas, Saville et al. 2004). Sumoylation is also similar to ubiquitylation in that an isopeptide bond is formed between the C-terminal carboxy group of the small ubiquitin-like protein SUMO1 and the ε-amino group of Lys386 (Gostissa, Hengstermann et al. 1999; Rodriguez, Desterro et al. 1999). Sumoylation was reported to positively modulate p53 transcriptional activity (Gostissa, Hengstermann et al. 1999; Rodriguez, Desterro et al. 1999). Other data indicate that sumoylation of p53 has a repressive effect (Buschmann, Fuchs et al. 2000; Chen and Chen

2003), leaving the issue of whether sumoylation of p53 results in activation or repression of p53 activities controversial. Although the effect on p53 is not completely elucidated, several other key regulators of p53 activation are subjected to sumoylation, including PML, MDM2, HIPK2 and p300 (Kim, Choi et al. 1999; Xirodimas, Chisholm et al. 2002; Girdwood, Bumpass et al. 2003). In particular sumoylation of PML is important in the formation of the nuclear bodies (Ishov, Sotnikov et al. 1999).

b.3) Regulation of p53 by localization at PML-NBs

The promyelocytic leukaemia protein (PML) is a tumor suppressor originally identified in acute promyelocytic leukaemia patients with a reciprocal t(15;17) chromosomal translocation where PML is fused to the retinoic acid receptor-α (RARα) gene (Kakizuka, Miller et al. 1991; Melnick and Licht 1999; Bernardi and Pandolfi 2003). PML localizes to and is responsible for the formation of punctuate nuclear structures that are interspersed between chromatin, known as PML nuclear bodies (PML-NBs) (Ishov, Sotnikov et al. 1999; Salomoni and Pandolfi 2002). PML-NBs are discrete nuclear foci, 0,2-1,0 μm wide, that are present in most mammalian cell nuclei and typically number 1-30 bodies per nucleus, depending on the cell type, cell-cycle phase and differentiation stage (Dellaire and Bazett-Jones 2004).

Many proteins reside constitutively, but more often transiently, in PML-NBs. Consequently, PML-NBs have been implicated in the regulation of various cellular functions, such as the induction of apoptosis and cellular senescence, inhibition of proliferation, maintenance of genomic stability and antiviral responses (Bernardi and Pandolfi 2007). Althoug the physiological roles of PML and PML-NBs are still a matter of debate, their tumor suppressive role is common to all of these functions (Salomoni and Pandolfi 2002). Furthermore, PML-NBs and their implicated functions are frequently lost in both leukaemias and solid tumors (Koken, Linares-Cruz et al. 1995; Gambacorta, Flenghi et al. 1996; Gurrieri, Capodieci et al. 2004).

Among the proteins that localize in PML-NBs are p53 and several of its regulators, including acetyltransferases, deacetylases, MDM2 and small ubiquitin-like modifier 1 (SUMO1). Several studies suggested that PML is an upstream regulator of p53. $pml^{-/-}$ thymocytes are severely impaired in p53 transcriptional activity and p53 dependent apoptosis (Guo, Salomoni et al. 2000). In addition, cells derived from $pml^{-/-}$ mice are resistant to ionizing radiation-induced apoptosis (Wang, Ruggero et al. 1998). Conversely, p53-deficient cells are resistant to PML-induced arrest and apoptosis (de Stanchina, Querido et al. 2004), and after DNA

damage PML recruits p53 to PML-NBs which coincides with the increased transcription of p53 targets (Guo, Salomoni et al. 2000). This is thought to be due, in part, by the ability of PML to recruit CBP to PML-NBs which thereafter prompts p53 acetylation (Guo, Salomoni et al. 2000; Boisvert, Kruhlak et al. 2001). Interestingly, it was observed that only PML IV (PML3) isoform is able to regulate positively p53 through p53 binding (Fogal, Gostissa et al. 2000). PML is modified by SUMO and the formation of PML-NBs is regulated by this modification (Duprez, Saurin et al. 1999; Shen, Lin et al. 2006). SUMO modified PML results in transactivation and strong recruitment of p53 to NBs (Gostissa, Hengstermann et al. 1999). However, SUMO modification of PML or p53 is not required for colocalization or interaction. Instead, SUMO modification appears to be necessary for the observed PML dependent increase in transactivation of p53 (Gostissa, Hengstermann et al. 1999; Fogal, Gostissa et al. 2000). These findings, suggest that recruitment of p53 to NBs alone is insufficient for its activation, but that a second, NB dependent, event is required.

Moreover, PML can bind to MDM2 and p53 (Kurki, Latonen et al. 2003; Wei, Yu et al. 2003; Zhu, Wu et al. 2003), consequently stabilizing p53 by preventing MDM2-mediated p53 ubiquitylation and degradation (Kurki, Latonen et al. 2003; Louria-Hayon, Grossman et al. 2003), and sequestering MDM2 within the nucleolus after DNA damage (Bernardi, Scaglioni et al. 2004).

HIPK2 interacts with both p53 and CBP, and is also recruited to PML-NBs after DNA damage, favoring p53 acetylation and transcriptional activity (D'Orazi, Cecchinelli et al. 2002; Moller, Sirma et al. 2003).

As was mentioned above, PML was originally implicated in the induction of senescence because it is transcriptionally induced upon overexpression of the Ras-V12 oncogene and because it facilitates p53 activation by mediating its acetylation.

More recently, the PML gene was shown to be p53-responsive, suggesting that PML is an integral component of the p53 response, and indeed may act in a positive feedback loop (de Stanchina, Querido et al. 2004), contributing to the tumor suppressor functions of p53, including p53-mediated senescence, cell-cycle arrest and apoptosis. The precise mechanisms by which PML mediates these effectors functions are still to be determined, however it is quite clear that PML is a crucial component in the p53 functional network.

c) p53 cellular functions

There are three main outcomes after the activation of p53: apoptosis, senescence or cell-cycle arrest. The first two are terminal for the cell, whereas cell-cycle arrest permits repair processes to act and damage to be reversed, so that the cell survives. How the choice can be driven toward a specific response depends on different factors. First, the induction of p53 activation has different outcomes depending on cell type. In primary fibroblasts it is in fact usually associated with cell cycle arrest and senescence (Kuerbitz, Plunkett et al. 1992; Di Leonardo, Linke et al. 1994) whereas the activation of p53 in hematopoietic cells (e.g., thymocytes) generally results in apoptosis (Lowe, Schmitt et al. 1993). Moreover, even within a particular cell type, the p53 response can be influenced by many factors such as the nature of the stimuli that activate p53 and a plethora of protein partners that affect its stability and activity.

c.1) Cell-cycle arrest and DNA damage repair

The ability of p53 to induce cell-cycle arrest mostly depends on three critical target genes: p21, 14-3-3σ and GADD45 (Kastan, Zhan et al. 1992; el-Deiry, Tokino et al. 1993; Hermeking, Lengauer et al. 1997). The cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$ was the first transcriptional target identified and its transactivation results in cell cycle arrest in G1 phase due to inhibition of cyclinE/CDK2, cyclinA/CDK2 and cyclinD/CDK4 (Harper, Adami et al. 1993; Prives and Hall 1999), that leads to an accumulation of hypophosphorylated Rb that in turn binds and inhibits the transcription function of E2F1, blocking the S-phase entry and consequently the cell-cycle progression. The role of p21 in G1-arrest is further underlined by the observation that cells lacking p21 fail to arrest in response to DNA-damage, yet they can still undergo cell death (Brugarolas, Chandrasekaran et al. 1995). Although the most prominent function of p21 is the mediation of G1 arrest, evidence has been presented that it also participates in the G2/M arrest after DNA damage, presumably by blocking PCNA function at replication forks (Bunz, Dutriaux et al. 1998; Ando, Kawabe et al. 2001). However, the p53-induced G2 arrest is mostly mediated by the activation of genes such as GADD45 and 14-3-3σ. 14-3-3σ has been shown to prevent nuclear import of cyclinB1 and CDC2, through their sequestration in the cytoplasm (Chan, Hermeking et al. 1999), whereas GADD45 destabilizes CDC2/cyclinB complexes and these two processes cooperate to prevent initiation of mitosis (Zhan, Antinore et al. 1999; Jin, Tong et al. 2002).

Part of tumor suppressor functions of p53 is exerted by preventing propagation of deleterious mutations arising from DNA damage. Indeed, p53 plays an indirect role also in DNA repair through the induction of ribonucleotide reductase subunits (Hwang, Ford et al. 1999; Xue, Zhou et al. 2003). Another p53-regulated gene, GADD45, that was originally proposed to participate in global genomic repair downstream of p53 (Smith, Chen et al. 1994) has been later reported to have more likely functions in remodelling chromatin to give access to the sites of damaged DNA (Smith, Ford et al. 2000). Furthermore, two mismatch repair genes MLH1 and PMS2 have recently been shown to contain p53-response elements within their first intron and to be responsive to p53 activation after DNA-damage. These two genes may provide a sensor in DNA repair mechanisms and constitute a critical determinant for the decision between cell-cycle arrest and apoptosis (Chen and Sadowski 2005).

A direct participation of p53 in DNA repair was suggested by a number of biochemical observations. For instance, the C-terminal 30 amino acids of p53 were shown to recognise several DNA damage-related structures, such as DNA ends, gaps, and insertion/deletion mismatches (Bakalkin, Selivanova et al. 1995; Jayaraman and Prives 1995; Lee, Elenbaas et al. 1995). p53 was also demonstrated to catalyze reannealing of short stretches of single- and double-stranded DNA and to promote strand exchange between them (Oberosler, Hloch et al. 1993; Brain and Jenkins 1994). In addition to p53's biochemical activities, numerous reports on physical and functional protein interactions further strengthened the proposal of a direct role of p53 in nucleotide excision repair (NER), base excision repair (BER), and double-strand break (DSB) repair (Albrechtsen, Dornreiter et al. 1999; Bertrand, Saintigny et al. 2004).

Although it is well documented that efficient nucleotide excision repair (NER) requires p53, its exact role has been difficult to define (Hanawalt 2001). It has been suggested that p53 may function in NER by facilitating access to the chromatin to the repair machinery thus favoring DNA repair. It was further demonstrated that p53 is required for global chromatin relaxation induced by UV-irradiation (Rubbi and Milner 2003). In concordance with this, the histone deacetylase inhibitor trichostatin A overcomes the requirement for p53 suggesting that p53 may induce global chromatin relaxation through changes in histone acetylation (Rubbi and Milner 2003). Moreover, the histone acetyltransferase p300 binds and co-localizes with p53 to sites of NER and inhibition of p300 by antibody microinjection inhibits NER, suggesting that p53-dependent recruitment of p300 histone acetyl-transferase (HAT) activity may be mechanistically involved in the ability of p53 to induce global chromatin relaxation to foster DNA repair (Rapic-Otrin, McLenigan et al. 2002).

p53 is also directly involved in inhibiting homologous recombination (HR). Two studies demonstrated that p53 inhibits HR in response to replication fork stalling (Janz and Wiesmuller 2002; Saintigny and Lopez 2002). Consistently, it was further noticed that p53 prevents the accumulation of DSBs at stalled-replication forks induced by UV or hydroxyurea treatment (Kumari, Schultz et al. 2004; Squires, Coates et al. 2004). When DNA replication is blocked, p53 becomes phosphorylated on serine 15 and associates with key enzymes of HR (Zink, Mayr et al. 2002; Linke, Sengupta et al. 2003; Sengupta, Linke et al. 2003). Notably, during replication arrest p53 remains inactive in transcriptional transactivation (Gottifredi, Shieh et al. 2001; Restle, Janz et al. 2005) supporting the idea that p53 is involved in HR regulatory functions unrelated to transcriptional activities.

c.2) Apoptosis

Clearly the main role of p53 is to prevent the outgrowth of damaged or stressed cells that may develop into malignancies if left unchallenged. This can be achieved by eliminating any aberrant cells through apoptosis. Indeed, the best known transcriptional targets of p53 include a large number of pro-apoptotic genes that can be divided into several categories depending on their specific functions. These target genes are generally classified on the basis of their involvement either in the extrinsic or in the intrinsic apoptotic pathway. The extrinsic apoptotic pathway is triggered upon the engagement of particular death-receptors belonging to TNF-receptor family to their specific ligands and leads to the induction of a cascade of caspase activation which in turn induces apoptosis (Nagata and Golstein 1995; Wu, Burns et al. 1997; Attardi, Reczek et al. 2000). The intrinsic pathway instead is activated in response to different signals such as DNA damage, oncogenic signalling, hypoxia or endoplasmic reticulum stress, and is associated with mitochondrial depolarization and release of cytochrome c (cyt c) from the mitochondrial intermembrane space into the cytoplasm. This event leads to the formation of the apoptosome, a complex of cyt c, APAF-1 and pro-caspase-9, that activates the caspase cascade, thus converging in the effector phase with the extrinsic pathway (Cory and Adams 2002).

p53 can promote apoptosis via the extrinsic pathway by activating the transcription of the death receptors located at the plasma membrane, including Fas, DR4 and KILLER/DR5, PERP and PIDD. Both DR5 and DR4 can trigger apoptosis or enhance apoptosis induced by their ligand TRAIL and by chemotherapeutic agents (Liu, Yue et al. 2004). Fas can be activated by p53, yet its induction upon DNA damage is tissue specific and often does not

require p53 (Bouvard, Zaitchouk et al. 2000). Moreover, Fas is dispensable for p53-dependent apoptosis in most tissues (O'Connor, Harris et al. 2000). PERP, a PMP-22/gas family protein, is activated in transformed MEFs following DNA damage (Attardi, Reczek et al. 2000) and contributes to the p53-dependent apoptosis induced by γ-irradiation in thymocytes and neurons, but not to that induced by oncogene activation (Ihrie, Reczek et al. 2003; Reczek, Flores et al. 2003). PIDD was identified as a p53-regulated gene in mouse erythroleukemia cells and shown to promote apoptosis. Its induction by ionizing radiation is p53-dependent also in MEFs (Lin, Ma et al. 2000). It has been described that PIDD can form an activating complex with caspase 2 (Tinel and Tschopp 2004). Yet, it remains unclear whether PIDD is required for p53-dependent apoptosis as caspase 2 deficiency does not abrogate p53 responses *in vivo*.

Several other p53-regulated genes such as Bax, Noxa and PUMA enhance the release of Cyt c into cytoplasm from mitochondria to initiate the intrinsic apoptotic pathway. Bax was the first identified p53-regulated pro-apoptotic Bcl-2 family member (Miyashita and Reed 1995). Loss of Bax accounts for nearly half of the accelerated tumor growth which resulted from the loss of p53 in a brain tumor model (Schmitt, Fridman et al. 2002). Bax is also responsible for nearly half of p53-dependent apoptosis induced by 5-FU in colorectal cancer cells (Zhang, Yu et al. 2000). Nevertheless, Bax is dispensable for the apoptosis induced by γ-irradiation in thymocytes and intestinal epithelial cells and its induction is not strictly dependent on p53 in many tissues (Bouvard, Zaitchouk et al. 2000).

p53 regulates the expression of several BH3 domain-only proteins that function upstream of Bax to induce apoptosis. Some of these proteins are shown to be critical mediators of p53-dependent apoptosis. PUMA and Noxa are activated in a p53-dependent manner following DNA damage (Han, Flemington et al. 2001; Nakano and Vousden 2001; Yu, Zhang et al. 2001). PUMA mediates apoptosis induced by p53 in response to hypoxia, DNA damaging agents, and endoplasmic reticulum (ER) stress in human colorectal cancer cells (Reimertz, Kogel et al. 2003; Yu, Wang et al. 2003). Remarkably, PUMA-knockout mice recapitulated several key apoptotic deficiencies observed in the p53-knockout mice, including deficiencies in the apoptosis induced by γ-irradiation in thymocytes, by oncogenes in MEFs, and by DNA damage in developing neurons (Jeffers, Parganas et al. 2003; Villunger, Michalak et al. 2003). This suggests that the ability of PUMA to mediate apoptosis and tumor suppression is context-dependent. Indeed, other studies demonstrated that the capability of PUMA to act as a tumor suppressor can be dependent on other oncogenic events such as myc or E1A activation. Notably, PUMA depletion cooperated with myc and E1A induced tumorigenesis but not with

Ras as this last oncogene induces a senescence response rather than apoptosis. Similarly, Noxa-deficient mice develop normally but their MEFs are strongly resistant to apoptosis induced by oncogenes and UV radiation while only slightly resistant to etoposide induced cell death. *In vivo*, the absence of Noxa resulted in resistance to X-ray-induced apoptosis in the small intestinal crypts (Shibue, Takeda et al. 2003; Villunger, Michalak et al. 2003). Moreover, p53 contributes to the formation of the apoptosome also through the transcriptional activation of APAF-1 (Kannan, Kaminski et al. 2001; Moroni, Hickman et al. 2001) and is involved in the more downstream phases of apoptosis by activating the transcription of caspase-6 (MacLachlan and El-Deiry 2002).

p53 may sense the alterations in ROS levels and can activate numerous REDOX genes like PIG3, POX2/PIG6 and ferredoxin reductase (Donald, Sun et al. 2001; Hwang, Bunz et al. 2001). This results in an increased generation of ROS and originates a positive loop feeding p53 activation that further contributes to apoptosis (Johnson, Yu et al. 1996; Polyak, Xia et al. 1997; Li, Dietz et al. 1999; Martindale and Holbrook 2002). Moreover, p53 can contribute to the apoptotic pathway also through repressing the transactivation of anti-apoptotic genes such as Bcl-2 (Haldar, Negrini et al. 1994; Miyashita, Harigai et al. 1994; Miyashita, Krajewski et al. 1994), Bcl-X_L (Cherbonnel-Lasserre and Dosanjh 1997) and survivin (Hoffman, Biade et al. 2002).

Interestingly, p53 appears also to provide a connection between the extrinsic death receptor pathway and the triggering of mitochondrial disruption processes through the activity of its transcriptional target Bid. In fact Bid is activated by caspase-8 upon triggering of the extrinsic pathway and translocates to the mitochondria where it activates Bax and consequently the initiation of the intrinsic pathway (Sax, Fei et al. 2002). Among the pro-apoptotic p53 target proteins Scotin (Bourdon, Renzing et al. 2002), that is located in the ER and the nuclear membrane, has been shown to be required for ER-stress mediated apoptosis. As a protein-folding compartment, the ER is extremely sensitive to alterations in homeostasis that disrupt its functions (Kaufman 1999; Ferri and Kroemer 2001). Prolonged ER stress can result in the activation of caspase-12 that in turn activates caspase-9 thus executing apoptosis. It has been proposed that, upon ER-stress, the release of calcium from the ER triggers mitochondrial depolarization thus allowing the release of Cyt c to the cytosol and the activation of the caspase cascade (Ichas, Jouaville et al. 1997; Ichas and Mazat 1998; Jouaville, Ichas et al. 1998). Notably, upon ER-stress p53 is up-regulated and induces transcription of two other pro-apoptotic genes, Noxa and PUMA (Li, Lee et al. 2006). These evidences clearly suggest

that p53 mediates the apoptotic response at multiple levels and that the apoptotic pathways are not independent but rather involved in a complex interplay.

p53 participates in apoptosis also in a transcription-independent fashion by acting directly at mitochondria where it can perturb or modulate the functions of proteins implicated in the apoptotic machinery. This was thought to account for the reported ability of a transactivation incompetent p53 mutant to trigger apoptosis via the mitochondrial death pathway (Regula and Kirshenbaum 2001). A fraction of p53 protein rapidly translocates to mitochondria in response to genotoxic, hypoxic, and oxidative stresses (Marchenko, Zaika et al. 2000) and it has recently been demonstrated that this translocation is dependent on its monoubiquitination by MDM2 (Marchenko, Wolff et al. 2007). At the mitochondria, p53 has been found to interact with the Bcl-X_L and Bcl-2 protective proteins and to prevent them from inhibiting on the oligomerization among Bax and Bak (Mihara, Erster et al. 2003). There is a crosstalk among p53 functions at the mitochondria and its transcriptional activity, in fact upon stress-induced stabilization and activation within the nucleus, p53 induces the transcription of PUMA and this one is able to release cytoplasmic p53 from the inhibitory interaction with Bcl-XL, thus allowing it to directly activate Bax (Chipuk, Bouchier-Hayes et al. 2005). p53 interacts also with Bad and the mitochondrial p53/Bad complex promotes apoptosis via activation and oligomerization of Bak (Jiang, Du et al. 2006). Moreover, p53 acts directly on the pro-apoptotic Bak promoting its dissociation from the anti-apoptotic protein MCL1 (Leu, Dumont et al. 2004). Once the inhibitory interactions upon Bax and Bak are relieved, they oligomerize to form a transmembrane pore for the release of Cyt c from mitochondria. Notably, upon translocation at mitochondria, p53 has been shown to play a role in maintaining mitochondrial genetic stability. Indeed, in response to mitochondrial damage p53 interacts with Polγ and enhances its DNA replication functions (Achanta, Sasaki et al. 2005) . Moreover it has been proposed that p53 might interact with mtDNA (Heyne, Mannebach et al. 2004) and that it might participate in mitochondrial base excision repair (mtBER) (de Souza-Pinto, Harris et al. 2004).

The precise contribution of the transcriptional-independent apoptotic activities of p53 to the overall apoptotic response needs to be figured out much more in detail. However, they seem to be important, as demonstrated by the fact that the stronger capacity of the isoform p53Arg72 to activate apoptosis as compared to the Pro72 form, is associated at least in part with the greater ability of Arg72 isoform to localize to mitochondria (Dumont, Leu et al. 2003).

Recent reports showed that p53 can also modulate autophagy, a cellular process triggered by nutrient starvation and genotoxic agents that may function in different contexts to either promote or inhibit cell survival (Crighton, Wilkinson et al. 2006; Crighton, Wilkinson et al. 2007). DRAM (damage-regulated autophagy modulator), a p53-induced protein, has been identified to be a likely linker between p53-dependent apoptosis and autophagy. In fact p53 induces autophagy in a DRAM-dependent manner and DRAM, which has been found to be down regulated in human cancer, has proven to be also critical for p53-induced cell death. Nevertheless, other works suggested that p53 downregulation induces autophagy in different cell types and that cytoplasmic p53 inhibits autophagy in p53 knockdown cells (Tasdemir, Maiuri et al. 2008).

c.3) Senescence

Cellular senescence is a program leading to an irreversible cell cycle arrest that is accompanied by a characteristic set of phenotypic changes in the cell. Senescence was first observed as the limited ability of normal cells to grow in culture; after several cycles of cell division, proliferation declines and cells enters in a permanent arrested state (Hayflick 1965). Since this process seems to be regulated by several tumor suppressors and oncogenes in normal cells and mechanisms that bypass senescence appear to be important in cancer development, senescence was proposed as a tumor suppressive mechanism (Campisi 2001; Wright and Shay 2001; Dimri 2005; Braig and Schmitt 2006). Senescence response is elicited by DNA damage and oncogenic and mitogenic signals, so in this scenario senescence acts as a failsafe mechanism.

Senescent cells acquire some characteristic features or senescent phenotype. When normal cells are exposed to potentially oncogenic insults, they irreversibly cease to proliferate (**growth arrest**), and often become resistant to apoptosis (**apoptosis resistance**) (Chen, Liu et al. 2000; Hampel, Malisan et al. 2004; Marcotte, Lacelle et al. 2004; Murata, Wakoh et al. 2006), and show changes in gene expression (**altered gene expression**) (Zhang, Pan et al. 2003; Yoon, Kim et al. 2004; Trougakos, Saridaki et al. 2006), including changes in known cell-cycle inhibitors and activators, like induction of p21 and p16 or repression of c-Fos, cyclin A, cyclin B and PCNA. Moreover, senescent cells can be identified in culture by morphological means like large cell size, flat vacuolated morphology or by several distinctive markers, like the incorporation of 5-bromodeoxyuridine or ^{3}H-thymidine, or the immunostaining for proteins such as PCNA and Ki-67, to detect the lack of DNA replication.

One of the first and classical marker of senescence, is the senescence-associated β-galactosidase (SA-βgal) that derives from the lysosomal β-galactosidase and reflects the increased lysosomal biogenesis that commonly occurs in senescent cells (Dimri, Lee et al. 1995; Lee, Han et al. 2006). More recently, other senescence markers have been identified, such as the expression of p16, an important regulator of senescence (Krishnamurthy, Torrice et al. 2004), and the detection of cytological markers like senescence-associated heterochromatin foci (SAHFs) (Narita, Nunez et al. 2003) and senescence-associated DNA-damage foci (SDFs) (d'Adda di Fagagna, Reaper et al. 2003; Takai, Smogorzewska et al. 2003; Herbig, Jobling et al. 2004; Di Micco, Fumagalli et al. 2006). SAHFs which silence the genes that are needed for proliferation are detected by preferential binding of DNA dyes and the presence of certain heterochromatin-associated histone modifications (for example, H3 lys9 methylation) and proteins (for example, heterchromatin protein-1 (HP1)). SDFs contain proteins that are associated with DNA damage, and can be visualized by staining of such proteins, for example phosphorylated histone H2AX (γ-H2AX) and p53-binding protein-1 (53BP1).

Senescence can be triggered by different stimuli, like the shortening of telomeres due to proliferation (**replicative senescence**) (Harley, Futcher et al. 1990; Herbig, Jobling et al. 2004) or by other exogenous or endogenous acute and chronic stress signals (**telomere-independent or premature senescence**) such as cytokine signalling (TGFβ) oxidative damage (Di Leonardo, Linke et al. 1994; Chen, Liu et al. 2000), or by mitogenic oncogene overexpression (Ras, Raf) (Serrano, Lin et al. 1997; Zhu, Woods et al. 1998; Ferbeyre, de Stanchina et al. 2000; Michaloglou, Vredeveld et al. 2005), loss of anti-oncogenic tumor suppressors (PTEN) (Chen, Trotman et al. 2005) or supra-physiological mitogenic signals (overexpressed MAPK or E2F1) (Lin, Barradas et al. 1998; Dimri, Itahana et al. 2000) (**oncogene-induced senescence**). In both replicative, premature or oncogene-induced senescence, a key role is mediated by tumor suppressor pathways involving p53 and $p16^{INK4a}$-pRB (Shay, Pereira-Smith et al. 1991; Serrano, Lin et al. 1997) as demonstrated by a general refractoriness of human cells to multiple senescence-inducing stimuli upon loss of p53 and pRB function (Dimri, Itahana et al. 2000; Beausejour, Krtolica et al. 2003). These two pathways interact but can also independently halt cell-cycle progression and to some extent they respond to different stimuli in a cell-type or species-specific fashion. Most studies suggest that p21 induction by p53 inhibits CDK2/Cyclin E activity. Activity of CDK4/Cyclin Ds can also be inhibited by p21. Inhibition of activity of CDKs by p21 results in hypophosphorylation of pRB, which very likely mediates arrest during senescence (Itahana,

Campisi et al. 2004). In contrast to p53, the role of pRB in cellular senescence is less clear. pRB remains constitutively hypophosphorylated in senescent cells (Stein, Drullinger et al. 1999), suggesting downregulation of the activity of pRB kinases during senescence. The $p16^{INK4a}$, an inhibitor of CDK4 and CDK6 activity, is upregulated during senescence (Alcorta, Xiong et al. 1996; Stein, Drullinger et al. 1999), accounting for the hypophosphorylation of pRB. Nevertheless, it was reported that downregulation of p16 may not be functionally equivalent to pRB inactivation (Wei, Herbig et al. 2003), leaving the possibility that other CDKs inhibitors might play a surrogate role in cellular senescence. Since p21 induction can also lead to the inhibition of pRB phosphorylation by inhibiting CDK2/Cyclin E activity, p16 and p21 are likely to cooperate to keep pRB in a hypophosphorylated form during senescence. Alternatively, it is possible that p21 initially and temporarily keeps pRB in its inhibitory form, while p16 ensures permanent hypophosphorylation of pRB (Beausejour, Krtolica et al. 2003). The p16-pRB pathway is crucial for generating SAHFs (Narita, Nunez et al. 2003). SAHFs contains chromatin modifiers proteins like HIRA (histone repressor A), ASF1a (anti-silencing function-1a) and HP1, and finally after formation they are composed of a single condensed chromosome, which is depleted for the linker histone H1 and enriched for HP1 and histone variant macroH2A (Zhang, Poustovoitov et al. 2005; Zhang, Chen et al. 2007). Like the growth arrest, once established, SAHFs no longer require p16 or pRB for maintenaice. This suggest that the p16-pRB pathway can establish self-maintaining senescence-associated heterochromatin (Campisi and d'Adda di Fagagna 2007).

Recent work has revealed the importance of DNA-damage response (DDR) in initiating and maintaining cellular senescence (d'Adda di Fagagna 2008). DDR is a pathway that senses DNA damage and triggers an amplified cascade of events that coordinates cell cycle arrest with DNA repair (Fig. K) (Nyberg, Michelson et al. 2002). DDR is activated in response to generation of single-stranded DNA and/or DNA double strand breaks (DSBs), that are sensed by large protein complexes that recruit and activate ataxia telangiectasia and Rad3-related (ATR) or ataxia-telangiectasia mutated (ATM), respectively, at the site of the DNA lesion. These kinases then phosphorylate the histone H2AX, leading to further recruitment of ATM complexes in a positive feedback loop, thus engaging a signal cascade mediated by different DNA-damage mediators (for example, MDC1, 53BP1 and TOPBP1) and other downstream kinases, like CHK1 and CHK2. These two kinases finally activate the effectors of the DDR signalling, p53 and CDC25. Dysfunctional telomeres trigger a classical DDR, the progressive telomere shortening eventually cause chromosome ends to be recognized as DNA breaks, consequently activating the ATM-p53-p21 branch of DDR and enforcing senescence (d'Adda

di Fagagna, Reaper et al. 2003; Herbig, Jobling et al. 2004). Stimuli that produce a DDR can also engage the p16-pRB pathway, but this usually occurs secondary to engagement of the p53 pathway (Stein, Drullinger et al. 1999; Jacobs and de Lange 2004; Jacobs and de Lange 2005). A common signal in oncogene-induced senescence is the occurrence of double strand breaks caused by oncogene activation through aberrant DNA replication. Inactivation of key DDR gene products leads to senescence avoidance and cell proliferation and allows cell transformation (Bartkova, Rezaei et al. 2006; Di Micco, Fumagalli et al. 2006; Hemann and Narita 2007; Mallette, Gaumont-Leclerc et al. 2007).

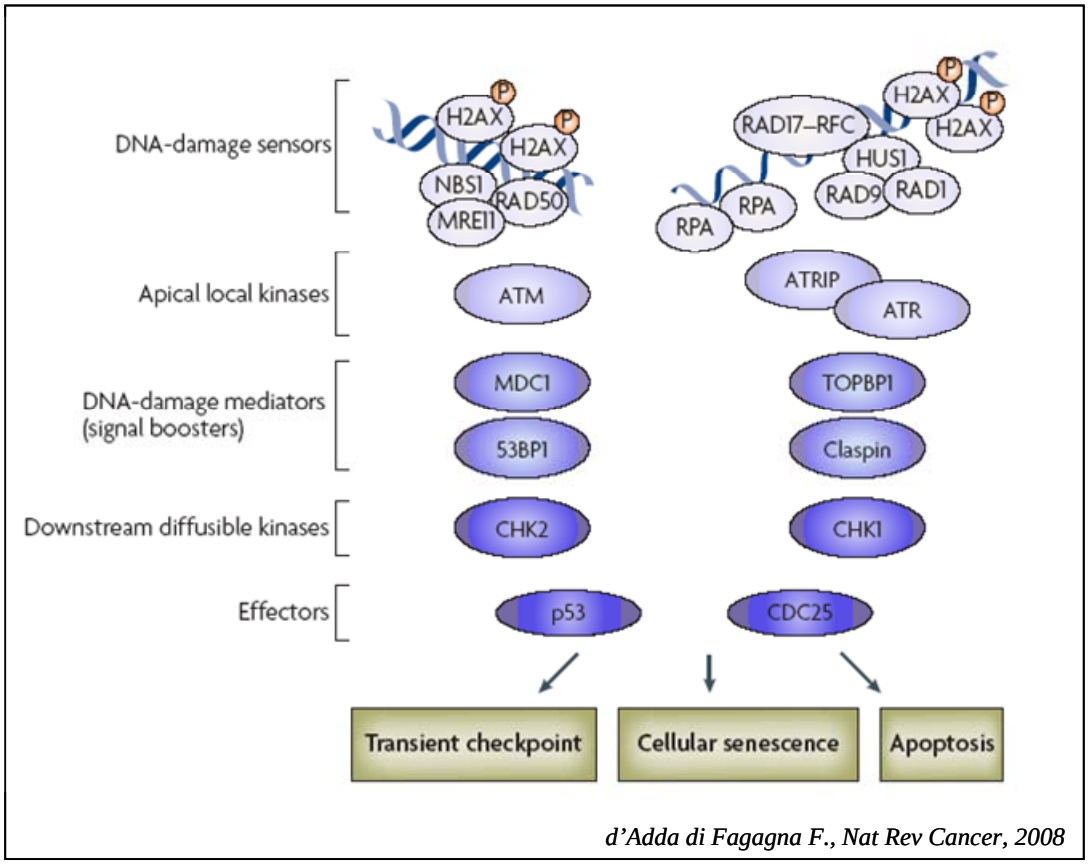

Fig. K: The DNA damage response (DDR) pathway

As previously described, p53 is positively and negatively regulated by a multitude of factors. The E3 ubiquitin ligases MDM2, PIRH2, and COP1 negatively regulates p53 by targeting it for proteosome-mediated degradation (Lu 2005). On the other hand, p53 is positively regulated by ARF, PML, PTEN, NPM, p33ING1, and other tumor suppressors, which

posttranslationally stabilize p53 (Weber, Taylor et al. 1999; Leung, Po et al. 2002; Freeman, Li et al. 2003; Bernardi, Scaglioni et al. 2004; Kurki, Peltonen et al. 2004). All these p53 regulators can also impact cellular senescence (Dimri 2005).

Among p53 regulators, promyelocytic leukemia protein (PML) has shown to be an important mediator of senescence. PML nuclear bodies (PML-NBs) are distinct nuclear structures that have multiple cellular functions (see later) and whose number increase during senescence. Actually, it was demonstrated that RAS-induced senescence is associated with increased PML levels leading to an increase in the size and the number of PML-NBs, high p16 levels, pRB hypophosphorylation, p53 phosphorylation at serine 15 and activation of p53 transcriptional targets (Ferbeyre, de Stanchina et al. 2000). Moreover, p53 is acetylated at lysine 382 upon RAS expression, and RAS induces relocalization of p53 and CBP acetyltransferase within the PML-NBs, favouring the formation of a trimeric p53-PML-CBP complex (Pearson, Carbone et al. 2000). Furthermore, the NAD-dependent histone deacetylase SIRT1, is recruited to PML-NBs upon overexpression of either PML or oncogenic RAS, inducing p53 deacetylation, and inhibiting PML- and p53-induced premature senescence (Langley, Pearson et al. 2002). More detailed studies have recently suggested that PML isoform IV (PML3), when overexpressed induces senescence in human fibroblast in a p53- and pRB- dependent manner (Bischof, Kirsh et al. 2002; Mallette, Goumard et al. 2004; Bischof, Nacerddine et al. 2005). Recently, PML-NBs have been shown to regulate another important aspect of cellular senescence: the formation of SAHFs. The chromatin remodelling process that occur during SAHFs formation is mediated by PML-NBs because, before localization to SAHFs, the histone chaperone HIRA translocates to PML-NBs. HIRA localization to PML-NBs is indeed one of the first steps in the senescence programme because it precedes all other markers of senescence and is a necessary event for the formation of SAHFs and the progression of senescence (Zhang, Poustovoitov et al. 2005; Ye, Zerlanko et al. 2007).

3. Aim of the thesis

Due to their high degree of sequence homology, MAGE-I proteins were considered for a long time as functionally redundant proteins. Nevertheless, in the last few years emerging data suggested that MAGE-I proteins could display specific functions possibly related to cell transformation. However, there is still little information about the potential and specific roles that individual MAGE-I proteins could play in these processes.

In this Thesis, we aimed to functionally characterize different MAGE-I genes, in particular MageA2 and MageB2 genes, in order to demonstrate their functional specificity, despite their sequence homology. The major starting readout to dissect the respective roles of the analyzed MAGE-I proteins was the p53 tumor suppression pathway.

The general framework aim was in fact to reconsider the specific cellular functions and the respective pathways that are targeted by the different family members to generate critical knowledge for exploiting MAGE-I genes as targets to be specifically inactivated in cancer cells.

4. Results

Part I: MAGE-A tumor antigens target p53 transactivation function through histone deacetylase recruitment and confer resistance to chemotherapeutic agents

MAGE-A proteins repress p53 function with different efficiency

At the beginning of this study, experiments carried out in collaboration with Dr. Monica Rodolfo (Istituto Nazionale Tumori, Milano, Italy) showed a strong correlation between MAGE-A proteins levels, in particular MageA2, A6 and A12, and resistance to chemotherapeutic drugs such as cisplatin (CP) and etoposide (ET) in primary melanoma cells harboring wild type p53 (Fig. 1A and 1B).

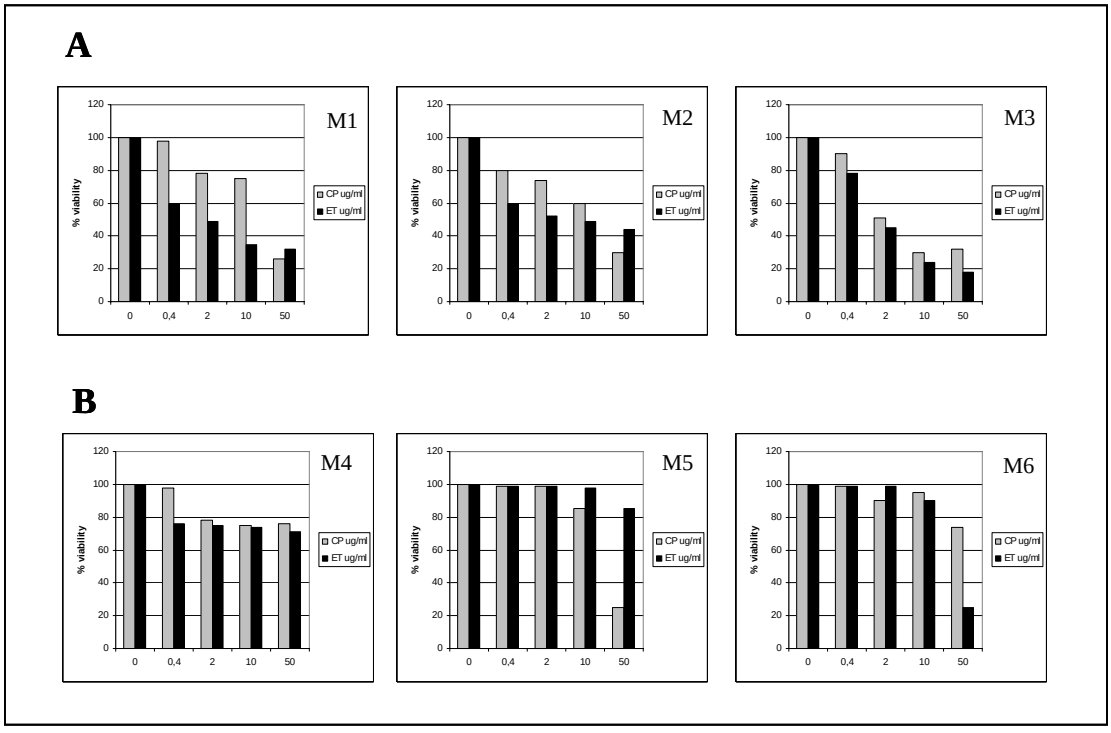

Fig. 1: *MAGE-A correlates with resistance to chemotherapeutic drugs in tumors harboring wild type p53*
Melanoma cell lines expressing No or Low MAGE-A levels (M1, M2 and M3) (**A**) and those expressing High MAGE-A levels (M4, M5 and M6) (**B**) were treated with different doses of ET or CP as indicated. Cell viability was calculated by MTT assay 72 hours after treatment.

Consequently, we asked whether MAGE-A proteins could have an effect on p53 function. We observed that MAGE-A proteins such as MageA1, A2 and A6 were able to repress p53 transactivation function in gene reporter assays using a vector containing p53 responsive sequences as synthetic promoter for the luciferase gene (pG13-Luc) (Fig. 2A). The assay indicated that MageA2 exerts the strongest repressive effect on p53 as compared with other MAGE-A proteins. For this reason, we decided to use MageA2 in the following studies. A similar analysis was performed using a panel of p53 responsive promoters, confirming the efficiency of MageA2 in down-regulating p53 activity (Fig. 2B). Expression of MageA2 did not alter p53 protein levels, as evaluated on the same lysates used for gene reporter assay (Fig. 2C).

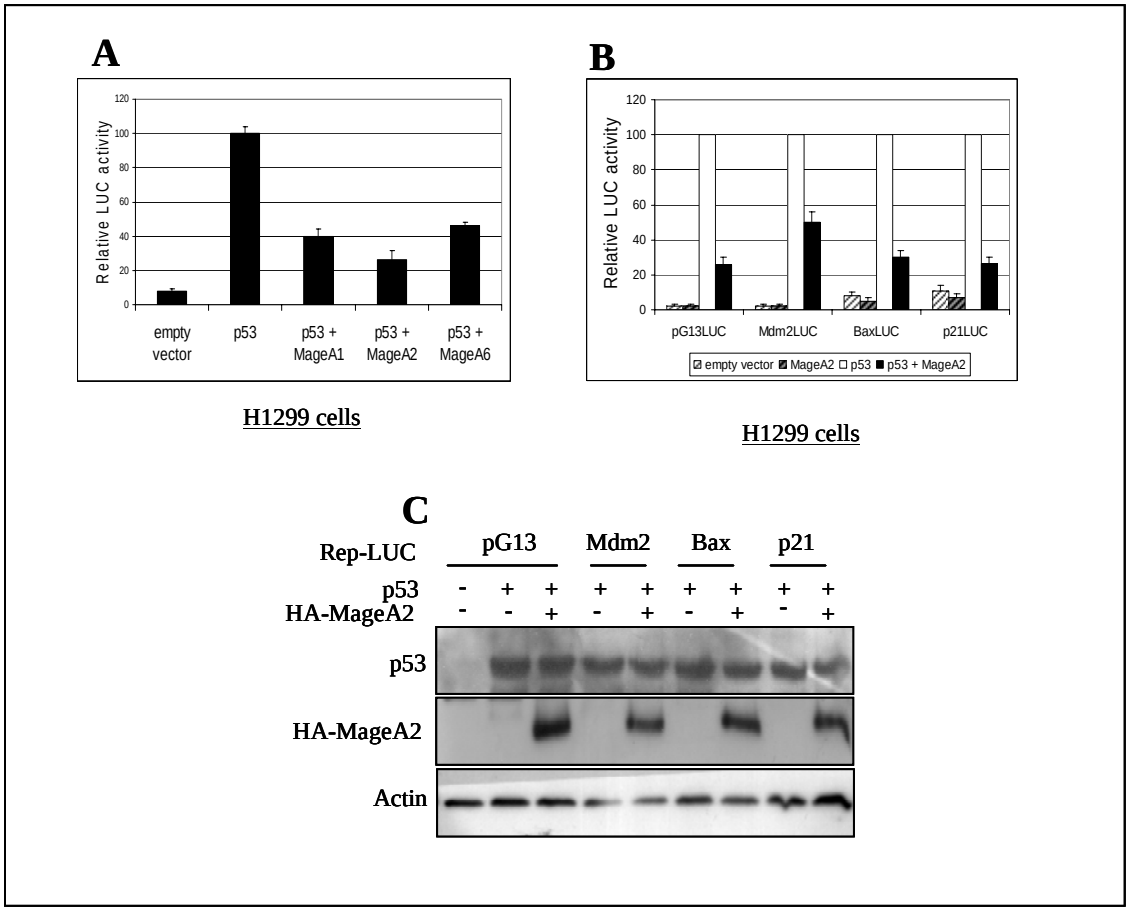

Fig. 2: *MageA2 expression represses p53 transactivation function*
A) p53-specific reporter gene assay performed in H1299 cells (p53 null) transfected with p53, or in combination with MageA1, MageA2 and MageA6 together with the p53-responsive promoter pG13LUC. Value corresponding to p53 transfection was reported to 100. **B)** p53-specific reporter gene assay as performed in "A" using p53 and MageA2 together with different p53-responsive promoters. **C**) The levels of transfected p53 and HA-MageA2 from lysates used in "B" were analyzed by western blot using DO-I and anti HA tag antibodies, respectively. Actin levels were used as loading control.

We then determined the effect of MageA2 expression on the endogenous levels of p53 target genes. We established the HA-MageA2 inducible U2OS cell line (named M20 cells) under the control of Ponasterone A (PonA). Although p53 protein levels were comparable in cells overexpressing or not HA-MageA2 upon treatment with ET, the endogenous levels of p53 targets, p21Waf-1 and Bax, were markedly reduced in cells expressing HA-MageA2 (Fig. 3A). Conversely, siRNA mediated knockdown of MageA2 in U2OS cells resulted in an earlier accumulation of p53 targets after DNA damage (Fig. 3B).

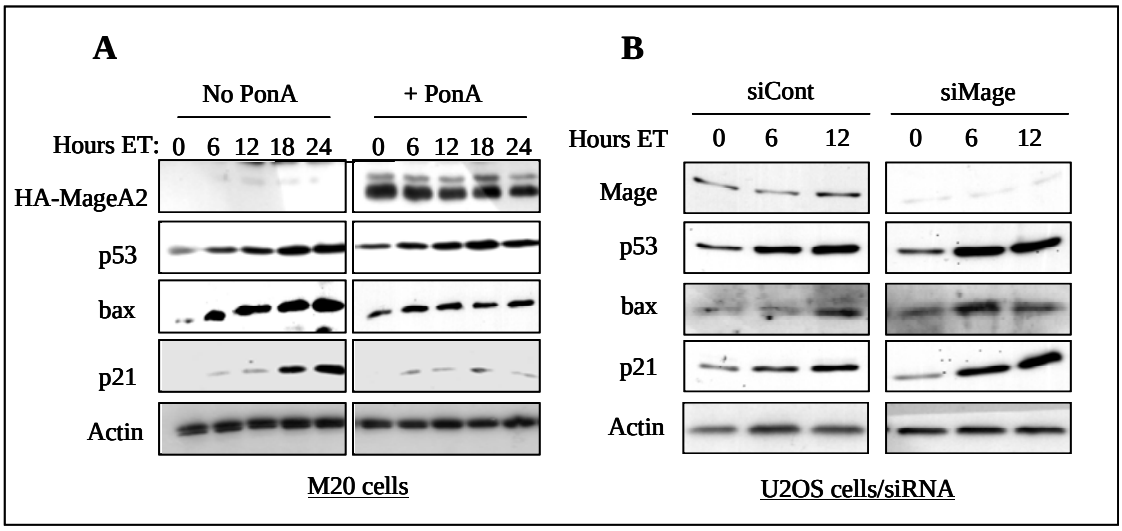

Fig.3: *MageA2 expression represses p53 targets expression without affecting p53 levels*
A) Western blot analysis of M20 cells (HA-MageA2 inducible U2OS cells) expressing (+PonA) or not (No PonA) HA-MageA2. p53, bax and p21 protein levels were determined at the indicated time-points after treatment with 10 uM ET. **B)** Determination of p53, bax and p21 protein levels after ET treatment in U2OS cells transfected with control siRNA (siCont) or Mage siRNA (siMage).

We then analyzed the effect of MageA2 on p53 biological functions, using DNA damage-induced growth arrest and apoptosis as readouts. Overexpression of MageA2 resulted in impaired DNA damage induced growth arrest as assessed by BrdU incorporation in M20 cells upon treatment with ET (Fig. 4A). Moreover, MageA2 expression correlated with resistance to ET treatment as evaluated in M20 cells overexpressing MageA2 (Fig. 4B) and in U2OS cells transfected with specific siRNA for knocking down MAGE-A levels (Fig. 4C). No significant protection to ET treatment was seen in p53-null Saos-2 cells by MageA2 expression, which, however, efficiently inhibited cell death induced by p53 overexpression (Fig. 4D). These data support the notion that after DNA damage the p53 dependent response is impaired in cells overexpressing MageA2.

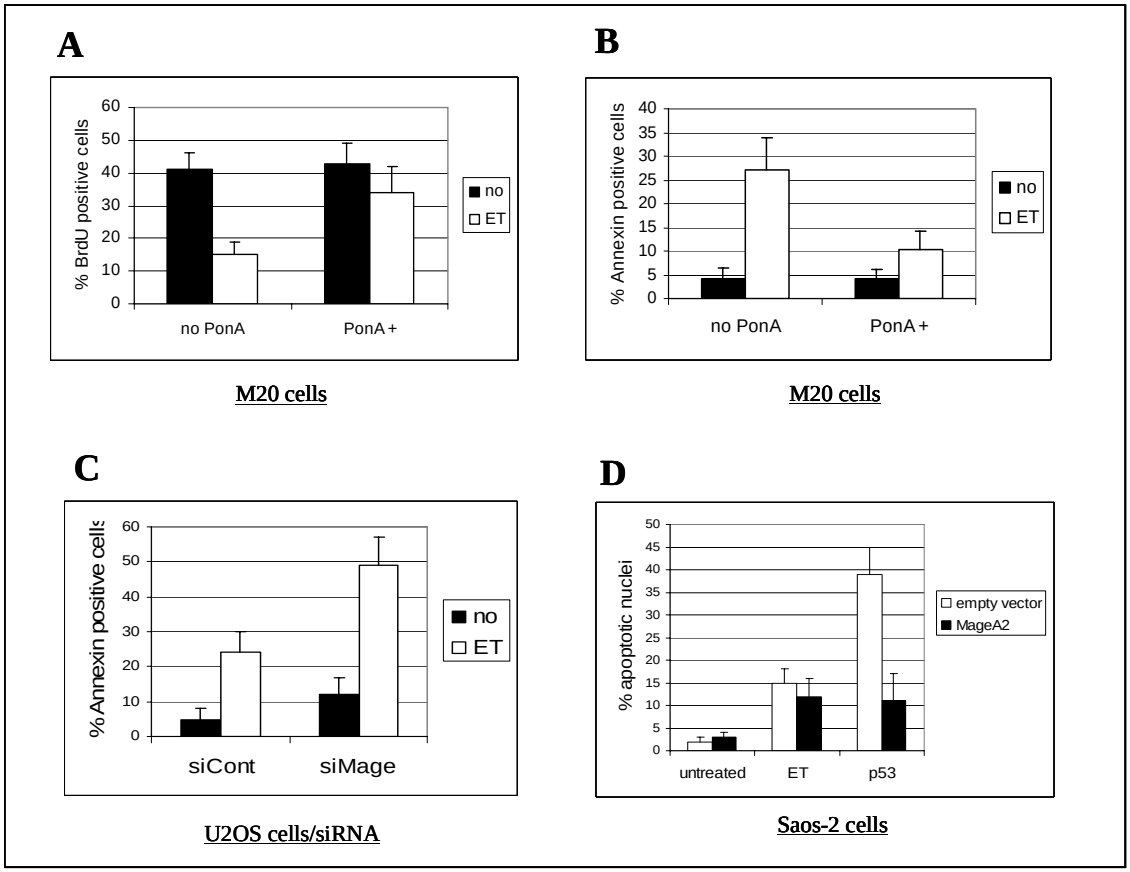

Fig. 4: *MageA2 expression impairs p53 dependent damage-induced growth arrest and apoptosis*
A) Growth arrest was determined by BrdU incorporation in M20 cells expressing (PonA+) or not (no PonA) HA-MageA2 after treatment with 10 uM ET for 16 hs. 200 cells were counted for at least 3 independent experiments. **B**) Apoptosis determination by Annexin V assay coupled to FACS analysis (10.000 counted cells for at least 3 independent experiments). M20 cells were treated with 20 uM ET for 36 hours. **C**) Similar experiment as shown in "B", but performed in U2OS cells previously silenced using siRNA as indicated. **D**) Apoptotic scoring in p53-null Saos-2 cells expressing or not HA-MageA2 treated with 20 uM ET for 36 hours (see ET) or overexpressing p53 for 48 hours (see p53). Apoptosis was determined by nuclear morphology on transfected cells using Hoechst dye. 200 cells were counted for at least 3 independent experiments.

MageA2 can physically interact with p53

In order to evaluate a potential interaction between Mage-A and p53 proteins, we performed immunoprecipitation assays in U2OS cells, which revealed an association between endogenous p53 and MAGE-A proteins (Fig. 5A). A set of p53 deletion mutants were used to map the interaction between MageA2 and p53, demonstrating that the complex formation *in vivo* required the p53-DNA-binding domain (p53DBD, amino acids 94-298), whereas the p53 transactivation domain (amino acids 1-98) and the p53-C-terminal domain (p53CTD, amino acids 298-393) were dispensable (Fig. 5B and 5C). We also performed *in vitro* pull-down experiments showing that recombinant GST-p53 associated with *in vitro* translated (IVT) ^{35}S-

labeled MageA2 (Fig. 5D) and that GST-MageA2 bound specifically to the p53DBD but not to p53CTD or the N-terminal domain (p53NTD, amino acids 1-98) (Fig. 5E), suggesting a direct interaction between these proteins.

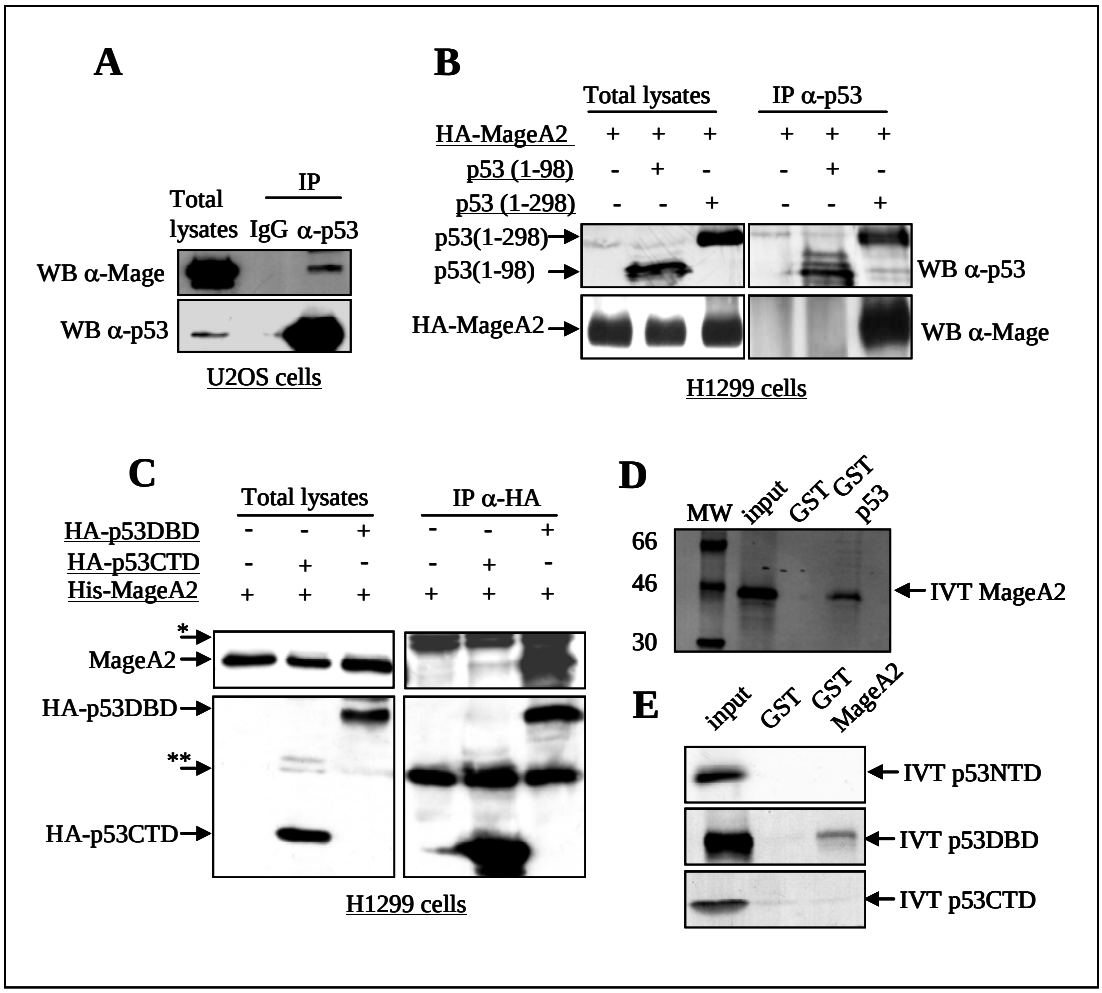

Fig. 5: *Interaction between MageA2 and p53*
A) Immunoprecipitation of endogenous p53/Mage protein complex in U2OS cells using anti-p53 polyclonal antibody or preimmune IgG. Western blot were performed by using p53 (DO1) and Mage (57B) antibodies. **B**) Immunoprecipitation in H1299 cells transfected with HA-MageA2 and p53 deletion mutants (p53 (1-98) and p53 (1-298)) using polyclonal anti-p53 antibody. DO1 (p53) and anti-HA were used in the Western blot. **C**) Immunoprecipitation assay similar to that in "B" but using p53 deletions expressing HA-p53 94-298 (HA-p53DBD) and HA-p53 298-393 (HA-p53CTD). MageA2 was used as His-tagged version. **D**) *In vitro* binding assay using recombinant/purified GST and GST-p53 fusion protein incubated with *in vitro* translated ^{35}S-labeled HA-MageA2 (IVT MageA2). **E**) I*n vitro* binding assay using recombinant/purified GST and GST-MageA2 fusion protein incubated with IVT ^{35}S-labeled domains of p53: p53NTD (1-98), p53DBD (94-298), and p53CTD (298-393) as indicated. * indicates immunoglobulin heavy chain, ** indicates immunoglobulin light chain.

MageA2 impairs p53 acetylation by assembling HDAC3/p53 complex

p53 function has been shown to be tightly regulated by many different mechanisms that include specific post-translational modifications, as phosphorylation, acetylation, ubiquitylation, neddylation, methylation and sumoylation (Bode and Dong 2004; Murray-Zmijewski, Slee et al. 2008). We analyzed the profile of p53 modifications, in particular phosphorylation and acetylation, in M20 cells after treatment with DNA damaging agents. ET treatment caused rapid p53 phosphorylation on Ser-15, independently of MageA2 induction, whereas acetylation of Lys-382 was severely impaired in MageA2 overexpressing M20 cells (Fig. 6A). Moreover, the use of HDAC inhibitor Trichostatin A (TSA) counteracted the effect of MageA2 expression on p53 acetylation (Fig. 6B), suggesting that a balance between histone acetyl-transferases (HATs)/HDACs activities could be affected in cells overexpressing MageA2.

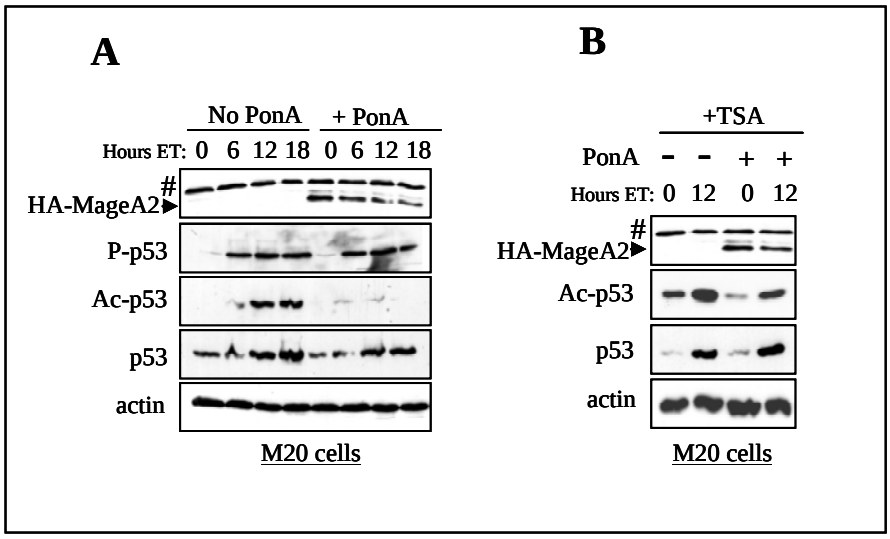

Fig. 6: *MageA2 induces p53 deacetylation*
A) Time-course experiment in M20 cells after addition of 10uM ET. PonA was added 15 hours before ET treatment. DO1 antibody was used for total p53, while anti-P-p53Ser15 (P-p53) and anti-Ac-p53Lys382 (Ac-p53) were used for specific phosphorylation and acetylation. HA-MageA2 expression was determined using anti-HA antibody.
B) Similar to "A" but using 300 nM TSA together with ET treatment on M20 cells expressing or not HA-MageA2.

Since it was previously reported that MageA1 can bind to HDAC1 (Laduron, Deplus et al. 2004), we decided to investigate whether MageA2 could associate with HDACs as a potential mechanism to control p53 activity. Induction of MageA2 in M20 cells and knocking-down of MAGE-A in U2OS cells, correlated with similar changes in HDAC3 protein levels with

respect to HDAC1 (Fig. 7A). Therefore, we focused our studies on the relationship between MageA2 and HDAC3. HA-MageA2 was immunoprecipitated from transiently transfected 293T cells where endogenous HDAC3 was found to form a complex with MageA2. As expected, endogenous p53 coimmunoprecipitated with MageA2 (Fig. 7B). By *in vitro* pull-down experiments we detected a weak but reproducible interaction between recombinant GST-MageA2 and IVT ^{35}S-labeled HDAC3 (Fig. 7C). The possibility that MageA2 could recruit HDACs to p53 was confirmed by the finding that Flag-HDAC3/endogenous p53 association was stronger in the presence of MageA2 (Fig. 7D).

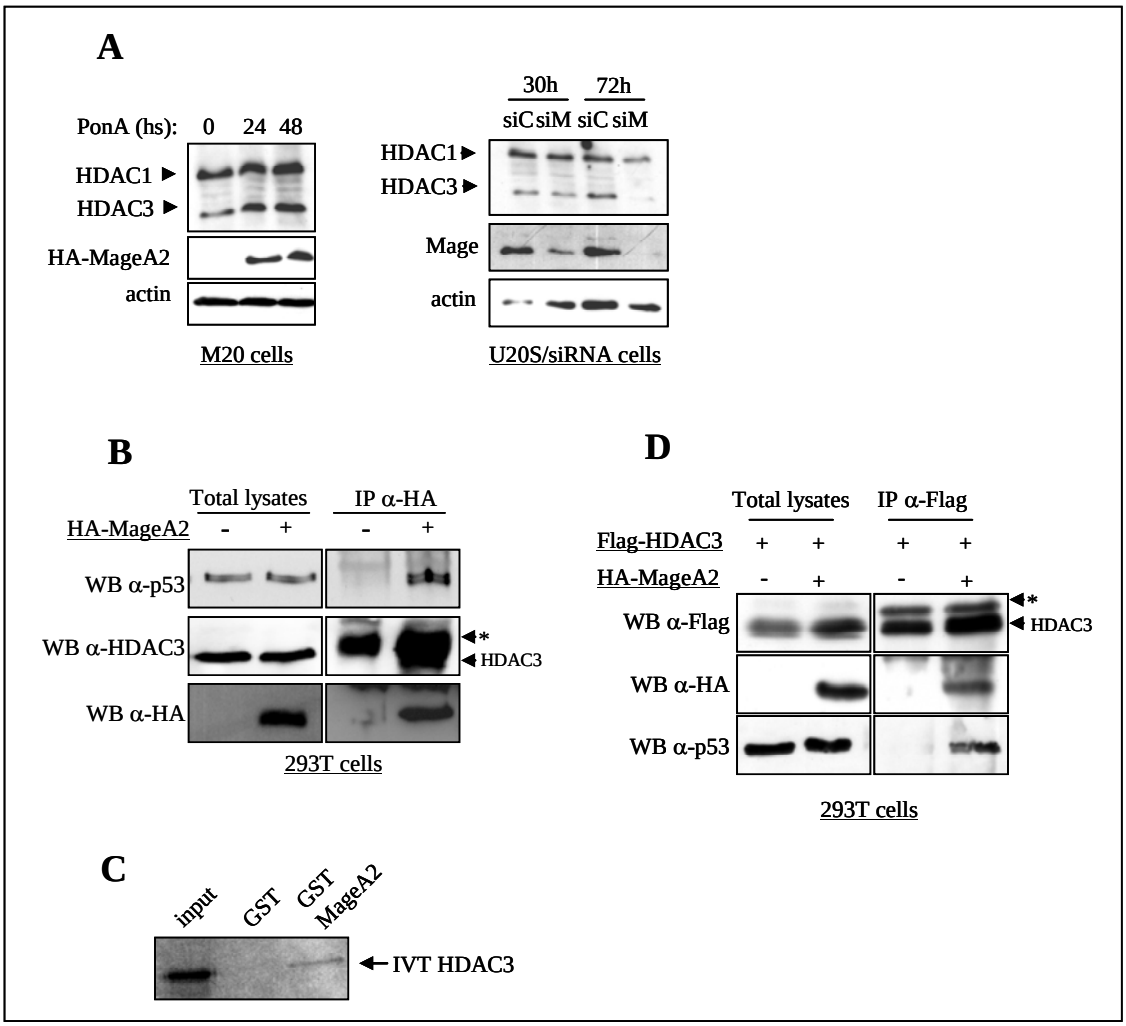

Fig. 7: *MageA2 recruits HDAC3 to p53*
A) Endogenous levels of HDAC1 and HDAC3 were assessed by western blot in M20 cells 24 and 48 hs after the induction of HA-MageA2 (PonA) *(left panel)*, and in U2OS cells transfected for 30 and 72 hs with control siRNA (siC) or Mage siRNA (siM) (*right panel*), using anti-HDAC1 and anti-HDAC3 polyclonal antibodies. HA-Mage and Mage were detected using anti-HA and anti-MageA monoclonal antibodies, respectively. **B**) Immunoprecipitation of transiently transfected 293T cells with HA-MageA2 using anti-HA antibody. Anti-p53 and anti-HDAC3 polyclonal antibodies and anti-HA were used for Western blot as indicated. **C**) *In vitro* binding assay using recombinant/purified GST and GST-MageA2 fusion protein incubated with *in vitro* translated ^{35}S-labeled HDAC3. **D**) Immunoprecipitation of transiently transfected 293T cells with HA-MageA2 and Flag-HDAC3 expression vectors using anti-Flag antibody as indicated. Endogenous p53 was detected using anti-p53 polyclonal antibody.

MageA2/HDAC3 complex can associate with p53 on its DNA-binding sites

To evaluate whether the MageA2/HDAC3/p53 complex could associate to a p53 responsive promoter, Chromatin Immunoprecipitation (ChIP) assay was performed after cotransfection of p53, MageA2 and HDAC3 in p53 null H1299 cells. Briefly, cells were transfected with pBax-Luc, p53 and Flag-MageA2 or Flag-HDAC3 as indicated in Figure 8B. Samples were immunoprecipitated with an anti-Flag antibody, followed by pBax-Luc PCR amplification using the Fw primer on the Bax promoter and the Rev primer on the Luc gene (Fig. 8A). We observed that MageA2 was able to bind the Bax promoter only when p53 was transfected, and that HDAC3 required p53/MageA2 complex to associate with this promoter (Fig. 8B).
To assess whether HDAC3 in complex with MageA2 conserved its deacetylating activity, we transfected H1299 cells with HA-MageA2 and Flag-HDAC3 and used the immuno-complex in an *in vitro* deacetylase assay by using ^{14}C-acetylated histones as substrate for the reaction. Autoradiography revealed strong *in vitro* deacetylase activity of the complex (Fig. 8C), indicating that MageA2 could assemble active HDAC3 to p53 complex as part of its repressive function.

HDAC3/MageA2/p53 complex induces histone hypoacetylation at p53-binding sites

Overexpression of MAGE-A genes was originally found in melanoma, a human tumor highly refractory to chemotherapy even though harboring wt-p53 (Albino, Vidal et al. 1994; Satyamoorthy, Chehab et al. 2000). Therefore, we analyzed the p53-dependent response in two human short-term cell lines obtained from melanoma biopsies expressing different MAGE-A levels and harboring wt-p53 (15392M and 13923M) (van Baren, Brasseur et al. 1999; Daniotti, Oggionni et al. 2004). 15392M cells express very low levels of MAGE-A while 13923M cells express high levels of MAGE-A. Upon ET treatment, the p53 response as well as p53 acetylation were severely impaired in 13923M cells expressing high MAGE-A levels when compared to 15392M cells expressing low MAGE-A levels (Fig. 9A and 9B). Importantly, both acetylation as well the p53 response to ET were restored, as determined both by p53 acetylation and p21 and bax protein levels, when TSA was used in combination with ET (Fig. 9C).

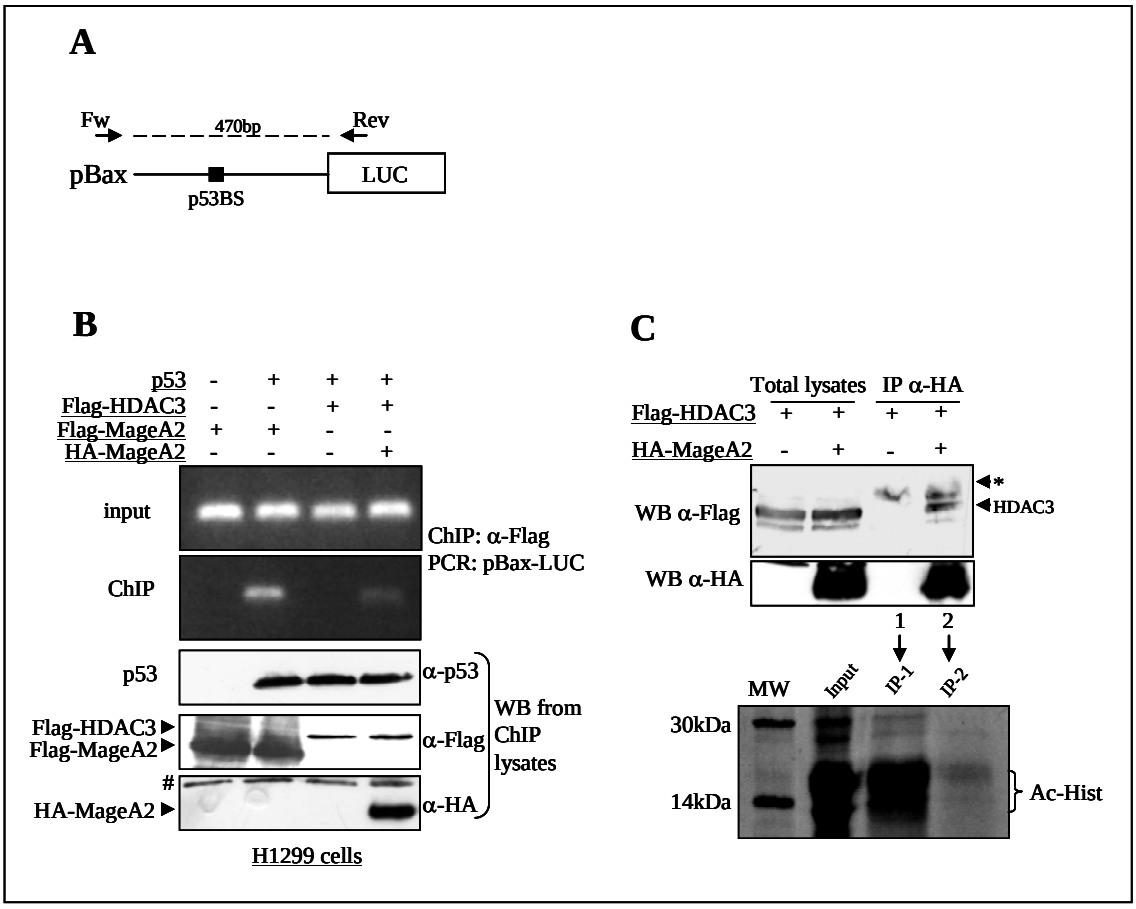

Fig. 8: *Active MageA2/HDAC3 complex is recruited to p53 binding sites on chromatin*
A) Diagram showing the PCR amplification approach of pBax-Luc DNA used for ChIP assay. **B**) *Upper panel*: ChIP performed in H1299 cells transfected with pBax-Luc, p53 and Flag-tagged construct as indicated. Samples were immunoprecipitated (ChIP) or not (input) using anti-Flag antibody, followed by pBax-Luc PCR amplification using a Fw primer recognizing the 5' of the transfected Bax promoter and a Rev primer recognizing the Luc gene (see diagram in "A"). *Lower panel*: control of the indicated transfections by western blot. **C**) *Upper panel*: Immunoprecipitation of transiently transfected H1299 cells with HA-MageA2 and Flag-HDAC3 using anti-HA antibody. Lane 1 and 2 show the absence and presence of MageA2/HDAC3 complex, respectively. *Lower panel*: *In vitro* deacetylation assay of ^{14}C-acetylated histones (Ac-Hist) by immunoprecipitated complex shown in Lane 1 (IP-1) and Lane 2 (IP-2) of the upper panel. Input indicates mock-treated ^{14}C-acetylated histones. MW is ^{14}C-labeled protein molecular weight. # indicates unspecific band. * indicates immunoglobulin heavy chain.

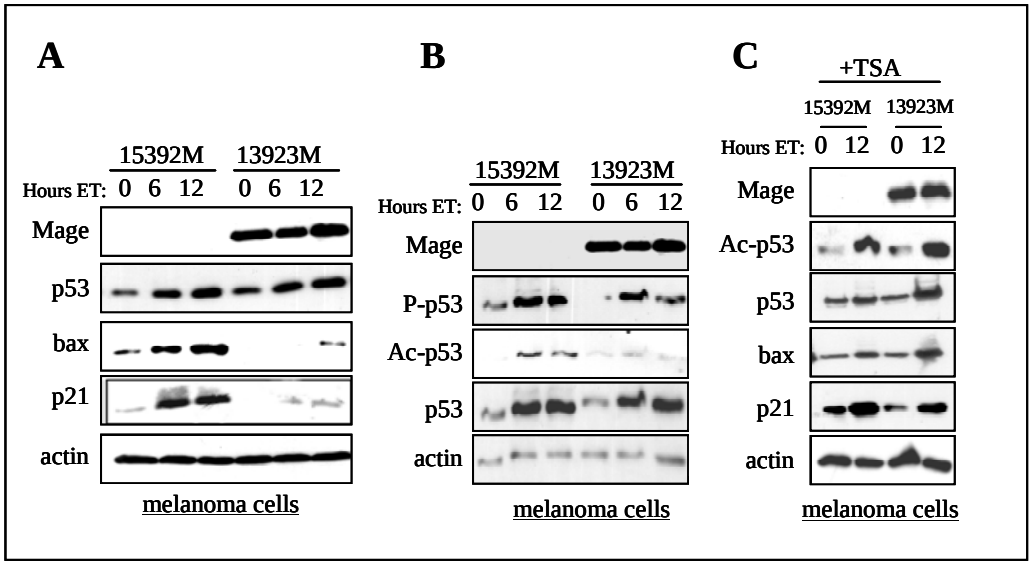

Fig. 9: *MAGE-A deacetylates and inhibits p53 in melanoma cells*
A) DNA-damage response in melanoma cells expressing low (15392M cells) or high (13923M) MAGE-A levels, after treatment with 20uM ET. MAGE-A, p53, bax and p21 protein levels were determined at the indicated time-points. **B**) Analysis of p53 modification in 15392M and 13923M melanoma cells after ET damage (20uM). p53 modifications were determined using anti-P-p53Ser15 (P-p53) and anti-Ac-p53Lys382 (Ac-p53). **C**) Similar to "B" but using 300 nM TSA together with ET (20uM) treatment in 15392M and 13923M melanoma cells.

We then asked whether deacetylation of histones surrounding p53 binding sites on promoter DNA could be an additional target for HDAC recruited by MageA2 to exert p53-dependent transcription repression. p53/MageA2/HDAC3 protein complex can be found assembled at p53 binding sites (Fig. 8B), suggesting that the inhibitory effect of such complex could be extended to other acetylation-dependent substrates critically involved in transcription regulation such as nucleosome histones. In fact, MageA2 was able to down-regulate the transcriptional activity of the non-acetylable p53 mutant, p539KR (Ard, Chatterjee et al. 2002) both alone or when cotransfected with the histone acetyl-transferase p300 (Fig. 10A and 10B), strongly suggesting that histone hypoacetylation could represent the main mechanism used by MageA2 for targeting p53 activity.

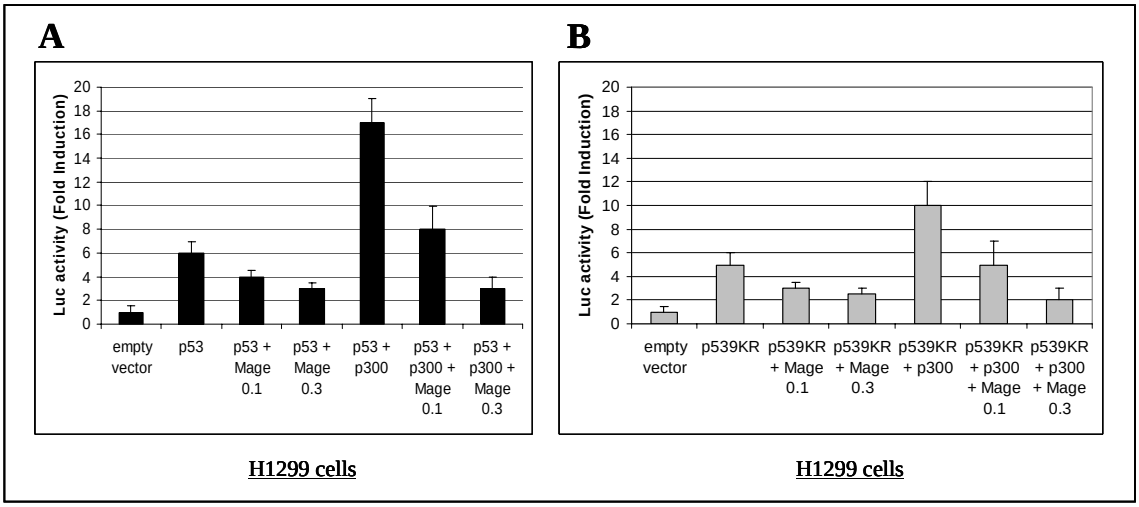

Fig. 10: *MageA2 repress the transcriptional activity of non-acetylable p53*
A) Analysis of MageA2 function on wt-p53 transcription activity with or without the addition of p300. H1299 cells (p53 null) were transiently transfected with expression vectors encoding p53, HA-MageA2 and HA-p300 combined as indicated, together with BAX promoter containing the *fire-fly* luciferase gene (pBax-Luc) as reporter. MageA2 was transfected in two different amounts: 100ng (Mage 0.1) or 300ng (Mage 0.3). LUC activity was assessed 24 hours later. pCMV-*renilla* luciferase vector was cotransfected to normalize transfection efficiency. **B)** Analysis of MageA2 function on mutated p53 (p539KR) transcription activity with or without the addition of p300 as performed in "A".

Using 15392M and 13923M melanoma cells as model, we analyzed both endogenous protein/chromatin complexes as well as chromatin acetylation status using a quantitative ChIP approach (ChIP followed by real time PCR) before and after DNA-damage. As shown in Fig. 11A, p53 was similarly recruited to p21 or Bax promoter after ET treatment independently of MAGE levels, as evidenced by the ratio between damaged/undamaged ChIP value, thus confirming that MAGE-A expression does not affect the DNA binding ability of p53. However, significant HDAC3 recruitment to p21 and Bax promoters after damage was clearly related to MAGE-A expression. On the contrary, histone H3 acetylation level increased more efficiently after damage in cells lacking MAGE-A when compared to cells expressing high MAGE-A levels. Addition of TSA caused a global histone acetylation status in both cell strains resulting in no difference (ratio next to 1) with respect to ET addition. Similar data was obtained when the MageA2-inducible M20 cell line was tested (Fig. 11B). Altogether our data support the notion that MAGE-A proteins could recruit HDACs to p53 causing hypoacetylation of histones surrounding p53 binding sites and of p53 protein itself, thus weakening the p53-dependent transactivation function.

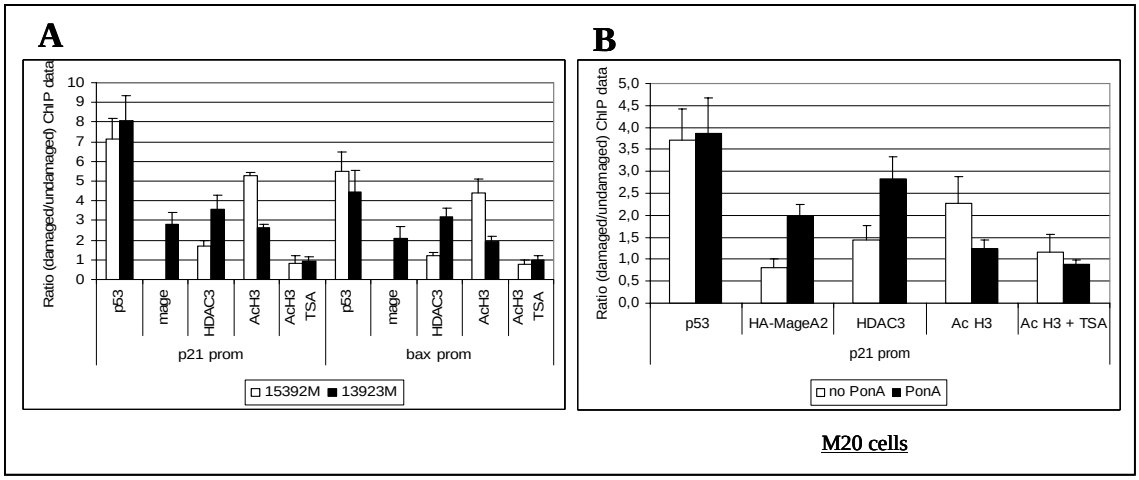

Fig. 11: *MageA2/HDAC complex deacetylates p53 and histones surrounding p53-binding sites*
A) ChIP assay followed by quantitative PCR analysis using real-time PCR. Protein/DNA complexes were immunoprecipitated from 15392M and 13923M melanoma cells before and after ET treatment using the antibodies indicated in the X axis. AcH3 is acetylated histone H3 antibody. TSA indicates TSA treatment. Data are expressed as the ratio between damaged/undamaged ChIP values, therefore indicating the enrichment of each protein to the indicated promoter after damage. **B)** ChIP assay followed by quantitative PCR analysis using real- time PCR as in "A". Protein/DNA complexes were immunoprecipitated from non induced (white) or PonA induced (black) HA-MageA2 expression in M20 cells before and after ET treatment. The antibodies are indicated in the X axis, MageA2 was immunoprecipitated using the HA anti-tag monoclonal antibody.

Melanoma cells expressing MAGE-A genes are refractory to DNA damage-induced apoptosis

By using melanoma cells as model, we evaluated the correlation between MAGE-A expression and resistance to apoptosis. ET treatment of 15392M and 13923M melanoma cells showed a dose-dependent induction of cell death in cell expressing low MAGE-A levels, while cells expressing high MAGE-A levels remained almost insensitive to such treatment (Fig. 12A). Importantly, addition of TSA (able to efficiently stimulate p53 function in cell expressing high levels of MAGE-A, see Fig. 9C) significantly sensitized 13923M cells to ET induced apoptosis (Fig. 12A). Similar effects were observed in MageA2-inducible M20 cell line (Fig. 12B). Melanoma cell lines harboring mutant p53 were found to be strongly resistant to ET/TSA treatment independently of MAGE-A expression as assessed in SK-Mel-28 (high MAGE-A expression) and BE cells (low MAGE-A expression) (Fig. 12C), supporting the hypothesis that MAGE-A proteins could exert their function by suppressing wt-p53 function.
The direct relevance of MAGE-A levels and wt-p53 activity in resistance to DNA-damaging agents was then analyzed in 13923M cells. MAGE-A levels were down-regulated by pSuper-Retro (pSR) retrovirus vector transducing siRNA designed for targeting a highly conserved sequence of several MAGE-A genes (pSRMage). p53 activity was controlled by using pBabe-

EGFP-TNVp53OD (Gostissa, Morelli et al. 2004) retroviral vector (p53OD) encoding a p53 oligomerization domain peptide (amino acids 322-355) able to repress p53 function. 13923M cells were co-infected with pSR or pSRMage (Puromycin resistance) and pBabe or pBabep53OD (Hygromycin resistance) as indicated. Puromycin/Hygromycin resistant cells were selected to perform the experiment. Significant increase in susceptibility to ET was observed in 13923M-pSRMage cells when compared to their control, 13923M-pSR cells. This effect was clearly reduced when p53 activity was inactivated through p53OD (Fig. 12D), indicating that down-regulation of MAGE-A genes triggers p53-dependent cell death in cells treated with ET.

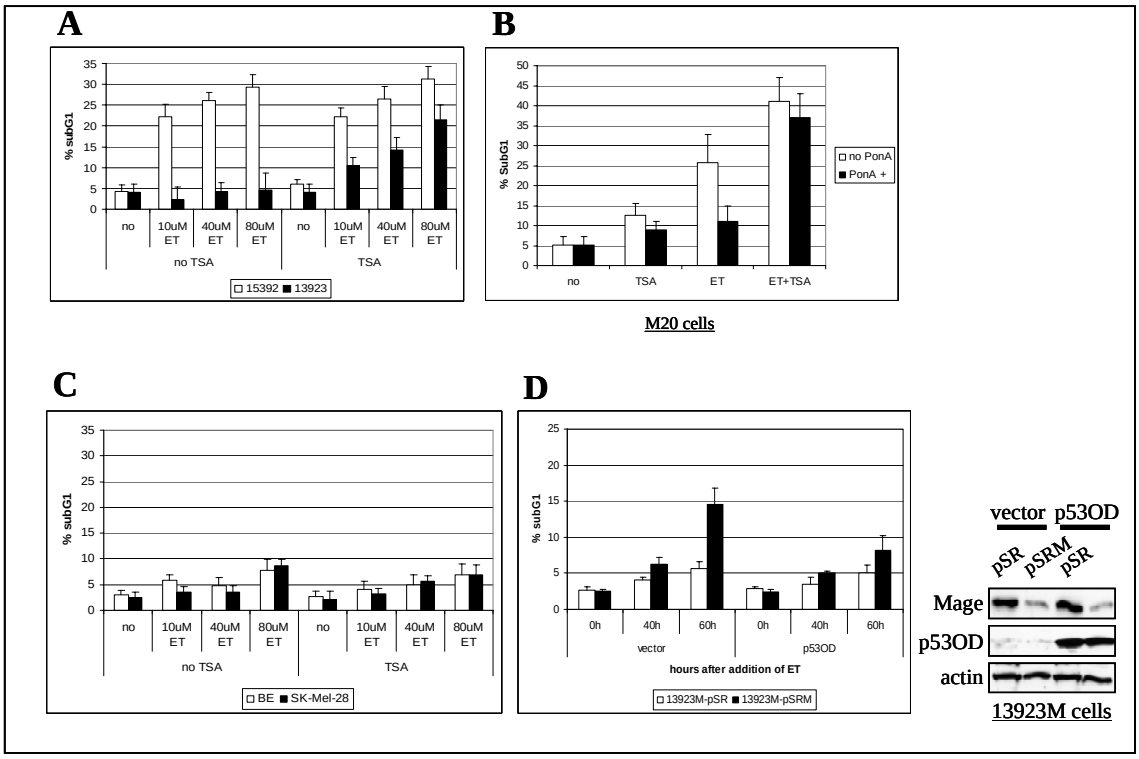

Fig. 12: *MAGE-A expression correlates with resistance to DNA damage-induced apoptosis*
A) Apoptosis determination by subG1 by FACS analysis (10.000 counted cells for at least 3 independent experiments) in 15392M and 13923M cells. Melanoma cells were treated with the indicated concentration of ET for 48hs. Where indicated, 300nM TSA was added together with ET. **B)** Similar to "A" but using M20 cells expressing (+PonA) or not (No PonA) HA-MageA2 and treated with 40uM ET for 36hs. **C)** Similar to "A" but using melanoma cell lines harboring mutant p53. **D)** *Left panel*: Apoptosis determination by subG1/FACS analysis in 13923M cells co-transduced with siRNA vector, pSR (empty) or pSRM (for Mage knock-down) and pBABE (empty) or pBABE.GFP-p53OD (for p53 inactivation) as indicated. Cells were treated with 40uM ET for the indicated time. *Right panel*: determination of MAGE-A protein levels and the expression of GFP-p53OD in 13923M-pSR and 13923M-pSRM cells using anti-MAGE and anti-GFP polyclonal antibodies, respectively.

In summary, in this part of the work we demonstrated that MageA2 protein confers resistance to chemotherapeutic drugs such as ET, by recruitment of HDAC3 to p53, thus repressing p53 transactivation function. The mechanism responsible for the repressive effect of MageA2, relies on an impaired acetylation of both p53 and histones surrounding p53 binding sites by MageA2/HDAC3 complexes (Fig. 13). Finally, the correlation between MAGE-A expression and resistance to apoptosis has been validated in short-term melanoma cell lines, where combined treatment with ET and TSA restores de p53 response and reverts chemoresistance of melanoma cells expressing high levels of MAGE-A.

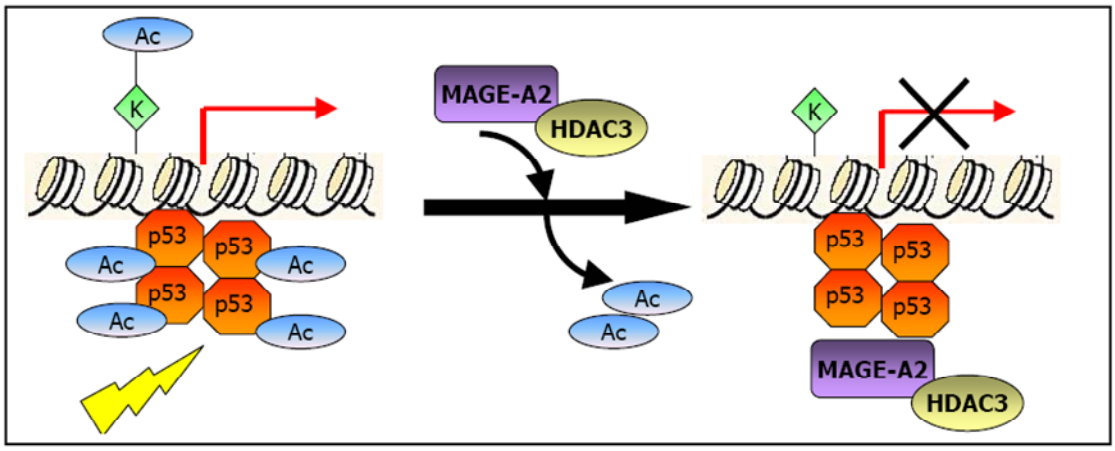

Fig. 13: *Proposed model for the repressive effect of MageA2 on p53 function*
Upon DNA damage, p53 becomes acetylated and activates the transcription of its targets genes (*left*); the formation of MageA2/HDAC3 complexes leads to the deacetylation of p53 and histones surrounding p53 binding sites, leading to transcriptional repression (*right*). Ac: acetyl groups. K: lysine residue.

Most of the data and results described in this Part I have been published in:

"MAGE-A tumor antigens target p53 transactivation function through histone deacetylase recruitment and confer resistance to chemotherapeutic agents". Monte M, Simonatto M*, Peche LY*, Bublik DR, Gobessi S, Pierotti MA, Rodolfo M, Schneider C. ***Proceedings of the National Academy of Sciences of the United States of America***; 130:11160-11165; 2006.

* Simonatto M and Peche LY equally contributed in this work

Part II: MageA2 regulates PML3-induced p53 activity

MageA2 impairs PML3 induced senescence

Loss of senescence is an early step during cell transformation. Here we have analyzed the potential role of MageA2 in interfering the PML3-p53 axis and its consequence on cellular senescence. Acetylation is a relevant modification for p53 activity, and several stimuli such as DNA damage are responsible for the induction of p53 acetylation (Bode and Dong 2004). Most specifically, p53 acetylation has also been associated with the promyelocytic leukemia (PML) tumor suppressor protein. In particular, it has been demonstrated that PML3 activates p53 (Fogal, Gostissa et al. 2000) inducing the recruitment of p53 to the PML-Nuclear Bodies (PML-NBs) where p53 becomes acetylated, leading to enhanced p53 activity for induction of premature senescence (Ferbeyre, de Stanchina et al. 2000; Pearson, Carbone et al. 2000).
Normal human fibroblast (Wi38 cells) were co-infected with PML3, and control or MageA2 expressing retroviruses. It has been reported that in this cellular system the prolonged expression of PML3 leads to a permanent arrest or premature senescence (Bischof, Kirsh et al. 2002). After ten days in culture, PML3 overexpressing cells showed all the features of the senescence process; they ceased to proliferate at sub-clonfluent densities and became flat and enlarged (Fig. 14A). Conversely, cells co-expressing PML3 and MageA2 did not show morphological changes respect to control cells (Fig. 14A). MageA2 overexpressing cells behaved in a manner that was indistinguishable from control cells. Analysis of the expression of senescence-associated β-galactosidase activity (SA-β-Gal), a well known marker of senescence, demonstrated that MageA2 expression reduced the levels of SA-β-Gal in PML3 expressing cells (Fig. 14A and 14B). Moreover, cells expressing MageA2 and PML3 showed higher levels of BrdU incorporation than PML3 overexpressing cells (Fig. 14C). These data suggest that PML3-induced senescence is impaired in cells expressing MageA2.

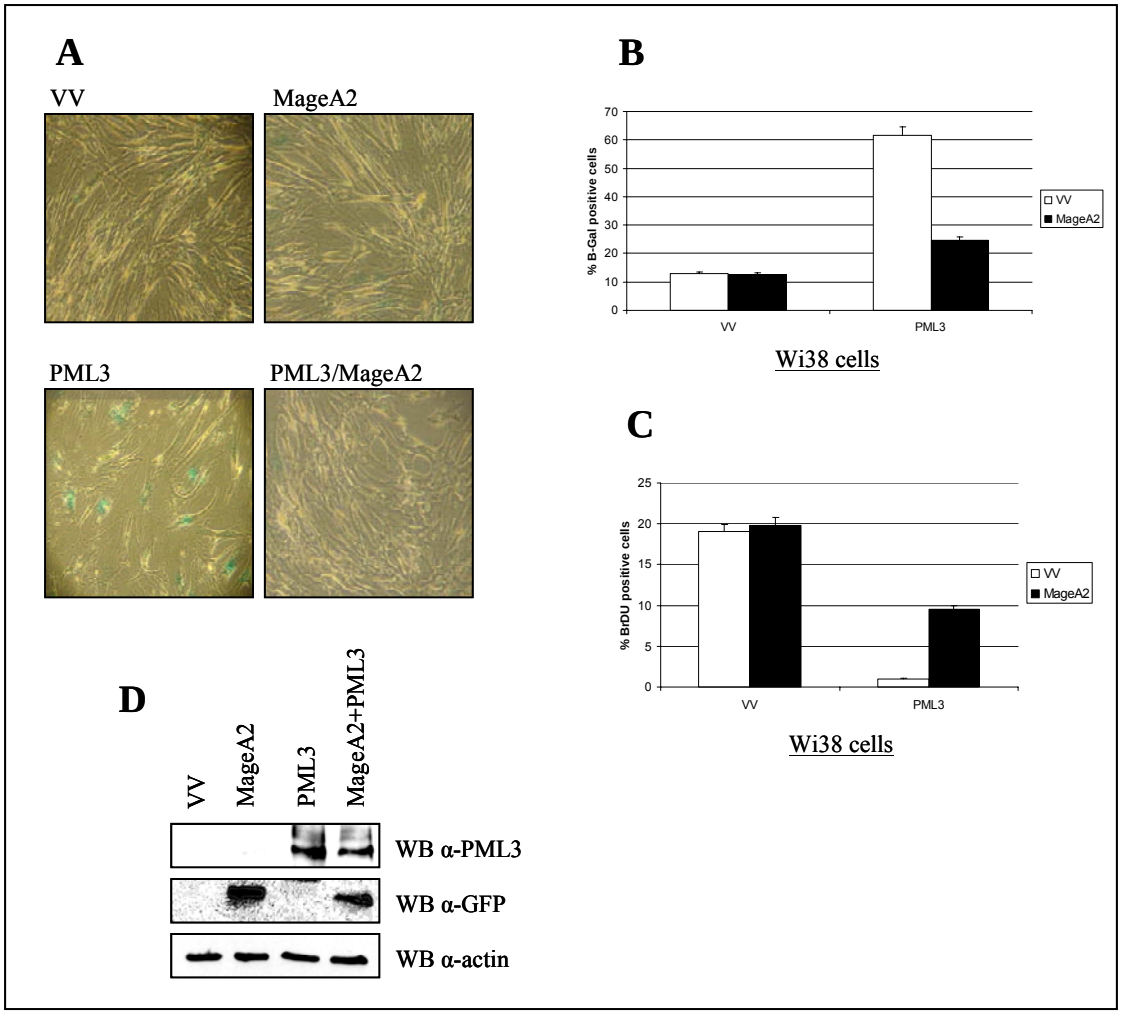

Fig. 14: *MageA2 impairs PML3 induced senescence*
A) Morphology of WI38 normal human fibroblasts infected with empty virus as control (VV), and GFP-MageA2 (MageA2) and/or PML3-expressing retroviruses. Cells were selected for ten days with puromycin and hygromycin, fixed and stained for SA-β-Gal activity and photographed under a phase contrast microscopy. **B**) Average of cells from "A" that stained positive for SA-β-Gal after counting 200 cells in at least three independent experiments. **C**) The same cells from "A" were grown for 3 hours in the presence of BrdU. The graph shows the average of nuclei positive for BrdU incorporation after counting 200 cells in at least three independent experiments. **D**) Western blot from lysates of cells in "A" to confirm the expression of PML and GFP-MageA2 using specific antibodies. Actin was used as loading control.

MageA2 relocalizes to PML3 NBs inducing deacetylation of p53 in these structures

Given that p53 becomes acetylated and activated within the PML3 NBs (Pearson, Carbone et al. 2000), and because we previously demonstrated the formation of a MageA2/p53 complex, we decided to evaluate whether MageA2 could be recruited with p53 to the NBs. Immunofluorescence analysis of U2OS cells showed nuclear staining of HA-MageA2 (Fig. 15A). Co-expression of PML3 with MageA2 induced the recruitment of MageA2 to PML3

NBs (Fig. 15B), where endogenous acetylated p53 was also seen to localize (Fig. 15C). Interestingly, HA-MageA2 co-localized with endogenous p53 at the NBs (Fig. 15D, *left panel*) and repressed p53 acetylation in these structures (Fig. 15D, *right panel*), as assessed by anti-Ac-p53Lys382 staining.

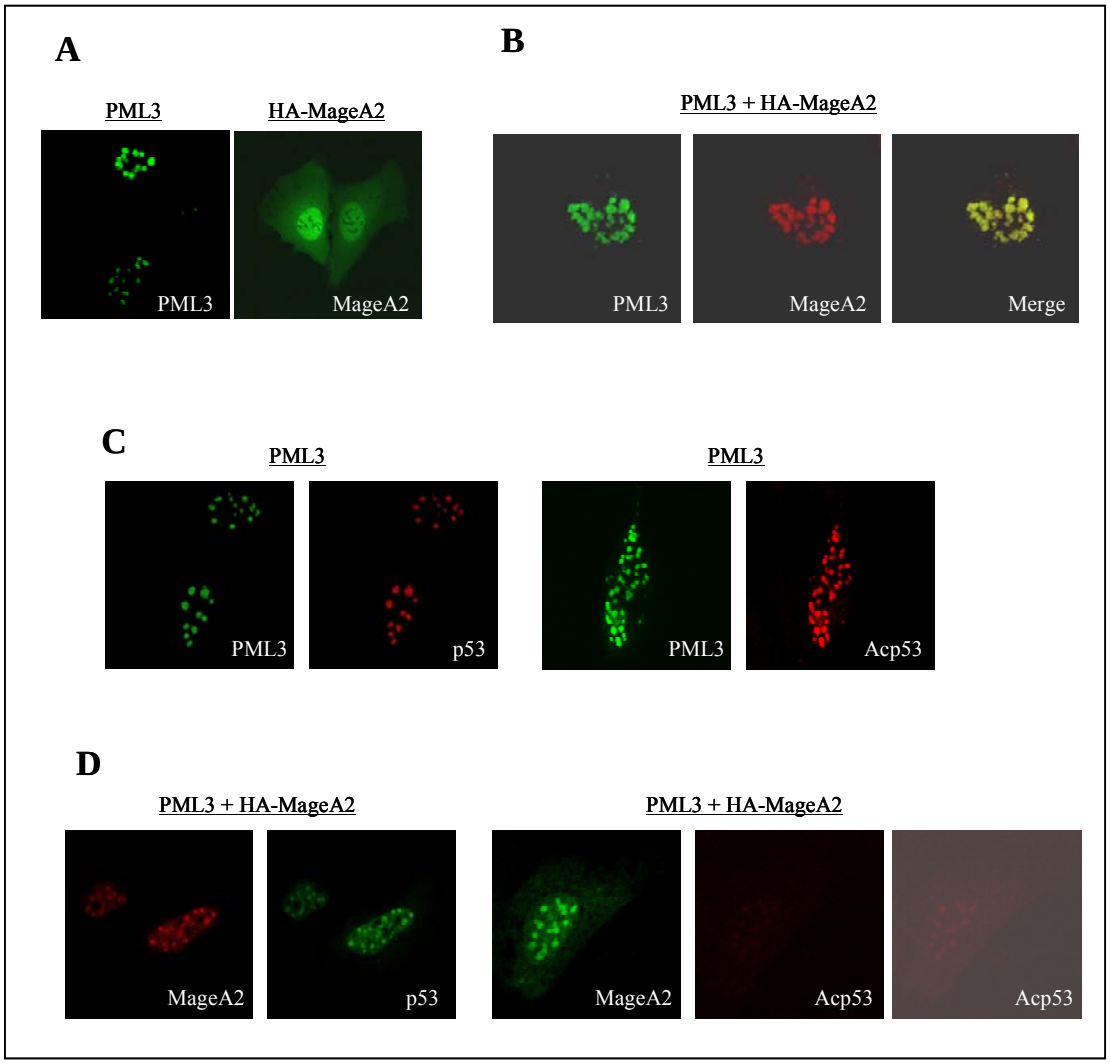

Fig. 15: *MageA2 relocalizes to PML nuclear bodies where deacetylates endogenous p53*
A) Immunofluorescence of PML3 or HA-MageA2 expressing U2OS cells. PML3 was detected with a monoclonal anti-PML antibody and MageA2 with an anti-HA monoclonal antibody. **B)** Immunofluorescence as in "A" but in U2OS cells co-expressing PML3 and HA-MageA2. **C)** Acetylation (*right panel-Acp53*) and co-localization (*left panel*) of endogenous p53 and PML3 at NBs. p53 was detected with anti DO-I and Acp53 with an anti-Ac-p53Lys382. **D)** Immunofluorescence to visualize the colocalization of endogenous p53, PML3 and MageA2 at NBs (*left panel*) and the acetylation status of endogenous p53 in such structures (*right panel*) using antibodies as in "C".

MageA2 represses PML3 induced p53 activity

Since relocalization of p53 to the NBs enhances its transactivation function (Fogal, Gostissa et al. 2000), we asked whether the expression of MageA2 could affect the activation of p53 by PML3. We used the promoter of the p53-target pig3 gene as reporter in luciferase assays (PIG3-Luc), since it was shown to be highly responsive to the activation of p53 by PML3 (Fogal, Gostissa et al. 2000). As expected, expression of MageA2 resulted in reduced activity of p53. Interestingly, a strong repressive effect of MageA2 on p53 was also observed when PML3 was used to induce p53 transcriptional activity (Fig. 16A). Also in this case MageA2 showed to be the most efficient MAGE-A member to repress p53 function upon the activation by PML3 (Fig. 16B).

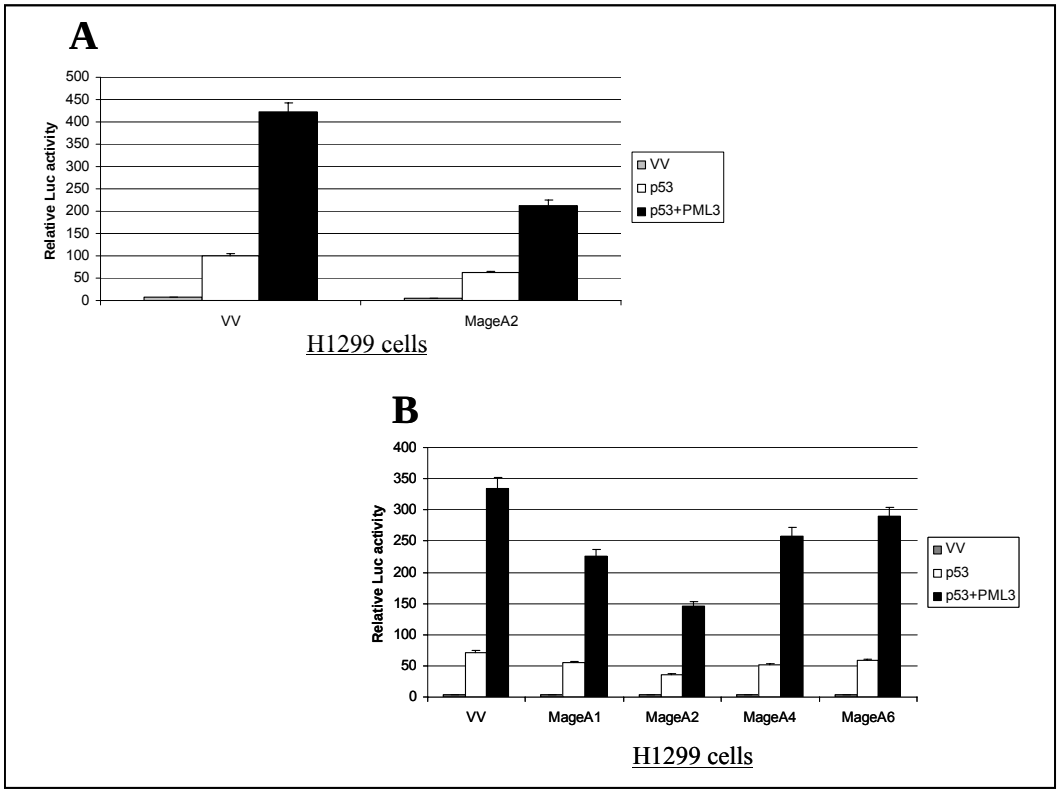

Fig. 16: *MageA2 represses PML3 induced p53 activity*
A) Luciferase gene reporter assay in H1299 cells transfected with p53, or in combination with PML3, in the presence or not of MageA2 together with the p53-responsive promoter pPIG3LUC. Value corresponding to p53 transfection was reported to 100. **B)** p53-specific reporter gene assay as performed in "A" but using different MageA proteins.

MAGE-A family members share high levels of sequence identity (Chomez, De Backer et al. 2001), nonetheless they do not seem to regulate p53 function to the same extent (Fig. 16B and Results I – Fig. 2A). Among MAGE-A members, MageA2 and MageA4 are the most phylogenetically distant (Chomez, De Backer et al. 2001), however, sequence alignment of these two proteins reveal that they are 67 % identical. Based on these evidence and in order to give specificity to MageA2 function on p53 and PML3 regulation, we decided to investigate the effect of MageA4 on PML3 induced senescence. Surprisingly, MageA4 only slightly affected the ability of PML3 to induce senescence, since cells expressing MageA4 and PML3 showed similar morphological changes than PML3 expressing senescent cells (Fig. 17A), and the SA-β-Gal staining and BrdU incorporation were comparable to cells expressing PML3 in the presence or not of MageA4 (Fig. 17B and 17C).

It has to be pointed out that, in contrast to MageA2, MageA4 showed a major cytoplasmic staining (Fig.18A), and did not co-localized with PML3 and p53 in NBs (Fig. 18B) nor induced p53 deacetylation in these structures as MageA2 does (Fig. 18C and see Fig. 15D).

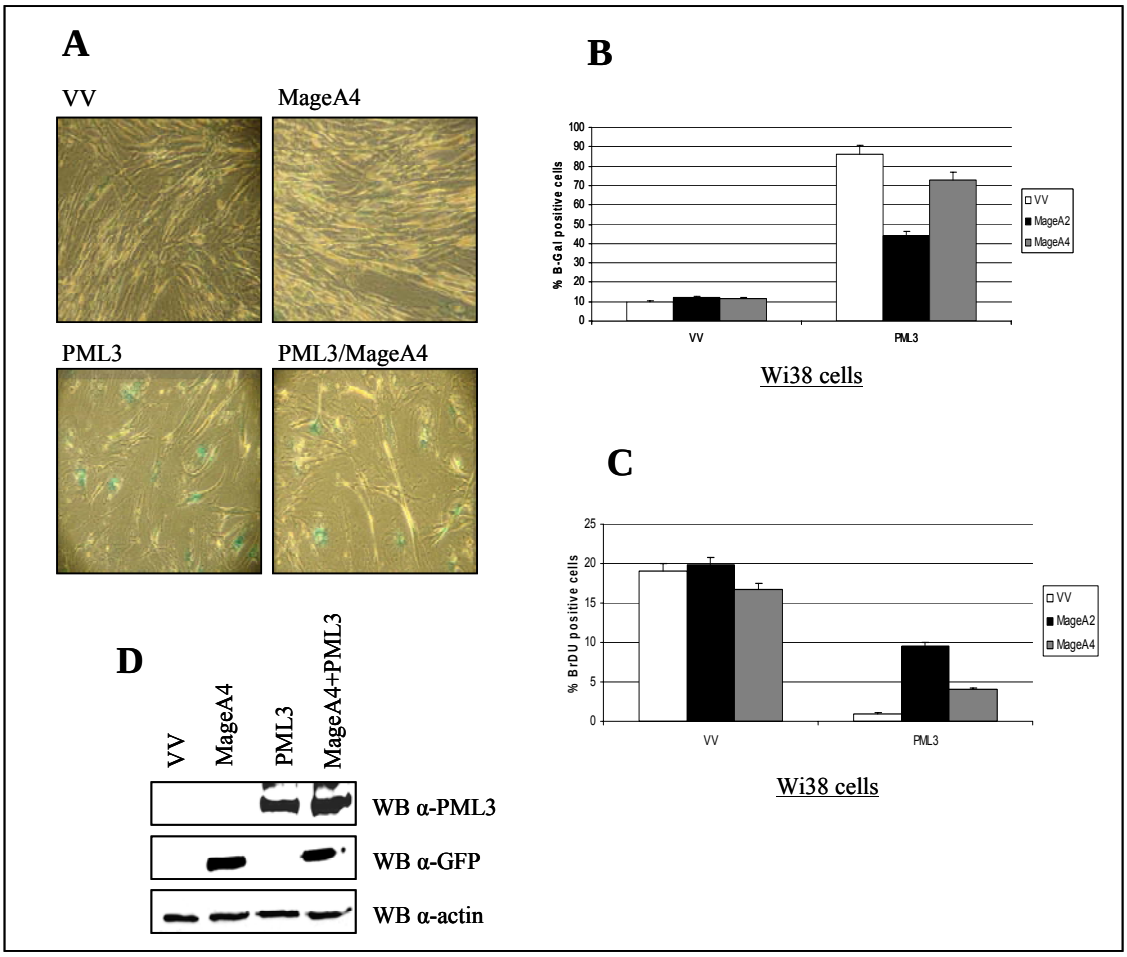

Fig. 17: *MageA4 does not impair PML3 induced senescence*
A) Morphology of WI38 normal human fibroblasts infected with empty virus as control (VV), and GFP-MageA2 (MageA2) or GFP-MageA4 (MageA4) and/or PML3-expressing retroviruses. Cells were selected for ten days with puromycin and hygromycin, fixed and stained for SA-β-Gal activity and photographed under a phase contrast microscopy. **B**) Average of cells from "A" that stained positive for SA-β-Gal after counting 200 cells in at least three independent experiments. **C**) The same cells from "A" were grown for 3 hours in the presence of BrdU. The graph shows the average of nuclei positive for BrdU incorporation after counting 200 cells in at least three independent experiments. **D**) Western blot from lysates of cells in "A" to confirm the expression of PML and GFP-MageA4 using specific antibodies. Actin was used as loading control.

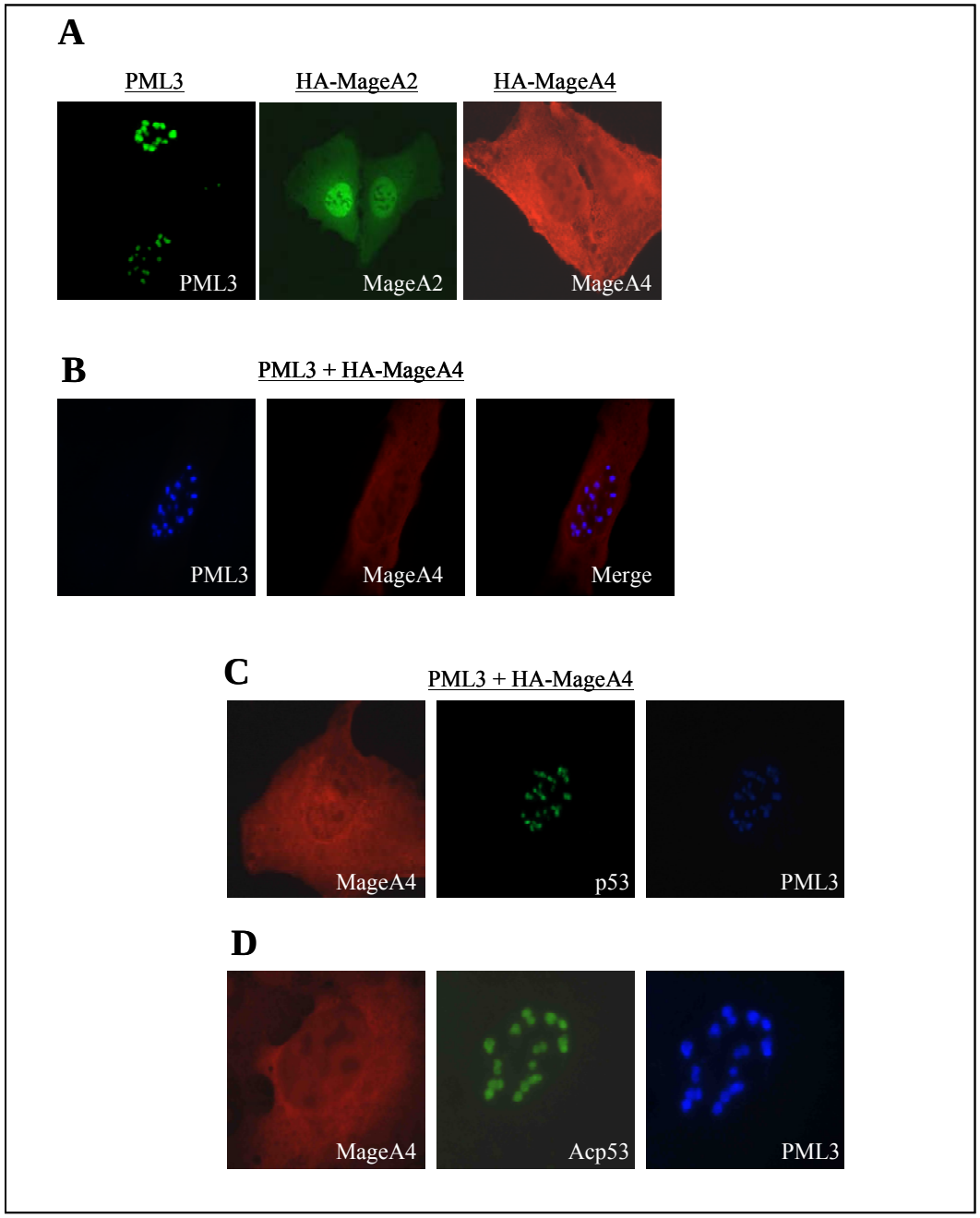

Fig. 18: *MageA4 does not relocalize to PML nuclear bodies nor induces deacetylation of endogenous p53*
A) Immunofluorescence of PML3 or HA-MageA2 or HA-MageA4 expressing U2OS cells. PML3 was detected with a monoclonal anti-PML antibody, MageA2 and MageA4 were identified with an anti-HA monoclonal antibody. **B)** Immunofluorescence as in "A" but in U2OS cells co-expressing PML3 and HA-MageA4. **C)** Immunofluorescence as in "B" to evaluate the colocalization of endogenous p53, PML3 and MageA4 at NBs. **D)** Immunofluorescence as in "B" showing the acetylation status of endogenous p53 at NBs. For Acp53 staining was used an anti-Ac-p53Lys382 antibody.

Finally, we analyzed the ability of MageA2 and MageA4 to bind PML3. Immunoprecipitation assays demonstrated that MageA2, but not MageA4, was able to interact with endogenous PML (Fig. 19A). For this experiment endogenous PML was immunoprecipitated with a monoclonal antibody that recognizes different isoforms of the protein. To determine whether MageA2 could bind specifically the PML3 isoform, 293T cells were transfected with PML3 and HA-MageA2 or HA-MageA4 expressing vectors, and immunoprecipitation using anti-HA antibody confirmed that only MageA2 could bind PML3 (Fig. 19B). Interestingly, *in vitro* pull-down experiments using recombinant MageA2 or MageA4 proteins showed direct binding between MageA2 and PML3 (Fig. 19C). All these data support the notion that MageA2 has a specific regulatory function on PML3 and p53 that is independent on sequence similarities with other members of the MAGE-A subfamily.

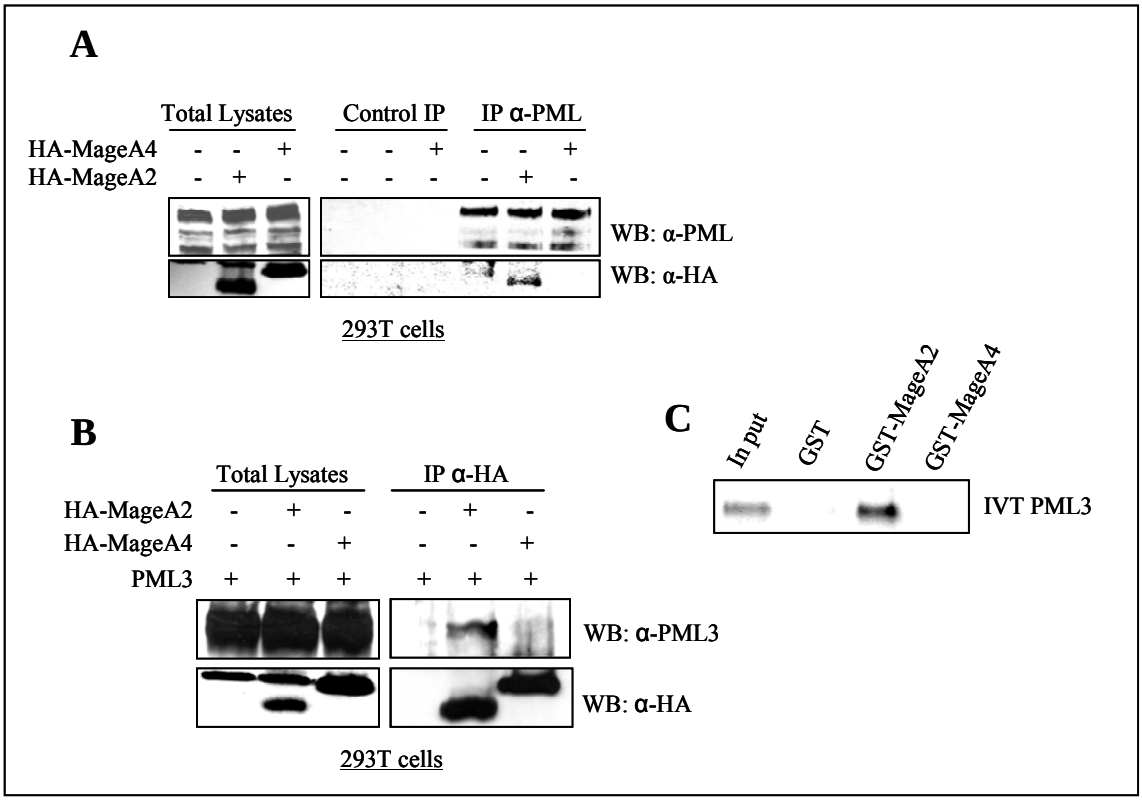

Fig. 19: *MageA2 interaction with PML3*
A) Immunoprecipitation of endogenous PML in 293T cells transfected with HA-MageA2 or HA-MageA4 using anti-PML monoclonal antibody or anti-Flag tag monoclonal antibody as control. Western blot was performed by using polyclonal anti-PML and monoclonal anti-HA antibodies. **B**) Immunoprecipitation in 293T cells transfected with PML3 and HA-MageA2 or HA-MageA4 using monoclonal anti-HA antibody. Western blot was performed as in A. **C**) *In vitro* binding assay using recombinant/purified GST, GST-MageA2 or GST-MageA4 fusion proteins incubated with *in vitro* translated ^{35}S-labeled PML3 (IVT PML3).

MageA2 binds to PML3 in a SUMO1 independent manner

Emerging evidence suggest that the NBs are PML-SUMO structures, where both PML sumoylation and PML-SUMO interaction are required for NB formation (Shen, Lin et al. 2006). PML-SUMO binding is mediated by a short motif (Sumo Binding Motif, SBM) that was recently identified in proteins that interact non-covalently with SUMO (Yoon, Kim et al. 2004). Given the high homology between MAGE-A proteins and their apparent different interference with the PML3-p53 axis, we asked whether small differences in the sequence of these proteins could account for functional specificity. Sequence analysis of different MAGE-A proteins (in particular MageA2 and MageA4) evidenced two potential SBMs present in MageA2 exclusively but absent in other MAGE-A proteins, as MageA4 (see Fig. 20A and 20B).

Although we observed direct binding of MageA2 and PML3, we wondered whether the presence of SBMs in MageA2 could facilitate the binding to PML3 or influence the repressive effect of MageA2 on p53 activation by PML3. To this purpose we generated three different MageA2 mutants, bearing mutations in each potential SBM (-SBM I and –SBM II), or alternatively mutated in both SBMs (-SBM I/II). Immunoprecipitation assays by using anti-HA antibody showed no differences in the binding of MageA2 to PML3 (Fig. 21A). Moreover, all three mutants demonstrated the same repressive ability on p53 in the presence or not of PML3 (Fig. 21B), suggesting that the SBMs in MageA2 are dispensable to its binding and function on the PML3-p53 axis.

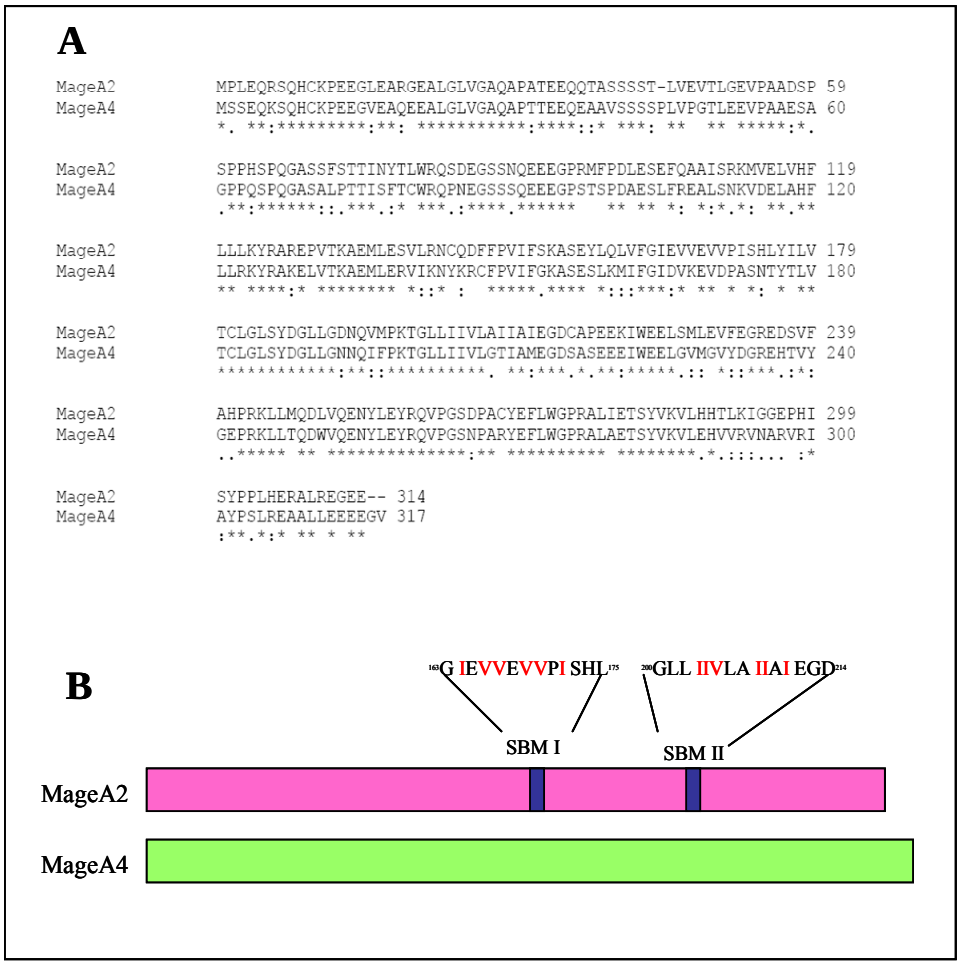

Fig. 20: A) Multiple sequence alignment of MageA2 and MageA4 proteins. **B**) Schematic representation of MageA2 and MageA4 proteins sequences showing the putative SBM sequences present in MageA2 but absent in MageA4.

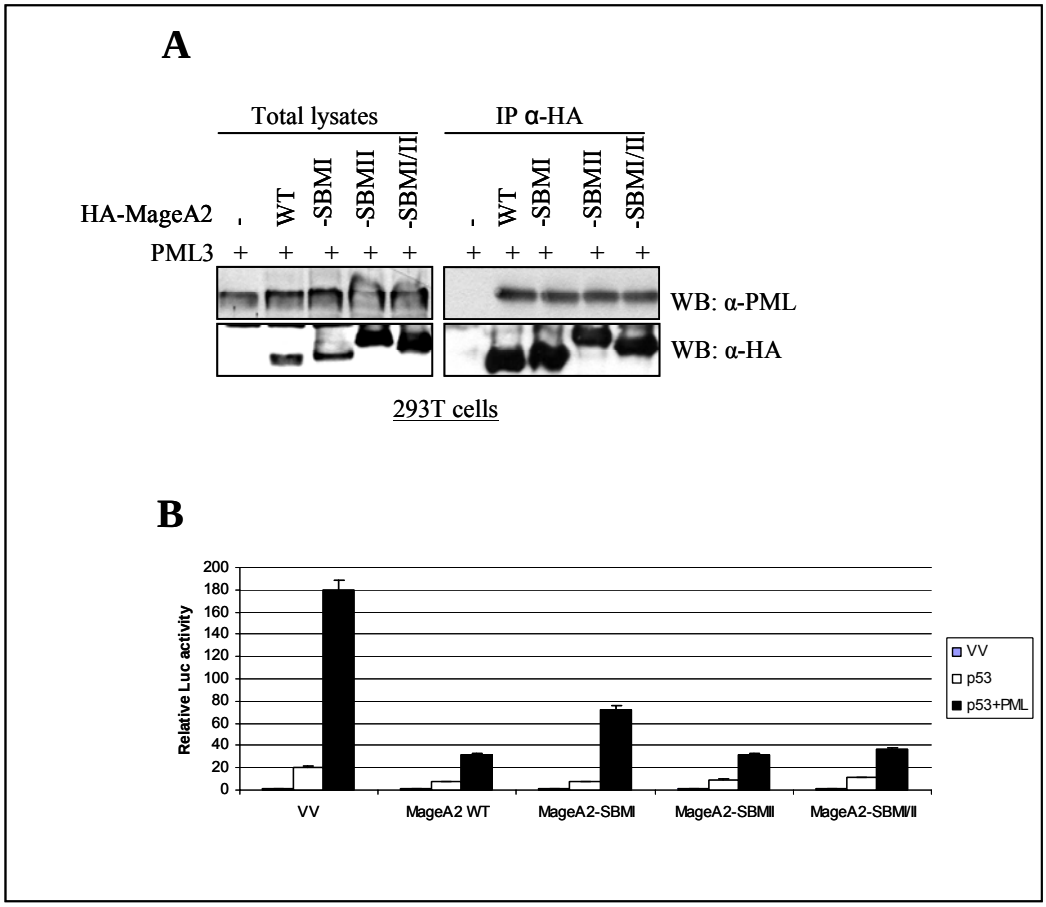

Fig. 21: *MageA2 SUMO binding motifs are dispensable for MageA2 function*
A) Immunoprecipitation of transiently transfected 293T cells with different HA-MageA2 mutants and PML3 expressing vectors, using anti-HA antibody. Anti-PML polyclonal and anti-HA were used for Western blot as indicated. **B**) Luciferase gene reporter assay in H1299 cells transfected with p53, or in combination with PML3, in the presence or not of different MageA2 SBM mutants together with the p53-responsive promoter pPIG3LUC.

MageA2 affects PML3 sumoylation status

PML is functionally regulated by posttranslational modifications (Bernardi and Pandolfi 2007). In particular, sumoylation is though to regulate the localization, and stability of PML, being one of the nucleation events for NB formation and function of PML (Shen, Lin et al. 2006). As a second possible mechanism for the repressive effect of MageA2 on PML3 activity, we therefore investigated whether MageA2 could affect PML3 sumoylation.
Western blot analysis of 293T cells transfected with HA-MageA2, PML3 and SUMO1 demonstrated that MageA2 expression impaired the sumoylation levels of PML3 (Fig. 22A). To confirm that the bands recognized by anti-SUMO corresponded effectively to sumoylated

PML3, we performed the same experiment followed by immunoprecipitation of PML3 obtaining similar results (Fig. 22B).

Recently, it has been demonstrated that PML can also be modified by acetylation, and this modification seems to favor its subsequent sumoylation (Hayakawa, Abe et al. 2008). In this context our results could indicate a possible role of MageA2 in the induction of PML3 deacetylation probably by recruitment of HDACs (similar to the mechanism observed for p53), thus favoring the consequent reduction of PML3 sumoylation levels.

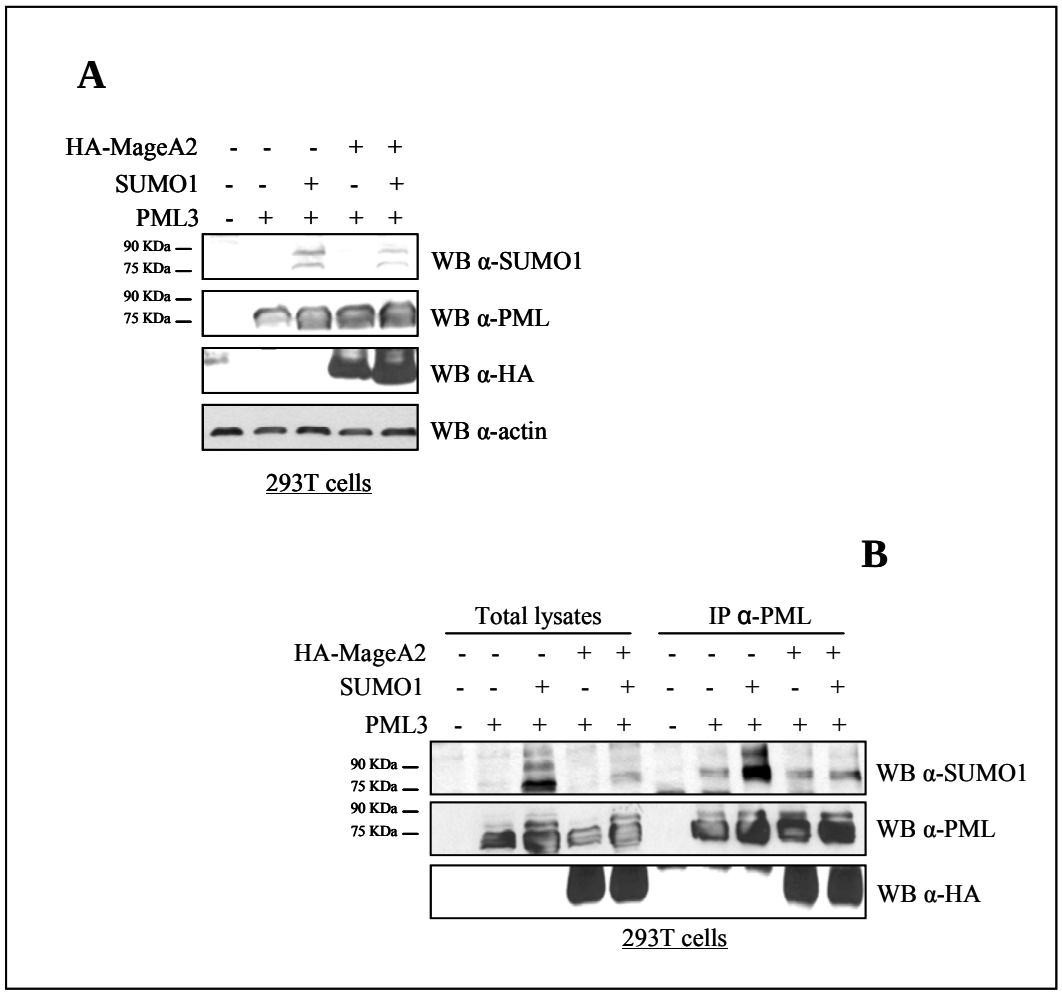

Fig.22: *MageA2 impairs PML3 sumoylation status*
A) Western Blot analysis of 293T cells transfected with PML3 in combination with MageA2 and/or SUMO1. The membrane was first incubated with an anti-SUMO1 monoclonal antibody, to detect sumoylated PML3 and subsequently with an anti-PML polyclonal antibody. HA-MageA2 was detected with an anti-HA monoclonal antibody. **B**) Immunoprecipitation performed in 293T cells transfected as in "A" using an anti-PML monoclonal antibody. Sumoylated and total PML3 was detected in western blot with an anti-SUMO1 and anti-PML polyclonal antibody, respectively. Anti-HA was used to detected HA-MageA2.

Altogether the data presented here strongly suggest that MageA2, in contrast to MageA4, is able to repress PML3-induced p53 activity in a specific manner, by affecting PML3 mediated p53 acetylation at the NBs. The relevance of this effect has been analyzed in a normal cellular context, in which PML3 induced premature senescence. In fact, we demonstrated that MageA2 impaired the senescence response associated to PML3 expression in normal human fibroblast. A possible mechanism for the inhibitory effect of MageA2 on PML3 is that MageA2 could interfere with PML3 post-translational modifications such as sumoylation, and potentially PML3 acetylation, through recruitment of HDACs as similarly ascertained for p53.

Part III: MAGE-I family diversity- MageB2

MageB2 is a nucleolar protein and translocates to the nucleoplasm upon ribotoxic stress

To extend our studies to other MAGE-I family members, we focused our work on the MageB2 protein, a member of the MAGE-B subfamily.

In order to initiate a functional characterization of MageB2 we first analyzed its cellular localization. By immunofluorescence experiments in U2OS cells, we observed that MageB2 localized predominantly in the nucleoli and nucleus of the cells, co-localizing with other nucleolar proteins such as nucleolin (NCL) and/or nucleophosmin (NPM) (Fig. 23A). Moreover, MageB2 localization seems to be independent of the cellular context, since the localization found in U2OS cells was confirmed in different normal and tumor cells (data not shown).

Stress conditions or ribotoxic drugs such as Actinomycin D (ActD) induce nucleolar stress with the consequent redistribution of some nucleolar proteins (like ribosomal protein L11, ribosomal protein L23 or NCL) to the nucleoplasm (Andersen, Lyon et al. 2002; Zhang, Wolf et al. 2003; Dai, Zeng et al. 2004). Upon induction of nucleolar stress by low dose of ActD in U2OS cells, HA-MageB2 became relocalized to the nucleoplasm, similar to NCL and NPM (Fig. 23B). This behavior was also observed after treatment with other drugs known to produce nucleolar disruption, such as low concentration of doxorubicin and UV (Fig. 23B). However, using 5-Fluorouridine (5-FUrd), an inhibitor of rRNA processing that also causes nucleolar stress, we could observe a nucleoplasm redistribution only for MageB2, whereas NCL and NPM retained its original localization (Fig. 23C). This suggested a degree of specificity in the behavior of MageB2 in response to various types of nucleolar stress.

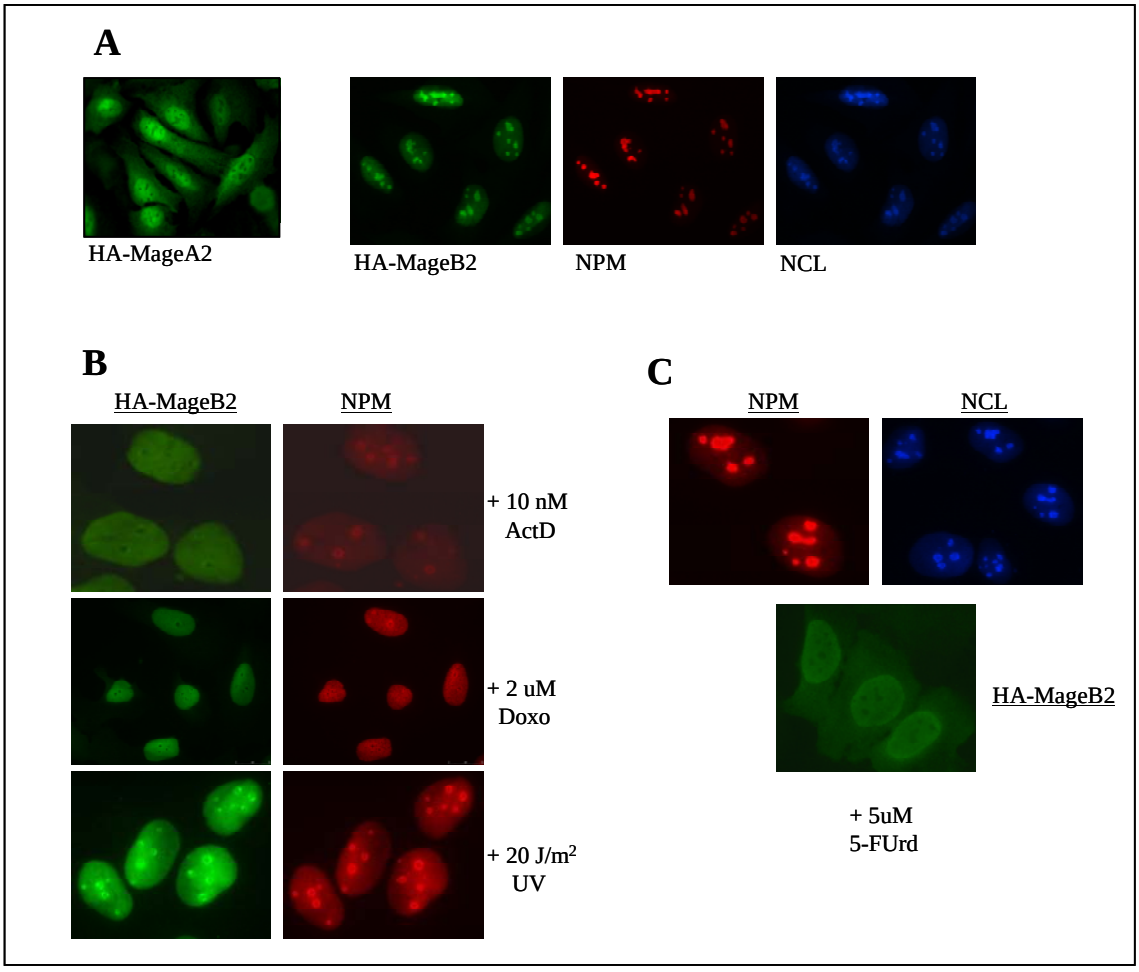

Fig. 23: *MageB2 is a nucleolar protein and translocates to the nucleoplasm upon ribotoxic stress*
A) Immunofluorescence of U2OS cells showing localization of transfected HA-MageA2 and HA-MageB2 using an anti-HA antibody. Monoclonal antibodies for nucleophosmin (NPM) and nucleolin (NCL) were used to visualize endogenous proteins. **B)** and **C)** Immunofluorescence was performed as in "A" but cells were previously treated for 5 hours with the indicated drugs, or UV radiation.

MageB2 does not repress p53 transactivation function and confers resistance to Actinomycin D in a p53 independent manner

In order to determine whether MageB2 could share redundant functions with MageA2 protein, we analyzed the possibility that MageB2 could also regulate p53 transactivation function. Luciferase reporter gene assay of a p53 responsive promoter demonstrated that MageB2 was not able to regulate p53 function (Fig. 24A). Moreover, immunoprecipitation experiments showed no binding between HA-MageB2 and p53 (Fig. 24B). With the purpose of better analyze the potential effects of inducible MageB2 expression, we obtained a Ponasterone A inducible HA-MageB2-U2OS cell line, which was named U24. Interestingly, HA-MageB2

induced expression in U24 cells, correlated with resistance of cells to the ribotoxic drug Actinomycin D (ActD). Moreover, HA-MageB2 induction led to a reduction in the apoptotic response of the cells after treatment with ActD, as determined by subG1 populations after FACS analysis (Fig. 24C). Furthermore, siRNA mediated down-regulation of p53 in U24 cells did not affect the resistance effect of MageB2 (Fig. 24D), suggesting that MageB2 confers resistance to ActD in a p53 independent manner.

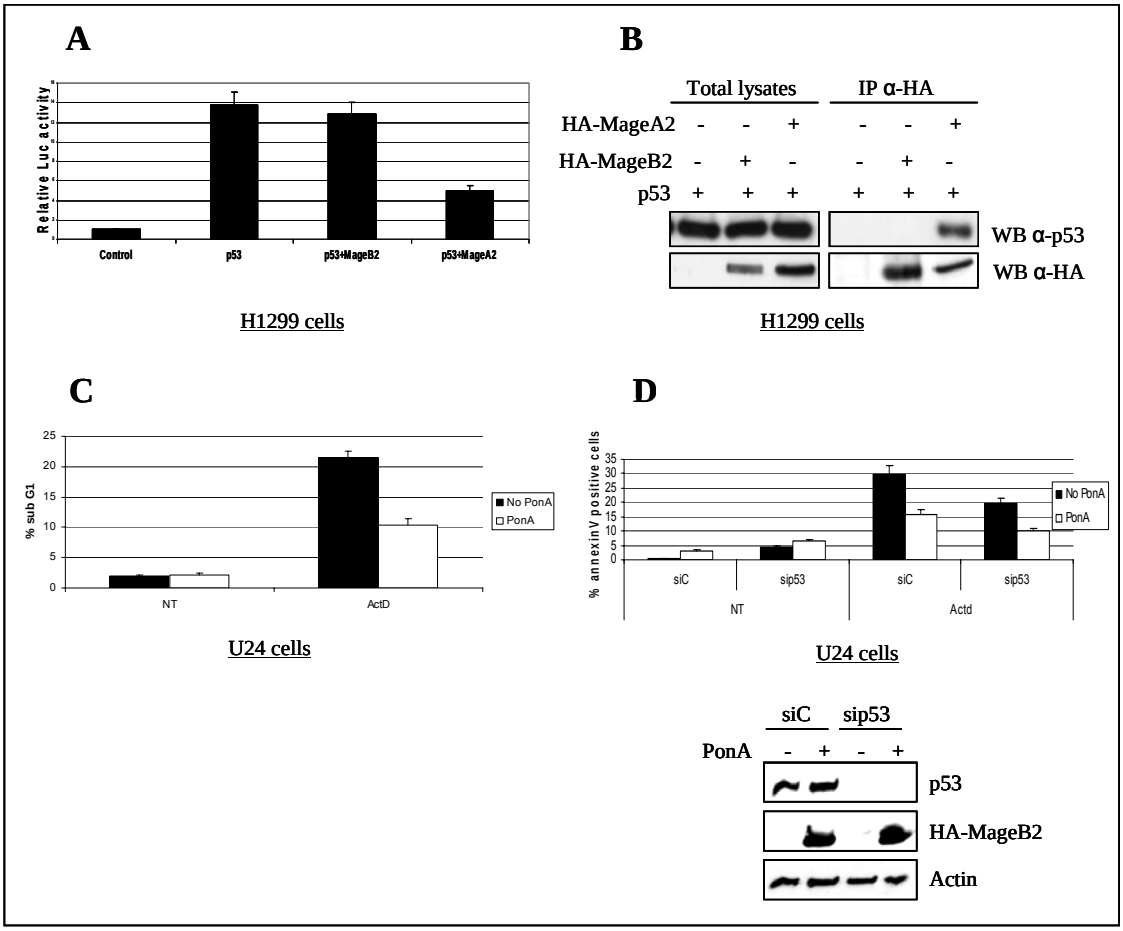

Fig. 24: *MageB2 does not regulate p53 function and confers resistance to ActD in a p53 independent manner*
A) Luciferase assay in H1299 cells (p53 null) transfected with p53, or in combination with MageB2 and MageA2 together with the p53-responsive promoter pG13LUC. **B**) Immunoprecipitation in H1299 cells transfected with HA-MageB2 or HA-MageA2 and p53, using an anti-HA monoclonal antibody. Western blot was performed by using p53 (DO1) and HA monoclonal antibodies. **C**) Apoptotic scoring in U24 (HA-MageB2 inducible U2OS) cells expressing (PonA) or not (No PonA) HA-MageB2, treated for 16 hours with 500 nM ActD. Apoptosis was determined as subG1 populations by FACS analysis (10.000 counted cells for at least 3 independent experiments). **D**) Apoptosis determination by Annexin V assay coupled to FACS analysis (10.000 counted cells for at least 3 independent experiments). U24 cells transfected with control siRNA (siC) or p53 siRNA (sip53), were treated as shown in "C" (*upper panel*). Western blot of U24 cells used for FACS analysis, anti-p53 (DO-I) and anti-HA antibodies were used to detect p53 and MageB2, respectively (*lower panel*).

MageB2 binding to ribonucleoproteins and ribosomal proteins is dependent on RNA

To gain insights into the functional characterization of MageB2, we performed an immunoaffinity purification assay to find MageB2 interactors in human cells. To this end, we generated a HeLa cell line stably expressing HA-MageB2. Briefly, cells were lysed and proteins were immunoprecipitated with an anti-HA monoclonal antibody. The precipitated complex was resolved by electrophoresis (SDS-PAGE) and proteins were visualized on the gel by colloidal blue coomassie staining. Finally, proteins were identified by in gel digestion and mass spectrometry. Sequence analysis of the most prominent bands gave rise to a list of MageB2 interactors that included ribonucleoproteins, such as heterogeneous nuclear ribonucleoprotein M (hnRPM) and NCL; transcription factors of the general transcription machinery (General Transcription Factor II-I, TFII-I); and ribosomal proteins like RP S8, S9, S13 and L27a (Fig. 25A).

Since MageB2 was originally detected as a nucleolar protein and a number of the proteins shown to associate with MageB2 localize in the nucleolus participating in the control of RNA metabolism, we wondered whether formation of MageB2 protein complexes was dependent on RNA. To test this hypotesis, U24 cells lysates were incubated or not with RNase and MageB2 protein complexes were subsequently immunoprecipitated. Treatment with RNase impaired the binding of almost all ribosomal proteins as assessed by HA-MageB2 immunoprecipitation of ^{35}S-Methionine pre-labelled proteins (Fig. 25B), and also the binding of NCL to MageB2 by performing the classical IP/western blot assay (Fig. 25C). Moreover, MageB2 nucleolar localization was lost after treatment of HeLa cells stably expressing HA-MageB2 with RNase, an effect that was also observed for NCL (Fig. 25D). These data demonstrate that MageB2 could be part of ribonucleoprotein complexes and that its localization depends on some specific RNAs, suggesting that MageB2 could participate in nucleolar or rRNA metabolism related functions.

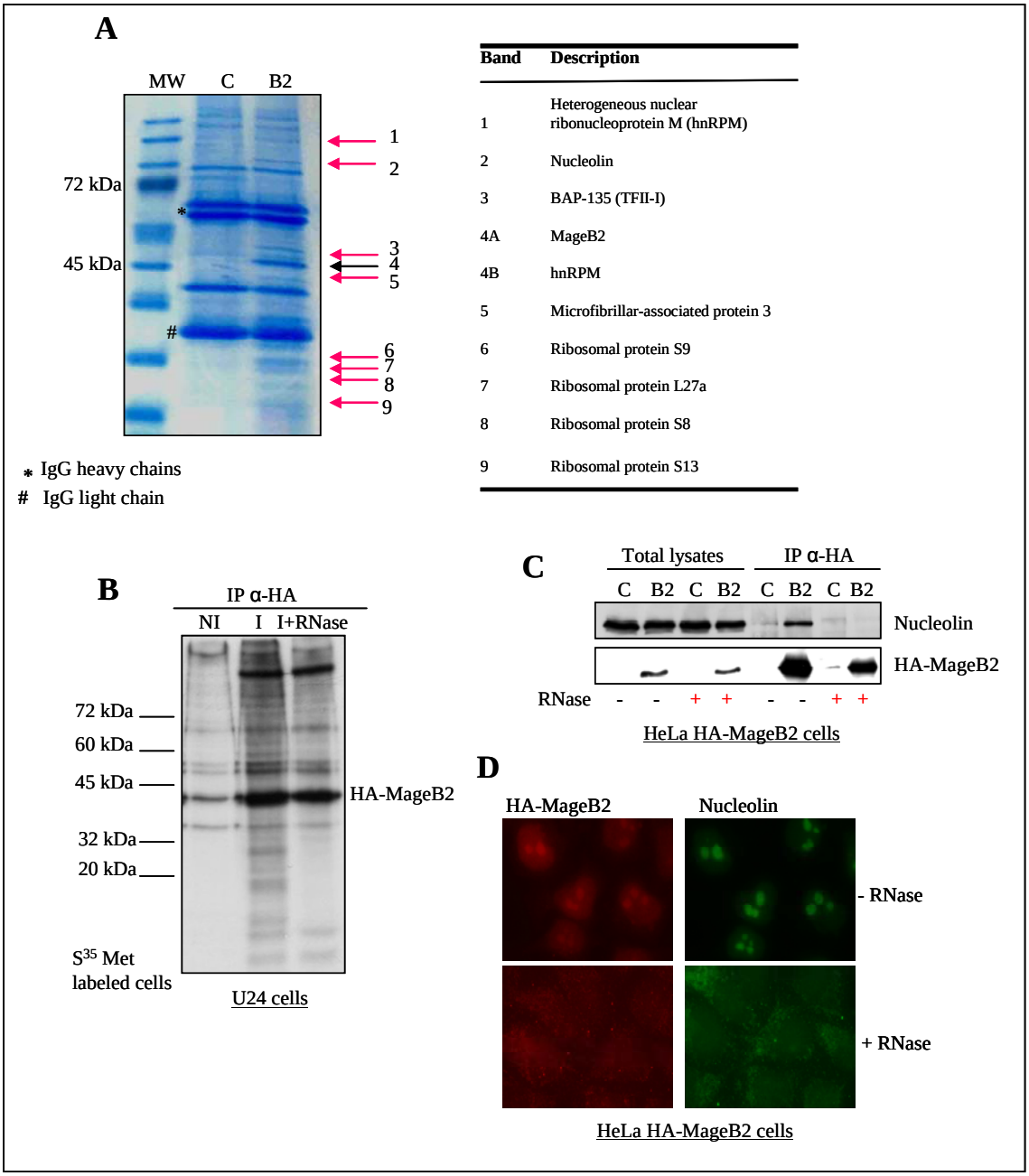

Fig. 25: *MageB2 binds to ribonucleoproteins and ribosomal proteins in a RNA dependent manner*
A) SDS-PAGE of HA-MageB2 immunoprecipitated complex from HeLa cells stably expressing HA-MageB2 (B2) or HeLa control cells (C). Purified proteins were separated and visualized by gel staining with colloidal blue coomassie. Differential bands between MageB2 and control IPs (1-9) were gel digested and sequenced by mass spectrometry. MW correspond to molecular weight marker. An anti-HA antibody was used in the immunoprecipitation (*left panel*). *Right panel* shows the list of the proteins that were precipitated and identified by excision of the bands showed on the left panel. **B)** IP of U24 cells with an anti-HA antibody was performed after pulse labeling of the cells with S35-Methionine for 16 hours. Prior to IP cell lysates were treated or not with RNase for 30 minutes. Immunoprecipitated complex was resolved by SDS-PAGE and visualized by autoradiography. **C)** IP as in "B" but using HeLa HA-MageB2 stable cells. After IP endogenous nucleolin was visualized with an anti-nucleolin monoclonal antibody by western blot. **D)** HA-MageB2 and endogenous nucleolin localization after treatment of cells with RNAse. Immunofluorescence was performed in HA-MageB2 HeLa cells.

MageB2 stimulates E2F1 activity and relocalizes pRB to nucleoli

During the characterization of MageB2 we observed that although it failed to regulate p53 transcriptional activity, it was able to significantly activate the transactivation function of the E2F1 transcription factor, in luciferase reporter gene assays using an E2F1 responsive promoter as reporter and growing amounts of MageB2 (Fig. 26A). In addition, MageB2 expression impaired the repressive effect of pRB on E2F1 activity (Fig. 26A). To determine whether MageB2 could inhibit the function of pRB by affecting its cellular localization, we performed immunofluorescence experiments. pRB is generally localized in the nucleus of the cell, however co-expression of pRB with HA-MageB2 induced pRB relocalization to the nucleolus (Fig. 26B, *upper panel*). Treatment of cells with a buffer that eliminates soluble proteins (CSK buffer), demonstrated more clearly the relocalization effect of MageB2 on pRB (Fig. 26B, *lower panel*). Altogether these data suggest that MageB2 could induce E2F1 transactivation function by sequestering pRB to the nucleolus.

It has been reported that E2F1 participates in the induction of rRNA transcription (Ayrault, Andrique et al. 2006). Since we showed that MageB2 enhances the transcriptional activity of E2F1, reporter gene assays were performed to explore whether MageB2 could modulate the activity of E2F1 using the rRNA promoter-Luc construct (pHrD-Luc). Expression of E2F1 produced the expected increase in the activity of the rRNA promoter, while co-expression of MageB2 led to a further enhancement of E2F1 activity on this promoter (Fig. 27A). Moreover, analysis of newly transcribed RNA by FUrd incorporation into nascent RNA revealed that E2F1 was able to increase RNA transcription as was observed by immunofluorescence using antibodies against halogenated nucleotides (Fig. 27B, *upper panel*). Interestingly, co-expression of E2F1 with HA-MageB2 resulted in an increased RNA transcription induction by E2F1 (Fig. 27B, *lower panel*). However, the single expression of MageB2 was not sufficient to stimulate RNA transcription (Fig. 27B, *middle panel*).

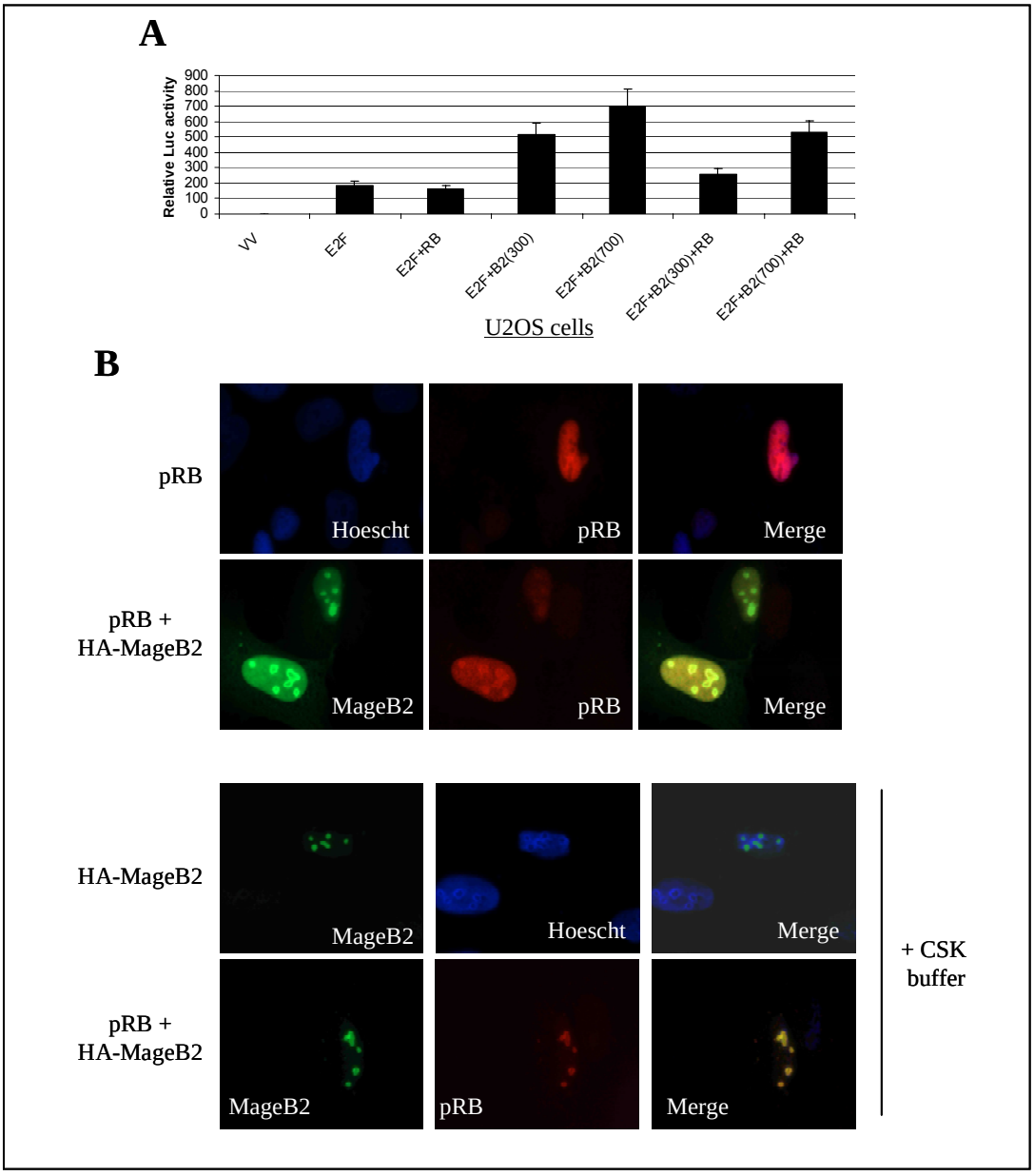

Fig. 26: *MageB2 stimulates E2F1 activity and relocalizes pRB to the nucleolus*
A) Luciferase assay in U2OS cells transfected with E2F1 in the presence or not of pRB, and growing quantities of MageB2 using an E2F1-responsive promoter. **B)** *Upper panel*, Immunofluorescence in U2OS cells transfected with pRB and/or HA-MageB2, using anti-pRB and anti-HA monoclonal antibodies. *Lower panel*, similar to upper panel but cells were treated with CSK buffer to eliminate soluble proteins prior to fixation.

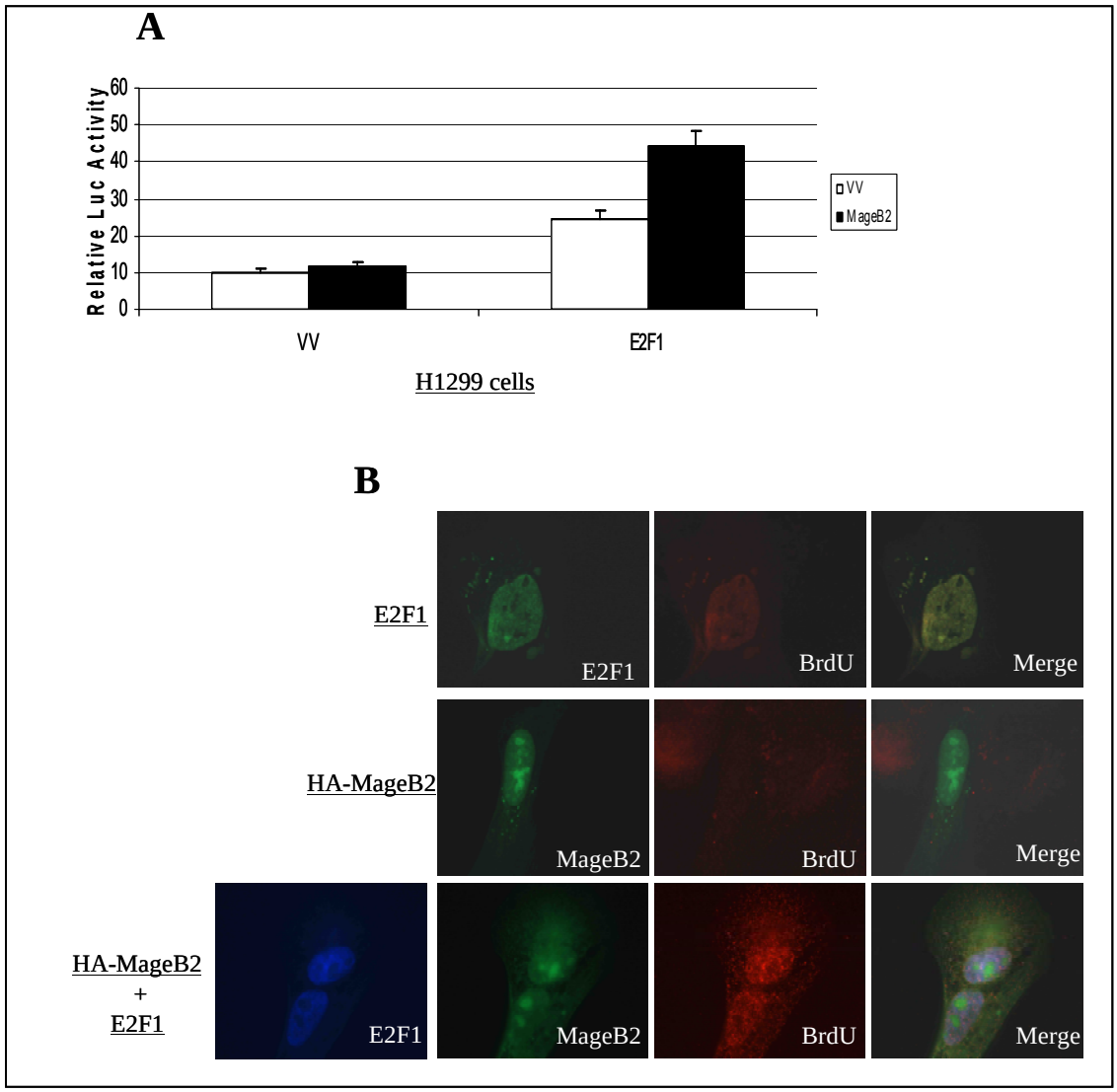

Fig. 27: *MageB2 colaborates with E2F1 to induce the transcription of rRNA*
A) Luciferase assay in H1299 cells transfected with E2F1 in the presence or not of MageB2 using the promoter of the human ribosomal DNA (pHrD) as reporter. **B**) Analysis of newly transcribed RNA in U2OS cells transiently transfected with E2F1 (*upper panel*), HA-MageB2 (*middle panel*) or the combination of them (*lower panel*). Cells were pulsed for 1 hour with 2 mM 5-FUrd, and nascent RNA visualized with antibodies against halogenated nucleotides (anti-BrdU). Anti-E2F1 and anti-HA monoclonal antibodies were used to detect E2F1 and MageB2, respectively.

This part of the work comprises a preliminary characterization of MageB2 protein, which clearly demonstrates its functional difference with respect to MageA2 protein. All the data obtained here suggest that MageB2 could function as a positive regulator of proliferation, probably by regulating the E2F1-pRB pathway and/or participating in rRNA metabolism

processes. Future experiments will be focused on determining the mechanism by which MageB2 expression controls such cellular functions.

5. Discussion

MAGE genes belong to a family of highly conserved genes sharing significant level of sequence homology (Chomez, De Backer et al. 2001). Type I MAGE genes are part of a growing group of genes termed Cancer Testis Antigens (CTAs), so named by their restricted expression in tumor and male germ cells (Simpson, Caballero et al. 2005). Expression of MAGE-I genes seems to be an early event in tumorigenesis and correlates with genome-wide hypomethylation, a frequently observed epigenetic event in carcinogenesis (De Smet, Lurquin et al. 1999). Since the discovery of MageA1 in the early 90's, the study of MAGE-I genes was focused on their application in cancer immunotherapy; the analysis of their biological functions remained poorly investigated. Due to their sequence homology, MAGE-I proteins were considered functionally redundant proteins: their expression being confined to their use as cancer-markers. Emerging evidence suggest however that MAGE-I proteins could display specific roles along the complexity of tumor progression.

In this Thesis, the aim was to functionally characterize specific MAGE-I genes, in particular MageA2 and MageB2 genes, in order to demonstrate their functional specificity. The main aim was to place their roles within different known pathways: the clearest picture emerging for the specificity of MageA2 on the p53 pathway, and the promising, still preliminary, activity of MageB2 in the Rb/E2F1 pathway.

MageA2 targets p53 transactivation function and confers resistance to chemotherapeutic agents

The p53 protein is a tumor suppressor, that is activated in response to several stimuli to regulate a multitude of cellular outcomes, which finally ensure the maintenance of genomic stability and prevent cell transformation. The relevance of p53 function is highlighted by the fact that the p53 gene is mutated in more than 50% of tumors or alternatively its function is affected by many different means in tumors expressing wild type p53. Moreover, p53 function is tightly regulated through different mechanisms, including post-translational modifications, cellular localization or the cell cycle phase (Murray-Zmijewski, Slee et al. 2008).

The first evidence we obtained that MAGE-A proteins regulate p53 function, came from experiments in which we observed that in tumors bearing wild type p53, the levels of MAGE-A genes correlate with resistance to chemotherapeutic drugs. More detailed experimental approaches demonstrated that all MAGE-A genes analyzed (MageA1, A2 and A6) are able to repress p53 transactivation function, but in particular MageA2 showed the strongest repressive activity. The differential response of MAGE-A proteins on p53 function seems to correlate with the different ability of these proteins to bind p53. Actually, we observed that there is a direct correlation between binding of MAGE-A proteins to p53 and their ability to repress its transactivation function. For instance, MageA2 binds and repress p53 more strongly than MageA1 or MageA6, while MageA4 does not bind nor repress p53 function (data not shown). MageA2 repressive effect on p53 also displays specificity with respect to the family members of p53 transcription factors, since we observed that MageA2 can repress p53 and p73, but not p63. This repressive effect seems to be associated with the ability of MageA2 to physically bind the specific transcription factor: while MageA2 can efficiently interact both with p53 and p73, it fails to associate with p63 (data not shown). This suggests that the specificity of MageA2 for the p53 family members is based on a direct link between protein-protein interaction and transcriptional repression.

Analysis of p53 endogenous targets demonstrated that expression of MageA2 leads to an impaired transcription of p53 targets, such as p21 and bax genes, after treatment of cells with etoposide (ET), without affecting p53 protein levels, and these effects correlate with a reduced ET-induced growth arrest and apoptosis. Conversely, siRNA mediated knock-down of MageA2 expression, was shown to restore the p53 dependent response. Moreover, expression of MageA2 in p53 null cell line does not induce survival after ET treatment, demonstrating the p53 dependence for MAGE-A effect.

In order to gain insights into the mechanism responsible for the MageA2 repressive effect on p53, the p53 post-translational modifications were analyzed showing that MageA2 represses damage-induced p53 acetylation, without affecting p53 phosphorylation on Ser15.

The reduction of p53 acetylation levels after expression of MageA2 seems to be dependent on the recruitment and assembling of HDAC3/p53 complexes. In fact immunoprecipitation and *in vitro* binding assays demonstrated that MageA2 binds directly both p53 and HDAC3, and that the p53 DBD is involved in this interaction. In addition, the deacetylating function of MageA2/HDAC3 complex remains fully active and it is found associated to p53 on p53 targets promoters. This suggests that p53/MageA2/HDAC3 complex can regulate other acetylation-dependent substrates such as nucleosomal histones. In fact, the transcriptional

activity of a non-acetylable p53 mutant (p539KR) is similarly repressed by MageA2. Finally, ChIP assays demonstrated that MageA2 does not affect p53 binding to chromatin upon damage, but instead favors the recruitment of HDAC3 and the consequent deacetylation of histones on p53 binding sites. All these data support the notion that the main mechanism by which MageA2 targets p53 activity is the induction of histone hypoacetylation on the chromatin surrounding p53 binding sites, in addition to deacetylating p53 itself, by selectively recruiting HDAC3.

The relevance of MageA2 expression on p53 function was highlighted by using cell lines obtained from melanoma biopsies expressing high levels of MAGE-A and wild type p53. In this context we confirmed that the DNA damage response is impaired in melanoma cells expressing high MAGE-A levels due to MageA2-dependent impairment of p53 transcriptional activity. Moreover, the chemoresistance effects of MAGE-A expression could be reverted by using inhibitors of HDACs, such as TSA. Finally, melanoma cell lines expressing high levels of MAGE-A but harboring mutant p53, or where p53 was depleted by retrovirus transducing siRNA, are refractory to ET/TSA treatment.

All these data demonstrate that MageA2 has a specific repressive function on p53, that relies on its ability to recruit HDACs, consequently inducing deacetylation of p53 and histones surrounding p53 binding sites thus conferring resistance to chemotherapeutic drugs.

MageA2 represses PML3-induced p53 senescence

The p53 tumor suppressor is a highly regulated protein. Several proteins were shown to specifically regulate p53 function. PML3 is such a p53 regulator, being responsible for recruiting p53 to PML-NBs, where it is stabilized and transcriptionally activated (Bode and Dong 2004).

The second part of the Thesis was devoted to analyze whether MageA2, by regulating p53 primarily via acetylation, could also participate in the control of PML3-induced p53 acetylation, a process that has been correlated with the induction of premature cellular senescence (Ferbeyre, de Stanchina et al. 2000; Pearson, Carbone et al. 2000).

Retroviral mediated transduction of normal human fibroblasts with PML3 results in the acquisition of a senescent phenotype as evidenced by the typical morphological changes, expression of SA-Gal and reduced incorporation of BrdU. Co-infection of PML3 with

MageA2 retroviruses resulted in markedly reduced signs of senescence, suggesting that MageA2 can prevent PML3 induced senescence.

Moreover, immunofluorescence assays demonstrated that MageA2 is recruited to PML-NBs where it co-localizes with endogenous p53, inducing p53 deacetylation in such structures. This effect could also be explained by a direct effect of MageA2 on PML3 restricting its ability to induce p53 mediated senescence. Importantly, MageA2 markedly represses PML3-induced p53 transactivation activity on a p53 responsive promoter, previously shown to be specifically regulated by p53 in response to PML3 expression. Also in this case, MageA2 is again the strongest repressor with respect to the other MAGE-A proteins assayed.

The most divergent MAGE-A proteins (MageA2 and MageA4: 67% identical) behave quite differently with respect to the PML3/p53 regulatory axis. In fact, MageA4 is incapable of interfering with the PML3-induced senescence response; cellular morphology, SA-β-Gal expression and BrdU incorporation are unaffected upon co-expression of MageA4 and PML3. Furthermore, MageA4 does not relocalize to PML-NBs, does not affect p53 transactivation function nor modifies p53 acetylation status. Moreover, contrary to MageA2, MageA4 is not able to interact with PML3 as shown both by *in vivo* and *in vitro* pull-down assays.

In silico analysis of the amino acid sequences of MAGE-A proteins revealed that MageA2 contains two potential SUMO binding motifs (SBMs) that are not present in MageA4. SUMO modification and binding to PML were shown to be required for the arrangement of PML-NBs (Shen, Lin et al. 2006) and for the function of PML3 on p53 activity (Fogal, Gostissa et al. 2000). To evaluate a potential involvement of SBMs in the mechanism of MageA2 repressive effect on PML3 function, we generated three different MageA2 constructs where the hydrophobic Ile and/or Val residues in the MageA2 SBMs were replaced with Ala residues. The respective mutants were analyzed for binding to PML3 by immunoprecipitation assays demonstrating that MageA2 SBMs are dispensable for binding to PML3. In fact, MageA2-SBMs mutants bind PML3 and are capable of repressing PML3-induced p53 transactivation function equally to wild type MageA2. These data suggest that the mechanism responsible for MageA2 dependent repression of PML3-induced p53 function is not dependent on the binding of MageA2 to sumoylated PML3.

A second possible mechanism examined, was the possibility that MageA2 could interfere with PML3 function on p53 through the balance of post-translational modifications of PML3. In fact, MageA2 expression results in significantly reduced levels of PML3 sumoylation. The correct PML3 sumoylation is in fact necessary for PML-NBs formation and p53 activation. Impairment of PML3 sumoylation by MageA2 could therefore constitute a potential

mechanism for the observed reduction of PML3 induced senescence in MageA2 expressing cells. Interestingly, a very recent work has demonstrated that PML3 acetylation favors subsequent PML3 sumoylation (Hayakawa, Abe et al. 2008). We are currently evaluating a potential role of MageA2 in the induction of PML3 deacetylation probably through the recruitment of HDACs as demonstrated for p53. This could therefore constitute part of the mechanism responsible for the consequent reduction on PML3 sumoylation which leads to a defective p53 activation within NBs.

Altogether the data presented in this second part support the notion that MageA2 specifically represses PML3-induced p53 activity, by interfering with the ability of PML3 to stimulate p53 acetylation at the NBs. The significance of the effect of MageA2 on PML3 activity has been analyzed in a normal cellular context (human diploid fibroblasts), in which PML3 was observed to induce premature senescence.

MageA2 expression can therefore control a specific step in carcinogenesis, namely the PML3-p53 axis responsible for commitment to cellular senescence, which constitutes one of the critical barriers for cellular transformation.

MageB2 has nucleolar associated functions, which are independent of p53 and possibly dependent on RB/E2F1

In the last part of this Thesis we have focused our work on another MAGE-I family member, the MageB2 protein. MAGE-B genes share 45-63% sequence identity with MAGE-A genes, and so far none of them has been yet characterized. Immunofluorescence experiments demonstrated that MageB2 shows a specific nucleolar localization. The nucleolus is a dynamic structure that responds to different stresses through a spatial reorganization of the nucleolar proteins. This leads to a rapid variation on nucleolar functions such as rRNA transcription, pre-rRNA processing and ribosome subunits assembly (Lam, Trinkle-Mulcahy et al. 2005). We observed that upon nucleolar disruption by treatment with different drugs such as Actinomycin D, low doses of doxorubicin or UV light, MageB2 behaves similarly to those nucleolar proteins (nucleolin (NCL) and nucleophosmin (NPM)) showing a rapid redistribution to the nucleoplasm. Nevertheless, treatment with 5-fluorouridine produces a specific redistribution of MageB2 to the nucleoplasm, without affecting the localization of NCL or NPM. These data suggest that MageB2 could be even more sensitive in the response

to specific ribotoxic drugs, since its leaves the nucleolus faster than nucleolin and nucleophosmin.

We could determine that MageB2 does not affect p53 transactivation function as assessed by reporter-gene assays. Moreover, immunoprecipitation approaches indicate that MageB2 is not able to bind p53. Interestingly, we were able to show that MageB2 confers resistance to the ribotoxic drug Actinomycin D in a p53 independent manner.

As part of the characterization of MageB2 we performed a proteomic approach to identify MageB2 interactors. Immunoaffinity purification of MageB2 molecular complexes demonstrated that MageB2 interacts with different ribonucleoproteins, such as heterogeneous nuclear ribonucleoprotein M (hnRPM) and NCL; transcription factors of the general transcription machinery (General Transcription Factor II-I, TFII-I); and ribosomal proteins like ribosomal protein S8, S9, S13 and L27a. For some of these proteins the binding to MageB2 seems to be dependent on RNA. In fact, MageB2 nucleolar localization itself depends on RNA, since after treatment of cells with RNase, MageB2 completely looses its nucleolar localization.

Another specific feature of MageB2 that clearly differentiates it from MAGE-A proteins, is the fact that MageB2 induces the transactivation activity of E2F1. E2F1 transactivation activity is required for the transcription of genes involved in the G1/S transition, and hence, for the correct progression through the cell-cycle. The transactivating activity of E2F1 is repressed through binding to pRB, which once phosphorylated by CDKs releases E2F1, which is then able to activate its target genes (Coller 2007). Reporter gene assays with E2F1 responsive promoters suggest that MageB2 collaborates with E2F1 in the transactivation of such promoters and inhibits in part the repressive effect of pRB on E2F1 activity, probably by promoting pRB relocalization to the nucleoli, as observed in immunofluorescence assays.

Interestingly, although MageB2 is not able to induce the transcription of rDNA by itself, it enhances E2F1 activity on the rDNA promoter as assessed by reporter gene assays and by the analysis of newly transcribed RNA.

The initial characterization data of MageB2, indicate that MageB2 seems to display functions related to its nucleolar localization. With respect to the proteins that it binds and the requirement of RNA for these interactions, it can be suggested that such functions could be related to the control of mechanisms such as RNA metabolism or protein synthesis. Moreover, since we observed that MageB2 induces E2F1 transactivation function, it could also have a role in the regulation of cell proliferation. Future efforts will be devoted to better clarify the specific roles of MageB2 both in nucleolar functions and consequent effects on cell

proliferation as well as the mechanisms by which MageB2 is capable of performing such functions.

Altogether the data presented in this Thesis strongly support the notion that, despite a significant level of sequence homology shared by the members of MAGE-I family, these proteins do not have redundant functions but have specificities with respect to functional pathways.

An important point to consider in the study of MAGE-I proteins is the mechanism responsible for the expression of these genes. MAGE-I genes expression seems to be an early event in tumorigenesis as well as during gametogenesis, both processes sharing important and interesting similarities. In fact, the discovery of a growing number of proteins that appear to be present only in germ cells, trophoblasts and tumors (the cancer testis antigens genes that include MAGE-I genes), led to the theory that aberrant expression of germline genes in cancer reflects the activation of the silenced gametogenic program in somatic cells, and that this acquisition could constitute one of the driving forces of tumorigenesis (Simpson, Caballero et al. 2005; Old 2007). Actually, recapitulation of portions of the germline gene expression program during oncogenesis, might contribute characteristic features to the neoplastic phenotype, including immortality, invasiveness, immune evasion, apoptosis resistance, hypomethylation. Normally oncogenic proliferative signals are coupled to a variety of growth-inhibitory processes, such as the induction of apoptosis, differentiation or senescence, each of which restricts subsequent clonal expansion and neoplastic evolution. Tumor progression occurs only in the very rare instances where these growth-inhibitory mechanisms are contrasted by compensatory mutations (Evan and Vousden 2001). Therefore, mutation or epigenetic activation of master switches orchestrating gametogenesis, could provide the cancer cell with a global compensatory behavior that could enforce the accumulated oncogenic events to become fixed and fully functional (Old 2007). Hypomethylation is known to accompany and characterize gametogenesis and carcinogenesis and is clearly involved in the anomalous expression of MAGE-I genes. The functional role of MAGE-I genes could therefore be selected within the global gametogenic switch program.

Although the role of MAGE-I genes is only beginning to be explored, emerging data suggest that they could have functions favoring the malignant phenotype, e.g. resistance to apoptosis, chemotherapy sensitivity/resistance, migration, proliferation. (Simpson, Caballero et al. 2005). Moreover, even though the expression of individual MAGE-I genes varies greatly among tumor types, they are frequently co-expressed in the same tumor, suggesting a possible

cooperative effect through the control of different processes acting synergically to enforce/maintain the full transformation spectrum.

The experiments shown in this Thesis demonstrate how the expression of MageA2 or MageB2 could confer resistance to apoptosis upon chemotherapeutic drugs treatments, by respectively p53 dependent and independent mechanisms. After the publication of our data indicating that MAGE-A proteins regulate p53 activity (Monte, Simonatto et al. 2006), other groups have started to study MAGE-I genes in different tumor models. Yang *et al*. have reported that siRNA mediated repression of MAGE-I proteins (MAGE-A, -B and –C) induces apoptosis and increases acetylation and activity of p53, and demonstrated that in the case of MAGE-A the induction of apoptosis requires p53 (Yang, O'Herrin et al. 2007). Zhu *et al*. showed that in particular MageA3 down-regulation results in p53 transcriptional induction in pituitary tumors (Zhu, Asa et al. 2008). Liu *et al*. demonstrated that overexpression of MageA3 leads to p21 down-regulation, accelerated cell-cycle progression, increased cell migration rate, and invasion in a murine model of thyroid cancer (Liu, Cheng et al. 2008).

We observed that the mechanism responsible for p53 repression by MageA2 is through the association with HDACs. Previous studies demonstrated that MageA1 acts as a transcriptional repressor by binding to SKIP and recruiting HDAC1 (Laduron, Deplus et al. 2004). Nevertheless, we have not observed strong repression of p53 by MageA1, this observation agreeing with our hypothesis that the differences in the sequence of MAGE-I proteins should account for functional and quantitative specificity. This hypothesis is also supported by the fact that we observed that MageA4 does not share any functional feature with MageA2. Actually, it has been demonstrated that MageA4 can display anti-oncogenic functions by blocking the activity of gankyrin and the transcription factor Miz-1, leading to apoptosis (Nagao, Higashitsuji et al. 2003; Sakurai, Itoh et al. 2004; Peikert, Specks et al. 2006). So far, among characterized MAGE-A members, MageA4 is the only one that seems to behave as a tumor suppressor, while the other analyzed MAGE-A proteins (for instance MageA1, MageA2 and MageA3) have reported pro-oncogenic functions.

We have also demonstrated that MageA2 impairs PML3 induced senescence by repressing PML3-induced p53 activity. It will be interesting to analyze a possible involvement of MageA2 in bypassing senescence induced by oncogenes such as Ras through mechanisms dependent on p53 or by affecting the DNA-damage response. On the other hand, our results suggest that MageB2 could exert functions related to cell-cycle progression and proliferation through control of the E2F1-pRB pathway.

Considering the complex scenario, where the coordinated expression of highly similar proteins could perform different functions, it is possible to hypothesize that the early expression of MageA2 and MageB2 could be involved in bypassing senescence by affecting both PML-p53 and Rb-E2F axis, respectively. Alternatively, in established tumors the expression of MageA2 and MageB2 could confer cells the ability to survive and resist chemotherapy (Fig. X).

Since the discovery of MAGE-A proteins, continuous efforts were concentrated in understanding the mechanisms responsible for the expression of these genes in tumors and the prognostic relevance of their differential expression. Due to their large number and sequence similarities, MAGE-I proteins have been mostly considered as epiphenomenon of cell transformation and used as targets for anti-cancer immunotherapies. Evidence presented in this Thesis, point to consider the specific cellular functions and the respective pathways that are targeted by this vast and heterogeneous family of genes. This will generate critical knowledge to potentially exploit MAGE-I genes as targets to be specifically inactivated in cancer cells.

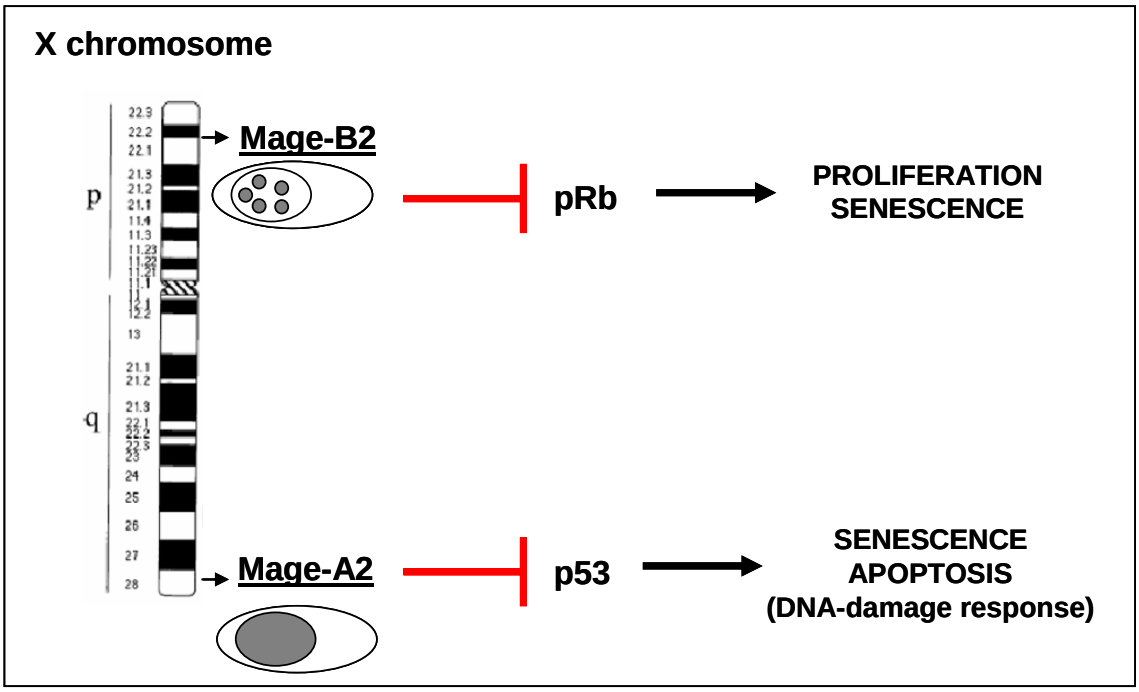

Fig. X: Schematic representation showing differences between the MAGE-I genes analyzed in the present work

6. Materials and methods

Cell lines and treatments

U2OS is a human osteosarcoma cell line; 293T is a derivative of the 293 kidney carcinoma cell line constitutively expressing the simian virus 40 (SV40) large T antigen; H1299 are human p53-null non–small cell lung carcinoma cells; HeLa are human cervical carcinoma cells; SaoS-2 is a human p53-null osteosarcoma cell line; 15392M and 13923M are human melanoma cell lines obtained from melanoma biopsies expressing, low and high, MAGE-A levels, respectively; BE and SK-Mel-28 are human melanoma cell lines bearing mutated p53; Wi38 are human primary lung fibroblasts. All cell lines were routinely cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml), with the exception of H1299, 15392M, 13923M, BE and SK-Mel-28 cell lines that were cultured in RPMI-1640 medium. M20 and U24 cell lines were maintained in medium containing zeocin and G418 (400 μg/ml). To induce the expression of HA-MageA2 or HA-MageB2, respectively, Ponasterone A, a synthetic analogue of ecdysone (Invitrogen) was added to the culture medium at a final concentration of 5 μM for 24 hr. Cisplatin, Etoposide, Trichostatin A, Actinomycin D, Doxorubicin and 5-Fluorouridine were purchased from Sigma and were used at the concentrations and for the indicated time.

Transfections and plasmids

Cells were transfected using Calcium Phosphate method, Lipofectamine 2000 Reagent (Invitrogen) or FuGENE 6 (Roche) according to the manufacturer's instructions. Unless stated otherwise, cells were analyzed 24 hr after transfection. MageA1, MageA2, MageA4, MageA6, p53, p53 deletion mutants, MageA2-SBM mutants, and MageB2 were HA tagged and cloned in pCDNA3 (Invitrogen). MageA2-SBM mutants were obtained by substitution of valine and/or isoleucine residues in the putative SBM sequences for alanines. His-MageA2 was cloned in pCDNA3.1 (Invitrogen). GST-p53, GST-MageA2, and GST-MageA4 were obtained by subcloning in pGEX-4T1 (Pharmacia). Flag-HDAC3 was obtained from C.Brancolini, DSTB University of Udine. Flag-MageA2 was obtained by subcloning in

p3XFLAG-CMV-10 (Sigma). pCDNA3-PML3 was kindly provided by Dr. Pandolfi PP. pCMV-HA-E2F1, pCMV-pRB were kindly provided by Dr. Giacca M.

For MAGE-I silencing we used a pool of four siRNA, based on a highly conserved MageA2 sequence (SMARTpool, Dharmacon Research). This reagent was able to knock-down all tested MAGE-I genes (MageA1, MageA2, MageA3, MageA6, data not shown). The control siRNA used was AACCUUUUUUUUUGGGGAAAA (siCONT). p53 silencing was from MWG and the mRNA target sequence was AAGACUCCAGUGGUAAUCUACTT. HDAC1 and HDAC3 siRNAs were from MWG. HDAC1 siRNA was GCAGAUGCAGAGAUUCAACTT and HDAC3 siRNA was GAUGCUGAACCAUGCACCUTT. Cells in mid-log growth phase were transfected with siRNA's using Oligofectamine Reagent (Invitrogen) or X-tremeGENE siRNA Transfection Reagent (Roche) as recommended by the manufacturer. Cells transfected with siRNA were analyzed after 36 hr.

Retroviral-mediated gene transfer

For retroviral infection, amphotrophic retrovirus packaging 293 cells were transfected by calcium-phosphate precipitation with the corresponding retroviral plasmid for 16 hr at 37°C. After 48 hr at 32°C, the virus-containing medium was filtered (0,45 µm filter) and supplemented with 4 µg/ml polybrene (Sigma). The culture medium of target cells was replaced by the appropriate viral supernatant, and then the cultures plates were centrifuged for 1 hr at 1500 rpm and incubated for 16-24 hr at 32°C. Finally, infected cell populations were purified using the appropriate selection: 2 µg/ml puromycin (Sigma) or 75 µg/ml hygromycin (Roche). Retroviral plasmids used were: pSuperRetro-Puro (pSR) vector (Oligoengine) encoding a sequence (5'-AACCAGCTATGTGAAAGTC-3') (pSRMage) with 100% matching to several MageA genes (A1, A2, A3, A4, A6, A7, A8 and A12). p53 activity was controlled by using pBabe-Hygro-EGFP-p53OD. pWZL-Hygro-PML3 was kindly provided by Dr. Ferbeyre, G. pLPC-EGF-MageA2 and pLPC-EGF-MageA4 were obtained by subcloning MageA2 and MageA4 cDNAs into pLPC-EGFP (Puromycin resistance).

Western blot analysis and antibodies

Western blot analysis was performed according to the standard procedures using the following primary antibodies: For p53: DO1, anti-p53 monoclonal (Santa Cruz Biotechnology), anti-acetyl p53 (Lys382) and anti-phospho p53 (Ser15) (16G8) (Cell Signaling). For Mage detection, affinity purified anti-MageA6 raised against GST-MageA6,

FL-309 anti-MAGE polyclonal antibody (Santa Cruz Biotechnology) or anti-Mage 57B monoclonal antibody (a gift from Spagnoli G., Basel). Other primary antibodies: anti-actin polyclonal antibody (Sigma), anti-Bax polyclonal antibody (Cell Signaling), anti-p21Waf1 polyclonal antibody (Santa Cruz Biotechnology), anti-HDAC3 polyclonal antibody (Cell Signaling), anti-HDAC1 polyclonal antibody (Cell Signaling), anti-PML polyclonal and monoclonal (PG-M3) antibodies (Santa Cruz Biotechnology), anti-SUMO1 monoclonal antibody (Zymed Laboratories), anti-NCL monoclonal antibody (Zymed Laboratories), anti-NPM monoclonal antibody (Zymed Laboratories), anti-pRB monoclonal antibody (Santa Cruz Biotechnology). For tags: anti-HA 12CA5 monoclonal antibody (Roche), anti-Flag M2 monoclonal antibody (Sigma), affinity purified anti-GFP raised against GST-GFP.

Immunoprecipitation

Cells were harvested in ice-cold lysis buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.1 mM sodium orthovanadate, 2 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM EDTA and Protease Inhibitor Cocktail (Sigma). After 10 min of rocking at 4°C, lysates were clarified by centrifugation and precleared with 25 µl of Protein A-Sepharose CL-4B or GammaBind G-Sepharose (Amersham Biosciences). Then, antibody prebound to 25 µl of Protein A-Sepharose CL-4B or GammaBind G-Sepharose was added and incubated at 4°C for 4 hr. The resin was then washed and bound proteins were eluted in SDS-PAGE sample buffer. For immunoprecipitation of endogenous PML, cells were harvested in RIPA buffer containing 50 mM Tris-HCl pH 7.8, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.5% NP-40, 0.2% deoxycholic acid, 0.1 mM sodium orthovanadate, 0.1 mM PMSF and Protease inhibitor cocktail (Sigma). For PML post-translational modifications analysis RIPA buffer was supplemented with 10 mM N-ethylmaleimide (NEM) for sumoylation analysis, or 5 mM butyric acid for acetylation analysis. Anti-PML monoclonal (PG-M3) antibody was prebound to Protein G-Sepharose and incubated with cells lysates for 16 hr at 4°C.

For *in vivo* labeling of proteins, cells were grown for 24 hr in complete medium, then it was replaced with methionine and cysteine free DMEM for 1 hr and 20 µCi/ml ^{35}S-Methionine (Amersham Biosciences) was then added to the medium for 16 hr. For RNase treatments, cell lysates were incubated with 50U RNase ONE (Promega) for 30 min at 37°C prior to immunoprecipitation.

In vitro pull-down assays

^{35}S-labeled proteins were *in vitro* translated using TNT Quick Coupled Transcription/Translation System (*in vitro* protein expression) (Promega) and incubated with purified GST, GST-MageA2, GST-MageA4 or GST-p53 (immobilized on glutathione-Sepharose 4B beads, Amersham Biosciences) in pull-down buffer (PBS, 0.1% NP-40, 10% Glycerol, 0.1 mM PMSF and Protease Inhibitor Cocktail). Bound proteins were eluted and resolved by SDS-PAGE.

Transient transactivation assays

For p53 reporters: cells were co-transfected in a 24-well plate with 100 ng of pG13LUC (an artificial promoter made of a tandem repeat of a fragment of the p21 promoter), p21LUC, Mdm2LUC, BaxLUC or PIG3LUC reporter constructs together with 50 ng of pCDNA3-p53 or pCDNA3-p539KR, either alone or with 150 ng of pCDNA3-HA-MageAs, pCDNA3-HA-MageB2 or pCDNA3-PML3 constructs.

For E2F1 reporters: cells were co-transfected in a 24-well plate with 500 ng of pGL3-TATA-6XE2F1-LUC reporter together with 10 ng of pCMV-HA-E2F1 and 10 ng pCMV-Myc-DP1, either alone or with 300 ng of pCDNA3-HA-MageB2 or 50 ng of pCMV-pRB. For the hrDNA reporter, cells were co-transfected with 200 ng of pIRES-LUC or pHrD-IRES-LUC, either alone or with 300 ng of pCMV-HA-E2F1 or 500 ng of pCDNA3-HA-MageB2. In this case, luciferase activity corresponded to the ratio (pHrD-IRES-LUC/pRL to pIRES-LUC/pRL) calculated from each set of experiments.

For normalization of transfection efficiency, 10 ng of pRL-CMV reporter (Promega), constitutively expressing the *Renilla reniformis* luciferase, was included. After 24 hrs, cells were lysed and assayed for luciferase activity using the Dual Luciferase kit (Promega).

Immunofluorescence analysis

Cells were plated on glass coverslips in 3 cm culture dishes. After washing with PBS, cells were fixed in 3% paraformaldehyde in PBS, treated with 1% glycine in PBS, and permeabilized in 0.1% Triton X-100 in PBS. The staining was performed using specific antibodies incubated in 5% bovine serum albumin in PBS at 37°C followed by fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate-conjugated secondary antibodies (Sigma) as indicated. Treatment with CSK buffer (100 mM NaCl, 300 mM sucrose, 10 mM Pipes, 3 mM $MgCl_2$, 1 mM EGTA, 15% glycerol, 1 mM PMSF, plus 0.5% Triton X-100) was performed for 10 minutes before fixation with paraformaldehyde. For RNase treatment,

previous to fixation, cells were treated with CSK buffer for 5 min at RT, and then treated with 1 mg/ml RNase A in PBS for 10 min a RT. For immunodetection of nascent RNA, cells were pulsed with 2 mM 5-Fluorouridine for 1 hr, fixed in 1% paraformaldehyde in PBS and permeabilized in PBS containing 0.5% Triton X-100 for 5 min. Antibodies against halogenated UTP (anti-BrdU) were used to label nascent RNA. Glass slides were analyzed using a laser scan confocal microscope (Zeiss).

BrdU incorporation assay

Cells were pulsed with 30 μM bromodeoxyuridine (BrdU, Sigma) for 1 hr (tumor cells) or 3 hr (fibroblasts). After pulsing, cells were fixed with 3% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS, and RNA denatured with 50 mM NaOH for 20 sec. BrdU incorporation was measured by immunofluorescence using an anti-BrdU antibody (GE Healthcare) and the nuclei were stained with Hoechst. Stained cells were visualized under a fluorescence microscope and at least 200 cells were scored for BrdU incorporation.

Cell viability and flow cytometry

Cells were harvested by trypsin treatment, permeabilized in 0.1% NP-40 in PBS containing RNase A (200 μg/ml) followed by Propidium Iodide (25 μg/ml) and/or Annexin V (Sigma) staining according to manufacturer's instructions. Apoptosis scoring was performed by subG1 determination or Annexin V staining. In both cases at least 10.000 cells were counted in independent experiments using a FACS (FACSCalibur, Becton-Dickinson). Cell viability was evaluated by a colorimetric assay using tetrazolium salt (MTT, Sigma) and reading absorbance at 550 nm on a multiwell scanning spectrophotometer after cell lysis with 33% DMF in SDS 30%.

Chromatin immunoprecipitation

Cells were crosslinked in 1% formaldehyde, washed in 125 mM glycine/PBS and harvested in Lysis Buffer (5 mM PIPES, 85 mM KCl, 0.5% NP-40). Pelleted cells were resuspended in RIPA-100 buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.5% Deoxycolate, 0.1% SDS). Sonicated chromatin (0.5Kb on average) (Bioruptor, Diagenode) was centrifugated. An aliquot of supernatant was used as input and the remainder was subjected to immunoprecipitation overnight at 4°C. Antibodies: for p53 immunoprecipitation a mix of PAb 240 and PAb 421 monoclonal antibodies, for HDAC3: anti-HDAC3 (Cell

Signaling), for Mage: a mix of affinity purified anti-Mage polyclonal antibodies, anti-MAGE monoclonal antibody (6C1) (Santa Cruz Biotechnology) and anti-Mage 57B monoclonal antibody, for Flag-tagged proteins: anti-Flag M2 monoclonal antibody (Sigma) and for Ac-Histone H3: Anti-acetylated Histone H3 polyclonal antibody (Upstate Biotechnology). DNA-protein complexes were recovered by 2 hr incubation with protein A/G PLUS-Agarose (Santa Cruz Biotechnology). Precipitates were extensively washed sequentially using RIPA-100 buffer, RIPA-250 (as RIPA-100 but using 250 mM NaCl) and LiCl solution (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 250 mM LiCl, 0.5% deoxycolate, 0.5% NP-40). Samples were then subjected to RNAse A treatment and decrosslinked overnight at 68°C in proteinase K solution (0.5% SDS, 100 mM NaCl, 150 ug/ml Prot-K). DNA was recovered by standard phenol-chloroform purification and sodium-acetate/ethanol precipitation. Quantitative PCR was performed on ABI PRISM 7000 PCR machine, using TaqMan Universal PCR Master Mix (Applied Biosystems). Primers and probes were obtained from Applied Biosystems and amplification settings were performed as described.

In vitro acetylation/deacetylation assay

500 ng of Histones (Sigma Chemical Co) were acetylated by incubating in acetylation buffer (50 mM TRIS-HCl pH7.5, 10% glycerol, 1 mM DTT) with 2ug of purified recombinant Histone Acetylase Transferase domain of p300 (GST-p300HAT) and ^{14}C-AcetylCoA (Amersham) shaking for 1 hour at 30°C.

Deacetylation assay was performed by incubating immunoprecipitated complex in deacetylation buffer (10 mM TRIS-HCl pH 8.0, 150 mM NaCl, 10% glycerol) with 200ng of ^{14}C-labeled histones, shaking for 16 hours at 30°C. Then, SDS-PAGE electrophoresis and autoradiography were performed for ^{14}C-labeled histones detection.

Analysis of SA-β-Galactosidase activity

Cells were fixed in 0.5% glutaraldehyde in PBS/2 mM $MgCl_2$ at pH 6.0 and then stained with staining solution containing 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$ in PBS/2 mM $MgCl_2$ at pH 6.0 plus 1 mg/ml X-Gal, for 16 hr at 37°C. Cells were then washed extensively with H_2O and observed under a bright field microscope.

Immunoaffinity purification

$2x10^8$ HeLa cells stably expressing HA-MageB2 were harvested in lysis buffer containing 50 mM Tris-HCl pH 8.0, 5 mM EDTA, 150 mM NaCl, 0,5% NP-40 and Protease Inhibitor

Cocktail (Sigma). Lysates were precleared for 30 min with Protein A-Sepharose at 4°C and then incubated with prebound Protein A-sepharose to anti-HA monoclonal antibody (12CA5 Roche) for 4 hr at 4°C. Beads were subsequently washed with SNNTE buffer containing 5% sucrose, 5 mM Tris-HCl pH 7.5, 5 mM EDTA, 200 mM NaCl, 1% NP-40 and Protease Inhibitor Cocktail. Immunoprecipitated complexes were resolved by SDS-PAGE and proteins visualized with colloidal blue coomassie. Bands then were isolated by in gel digestion and analyzed by mass spectrometry (Proteomic Facility at ICGEB, Trieste, Italy).

7. References

Achanta, G., R. Sasaki, et al. (2005). "Novel role of p53 in maintaining mitochondrial genetic stability through interaction with DNA Pol gamma." Embo J **24**(19): 3482-92.

Ahomadegbe, J. C., S. Tourpin, et al. (2000). "Loss of heterozygosity, allele silencing and decreased expression of p73 gene in breast cancers: prevalence of alterations in inflammatory breast cancers." Oncogene **19**(47): 5413-8.

Aizawa, T., K. Maruyama, et al. (1992). "Expression of necdin, an embryonal carcinoma-derived nuclear protein, in developing mouse brain." Brain Res Dev Brain Res **68**(2): 265-74.

Albino, A. P., M. J. Vidal, et al. (1994). "Mutation and expression of the p53 gene in human malignant melanoma." Melanoma Res **4**(1): 35-45.

Albrechtsen, N., I. Dornreiter, et al. (1999). "Maintenance of genomic integrity by p53: complementary roles for activated and non-activated p53." Oncogene **18**(53): 7706-17.

Alcorta, D. A., Y. Xiong, et al. (1996). "Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts." Proc Natl Acad Sci U S A **93**(24): 13742-7.

Andersen, J. S., C. E. Lyon, et al. (2002). "Directed proteomic analysis of the human nucleolus." Curr Biol **12**(1): 1-11.

Ando, T., T. Kawabe, et al. (2001). "Involvement of the interaction between p21 and proliferating cell nuclear antigen for the maintenance of G2/M arrest after DNA damage." J Biol Chem **276**(46): 42971-7.

Andrieu, D., H. Meziane, et al. (2006). "Sensory defects in Necdin deficient mice result from a loss of sensory neurons correlated within an increase of developmental programmed cell death." BMC Dev Biol **6**: 56.

Appella, E. and C. W. Anderson (2001). "Post-translational modifications and activation of p53 by genotoxic stresses." Eur J Biochem **268**(10): 2764-72.

Ard, P. G., C. Chatterjee, et al. (2002). "Transcriptional regulation of the mdm2 oncogene by p53 requires TRRAP acetyltransferase complexes." Mol Cell Biol **22**(16): 5650-61.

Attardi, L. D., E. E. Reczek, et al. (2000). "PERP, an apoptosis-associated target of p53, is a novel member of the PMP-22/gas3 family." Genes Dev **14**(6): 704-18.

Avantaggiati, M. L., V. Ogryzko, et al. (1997). "Recruitment of p300/CBP in p53-dependent signal pathways." Cell **89**(7): 1175-84.

Ayed, A., F. A. Mulder, et al. (2001). "Latent and active p53 are identical in conformation." Nat Struct Biol **8**(9): 756-60.

Ayrault, O., L. Andrique, et al. (2006). "Involvement of the transcriptional factor E2F1 in the regulation of the rRNA promoter." Exp Cell Res **312**(7): 1185-93.

Bai, S., B. He, et al. (2005). "Melanoma antigen gene protein MAGE-11 regulates androgen receptor function by modulating the interdomain interaction." Mol Cell Biol **25**(4): 1238-57.

Bai, S. and E. M. Wilson (2008). "Epidermal-growth-factor-dependent phosphorylation and ubiquitinylation of MAGE-11 regulates its interaction with the androgen receptor." Mol Cell Biol **28**(6): 1947-63.

Bakalkin, G., G. Selivanova, et al. (1995). "p53 binds single-stranded DNA ends through the C-terminal domain and internal DNA segments via the middle domain." Nucleic Acids Res **23**(3): 362-9.

Banin, S., L. Moyal, et al. (1998). "Enhanced phosphorylation of p53 by ATM in response to DNA damage." Science **281**(5383): 1674-7.

Barker, P. A. (1998). "p75NTR: A study in contrasts." Cell Death Differ **5**(5): 346-56.

Barker, P. A. and A. Salehi (2002). "The MAGE proteins: emerging roles in cell cycle progression, apoptosis, and neurogenetic disease." J Neurosci Res **67**(6): 705-12.

Barlev, N. A., L. Liu, et al. (2001). "Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferases." Mol Cell **8**(6): 1243-54.

Barrett, G. L. (2000). "The p75 neurotrophin receptor and neuronal apoptosis." Prog Neurobiol **61**(2): 205-29.

Bartkova, J., N. Rezaei, et al. (2006). "Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints." Nature **444**(7119): 633-7.

Baylin, S. B. and J. G. Herman (2000). "DNA hypermethylation in tumorigenesis: epigenetics joins genetics." Trends Genet **16**(4): 168-74.

Beausejour, C. M., A. Krtolica, et al. (2003). "Reversal of human cellular senescence: roles of the p53 and p16 pathways." Embo J **22**(16): 4212-22.

Bendall, A. J. and C. Abate-Shen (2000). "Roles for Msx and Dlx homeoproteins in vertebrate development." Gene **247**(1-2): 17-31.

Bergamaschi, D., Y. Samuels, et al. (2006). "iASPP preferentially binds p53 proline-rich region and modulates apoptotic function of codon 72-polymorphic p53." Nat Genet **38**(10): 1133-41.

Berger, M., N. Stahl, et al. (2005). "Mutations in proline 82 of p53 impair its activation by Pin1 and Chk2 in response to DNA damage." Mol Cell Biol **25**(13): 5380-8.

Berger, M., R. Vogt Sionov, et al. (2001). "A role for the polyproline domain of p53 in its regulation by Mdm2." J Biol Chem **276**(6): 3785-90.

Bernardi, R. and P. P. Pandolfi (2003). "Role of PML and the PML-nuclear body in the control of programmed cell death." Oncogene **22**(56): 9048-57.

Bernardi, R. and P. P. Pandolfi (2007). "Structure, dynamics and functions of promyelocytic leukaemia nuclear bodies." Nat Rev Mol Cell Biol **8**(12): 1006-16.

Bernardi, R., P. P. Scaglioni, et al. (2004). "PML regulates p53 stability by sequestering Mdm2 to the nucleolus." Nat Cell Biol **6**(7): 665-72.

Berns, K., E. M. Hijmans, et al. (2004). "A large-scale RNAi screen in human cells identifies new components of the p53 pathway." Nature **428**(6981): 431-7.

Bertram, J., K. Palfner, et al. (1998). "Elevated expression of S100P, CAPL and MAGE 3 in doxorubicin-resistant cell lines: comparison of mRNA differential display reverse transcription-polymerase chain reaction and subtractive suppressive hybridization for the analysis of differential gene expression." Anticancer Drugs **9**(4): 311-7.

Bertrand, P., Y. Saintigny, et al. (2004). "p53's double life: transactivation-independent repression of homologous recombination." Trends Genet **20**(6): 235-43.

Bischof, O., O. Kirsh, et al. (2002). "Deconstructing PML-induced premature senescence." Embo J **21**(13): 3358-69.

Bischof, O., K. Nacerddine, et al. (2005). "Human papillomavirus oncoprotein E7 targets the promyelocytic leukemia protein and circumvents cellular senescence via the Rb and p53 tumor suppressor pathways." Mol Cell Biol **25**(3): 1013-24.

Blattner, C., E. Tobiasch, et al. (1999). "DNA damage induced p53 stabilization: no indication for an involvement of p53 phosphorylation." Oncogene **18**(9): 1723-32.

Blaydes, J. P., M. G. Luciani, et al. (2001). "Stoichiometric phosphorylation of human p53 at Ser315 stimulates p53-dependent transcription." J Biol Chem **276**(7): 4699-708.

Boccaccio, I., H. Glatt-Deeley, et al. (1999). "The human MAGEL2 gene and its mouse homologue are paternally expressed and mapped to the Prader-Willi region." Hum Mol Genet **8**(13): 2497-505.

Bode, A. M. and Z. Dong (2004). "Post-translational modification of p53 in tumorigenesis." Nat Rev Cancer **4**(10): 793-805.

Boisvert, F. M., M. J. Kruhlak, et al. (2001). "The transcription coactivator CBP is a dynamic component of the promyelocytic leukemia nuclear body." J Cell Biol **152**(5): 1099-106.

Boon, T. and L. J. Old (1997). "Cancer Tumor antigens." Curr Opin Immunol **9**(5): 681-3.

Bottger, A., V. Bottger, et al. (1997). "Molecular characterization of the hdm2-p53 interaction." J Mol Biol **269**(5): 744-56.

Bourdon, J. C., J. Renzing, et al. (2002). "Scotin, a novel p53-inducible proapoptotic protein located in the ER and the nuclear membrane." J Cell Biol **158**(2): 235-46.

Bouvard, V., T. Zaitchouk, et al. (2000). "Tissue and cell-specific expression of the p53-target genes: bax, fas, mdm2 and waf1/p21, before and following ionising irradiation in mice." Oncogene **19**(5): 649-60.

Braig, M. and C. A. Schmitt (2006). "Oncogene-induced senescence: putting the brakes on tumor development." Cancer Res **66**(6): 2881-4.

Brain, R. and J. R. Jenkins (1994). "Human p53 directs DNA strand reassociation and is photolabelled by 8-azido ATP." Oncogene **9**(6): 1775-80.

Brooks, C. L. and W. Gu (2003). "Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation." Curr Opin Cell Biol **15**(2): 164-71.

Brooks, C. L., M. Li, et al. (2007). "The p53--Mdm2--HAUSP complex is involved in p53 stabilization by HAUSP." Oncogene **26**(51): 7262-6.

Brugarolas, J., C. Chandrasekaran, et al. (1995). "Radiation-induced cell cycle arrest compromised by p21 deficiency." Nature **377**(6549): 552-7.

Brunner, H. G., B. C. Hamel, et al. (2002). "The p63 gene in EEC and other syndromes." J Med Genet **39**(6): 377-81.

Bulavin, D. V., S. Saito, et al. (1999). "Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation." Embo J **18**(23): 6845-54.

Bullock, A. N., J. Henckel, et al. (1997). "Thermodynamic stability of wild-type and mutant p53 core domain." Proc Natl Acad Sci U S A **94**(26): 14338-42.

Bullock, A. N., J. Henckel, et al. (2000). "Quantitative analysis of residual folding and DNA binding in mutant p53 core domain: definition of mutant states for rescue in cancer therapy." Oncogene **19**(10): 1245-56.

Bunz, F., A. Dutriaux, et al. (1998). "Requirement for p53 and p21 to sustain G2 arrest after DNA damage." Science **282**(5393): 1497-501.

Buschmann, T., S. Y. Fuchs, et al. (2000). "SUMO-1 modification of Mdm2 prevents its self-ubiquitination and increases Mdm2 ability to ubiquitinate p53." Cell **101**(7): 753-62.

Bush, J. R. and R. Wevrick (2008). "The Prader-Willi syndrome protein necdin interacts with the E1A-like inhibitor of differentiation EID-1 and promotes myoblast differentiation." Differentiation.

Cahilly-Snyder, L., T. Yang-Feng, et al. (1987). "Molecular analysis and chromosomal mapping of amplified genes isolated from a transformed mouse 3T3 cell line." Somat Cell Mol Genet **13**(3): 235-44.

Campisi, J. (2001). "Cellular senescence as a tumor-suppressor mechanism." Trends Cell Biol **11**(11): S27-31.

Campisi, J. and F. d'Adda di Fagagna (2007). "Cellular senescence: when bad things happen to good cells." Nat Rev Mol Cell Biol **8**(9): 729-40.

Coller, H. A. (2007). "What's taking so long? S-phase entry from quiescence versus proliferation." Nat Rev Mol Cell Biol **8**(8): 667-70.

Cory, S. and J. M. Adams (2002). "The Bcl2 family: regulators of the cellular life-or-death switch." Nat Rev Cancer **2**(9): 647-56.

Crighton, D., S. Wilkinson, et al. (2006). "DRAM, a p53-induced modulator of autophagy, is critical for apoptosis." Cell **126**(1): 121-34.

Crighton, D., S. Wilkinson, et al. (2007). "DRAM links autophagy to p53 and programmed cell death." Autophagy **3**(1): 72-4.

Chan, T. A., H. Hermeking, et al. (1999). "14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage." Nature **401**(6753): 616-20.

Chehab, N. H., A. Malikzay, et al. (1999). "Phosphorylation of Ser-20 mediates stabilization of human p53 in response to DNA damage." Proc Natl Acad Sci U S A **96**(24): 13777-82.

Chen, D., N. Kon, et al. (2005). "ARF-BP1/Mule is a critical mediator of the ARF tumor suppressor." Cell **121**(7): 1071-83.

Chen, J., J. Lin, et al. (1995). "Regulation of transcription functions of the p53 tumor suppressor by the mdm-2 oncogene." Mol Med **1**(2): 142-52.

Chen, J. and I. Sadowski (2005). "Identification of the mismatch repair genes PMS2 and MLH1 as p53 target genes by using serial analysis of binding elements." Proc Natl Acad Sci U S A **102**(13): 4813-8.

Chen, J., X. Wu, et al. (1996). "mdm-2 inhibits the G1 arrest and apoptosis functions of the p53 tumor suppressor protein." Mol Cell Biol **16**(5): 2445-52.

Chen, L. and J. Chen (2003). "MDM2-ARF complex regulates p53 sumoylation." Oncogene **22**(34): 5348-57.

Chen, Q. M., J. Liu, et al. (2000). "Apoptosis or senescence-like growth arrest: influence of cell-cycle position, p53, p21 and bax in H2O2 response of normal human fibroblasts." Biochem J **347**(Pt 2): 543-51.

Chen, Y. T., A. O. Gure, et al. (1998). "Identification of multiple cancer/testis antigens by allogeneic antibody screening of a melanoma cell line library." Proc Natl Acad Sci U S A **95**(12): 6919-23.

Chen, Y. T., M. J. Scanlan, et al. (1997). "A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening." Proc Natl Acad Sci U S A **94**(5): 1914-8.

Chen, Z., L. C. Trotman, et al. (2005). "Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis." Nature **436**(7051): 725-30.

Cherbonnel-Lasserre, C. and M. K. Dosanjh (1997). "Suppression of apoptosis by overexpression of Bcl-2 or Bcl-xL promotes survival and mutagenesis after oxidative damage." Biochimie **79**(9-10): 613-7.

Chipuk, J. E., L. Bouchier-Hayes, et al. (2005). "PUMA couples the nuclear and cytoplasmic proapoptotic function of p53." Science **309**(5741): 1732-5.

Chipuk, J. E. and D. R. Green (2004). "Cytoplasmic p53: bax and forward." Cell Cycle **3**(4): 429-31.

Chipuk, J. E., T. Kuwana, et al. (2004). "Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis." Science **303**(5660): 1010-4.

Cho, Y., S. Gorina, et al. (1994). "Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations." Science **265**(5170): 346-55.

Chomez, P., O. De Backer, et al. (2001). "An overview of the MAGE gene family with the identification of all human members of the family." Cancer Res **61**(14): 5544-51.

Chuikov, S., J. K. Kurash, et al. (2004). "Regulation of p53 activity through lysine methylation." Nature **432**(7015): 353-60.

d'Adda di Fagagna, F. (2008). "Living on a break: cellular senescence as a DNA-damage response." Nat Rev Cancer **8**(7): 512-22.

d'Adda di Fagagna, F., P. M. Reaper, et al. (2003). "A DNA damage checkpoint response in telomere-initiated senescence." Nature **426**(6963): 194-8.

D'Orazi, G., B. Cecchinelli, et al. (2002). "Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser 46 and mediates apoptosis." Nat Cell Biol **4**(1): 11-9.

Dai, M. S., S. X. Zeng, et al. (2004). "Ribosomal protein L23 activates p53 by inhibiting MDM2 function in response to ribosomal perturbation but not to translation inhibition." Mol Cell Biol **24**(17): 7654-68.

Damalas, A., S. Kahan, et al. (2001). "Deregulated beta-catenin induces a p53- and ARF-dependent growth arrest and cooperates with Ras in transformation." Embo J **20**(17): 4912-22.

Daniotti, M., M. Oggionni, et al. (2004). "BRAF alterations are associated with complex mutational profiles in malignant melanoma." Oncogene **23**(35): 5968-77.

Danovi, D., E. Meulmeester, et al. (2004). "Amplification of Mdmx (or Mdm4) directly contributes to tumor formation by inhibiting p53 tumor suppressor activity." Mol Cell Biol **24**(13): 5835-43.

Davison, T. S., P. Yin, et al. (1998). "Characterization of the oligomerization defects of two p53 mutants found in families with Li-Fraumeni and Li-Fraumeni-like syndrome." Oncogene **17**(5): 651-6.

De Plaen, E., K. Arden, et al. (1994). "Structure, chromosomal localization, and expression of 12 genes of the MAGE family." Immunogenetics **40**(5): 360-9.

De Smet, C., O. De Backer, et al. (1996). "The activation of human gene MAGE-1 in tumor cells is correlated with genome-wide demethylation." Proc Natl Acad Sci U S A **93**(14): 7149-53.

De Smet, C., A. Loriot, et al. (2004). "Promoter-dependent mechanism leading to selective hypomethylation within the 5' region of gene MAGE-A1 in tumor cells." Mol Cell Biol **24**(11): 4781-90.

De Smet, C., C. Lurquin, et al. (1999). "DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter." Mol Cell Biol **19**(11): 7327-35.

de Souza-Pinto, N. C., C. C. Harris, et al. (2004). "p53 functions in the incorporation step in DNA base excision repair in mouse liver mitochondria." Oncogene **23**(39): 6559-68.

de Stanchina, E., E. Querido, et al. (2004). "PML is a direct p53 target that modulates p53 effector functions." Mol Cell **13**(4): 523-35.

DeLeo, A. B., G. Jay, et al. (1979). "Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse." Proc Natl Acad Sci U S A **76**(5): 2420-4.

Dellaire, G. and D. P. Bazett-Jones (2004). "PML nuclear bodies: dynamic sensors of DNA damage and cellular stress." Bioessays **26**(9): 963-77.

Deponti, D., S. Francois, et al. (2007). "Necdin mediates skeletal muscle regeneration by promoting myoblast survival and differentiation." J Cell Biol **179**(2): 305-19.

Di Certo, M. G., N. Corbi, et al. (2007). "NRAGE associates with the anti-apoptotic factor Che-1 and regulates its degradation to induce cell death." J Cell Sci **120**(Pt 11): 1852-8.

Di Leonardo, A., S. P. Linke, et al. (1994). "DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts." Genes Dev **8**(21): 2540-51.

Di Micco, R., M. Fumagalli, et al. (2006). "Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication." Nature **444**(7119): 638-42.

Dimri, G. P. (2005). "What has senescence got to do with cancer?" Cancer Cell **7**(6): 505-12.

Dimri, G. P., K. Itahana, et al. (2000). "Regulation of a senescence checkpoint response by the E2F1 transcription factor and p14(ARF) tumor suppressor." Mol Cell Biol **20**(1): 273-85.

Dimri, G. P., X. Lee, et al. (1995). "A biomarker that identifies senescent human cells in culture and in aging skin in vivo." Proc Natl Acad Sci U S A **92**(20): 9363-7.

Donald, S. P., X. Y. Sun, et al. (2001). "Proline oxidase, encoded by p53-induced gene-6, catalyzes the generation of proline-dependent reactive oxygen species." Cancer Res **61**(5): 1810-5.

Dornan, D. and T. R. Hupp (2001). "Inhibition of p53-dependent transcription by BOX-I phospho-peptide mimetics that bind to p300." EMBO Rep **2**(2): 139-44.

Dornan, D., H. Shimizu, et al. (2003). "The proline repeat domain of p53 binds directly to the transcriptional coactivator p300 and allosterically controls DNA-dependent acetylation of p53." Mol Cell Biol **23**(23): 8846-61.

Dornan, D., H. Shimizu, et al. (2003). "DNA-dependent acetylation of p53 by the transcription coactivator p300." J Biol Chem **278**(15): 13431-41.

Dornan, D., I. Wertz, et al. (2004). "The ubiquitin ligase COP1 is a critical negative regulator of p53." Nature **429**(6987): 86-92.

Doyon, Y., W. Selleck, et al. (2004). "Structural and functional conservation of the NuA4 histone acetyltransferase complex from yeast to humans." Mol Cell Biol **24**(5): 1884-96.

Duan, Z., Y. Duan, et al. (2003). "Overexpression of MAGE/GAGE genes in paclitaxel/doxorubicin-resistant human cancer cell lines." Clin Cancer Res **9**(7): 2778-85.

Dumaz, N. and D. W. Meek (1999). "Serine15 phosphorylation stimulates p53 transactivation but does not directly influence interaction with HDM2." Embo J **18**(24): 7002-10.

Dumaz, N., D. M. Milne, et al. (2001). "Critical roles for the serine 20, but not the serine 15, phosphorylation site and for the polyproline domain in regulating p53 turnover." Biochem J **359**(Pt 2): 459-64.

Dumaz, N., D. M. Milne, et al. (1999). "Protein kinase CK1 is a p53-threonine 18 kinase which requires prior phosphorylation of serine 15." FEBS Lett **463**(3): 312-6.

Dumont, P., J. I. Leu, et al. (2003). "The codon 72 polymorphic variants of p53 have markedly different apoptotic potential." Nat Genet **33**(3): 357-65.

Duprez, E., A. J. Saurin, et al. (1999). "SUMO-1 modification of the acute promyelocytic leukaemia protein PML: implications for nuclear localisation." J Cell Sci **112 (Pt 3)**: 381-93.

el-Deiry, W. S., S. E. Kern, et al. (1992). "Definition of a consensus binding site for p53." Nat Genet **1**(1): 45-9.

el-Deiry, W. S., T. Tokino, et al. (1993). "WAF1, a potential mediator of p53 tumor suppression." Cell **75**(4): 817-25.

Espinosa, J. M. and B. M. Emerson (2001). "Transcriptional regulation by p53 through intrinsic DNA/chromatin binding and site-directed cofactor recruitment." Mol Cell **8**(1): 57-69.

Espinosa, J. M., R. E. Verdun, et al. (2003). "p53 functions through stress- and promoter-specific recruitment of transcription initiation components before and after DNA damage." Mol Cell **12**(4): 1015-27.

Esser, C., M. Scheffner, et al. (2005). "The chaperone-associated ubiquitin ligase CHIP is able to target p53 for proteasomal degradation." J Biol Chem **280**(29): 27443-8.

Evan, G. I. and K. H. Vousden (2001). "Proliferation, cell cycle and apoptosis in cancer." Nature **411**(6835): 342-8.

Ferbeyre, G., E. de Stanchina, et al. (2000). "PML is induced by oncogenic ras and promotes premature senescence." Genes Dev **14**(16): 2015-27.

Ferri, K. F. and G. Kroemer (2001). "Organelle-specific initiation of cell death pathways." Nat Cell Biol **3**(11): E255-63.

Fields, S. and S. K. Jang (1990). "Presence of a potent transcription activating sequence in the p53 protein." Science **249**(4972): 1046-9.

Finch, R. A., D. B. Donoviel, et al. (2002). "mdmx is a negative regulator of p53 activity in vivo." Cancer Res **62**(11): 3221-5.

Fiscella, M., S. J. Ullrich, et al. (1993). "Mutation of the serine 15 phosphorylation site of human p53 reduces the ability of p53 to inhibit cell cycle progression." Oncogene **8**(6): 1519-28.

Flores, E. R., S. Sengupta, et al. (2005). "Tumor predisposition in mice mutant for p63 and p73: evidence for broader tumor suppressor functions for the p53 family." Cancer Cell **7**(4): 363-73.

Flores, E. R., K. Y. Tsai, et al. (2002). "p63 and p73 are required for p53-dependent apoptosis in response to DNA damage." Nature **416**(6880): 560-4.

Fogal, V., M. Gostissa, et al. (2000). "Regulation of p53 activity in nuclear bodies by a specific PML isoform." Embo J **19**(22): 6185-95.

Fogal, V., J. K. Hsieh, et al. (2005). "Cell cycle-dependent nuclear retention of p53 by E2F1 requires phosphorylation of p53 at Ser315." Embo J **24**(15): 2768-82.

Fojta, M., H. Pivonkova, et al. (2004). "Investigations of the supercoil-selective DNA binding of wild type p53 suggest a novel mechanism for controlling p53 function." Eur J Biochem **271**(19): 3865-76.

Freedman, D. A., C. B. Epstein, et al. (1997). "A genetic approach to mapping the p53 binding site in the MDM2 protein." Mol Med **3**(4): 248-59.

Freeman, D. J., A. G. Li, et al. (2003). "PTEN tumor suppressor regulates p53 protein levels and activity through phosphatase-dependent and -independent mechanisms." Cancer Cell **3**(2): 117-30.

Friedman, P. N., X. Chen, et al. (1993). "The p53 protein is an unusually shaped tetramer that binds directly to DNA." Proc Natl Acad Sci U S A **90**(8): 3319-23.

Fu, X. Y., H. Y. Wang, et al. (2002). "Overexpression of p28/gankyrin in human hepatocellular carcinoma and its clinical significance." World J Gastroenterol **8**(4): 638-43.

Fuchs, B., D. O'Connor, et al. (1995). "p53 phosphorylation mutants retain transcription activity." Oncogene **10**(4): 789-93.

Fuster, J. J., S. M. Sanz-Gonzalez, et al. (2007). "Classic and novel roles of p53: prospects for anticancer therapy." Trends Mol Med **13**(5): 192-9.

Gambacorta, M., L. Flenghi, et al. (1996). "Heterogeneous nuclear expression of the promyelocytic leukemia (PML) protein in normal and neoplastic human tissues." Am J Pathol **149**(6): 2023-35.

Gatti, A., H. H. Li, et al. (2000). "Phosphorylation of human p53 on Thr-55." Biochemistry **39**(32): 9837-42.

Gaugler, B., B. Van den Eynde, et al. (1994). "Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocytes." J Exp Med **179**(3): 921-30.

Gerard, M., L. Hernandez, et al. (1999). "Disruption of the mouse necdin gene results in early post-natal lethality." Nat Genet **23**(2): 199-202.

Girdwood, D., D. Bumpass, et al. (2003). "P300 transcriptional repression is mediated by SUMO modification." Mol Cell **11**(4): 1043-54.

Glynn, S. A., P. Gammell, et al. (2004). "A new superinvasive in vitro phenotype induced by selection of human breast carcinoma cells with the chemotherapeutic drugs paclitaxel and doxorubicin." Br J Cancer **91**(10): 1800-7.

Gostissa, M., A. Hengstermann, et al. (1999). "Activation of p53 by conjugation to the ubiquitin-like protein SUMO-1." Embo J **18**(22): 6462-71.

Gostissa, M., M. Morelli, et al. (2004). "The transcriptional repressor hDaxx potentiates p53-dependent apoptosis." J Biol Chem **279**(46): 48013-23.

Gottifredi, V., S. Shieh, et al. (2001). "p53 accumulates but is functionally impaired when DNA synthesis is blocked." Proc Natl Acad Sci U S A **98**(3): 1036-41.

Grossman, H. B. (2001). "Biomarkers for transitional cell carcinoma-pro." Urology **57**(5): 847-8.

Grossman, S. R., M. E. Deato, et al. (2003). "Polyubiquitination of p53 by a ubiquitin ligase activity of p300." Science **300**(5617): 342-4.

Gu, J., L. Nie, et al. (2001). "Identification of p53 sequence elements that are required for MDM2-mediated nuclear export." Mol Cell Biol **21**(24): 8533-46.

Gu, W. and R. G. Roeder (1997). "Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain." Cell **90**(4): 595-606.

Guo, A., P. Salomoni, et al. (2000). "The function of PML in p53-dependent apoptosis." Nat Cell Biol **2**(10): 730-6.

Gure, A. O., E. Stockert, et al. (2000). "CT10: a new cancer-testis (CT) antigen homologous to CT7 and the MAGE family, identified by representational-difference analysis." Int J Cancer **85**(5): 726-32.

Gurrieri, C., P. Capodieci, et al. (2004). "Loss of the tumor suppressor PML in human cancers of multiple histologic origins." J Natl Cancer Inst **96**(4): 269-79.

Haldar, S., M. Negrini, et al. (1994). "Down-regulation of bcl-2 by p53 in breast cancer cells." Cancer Res **54**(8): 2095-7.

Hampel, B., F. Malisan, et al. (2004). "Differential regulation of apoptotic cell death in senescent human cells." Exp Gerontol **39**(11-12): 1713-21.

Han, J., C. Flemington, et al. (2001). "Expression of bbc3, a pro-apoptotic BH3-only gene, is regulated by diverse cell death and survival signals." Proc Natl Acad Sci U S A **98**(20): 11318-23.

Hanawalt, P. C. (2001). "Controlling the efficiency of excision repair." Mutat Res **485**(1): 3-13.

Harley, C. B., A. B. Futcher, et al. (1990). "Telomeres shorten during ageing of human fibroblasts." Nature **345**(6274): 458-60.

Harper, J. W., G. R. Adami, et al. (1993). "The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases." Cell **75**(4): 805-16.

Hasegawa, K. and K. Yoshikawa (2008). "Necdin regulates p53 acetylation via Sirtuin1 to modulate DNA damage response in cortical neurons." J Neurosci **28**(35): 8772-84.

Haupt, Y., Y. Barak, et al. (1996). "Cell type-specific inhibition of p53-mediated apoptosis by mdm2." Embo J **15**(7): 1596-606.

Haupt, Y., R. Maya, et al. (1997). "Mdm2 promotes the rapid degradation of p53." Nature **387**(6630): 296-9.

Hayakawa, F., A. Abe, et al. (2008). "Acetylation of PML is involved in histone deacetylase inhibitor-mediated apoptosis." J Biol Chem **283**(36): 24420-5.

Hayashi, Y., K. Matsuyama, et al. (1995). "Arrest of cell growth by necdin, a nuclear protein expressed in postmitotic neurons." Biochem Biophys Res Commun **213**(1): 317-24.

Hayflick, L. (1965). "The Limited in Vitro Lifetime of Human Diploid Cell Strains." Exp Cell Res **37**: 614-36.

Hemann, M. T. and M. Narita (2007). "Oncogenes and senescence: breaking down in the fast lane." Genes Dev **21**(1): 1-5.

Herbig, U., W. A. Jobling, et al. (2004). "Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a)." Mol Cell **14**(4): 501-13.

Hermeking, H., C. Lengauer, et al. (1997). "14-3-3 sigma is a p53-regulated inhibitor of G2/M progression." Mol Cell **1**(1): 3-11.

Hersey, P., G. M. Halliday, et al. (2008). "Phase I/II study of treatment with matured dendritic cells with or without low dose IL-2 in patients with disseminated melanoma." Cancer Immunol Immunother **57**(7): 1039-51.

Heyne, K., S. Mannebach, et al. (2004). "Identification of a putative p53 binding sequence within the human mitochondrial genome." FEBS Lett **578**(1-2): 198-202.

Higashitsuji, H., H. Higashitsuji, et al. (2005). "The oncoprotein gankyrin binds to MDM2/HDM2, enhancing ubiquitylation and degradation of p53." Cancer Cell **8**(1): 75-87.

Higashitsuji, H., K. Itoh, et al. (2000). "Reduced stability of retinoblastoma protein by gankyrin, an oncogenic ankyrin-repeat protein overexpressed in hepatomas." Nat Med **6**(1): 96-9.

Hoffman, W. H., S. Biade, et al. (2002). "Transcriptional repression of the anti-apoptotic survivin gene by wild type p53." J Biol Chem **277**(5): 3247-57.

Hofmann, T. G., A. Moller, et al. (2002). "Regulation of p53 activity by its interaction with homeodomain-interacting protein kinase-2." Nat Cell Biol **4**(1): 1-10.

Honda, R. and H. Yasuda (1999). "Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53." Embo J **18**(1): 22-7.

Huang, J., L. Perez-Burgos, et al. (2006). "Repression of p53 activity by Smyd2-mediated methylation." Nature **444**(7119): 629-32.

Huang, J., R. Sengupta, et al. (2007). "p53 is regulated by the lysine demethylase LSD1." Nature **449**(7158): 105-8.

Hwang, B. J., J. M. Ford, et al. (1999). "Expression of the p48 xeroderma pigmentosum gene is p53-dependent and is involved in global genomic repair." Proc Natl Acad Sci U S A **96**(2): 424-8.

Hwang, P. M., F. Bunz, et al. (2001). "Ferredoxin reductase affects p53-dependent, 5-fluorouracil-induced apoptosis in colorectal cancer cells." Nat Med **7**(10): 1111-7.

Ichas, F., L. S. Jouaville, et al. (1997). "Mitochondria are excitable organelles capable of generating and conveying electrical and calcium signals." Cell **89**(7): 1145-53.

Ichas, F. and J. P. Mazat (1998). "From calcium signaling to cell death: two conformations for the mitochondrial permeability transition pore. Switching from low- to high-conductance state." Biochim Biophys Acta **1366**(1-2): 33-50.

Ihrie, R. A., E. Reczek, et al. (2003). "Perp is a mediator of p53-dependent apoptosis in diverse cell types." Curr Biol **13**(22): 1985-90.

Insinga, A., S. Monestiroli, et al. (2004). "Impairment of p53 acetylation, stability and function by an oncogenic transcription factor." Embo J **23**(5): 1144-54.

Irwin, M. S. and W. G. Kaelin, Jr. (2001). "Role of the newer p53 family proteins in malignancy." Apoptosis **6**(1-2): 17-29.

Ishov, A. M., A. G. Sotnikov, et al. (1999). "PML is critical for ND10 formation and recruits the PML-interacting protein daxx to this nuclear structure when modified by SUMO-1." J Cell Biol **147**(2): 221-34.

Itahana, K., J. Campisi, et al. (2004). "Mechanisms of cellular senescence in human and mouse cells." Biogerontology **5**(1): 1-10.

Ito, A., Y. Kawaguchi, et al. (2002). "MDM2-HDAC1-mediated deacetylation of p53 is required for its degradation." Embo J **21**(22): 6236-45.

Ito, A., C. H. Lai, et al. (2001). "p300/CBP-mediated p53 acetylation is commonly induced by p53-activating agents and inhibited by MDM2." Embo J **20**(6): 1331-40.

Jackson, M. W. and S. J. Berberich (2000). "MdmX protects p53 from Mdm2-mediated degradation." Mol Cell Biol **20**(3): 1001-7.

Jacobs, J. J. and T. de Lange (2004). "Significant role for p16INK4a in p53-independent telomere-directed senescence." Curr Biol **14**(24): 2302-8.

Jacobs, J. J. and T. de Lange (2005). "p16INK4a as a second effector of the telomere damage pathway." Cell Cycle **4**(10): 1364-8.

James, S. R., P. A. Link, et al. (2006). "Epigenetic regulation of X-linked cancer/germline antigen genes by DNMT1 and DNMT3b." Oncogene **25**(52): 6975-85.

Jang, S. J., J. C. Soria, et al. (2001). "Activation of melanoma antigen tumor antigens occurs early in lung carcinogenesis." Cancer Res **61**(21): 7959-63.

Janz, C. and L. Wiesmuller (2002). "Wild-type p53 inhibits replication-associated homologous recombination." Oncogene **21**(38): 5929-33.

Jay, P., C. Rougeulle, et al. (1997). "The human necdin gene, NDN, is maternally imprinted and located in the Prader-Willi syndrome chromosomal region." Nat Genet **17**(3): 357-61.

Jayaraman, J. and C. Prives (1995). "Activation of p53 sequence-specific DNA binding by short single strands of DNA requires the p53 C-terminus." Cell **81**(7): 1021-9.

Jeffers, J. R., E. Parganas, et al. (2003). "Puma is an essential mediator of p53-dependent and -independent apoptotic pathways." Cancer Cell **4**(4): 321-8.

Jeffrey, P. D., S. Gorina, et al. (1995). "Crystal structure of the tetramerization domain of the p53 tumor suppressor at 1.7 angstroms." Science **267**(5203): 1498-502.

Jiang, P., W. Du, et al. (2006). "The Bad guy cooperates with good cop p53: Bad is transcriptionally up-regulated by p53 and forms a Bad/p53 complex at the mitochondria to induce apoptosis." Mol Cell Biol **26**(23): 9071-82.

Jin, S., T. Tong, et al. (2002). "GADD45-induced cell cycle G2-M arrest associates with altered subcellular distribution of cyclin B1 and is independent of p38 kinase activity." Oncogene **21**(57): 8696-704.

Jin, Y., S. X. Zeng, et al. (2002). "MDM2 inhibits PCAF (p300/CREB-binding protein-associated factor)-mediated p53 acetylation." J Biol Chem **277**(34): 30838-43.

Johnson, T. M., Z. X. Yu, et al. (1996). "Reactive oxygen species are downstream mediators of p53-dependent apoptosis." Proc Natl Acad Sci U S A **93**(21): 11848-52.

Jones, S. N., A. E. Roe, et al. (1995). "Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53." Nature **378**(6553): 206-8.

Jordan, B. W., D. Dinev, et al. (2001). "Neurotrophin receptor-interacting mage homologue is an inducible inhibitor of apoptosis protein-interacting protein that augments cell death." J Biol Chem **276**(43): 39985-9.

Jouaville, L. S., F. Ichas, et al. (1998). "Modulation of cell calcium signals by mitochondria." Mol Cell Biochem **184**(1-2): 371-6.

Juan, L. J., W. J. Shia, et al. (2000). "Histone deacetylases specifically down-regulate p53-dependent gene activation." J Biol Chem **275**(27): 20436-43.

Kaghad, M., H. Bonnet, et al. (1997). "Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers." Cell **90**(4): 809-19.

Kakizuka, A., W. H. Miller, Jr., et al. (1991). "Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RAR alpha with a novel putative transcription factor, PML." Cell **66**(4): 663-74.

Kannan, K., N. Kaminski, et al. (2001). "DNA microarray analysis of genes involved in p53 mediated apoptosis: activation of Apaf-1." Oncogene **20**(26): 3449-55.

Kaplan, D. R. and F. D. Miller (2000). "Neurotrophin signal transduction in the nervous system." Curr Opin Neurobiol **10**(3): 381-91.

Karpf, A. R., A. W. Lasek, et al. (2004). "Limited gene activation in tumor and normal epithelial cells treated with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine." Mol Pharmacol **65**(1): 18-27.

Kastan, M. B., Q. Zhan, et al. (1992). "A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia." Cell **71**(4): 587-97.

Katayama, H., K. Sasai, et al. (2004). "Phosphorylation by aurora kinase A induces Mdm2-mediated destabilization and inhibition of p53." Nat Genet **36**(1): 55-62.

Kaufman, R. J. (1999). "Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls." Genes Dev **13**(10): 1211-33.

Kay, B. K., M. P. Williamson, et al. (2000). "The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains." Faseb J **14**(2): 231-41.

Keller, D. M., X. Zeng, et al. (2001). "A DNA damage-induced p53 serine 392 kinase complex contains CK2, hSpt16, and SSRP1." Mol Cell **7**(2): 283-92.

Kholmanskikh, O., A. Loriot, et al. (2008). "Expression of BORIS in melanoma: lack of association with MAGE-A1 activation." Int J Cancer **122**(4): 777-84.

Kim, C. A. and J. U. Bowie (2003). "SAM domains: uniform structure, diversity of function." Trends Biochem Sci **28**(12): 625-8.

Kim, Y. H., C. Y. Choi, et al. (1999). "Covalent modification of the homeodomain-interacting protein kinase 2 (HIPK2) by the ubiquitin-like protein SUMO-1." Proc Natl Acad Sci U S A **96**(22): 12350-5.

Kimmins, S. and P. Sassone-Corsi (2005). "Chromatin remodelling and epigenetic features of germ cells." Nature **434**(7033): 583-9.

Klenova, E. M., H. C. Morse, 3rd, et al. (2002). "The novel BORIS + CTCF gene family is uniquely involved in the epigenetics of normal biology and cancer." Semin Cancer Biol **12**(5): 399-414.

Knights, C. D., J. Catania, et al. (2006). "Distinct p53 acetylation cassettes differentially influence gene-expression patterns and cell fate." J Cell Biol **173**(4): 533-44.

Knuth, A., B. Danowski, et al. (1984). "T-cell-mediated cytotoxicity against autologous malignant melanoma: analysis with interleukin 2-dependent T-cell cultures." Proc Natl Acad Sci U S A **81**(11): 3511-5.

Knuth, A., T. Wolfel, et al. (1989). "Cytolytic T-cell clones against an autologous human melanoma: specificity study and definition of three antigens by immunoselection." Proc Natl Acad Sci U S A **86**(8): 2804-8.

Kobayashi, M., H. Taniura, et al. (2002). "Ectopic expression of necdin induces differentiation of mouse neuroblastoma cells." J Biol Chem **277**(44): 42128-35.

Kobet, E., X. Zeng, et al. (2000). "MDM2 inhibits p300-mediated p53 acetylation and activation by forming a ternary complex with the two proteins." Proc Natl Acad Sci U S A **97**(23): 12547-52.

Koken, M. H., G. Linares-Cruz, et al. (1995). "The PML growth-suppressor has an altered expression in human oncogenesis." Oncogene **10**(7): 1315-24.

Kozlov, S. V., J. W. Bogenpohl, et al. (2007). "The imprinted gene Magel2 regulates normal circadian output." Nat Genet **39**(10): 1266-72.

Krishnamurthy, J., C. Torrice, et al. (2004). "Ink4a/Arf expression is a biomarker of aging." J Clin Invest **114**(9): 1299-307.

Kruit, W. H., H. H. van Ojik, et al. (2005). "Phase 1/2 study of subcutaneous and intradermal immunization with a recombinant MAGE-3 protein in patients with detectable metastatic melanoma." Int J Cancer **117**(4): 596-604.

Kubbutat, M. H., S. N. Jones, et al. (1997). "Regulation of p53 stability by Mdm2." Nature **387**(6630): 299-303.

Kuerbitz, S. J., B. S. Plunkett, et al. (1992). "Wild-type p53 is a cell cycle checkpoint determinant following irradiation." Proc Natl Acad Sci U S A **89**(16): 7491-5.

Kumari, A., N. Schultz, et al. (2004). "p53 protects from replication-associated DNA double-strand breaks in mammalian cells." Oncogene **23**(13): 2324-9.

Kurita, M., T. Kuwajima, et al. (2006). "Necdin downregulates CDC2 expression to attenuate neuronal apoptosis." J Neurosci **26**(46): 12003-13.

Kurki, S., L. Latonen, et al. (2003). "Cellular stress and DNA damage invoke temporally distinct Mdm2, p53 and PML complexes and damage-specific nuclear relocalization." J Cell Sci **116**(Pt 19): 3917-25.

Kurki, S., K. Peltonen, et al. (2004). "Nucleolar protein NPM interacts with HDM2 and protects tumor suppressor protein p53 from HDM2-mediated degradation." Cancer Cell **5**(5): 465-75.

Kuwajima, T., I. Nishimura, et al. (2006). "Necdin promotes GABAergic neuron differentiation in cooperation with Dlx homeodomain proteins." J Neurosci **26**(20): 5383-92.

Kuwako, K., A. Hosokawa, et al. (2005). "Disruption of the paternal necdin gene diminishes TrkA signaling for sensory neuron survival." J Neurosci **25**(30): 7090-9.

Kuwako, K., H. Taniura, et al. (2004). "Necdin-related MAGE proteins differentially interact with the E2F1 transcription factor and the p75 neurotrophin receptor." J Biol Chem **279**(3): 1703-12.

Laduron, S., R. Deplus, et al. (2004). "MAGE-A1 interacts with adaptor SKIP and the deacetylase HDAC1 to repress transcription." Nucleic Acids Res **32**(14): 4340-50.

Lam, Y. W., L. Trinkle-Mulcahy, et al. (2005). "The nucleolus." J Cell Sci **118**(Pt 7): 1335-7.

Lambert, P. F., F. Kashanchi, et al. (1998). "Phosphorylation of p53 serine 15 increases interaction with CBP." J Biol Chem **273**(49): 33048-53.

Lane, D. P. and L. V. Crawford (1979). "T antigen is bound to a host protein in SV40-transformed cells." Nature **278**(5701): 261-3.

Langley, E., M. Pearson, et al. (2002). "Human SIR2 deacetylates p53 and antagonizes PML/p53-induced cellular senescence." Embo J **21**(10): 2383-96.

Lee, B. Y., J. A. Han, et al. (2006). "Senescence-associated beta-galactosidase is lysosomal beta-galactosidase." Aging Cell **5**(2): 187-95.

Lee, S., B. Elenbaas, et al. (1995). "p53 and its 14 kDa C-terminal domain recognize primary DNA damage in the form of insertion/deletion mismatches." Cell **81**(7): 1013-20.

Lee, S., S. Kozlov, et al. (2000). "Expression and imprinting of MAGEL2 suggest a role in Prader-willi syndrome and the homologous murine imprinting phenotype." Hum Mol Genet **9**(12): 1813-9.

Lee, W., T. S. Harvey, et al. (1994). "Solution structure of the tetrameric minimum transforming domain of p53." Nat Struct Biol **1**(12): 877-90.

Legube, G., L. K. Linares, et al. (2002). "Tip60 is targeted to proteasome-mediated degradation by Mdm2 and accumulates after UV irradiation." Embo J **21**(7): 1704-12.

Legube, G., L. K. Linares, et al. (2004). "Role of the histone acetyl transferase Tip60 in the p53 pathway." J Biol Chem **279**(43): 44825-33.

Leu, J. I., P. Dumont, et al. (2004). "Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex." Nat Cell Biol **6**(5): 443-50.

Leung, K. M., L. S. Po, et al. (2002). "The candidate tumor suppressor ING1b can stabilize p53 by disrupting the regulation of p53 by MDM2." Cancer Res **62**(17): 4890-3.

Li, H. and X. Wu (2004). "Histone deacetylase inhibitor, Trichostatin A, activates p21WAF1/CIP1 expression through downregulation of c-myc and release of the

repression of c-myc from the promoter in human cervical cancer cells." Biochem Biophys Res Commun **324**(2): 860-7.

Li, H. H., X. Cai, et al. (2007). "A specific PP2A regulatory subunit, B56gamma, mediates DNA damage-induced dephosphorylation of p53 at Thr55." Embo J **26**(2): 402-11.

Li, J., B. Lee, et al. (2006). "Endoplasmic reticulum stress-induced apoptosis: multiple pathways and activation of p53-up-regulated modulator of apoptosis (PUMA) and NOXA by p53." J Biol Chem **281**(11): 7260-70.

Li, J. and M. D. Tsai (2002). "Novel insights into the INK4-CDK4/6-Rb pathway: counter action of gankyrin against INK4 proteins regulates the CDK4-mediated phosphorylation of Rb." Biochemistry **41**(12): 3977-83.

Li, M., C. L. Brooks, et al. (2004). "A dynamic role of HAUSP in the p53-Mdm2 pathway." Mol Cell **13**(6): 879-86.

Li, M., C. L. Brooks, et al. (2003). "Mono- versus polyubiquitination: differential control of p53 fate by Mdm2." Science **302**(5652): 1972-5.

Li, M., D. Chen, et al. (2002). "Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization." Nature **416**(6881): 648-53.

Li, P. F., R. Dietz, et al. (1999). "p53 regulates mitochondrial membrane potential through reactive oxygen species and induces cytochrome c-independent apoptosis blocked by Bcl-2." Embo J **18**(21): 6027-36.

Lill, N. L., S. R. Grossman, et al. (1997). "Binding and modulation of p53 by p300/CBP coactivators." Nature **387**(6635): 823-7.

Lin, A. W., M. Barradas, et al. (1998). "Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling." Genes Dev **12**(19): 3008-19.

Lin, Y., W. Ma, et al. (2000). "Pidd, a new death-domain-containing protein, is induced by p53 and promotes apoptosis." Nat Genet **26**(1): 122-7.

Linares, L. K., A. Hengstermann, et al. (2003). "HdmX stimulates Hdm2-mediated ubiquitination and degradation of p53." Proc Natl Acad Sci U S A **100**(21): 12009-14.

Linke, S. P., S. Sengupta, et al. (2003). "p53 interacts with hRAD51 and hRAD54, and directly modulates homologous recombination." Cancer Res **63**(10): 2596-605.

Linzer, D. I. and A. J. Levine (1979). "Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells." Cell **17**(1): 43-52.

Liu, G., T. Xia, et al. (2003). "The activation domains, the proline-rich domain, and the C-terminal basic domain in p53 are necessary for acetylation of histones on the proximal p21 promoter and interaction with p300/CREB-binding protein." J Biol Chem **278**(19): 17557-65.

Liu, L., D. M. Scolnick, et al. (1999). "p53 sites acetylated in vitro by PCAF and p300 are acetylated in vivo in response to DNA damage." Mol Cell Biol **19**(2): 1202-9.

Liu, Q., S. Kaneko, et al. (2004). "Aurora-A abrogation of p53 DNA binding and transactivation activity by phosphorylation of serine 215." J Biol Chem **279**(50): 52175-82.

Liu, W., S. Cheng, et al. (2008). "The melanoma-associated antigen A3 mediates fibronectin-controlled cancer progression and metastasis." Cancer Res **68**(19): 8104-12.

Liu, X., C. W. Miller, et al. (1993). "The p53 activation domain binds the TATA box-binding polypeptide in Holo-TFIID, and a neighboring p53 domain inhibits transcription." Mol Cell Biol **13**(6): 3291-300.

Liu, X., P. Yue, et al. (2004). "p53 upregulates death receptor 4 expression through an intronic p53 binding site." Cancer Res **64**(15): 5078-83.

Longhese, M. P. (2008). "DNA damage response at functional and dysfunctional telomeres." Genes Dev **22**(2): 125-40.

Loriot, A., E. De Plaen, et al. (2006). "Transient down-regulation of DNMT1 methyltransferase leads to activation and stable hypomethylation of MAGE-A1 in melanoma cells." J Biol Chem **281**(15): 10118-26.

Loukinov, D. I., E. Pugacheva, et al. (2002). "BORIS, a novel male germ-line-specific protein associated with epigenetic reprogramming events, shares the same 11-zinc-finger domain with CTCF, the insulator protein involved in reading imprinting marks in the soma." Proc Natl Acad Sci U S A **99**(10): 6806-11.

Louria-Hayon, I., T. Grossman, et al. (2003). "The promyelocytic leukemia protein protects p53 from Mdm2-mediated inhibition and degradation." J Biol Chem **278**(35): 33134-41.

Lowe, S. W. (1999). "Activation of p53 by oncogenes." Endocr Relat Cancer **6**(1): 45-8.

Lowe, S. W., E. M. Schmitt, et al. (1993). "p53 is required for radiation-induced apoptosis in mouse thymocytes." Nature **362**(6423): 847-9.

Lu, X. (2005). "p53: a heavily dictated dictator of life and death." Curr Opin Genet Dev **15**(1): 27-33.

Lucas, S., F. Brasseur, et al. (1999). "A new MAGE gene with ubiquitous expression does not code for known MAGE antigens recognized by T cells." Cancer Res **59**(16): 4100-3.

Lucas, S., C. De Smet, et al. (1998). "Identification of a new MAGE gene with tumor-specific expression by representational difference analysis." Cancer Res **58**(4): 743-52.

Luo, J., A. Y. Nikolaev, et al. (2001). "Negative control of p53 by Sir2alpha promotes cell survival under stress." Cell **107**(2): 137-48.

Luo, J., F. Su, et al. (2000). "Deacetylation of p53 modulates its effect on cell growth and apoptosis." Nature **408**(6810): 377-81.

Ma, B., Y. Pan, et al. (2005). "Comparison of the protein-protein interfaces in the p53-DNA crystal structures: towards elucidation of the biological interface." Proc Natl Acad Sci U S A **102**(11): 3988-93.

MacDonald, H. R. and R. Wevrick (1997). "The necdin gene is deleted in Prader-Willi syndrome and is imprinted in human and mouse." Hum Mol Genet **6**(11): 1873-8.

MacLachlan, T. K. and W. S. El-Deiry (2002). "Apoptotic threshold is lowered by p53 transactivation of caspase-6." Proc Natl Acad Sci U S A **99**(14): 9492-7.

Mallette, F. A., M. F. Gaumont-Leclerc, et al. (2007). "The DNA damage signaling pathway is a critical mediator of oncogene-induced senescence." Genes Dev **21**(1): 43-8.

Mallette, F. A., S. Goumard, et al. (2004). "Human fibroblasts require the Rb family of tumor suppressors, but not p53, for PML-induced senescence." Oncogene **23**(1): 91-9.

Mantovani, F., F. Tocco, et al. (2007). "The prolyl isomerase Pin1 orchestrates p53 acetylation and dissociation from the apoptosis inhibitor iASPP." Nat Struct Mol Biol **14**(10): 912-20.

Marcotte, R., C. Lacelle, et al. (2004). "Senescent fibroblasts resist apoptosis by downregulating caspase-3." Mech Ageing Dev **125**(10-11): 777-83.

Marchand, M., N. van Baren, et al. (1999). "Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene MAGE-3 and presented by HLA-A1." Int J Cancer **80**(2): 219-30.

Marchenko, N. D., S. Wolff, et al. (2007). "Monoubiquitylation promotes mitochondrial p53 translocation." Embo J **26**(4): 923-34.

Marchenko, N. D., A. Zaika, et al. (2000). "Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling." J Biol Chem **275**(21): 16202-12.

Martindale, J. L. and N. J. Holbrook (2002). "Cellular response to oxidative stress: signaling for suicide and survival." J Cell Physiol **192**(1): 1-15.

Maruyama, K., M. Usami, et al. (1991). "A novel brain-specific mRNA encoding nuclear protein (necdin) expressed in neurally differentiated embryonal carcinoma cells." Biochem Biophys Res Commun **178**(1): 291-6.

Masuda, Y., A. Sasaki, et al. (2001). "Dlxin-1, a novel protein that binds Dlx5 and regulates its transcriptional function." J Biol Chem **276**(7): 5331-8.

Matsuda, T., H. Suzuki, et al. (2003). "The receptor tyrosine kinase Ror2 associates with the melanoma-associated antigen (MAGE) family protein Dlxin-1 and regulates its intracellular distribution." J Biol Chem **278**(31): 29057-64.

Mayo, L. D., J. E. Dixon, et al. (2002). "PTEN protects p53 from Mdm2 and sensitizes cancer cells to chemotherapy." J Biol Chem **277**(7): 5484-9.

Mayo, L. D. and D. B. Donner (2002). "The PTEN, Mdm2, p53 tumor suppressor-oncoprotein network." Trends Biochem Sci **27**(9): 462-7.

Mayo, L. D., Y. R. Seo, et al. (2005). "Phosphorylation of human p53 at serine 46 determines promoter selection and whether apoptosis is attenuated or amplified." J Biol Chem **280**(28): 25953-9.

McKinney, K., M. Mattia, et al. (2004). "p53 linear diffusion along DNA requires its C terminus." Mol Cell **16**(3): 413-24.

McKinney, K. and C. Prives (2002). "Efficient specific DNA binding by p53 requires both its central and C-terminal domains as revealed by studies with high-mobility group 1 protein." Mol Cell Biol **22**(19): 6797-808.

Melnick, A. and J. D. Licht (1999). "Deconstructing a disease: RARalpha, its fusion partners, and their roles in the pathogenesis of acute promyelocytic leukemia." Blood **93**(10): 3167-215.

Michaloglou, C., L. C. Vredeveld, et al. (2005). "BRAFE600-associated senescence-like cell cycle arrest of human naevi." Nature **436**(7051): 720-4.

Migliorini, D., E. Lazzerini Denchi, et al. (2002). "Mdm4 (Mdmx) regulates p53-induced growth arrest and neuronal cell death during early embryonic mouse development." Mol Cell Biol **22**(15): 5527-38.

Mihara, M., S. Erster, et al. (2003). "p53 has a direct apoptogenic role at the mitochondria." Mol Cell **11**(3): 577-90.

Milutinovic, S., A. C. D'Alessio, et al. (2007). "Valproate induces widespread epigenetic reprogramming which involves demethylation of specific genes." Carcinogenesis **28**(3): 560-71.

Minsky, N. and M. Oren (2004). "The RING domain of Mdm2 mediates histone ubiquitylation and transcriptional repression." Mol Cell **16**(4): 631-9.

Miyashita, T., M. Harigai, et al. (1994). "Identification of a p53-dependent negative response element in the bcl-2 gene." Cancer Res **54**(12): 3131-5.

Miyashita, T., S. Krajewski, et al. (1994). "Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo." Oncogene **9**(6): 1799-805.

Miyashita, T. and J. C. Reed (1995). "Tumor suppressor p53 is a direct transcriptional activator of the human bax gene." Cell **80**(2): 293-9.

Moller, A., H. Sirma, et al. (2003). "PML is required for homeodomain-interacting protein kinase 2 (HIPK2)-mediated p53 phosphorylation and cell cycle arrest but is dispensable for the formation of HIPK domains." Cancer Res **63**(15): 4310-4.

Momand, J., D. Jung, et al. (1998). "The MDM2 gene amplification database." Nucleic Acids Res **26**(15): 3453-9.

Momand, J., G. P. Zambetti, et al. (1992). "The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation." Cell **69**(7): 1237-45.

Monte, M., M. Simonatto, et al. (2006). "MAGE-A tumor antigens target p53 transactivation function through histone deacetylase recruitment and confer resistance to chemotherapeutic agents." Proc Natl Acad Sci U S A **103**(30): 11160-5.

Montes de Oca Luna, R., D. S. Wagner, et al. (1995). "Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53." Nature **378**(6553): 203-6.

Moroni, M. C., E. S. Hickman, et al. (2001). "Apaf-1 is a transcriptional target for E2F and p53." Nat Cell Biol **3**(6): 552-8.

Mujtaba, S., Y. He, et al. (2004). "Structural mechanism of the bromodomain of the coactivator CBP in p53 transcriptional activation." Mol Cell **13**(2): 251-63.

Murata, Y., T. Wakoh, et al. (2006). "Death-associated protein 3 regulates cellular senescence through oxidative stress response." FEBS Lett **580**(26): 6093-9.

Murphy, M., J. Ahn, et al. (1999). "Transcriptional repression by wild-type p53 utilizes histone deacetylases, mediated by interaction with mSin3a." Genes Dev **13**(19): 2490-501.

Murray-Zmijewski, F., E. A. Slee, et al. (2008). "A complex barcode underlies the heterogeneous response of p53 to stress." Nat Rev Mol Cell Biol **9**(9): 702-12.

Muscatelli, F., D. N. Abrous, et al. (2000). "Disruption of the mouse Necdin gene results in hypothalamic and behavioral alterations reminiscent of the human Prader-Willi syndrome." Hum Mol Genet **9**(20): 3101-10.

Muscatelli, F., A. P. Walker, et al. (1995). "Isolation and characterization of a MAGE gene family in the Xp21.3 region." Proc Natl Acad Sci U S A **92**(11): 4987-91.

Nagao, T., H. Higashitsuji, et al. (2003). "MAGE-A4 interacts with the liver oncoprotein gankyrin and suppresses its tumorigenic activity." J Biol Chem **278**(12): 10668-74.

Nagata, S. and P. Golstein (1995). "The Fas death factor." Science **267**(5203): 1449-56.

Nakada, Y., H. Taniura, et al. (1998). "The human chromosomal gene for necdin, a neuronal growth suppressor, in the Prader-Willi syndrome deletion region." Gene **213**(1-2): 65-72.

Nakano, K. and K. H. Vousden (2001). "PUMA, a novel proapoptotic gene, is induced by p53." Mol Cell **7**(3): 683-94.

Narita, M., S. Nunez, et al. (2003). "Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence." Cell **113**(6): 703-16.

Niinobe, M., K. Koyama, et al. (2000). "Cellular and subcellular localization of necdin in fetal and adult mouse brain." Dev Neurosci **22**(4): 310-9.

Nomoto, S., N. Haruki, et al. (1998). "Search for mutations and examination of allelic expression imbalance of the p73 gene at 1p36.33 in human lung cancers." Cancer Res **58**(7): 1380-3.

Nyberg, K. A., R. J. Michelson, et al. (2002). "Toward maintaining the genome: DNA damage and replication checkpoints." Annu Rev Genet **36**: 617-56.

O'Connor, L., A. W. Harris, et al. (2000). "CD95 (Fas/APO-1) and p53 signal apoptosis independently in diverse cell types." Cancer Res **60**(5): 1217-20.

Oberosler, P., P. Hloch, et al. (1993). "p53-catalyzed annealing of complementary single-stranded nucleic acids." Embo J **12**(6): 2389-96.

Oda, K., H. Arakawa, et al. (2000). "p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53." Cell **102**(6): 849-62.

Old, L. J. (1981). "Cancer immunology: the search for specificity--G. H. A. Clowes Memorial lecture." Cancer Res **41**(2): 361-75.

Old, L. J. (2007). "Cancer is a somatic cell pregnancy." Cancer Immun **7**: 19.

Oliner, J. D., J. A. Pietenpol, et al. (1993). "Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53." Nature **362**(6423): 857-60.

Olivier, M., S. P. Hussain, et al. (2004). "TP53 mutation spectra and load: a tool for generating hypotheses on the etiology of cancer." IARC Sci Publ(157): 247-70.

Otte, M., M. Zafrakas, et al. (2001). "MAGE-A gene expression pattern in primary breast cancer." Cancer Res **61**(18): 6682-7.

Palecek, E., V. Brazda, et al. (2004). "Enhancement of p53 sequence-specific binding by DNA supercoiling." Oncogene **23**(12): 2119-27.

Pan, Y. and J. Chen (2003). "MDM2 promotes ubiquitination and degradation of MDMX." Mol Cell Biol **23**(15): 5113-21.

Parant, J., A. Chavez-Reyes, et al. (2001). "Rescue of embryonic lethality in Mdm4-null mice by loss of Trp53 suggests a nonoverlapping pathway with MDM2 to regulate p53." Nat Genet **29**(1): 92-5.

Park, B. J., S. J. Lee, et al. (2000). "Frequent alteration of p63 expression in human primary bladder carcinomas." Cancer Res **60**(13): 3370-4.

Park, H. R., Y. W. Kim, et al. (2004). "Low expression of p63 and p73 in osteosarcoma." Tumori **90**(2): 239-43.

Park, J. H., G. H. Kong, et al. (2002). "hMAGE-A1 overexpression reduces TNF-alpha cytotoxicity in ME-180 cells." Mol Cells **14**(1): 122-9.

Pearson, M., R. Carbone, et al. (2000). "PML regulates p53 acetylation and premature senescence induced by oncogenic Ras." Nature **406**(6792): 207-10.

Peikert, T., U. Specks, et al. (2006). "Melanoma antigen A4 is expressed in non-small cell lung cancers and promotes apoptosis." Cancer Res **66**(9): 4693-700.

Pellegrini, G., E. Dellambra, et al. (2001). "p63 identifies keratinocyte stem cells." Proc Natl Acad Sci U S A **98**(6): 3156-61.

Pietenpol, J. A., T. Tokino, et al. (1994). "Sequence-specific transcriptional activation is essential for growth suppression by p53." Proc Natl Acad Sci U S A **91**(6): 1998-2002.

Pluquet, O., L. K. Qu, et al. (2005). "Endoplasmic reticulum stress accelerates p53 degradation by the cooperative actions of Hdm2 and glycogen synthase kinase 3beta." Mol Cell Biol **25**(21): 9392-405.

Pold, M., J. Zhou, et al. (1999). "Identification of a new, unorthodox member of the MAGE gene family." Genomics **59**(2): 161-7.

Polyak, K., Y. Xia, et al. (1997). "A model for p53-induced apoptosis." Nature **389**(6648): 300-5.

Prives, C. and P. A. Hall (1999). "The p53 pathway." J Pathol **187**(1): 112-26.

Puig, P., P. Capodieci, et al. (2003). "p73 Expression in human normal and tumor tissues: loss of p73alpha expression is associated with tumor progression in bladder cancer." Clin Cancer Res **9**(15): 5642-51.

Qu, L., S. Huang, et al. (2004). "Endoplasmic reticulum stress induces p53 cytoplasmic localization and prevents p53-dependent apoptosis by a pathway involving glycogen synthase kinase-3beta." Genes Dev **18**(3): 261-77.

Qu, L. and A. E. Koromilas (2004). "Control of tumor suppressor p53 function by endoplasmic reticulum stress." Cell Cycle **3**(5): 567-70.

Rapic-Otrin, V., M. P. McLenigan, et al. (2002). "Sequential binding of UV DNA damage binding factor and degradation of the p48 subunit as early events after UV irradiation." Nucleic Acids Res **30**(11): 2588-98.

Reczek, E. E., E. R. Flores, et al. (2003). "Multiple response elements and differential p53 binding control Perp expression during apoptosis." Mol Cancer Res **1**(14): 1048-57.

Regula, K. M. and L. A. Kirshenbaum (2001). "p53 activates the mitochondrial death pathway and apoptosis of ventricular myocytes independent of de novo gene transcription." J Mol Cell Cardiol **33**(8): 1435-45.

Reimertz, C., D. Kogel, et al. (2003). "Gene expression during ER stress-induced apoptosis in neurons: induction of the BH3-only protein Bbc3/PUMA and activation of the mitochondrial apoptosis pathway." J Cell Biol **162**(4): 587-97.

Ren, J., S. Lee, et al. (2003). "Absence of Ndn, encoding the Prader-Willi syndrome-deleted gene necdin, results in congenital deficiency of central respiratory drive in neonatal mice." J Neurosci **23**(5): 1569-73.

Restle, A., C. Janz, et al. (2005). "Differences in the association of p53 phosphorylated on serine 15 and key enzymes of homologous recombination." Oncogene **24**(27): 4380-7.

Rinaldo, C., A. Prodosmo, et al. (2007). "MDM2-regulated degradation of HIPK2 prevents p53Ser46 phosphorylation and DNA damage-induced apoptosis." Mol Cell **25**(5): 739-50.

Robertson, K. D. and P. A. Jones (1998). "The human ARF cell cycle regulatory gene promoter is a CpG island which can be silenced by DNA methylation and down-regulated by wild-type p53." Mol Cell Biol **18**(11): 6457-73.

Rodriguez, M. S., J. M. Desterro, et al. (1999). "SUMO-1 modification activates the transcriptional response of p53." Embo J **18**(22): 6455-61.

Rogner, U. C., K. Wilke, et al. (1995). "The melanoma antigen gene (MAGE) family is clustered in the chromosomal band Xq28." Genomics **29**(3): 725-31.

Rubbi, C. P. and J. Milner (2003). "Disruption of the nucleolus mediates stabilization of p53 in response to DNA damage and other stresses." Embo J **22**(22): 6068-77.

Rubbi, C. P. and J. Milner (2003). "p53 is a chromatin accessibility factor for nucleotide excision repair of DNA damage." Embo J **22**(4): 975-86.

Sabbatini, P. and F. McCormick (2002). "MDMX inhibits the p300/CBP-mediated acetylation of p53." DNA Cell Biol **21**(7): 519-25.

Saintigny, Y. and B. S. Lopez (2002). "Homologous recombination induced by replication inhibition, is stimulated by expression of mutant p53." Oncogene **21**(3): 488-92.

Sakaguchi, K., J. E. Herrera, et al. (1998). "DNA damage activates p53 through a phosphorylation-acetylation cascade." Genes Dev **12**(18): 2831-41.

Sakamuro, D., P. Sabbatini, et al. (1997). "The polyproline region of p53 is required to activate apoptosis but not growth arrest." Oncogene **15**(8): 887-98.

Sakurai, T., K. Itoh, et al. (2004). "A cleaved form of MAGE-A4 binds to Miz-1 and induces apoptosis in human cells." J Biol Chem **279**(15): 15505-14.

Salehi, A. H., P. P. Roux, et al. (2000). "NRAGE, a novel MAGE protein, interacts with the p75 neurotrophin receptor and facilitates nerve growth factor-dependent apoptosis." Neuron **27**(2): 279-88.

Salomoni, P. and P. P. Pandolfi (2002). "The role of PML in tumor suppression." Cell **108**(2): 165-70.

Sasaki, A., Y. Masuda, et al. (2002). "A RING finger protein Praja1 regulates Dlx5-dependent transcription through its ubiquitin ligase activity for the Dlx/Msx-interacting MAGE/Necdin family protein, Dlxin-1." J Biol Chem **277**(25): 22541-6.

Satyamoorthy, K., N. H. Chehab, et al. (2000). "Aberrant regulation and function of wild-type p53 in radioresistant melanoma cells." Cell Growth Differ **11**(9): 467-74.

Sax, J. K., P. Fei, et al. (2002). "BID regulation by p53 contributes to chemosensitivity." Nat Cell Biol **4**(11): 842-9.

Schlessinger, J. (2000). "Cell signaling by receptor tyrosine kinases." Cell **103**(2): 211-25.

Schmitt, C. A., J. S. Fridman, et al. (2002). "Dissecting p53 tumor suppressor functions in vivo." Cancer Cell **1**(3): 289-98.

Sengupta, S., S. P. Linke, et al. (2003). "BLM helicase-dependent transport of p53 to sites of stalled DNA replication forks modulates homologous recombination." Embo J **22**(5): 1210-22.

Sergeant, J., E. Taylor, et al. (2005). "Composition and architecture of the Schizosaccharomyces pombe Rad18 (Smc5-6) complex." Mol Cell Biol **25**(1): 172-84.

Serrano, M., A. W. Lin, et al. (1997). "Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a." Cell **88**(5): 593-602.

Seth, P., I. Krop, et al. (2002). "Novel estrogen and tamoxifen induced genes identified by SAGE (Serial Analysis of Gene Expression)." Oncogene **21**(5): 836-43.

Sharp, D. A., S. A. Kratowicz, et al. (1999). "Stabilization of the MDM2 oncoprotein by interaction with the structurally related MDMX protein." J Biol Chem **274**(53): 38189-96.

Shay, J. W., O. M. Pereira-Smith, et al. (1991). "A role for both RB and p53 in the regulation of human cellular senescence." Exp Cell Res **196**(1): 33-9.

Shen, T. H., H. K. Lin, et al. (2006). "The mechanisms of PML-nuclear body formation." Mol Cell **24**(3): 331-9.

Shen, W. G., Q. Y. Xue, et al. (2007). "Melanoma-associated antigen family protein-D1 regulation of tumor cell migration, adhesion to endothelium, and actin structures reorganization in response to hypoxic stress." Cell Commun Adhes **14**(1): 21-31.

Shibue, T., K. Takeda, et al. (2003). "Integral role of Noxa in p53-mediated apoptotic response." Genes Dev **17**(18): 2233-8.

Shieh, S. Y., M. Ikeda, et al. (1997). "DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2." Cell **91**(3): 325-34.

Shvarts, A., W. T. Steegenga, et al. (1996). "MDMX: a novel p53-binding protein with some functional properties of MDM2." Embo J **15**(19): 5349-57.

Sigal, A. and V. Rotter (2000). "Oncogenic mutations of the p53 tumor suppressor: the demons of the guardian of the genome." Cancer Res **60**(24): 6788-93.

Simpson, A. J., O. L. Caballero, et al. (2005). "Cancer/testis antigens, gametogenesis and cancer." Nat Rev Cancer **5**(8): 615-25.

Smith, M. L., I. T. Chen, et al. (1994). "Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen." Science **266**(5189): 1376-80.

Smith, M. L., J. M. Ford, et al. (2000). "p53-mediated DNA repair responses to UV radiation: studies of mouse cells lacking p53, p21, and/or gadd45 genes." Mol Cell Biol **20**(10): 3705-14.

Squires, S., J. A. Coates, et al. (2004). "p53 prevents the accumulation of double-strand DNA breaks at stalled-replication forks induced by UV in human cells." Cell Cycle **3**(12): 1543-57.

Stad, R., N. A. Little, et al. (2001). "Mdmx stabilizes p53 and Mdm2 via two distinct mechanisms." EMBO Rep **2**(11): 1029-34.

Stambolic, V., D. MacPherson, et al. (2001). "Regulation of PTEN transcription by p53." Mol Cell **8**(2): 317-25.

Stein, G. H., L. F. Drullinger, et al. (1999). "Differential roles for cyclin-dependent kinase inhibitors p21 and p16 in the mechanisms of senescence and differentiation in human fibroblasts." Mol Cell Biol **19**(3): 2109-17.

Stiewe, T., S. Zimmermann, et al. (2002). "Transactivation-deficient DeltaTA-p73 acts as an oncogene." Cancer Res **62**(13): 3598-602.

Sui, G., B. Affar el, et al. (2004). "Yin Yang 1 is a negative regulator of p53." Cell **117**(7): 859-72.

Suzuki, T., K. Yoshida, et al. (2007). "Melanoma-associated antigen-A1 expression predicts resistance to docetaxel and paclitaxel in advanced and recurrent gastric cancer." Oncol Rep **18**(2): 329-36.

Tachibana, M., J. Ueda, et al. (2005). "Histone methyltransferases G9a and GLP form heteromeric complexes and are both crucial for methylation of euchromatin at H3-K9." Genes Dev **19**(7): 815-26.

Taira, N., K. Nihira, et al. (2007). "DYRK2 is targeted to the nucleus and controls p53 via Ser46 phosphorylation in the apoptotic response to DNA damage." Mol Cell **25**(5): 725-38.

Takai, H., A. Smogorzewska, et al. (2003). "DNA damage foci at dysfunctional telomeres." Curr Biol **13**(17): 1549-56.

Takazaki, R., I. Nishimura, et al. (2002). "Necdin is required for terminal differentiation and survival of primary dorsal root ganglion neurons." Exp Cell Res **277**(2): 220-32.

Tanimura, S., S. Ohtsuka, et al. (1999). "MDM2 interacts with MDMX through their RING finger domains." FEBS Lett **447**(1): 5-9.

Taniura, H., K. Matsumoto, et al. (1999). "Physical and functional interactions of neuronal growth suppressor necdin with p53." J Biol Chem **274**(23): 16242-8.

Taniura, H., N. Taniguchi, et al. (1998). "Necdin, a postmitotic neuron-specific growth suppressor, interacts with viral transforming proteins and cellular transcription factor E2F1." J Biol Chem **273**(2): 720-8.

Tasdemir, E., M. C. Maiuri, et al. (2008). "Regulation of autophagy by cytoplasmic p53." Nat Cell Biol **10**(6): 676-87.

Tcherpakov, M., F. C. Bronfman, et al. (2002). "The p75 neurotrophin receptor interacts with multiple MAGE proteins." J Biol Chem **277**(51): 49101-4.

Thomas, M. C. and C. M. Chiang (2006). "The general transcription machinery and general cofactors." Crit Rev Biochem Mol Biol **41**(3): 105-78.

Thompson, T., C. Tovar, et al. (2004). "Phosphorylation of p53 on key serines is dispensable for transcriptional activation and apoptosis." J Biol Chem **279**(51): 53015-22.

Thurner, B., I. Haendle, et al. (1999). "Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma." J Exp Med **190**(11): 1669-78.

Tian, X. X., D. Rai, et al. (2005). "BRCA2 suppresses cell proliferation via stabilizing MAGE-D1." Cancer Res **65**(11): 4747-53.

Tibbetts, R. S., K. M. Brumbaugh, et al. (1999). "A role for ATR in the DNA damage-induced phosphorylation of p53." Genes Dev **13**(2): 152-7.

Tinel, A. and J. Tschopp (2004). "The PIDDosome, a protein complex implicated in activation of caspase-2 in response to genotoxic stress." Science **304**(5672): 843-6.

Toledo, F., C. J. Lee, et al. (2007). "Mouse mutants reveal that putative protein interaction sites in the p53 proline-rich domain are dispensable for tumor suppression." Mol Cell Biol **27**(4): 1425-32.

Traversari, C., P. van der Bruggen, et al. (1992). "Transfection and expression of a gene coding for a human melanoma antigen recognized by autologous cytolytic T lymphocytes." Immunogenetics **35**(3): 145-52.

Trougakos, I. P., A. Saridaki, et al. (2006). "Identification of differentially expressed proteins in senescent human embryonic fibroblasts." Mech Ageing Dev **127**(1): 88-92.

Uhle, S., O. Medalia, et al. (2003). "Protein kinase CK2 and protein kinase D are associated with the COP9 signalosome." Embo J **22**(6): 1302-12.

Unger, T., T. Juven-Gershon, et al. (1999). "Critical role for Ser20 of human p53 in the negative regulation of p53 by Mdm2." Embo J **18**(7): 1805-14.

van Baren, N., F. Brasseur, et al. (1999). "Genes encoding tumor-specific antigens are expressed in human myeloma cells." Blood **94**(4): 1156-64.

van der Bruggen, P., C. Traversari, et al. (1991). "A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma." Science **254**(5038): 1643-7.

Van Pel, A., P. van der Bruggen, et al. (1995). "Genes coding for tumor antigens recognized by cytolytic T lymphocytes." Immunol Rev **145**: 229-50.

Vatolin, S., Z. Abdullaev, et al. (2005). "Conditional expression of the CTCF-paralogous transcriptional factor BORIS in normal cells results in demethylation and derepression

of MAGE-A1 and reactivation of other cancer-testis genes." Cancer Res **65**(17): 7751-62.

Vaziri, H., S. K. Dessain, et al. (2001). "hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase." Cell **107**(2): 149-59.

Venot, C., M. Maratrat, et al. (1998). "The requirement for the p53 proline-rich functional domain for mediation of apoptosis is correlated with specific PIG3 gene transactivation and with transcriptional repression." Embo J **17**(16): 4668-79.

Venot, C., M. Maratrat, et al. (1999). "Definition of a p53 transactivation function-deficient mutant and characterization of two independent p53 transactivation subdomains." Oncogene **18**(14): 2405-10.

Villunger, A., E. M. Michalak, et al. (2003). "p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa." Science **302**(5647): 1036-8.

Walker, K. K. and A. J. Levine (1996). "Identification of a novel p53 functional domain that is necessary for efficient growth suppression." Proc Natl Acad Sci U S A **93**(26): 15335-40.

Wang, Z. G., D. Ruggero, et al. (1998). "PML is essential for multiple apoptotic pathways." Nat Genet **20**(3): 266-72.

Waterman, M. J., E. S. Stavridi, et al. (1998). "ATM-dependent activation of p53 involves dephosphorylation and association with 14-3-3 proteins." Nat Genet **19**(2): 175-8.

Weber, J., M. Salgaller, et al. (1994). "Expression of the MAGE-1 tumor antigen is up-regulated by the demethylating agent 5-aza-2'-deoxycytidine." Cancer Res **54**(7): 1766-71.

Weber, J. D., L. J. Taylor, et al. (1999). "Nucleolar Arf sequesters Mdm2 and activates p53." Nat Cell Biol **1**(1): 20-6.

Wei, W., U. Herbig, et al. (2003). "Loss of retinoblastoma but not p16 function allows bypass of replicative senescence in human fibroblasts." EMBO Rep **4**(11): 1061-6.

Wei, X., Z. K. Yu, et al. (2003). "Physical and functional interactions between PML and MDM2." J Biol Chem **278**(31): 29288-97.

White, D. E., K. E. Talbott, et al. (2006). "Mouse double minute 2 associates with chromatin in the presence of p53 and is released to facilitate activation of transcription." Cancer Res **66**(7): 3463-70.

Wischnewski, F., K. Pantel, et al. (2006). "Promoter demethylation and histone acetylation mediate gene expression of MAGE-A1, -A2, -A3, and -A12 in human cancer cells." Mol Cancer Res **4**(5): 339-49.

Wong, K. B., B. S. DeDecker, et al. (1999). "Hot-spot mutants of p53 core domain evince characteristic local structural changes." Proc Natl Acad Sci U S A **96**(15): 8438-42.

Wright, W. E. and J. W. Shay (2001). "Cellular senescence as a tumor-protection mechanism: the essential role of counting." Curr Opin Genet Dev **11**(1): 98-103.

Wu, G. S., T. F. Burns, et al. (1997). "KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene." Nat Genet **17**(2): 141-3.

Wulf, G. M., Y. C. Liou, et al. (2002). "Role of Pin1 in the regulation of p53 stability and p21 transactivation, and cell cycle checkpoints in response to DNA damage." J Biol Chem **277**(50): 47976-9.

Xiao, J. and H. S. Chen (2004). "Biological functions of melanoma-associated antigens." World J Gastroenterol **10**(13): 1849-53.

Xirodimas, D. P., J. Chisholm, et al. (2002). "P14ARF promotes accumulation of SUMO-1 conjugated (H)Mdm2." FEBS Lett **528**(1-3): 207-11.

Xirodimas, D. P., M. K. Saville, et al. (2004). "Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity." Cell **118**(1): 83-97.

Xue, L., B. Zhou, et al. (2003). "Wild-type p53 regulates human ribonucleotide reductase by protein-protein interaction with p53R2 as well as hRRM2 subunits." Cancer Res **63**(5): 980-6.

Yang, A., M. Kaghad, et al. (2002). "On the shoulders of giants: p63, p73 and the rise of p53." Trends Genet **18**(2): 90-5.

Yang, A., M. Kaghad, et al. (1998). "p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities." Mol Cell **2**(3): 305-16.

Yang, A., R. Schweitzer, et al. (1999). "p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development." Nature **398**(6729): 714-8.

Yang, A., N. Walker, et al. (2000). "p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours." Nature **404**(6773): 99-103.

Yang, B., S. O'Herrin, et al. (2007). "Select cancer testes antigens of the MAGE-A, -B, and -C families are expressed in mast cell lines and promote cell viability in vitro and in vivo." J Invest Dermatol **127**(2): 267-75.

Yang, B., S. M. O'Herrin, et al. (2007). "MAGE-A, mMage-b, and MAGE-C proteins form complexes with KAP1 and suppress p53-dependent apoptosis in MAGE-positive cell lines." Cancer Res **67**(20): 9954-62.

Yang, W., L. M. Rozan, et al. (2007). "CARPs are ubiquitin ligases that promote MDM2-independent p53 and phospho-p53ser20 degradation." J Biol Chem **282**(5): 3273-81.

Yang, W. H., J. E. Kim, et al. (2006). "Modification of p53 with O-linked N-acetylglucosamine regulates p53 activity and stability." Nat Cell Biol **8**(10): 1074-83.

Ye, X., B. Zerlanko, et al. (2007). "Definition of pRB- and p53-dependent and -independent steps in HIRA/ASF1a-mediated formation of senescence-associated heterochromatin foci." Mol Cell Biol **27**(7): 2452-65.

Yoon, I. K., H. K. Kim, et al. (2004). "Exploration of replicative senescence-associated genes in human dermal fibroblasts by cDNA microarray technology." Exp Gerontol **39**(9): 1369-78.

Yoshikawa, K. (2000). "Cell cycle regulators in neural stem cells and postmitotic neurons." Neurosci Res **37**(1): 1-14.

Yu, J., Z. Wang, et al. (2003). "PUMA mediates the apoptotic response to p53 in colorectal cancer cells." Proc Natl Acad Sci U S A **100**(4): 1931-6.

Yu, J., L. Zhang, et al. (2001). "PUMA induces the rapid apoptosis of colorectal cancer cells." Mol Cell **7**(3): 673-82.

Zacchi, P., M. Gostissa, et al. (2002). "The prolyl isomerase Pin1 reveals a mechanism to control p53 functions after genotoxic insults." Nature **419**(6909): 853-7.

Zanella, S., F. Watrin, et al. (2008). "Necdin plays a role in the serotonergic modulation of the mouse respiratory network: implication for Prader-Willi syndrome." J Neurosci **28**(7): 1745-55.

Zhan, Q., M. J. Antinore, et al. (1999). "Association with Cdc2 and inhibition of Cdc2/Cyclin B1 kinase activity by the p53-regulated protein Gadd45." Oncogene **18**(18): 2892-900.

Zhang, H., K. H. Pan, et al. (2003). "Senescence-specific gene expression fingerprints reveal cell-type-dependent physical clustering of up-regulated chromosomal loci." Proc Natl Acad Sci U S A **100**(6): 3251-6.

Zhang, L., J. Yu, et al. (2000). "Role of BAX in the apoptotic response to anticancer agents." Science **290**(5493): 989-92.

Zhang, R., W. Chen, et al. (2007). "Molecular dissection of formation of senescence-associated heterochromatin foci." Mol Cell Biol **27**(6): 2343-58.

Zhang, R., M. V. Poustovoitov, et al. (2005). "Formation of MacroH2A-containing senescence-associated heterochromatin foci and senescence driven by ASF1a and HIRA." Dev Cell **8**(1): 19-30.

Zhang, Y., G. W. Wolf, et al. (2003). "Ribosomal protein L11 negatively regulates oncoprotein MDM2 and mediates a p53-dependent ribosomal-stress checkpoint pathway." Mol Cell Biol **23**(23): 8902-12.

Zhang, X. M., Y. F. Zhang, et al. (2008). "The anti-tumor immune response induced by a combination of MAGE-3/MAGE-n-derived peptides." Oncol Rep **20**(1): 245-52.

Zhou, M., L. Gu, et al. (2003). "PTEN reverses MDM2-mediated chemotherapy resistance by interacting with p53 in acute lymphoblastic leukemia cells." Cancer Res **63**(19): 6357-62.

Zhu, H., L. Wu, et al. (2003). "MDM2 and promyelocytic leukemia antagonize each other through their direct interaction with p53." J Biol Chem **278**(49): 49286-92.

Zhu, J., D. Woods, et al. (1998). "Senescence of human fibroblasts induced by oncogenic Raf." Genes Dev **12**(19): 2997-3007.

Zhu, X., S. L. Asa, et al. (2008). "Fibroblast growth factor 2 and estrogen control the balance of histone 3 modifications targeting MAGE-A3 in pituitary neoplasia." Clin Cancer Res **14**(7): 1984-96.

Zink, D., C. Mayr, et al. (2002). "Association of p53 and MSH2 with recombinative repair complexes during S phase." Oncogene **21**(31): 4788-800.

Zotchev, S. B., M. Protopopova, et al. (2000). "p53 C-terminal interaction with DNA ends and gaps has opposing effect on specific DNA binding by the core." Nucleic Acids Res **28**(20): 4005-12.