ISAS - INTERNATIONAL SCHOOL
FOR ADVANCED STUDIES

Novel mechanisms for modulating p53 activity

Thesis submitted for the Degree of Doctor Philosophiae

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INTRODUCTION 1

STRUCTURE AND BIOCHEMICAL FUNCTIONS OF THE P53 PROTEIN 3
1. The transactivation domain 4
2. The Proline-rich domain 5
3. The DNA binding domain 5
4. The tetramerization domain 7
5. The C-terminal regulatory domain 8

BIOLOGICAL RESPONSES INDUCED BY P53 ACTIVATION 9
1. Cell cycle arrest 10
2. Apoptosis 12
3. DNA repair 14

REGULATION OF P53 FUNCTION 16
1. Regulation of p53 stability 17
2. Activation of p53 sequence-specific transactivation 21
3. Determination of the selectivity of p53 response 24
4. Regulation of p53 activity by changes in subcellular distribution 27
5. Inhibition of p53 functions by viral proteins 30

RESULTS 31

PART 1 31
ACTIVATION OF P53 BY CONJUGATION TO THE UBIQUITIN-LIKE PROTEIN SUMO-1 31
Isolation of SUMO-1 as a candidate p53-interacting protein 31
SUMO-1 is conjugated to p53 in vitro 35
The lysine residue at position 386 of p53 is required for SUMO-1 modification 35
p53 is covalently modified by SUMO-1 in vivo 37
SUMO-1 conjugation to p53 in vivo requires lysine 386 38
The C terminus of p53 is sufficient to be targeted by SUMO-1 conjugation in vivo 40
SUMO-1 modification enhances p53-dependent transactivation 41
SUMO-1 and hUbc9 act in a cooperative way in modifying p53 45

PART 2 47
RECRUITMENT OF P53 INTO NUCLEAR BODIES BY A SPECIFIC PML ISOFORM MODULATES ITS TRANSCRIPTIONAL ACTIVITY AND AFFECTS CELL SURVIVAL 47
PML mediates the relocalization of p53 into Nuclear Bodies 50
Dissection of p53 region required for NBs localization 53
p53 binds to PML3 with the domain required for NBs targeting 56
Targeting of p53 to NBs depends on a specific PML isoform 57
Analysis of p53-containing nuclear bodies 59
PML3-dependent recruitment of p53 into NBs increases p53 transcriptional activity and affects cell survival 62

DISCUSSION 65

CONCLUSIONS 72

MATERIALS AND METHODS 73

REFERENCES 78

ACKNOWLEDGEMENTS 86

INTRODUCTION

Twenty years after its discovery, the p53 tumor suppressor has become one of the most intensively studied and best characterized molecules in cancer research.

Initially believed to function as an oncogene[1], it is now regarded as one of the more important cellular tumor suppressors, as underlined by the evidence that the p53 gene is mutated or deleted in more than 50% of human cancers[2].

The current model of the action of p53 suggests that it may work as a molecular "stress-responsive device". This tumor suppressor, in fact, is not required for proliferation of normal cells and is dispensable during mammalian development. Instead, its function in maintaining genetic stability become essential when cells are exposed to multiple environmental stresses, including DNA damage, hypoxia, activated oncogenes, ribonucleotide depletion, microtubule disruption and modulation of the redox potential[3,4].

All of these stimuli are believed to trigger signal transduction cascades, which converge on p53 and mediate its activation. Once activated, p53 is able to coordinate a complex cellular response aimed to prevent unrepaired DNA damage to turn into permanent mutation. Depending on the stress and cell type, however, the response evoked by p53 activation can be as different as reversible cell-cycle arrest, irreversible senescent-like state or apoptosis[5].

Despite the heterogeneity of the stimuli acting on p53 and of the biological responses that can be induced, two common events accompany p53 activation: (i) stabilization and accumulation of the protein in the nucleus of the damaged cell and (ii) conformational shift that allow the protein to bind to DNA and transcriptionally regulate a list of target genes[5].

The best studied function of p53 is, in fact, that of a transcription factor: when activated, p53 is able to bind to specific DNA sequences and to promote transcription of target genes involved in growth arrest, DNA repair and apoptosis[3,6]. Sequence-specific transactivation (SST), however, is not the only known function of p53, since it has been demonstrated to act also as a transcriptional repressor[6]. Moreover, transactivation-independent p53 functions can be performed by interacting with other cellular proteins[7].

The efficiency of p53 in controlling cell growth is indispensable to prevent malignant progression, but this activity must be tightly restrained in normal cells to allow physiological cell proliferation and development. It has been demonstrated that, while the absence of p53 is almost not affecting embryonic development, its deregulated activity has catastrophic effects in the embryo[8]. Efficient turnover of the protein in normal cells prevents p53 accumulation and its activation has to be kept under a stringent and complex control, which is mainly achieved through post-translational modifications[9].

In many tumors, p53 functions are inactivated by diverse mechanisms. In most of the cases, transformed cells express mutated p53 proteins that fail to bind to DNA and therefore to activate p53 target genes. However, also increased degradation of the protein as well as its sequestration out of the nucleus have been described[3,6]. Although loss of wild-type (wt) p53 function clearly contributes to tumor development, recent observations have elucidated an involvement of the mutant protein in tumor progression as well. Mutant p53 can contribute to inactivation of the wild type counterpart in a dominant-negative manner. Moreover, some mutants are capable of conferring increased tumorigenicity and metastatic potential, indicating a possible "gain of function" of these proteins[6].

Given the central role of p53 in controlling cell growth and transformation, it is important to increase our knowledge of the pathways modulating its activation, in order to allow the design of specific modulators of p53 activity and the development of novel anti-cancer therapies.

STRUCTURE AND BIOCHEMICAL FUNCTIONS OF THE p53 PROTEIN

The human p53 protein consists of 393 amino acids and can be divided from a structural and functional point of view into 5 domains. In addition, sequence comparison analysis between p53 from different organisms led to the identification of five regions that has been conserved during evolution (box 1-5; aa 13-23, 117-142, 171-181, 234-250 and 270-286), which, as expected, were shown to be crucial for the activity of the protein[6] (see Figure 1).

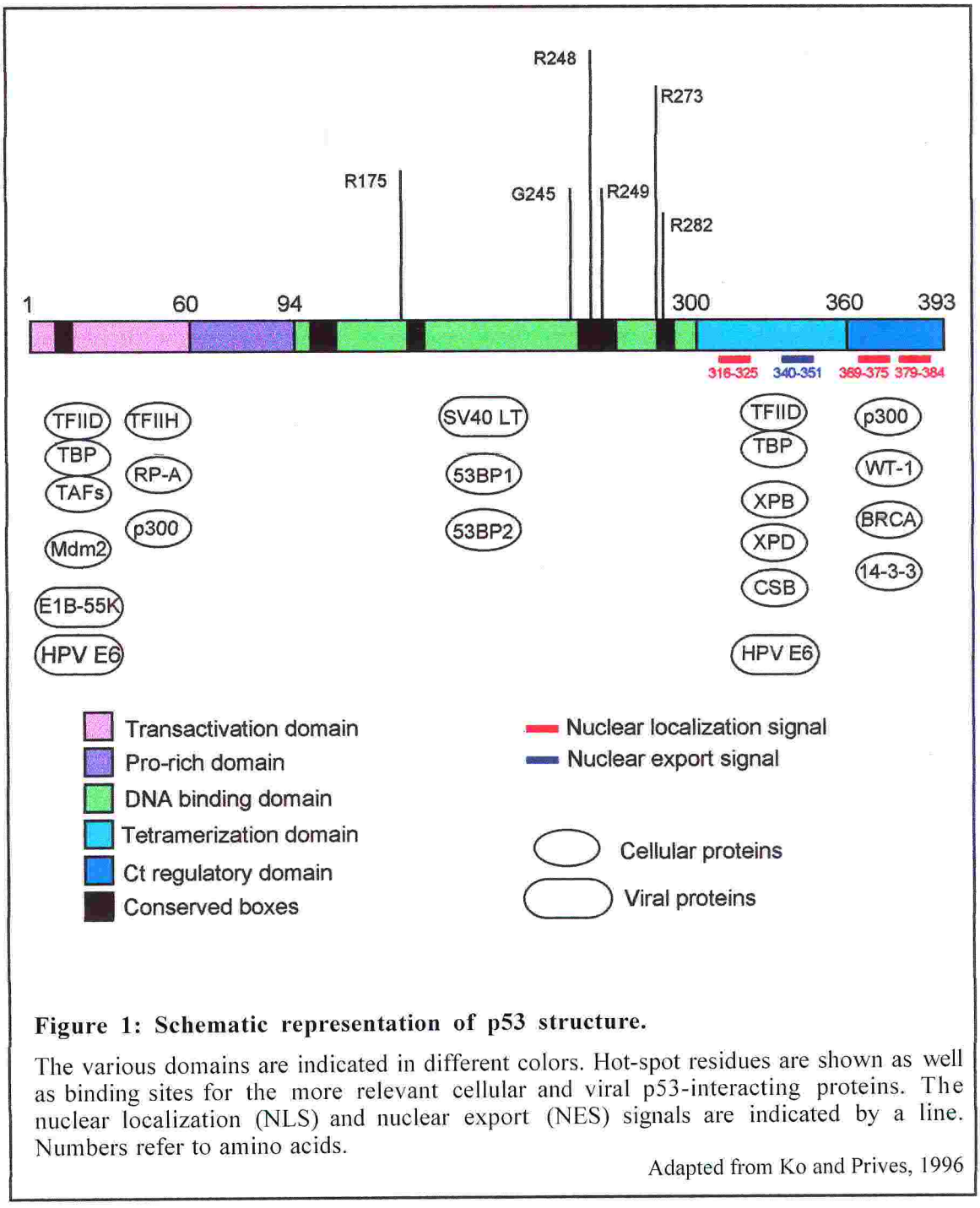


Figure 1: Schematic representation of p53 structure.

The various domains are indicated in different colors. Hot-spot residues are shown as well as binding sites for the more relevant cellular and viral p53-interacting proteins. The nuclear localization (NLS) and nuclear export (NES) signals are indicated by a line. Numbers refer to amino acids.

Adapted from Ko and Prives, 1996

1. The transactivation domain

The amino-terminal 42 residues of p53 contain an acidic domain, that is able to activate transcription as efficiently as the herpes virus protein VP16, which contains the strongest known activation domain[10,11]. This region allows the recruitment of the basal transcription machinery and, in particular, the amino acids comprised in the conserved box I have been shown to bind directly to components of TFIID, like TBP (TATA-binding protein) and several associated factors (TAFs)[12,13]. The N terminus of p53 interacts also with proteins involved in DNA repair, as the single-stranded DNA binding protein RP-A and the p62 subunit of the transcription/repair factor TFIIH[14,15] (see Figure 1). Residues 22 and 23, which are highly conserved, have been demonstrated to directly contact TAFII40 and TAFII60 and to be required for p53 sequence-specific transactivation (SST)[12,13]. Interestingly, the same residues are targeted by the competitive interaction with viral (E1B55K, HBX) or cellular (Mdm2) proteins that inhibit p53 SST, therefore resulting in abrogation of p53 functions and tumorigenesis[16-18]. In addition, binding to Mdm2 has also been proved to modulate p53 cellular localization and stability, underlining how the amino terminus of p53 is not only instrumental for the function of the protein as a transcription factor, but is also crucial in regulating its activity[19].

Recently this domain has been reported to bind to the transcriptional co-activator p300/CBP[20,21]. The current model is that p300, by binding to p53, is able to increase its transcriptional activity in two distinct ways: on one side, p300 HAT activity mediates hystones acetylation and chromatin rearrangement in the promoter region, therefore stimulating transcription. On the other side, p300 can directly acetylate p53 in the C-terminal region and increase its ability to bind to DNA[22].

Finally, several data demonstrated that p53 may repress transcription from a number of promoters lacking its consensus binding site, as c-fos, c-jun, c-myc, IL-6, hsc-70, MAP4 and others[6]. It is generally believed that this transrepression function does not involve p53 interaction with DNA but rather arise from the binding to TBP and the consequent sequestration of this important factor from the promoters. However, recent evidences suggest that also other mechanisms can be involved and in the case of cdc25c it has been showed that repression is due to direct p53 binding to the promoter, resulting in displacement of the Sp1 factor from its site (J. Manfredi, 10th p53 Workshop, 2000).

2. The Proline-rich domain

p53 residues between 60 and 94 contain five PXXP repeats (Figure 1), two of which are conserved in mouse[23]. The function of this region has only recently been explored and it has been demonstrated that the Pro-rich domain is somehow involved in inducing apoptosis and suppressing tumor cells growth[23,24]. Moreover, it is also required for the p53-dependent growth arrest mediated by Gas1, a protein highly expressed in cells arrested at the G0 phase[25]. Recent studies demonstrated that this domain, although not affecting the activity of several p53-responsive promoters, is instead required for specific transactivation of the pro-apoptotic gene PIG3 and is also involved in transcriptional repression[26].

Repeats of the PXXP motif represent docking sites for SH3 domain-containing proteins that are usually involved in signal transduction pathways: therefore it has been hypothesized that the Pro-rich domain may mediate interaction with such a protein. Despite preliminary reports, indicating possible interaction partners, like the non-receptor tyrosine kinase c-Abl or the adapter protein Grb2, a definitive evidence of a cellular protein that binds to p53 Pro-rich region is still missing.

Population analysis identified within this region of the p53 gene a sequence polymorphism resulting in either a Pro or an Arg residue at position 72, with the p53Arg variant having abolished the last PXXP repeat. Interestingly, genetic studies indicated a correlation between the presence of homozygous p53Arg genotype and the risk of development of human papilloma virus (HPV)-associated cervical cancers. Accordingly, the p53Arg variant appeared to be more susceptible than p53Pro to degradation mediated by the E6 protein from high risk HPV[27].

3. The DNA binding domain

The central part of p53, spanning residues from 100 to 300, corresponds to a proteolysis-resistant core domain, which has been shown to bind to DNA in a sequence-specific manner[28,29].

The p53 consensus binding site is composed by two copies of the palyndromic 10 bp motif: 5'PuPuPuC(A/T)(T/A)GPyPyPy3', separated by 0-13 nucleotides[28]. The internal symmetry of the four half-sites suggests that p53 binds to DNA as a tetramer and this evidence is confirmed by several experimental data and consistent with the known crystal structure of the protein[30].

When bound to DNA, through its ability to interact with components of the basal transcription machinery, p53 is able to assemble the initiation complex and to stimulate transcription even in the absence of a proper minimal promoter.

Underlining the importance of this region, more than 90% of the mutations that inactivate p53 in human cancers have been localized in the DNA binding domain. In particular, the observed mutations in p53 gene more frequently involve only some residues (so-called "hot-spots", aa 175, 245, 248, 273 and 282) that are believed to be crucial either for contacting DNA or maintaining the overall structure of the domain[2] (see Figure 1). Strikingly, the majority of mutant p53 proteins isolated from cancers are impaired in binding to DNA and in activating transcription of target genes, therefore being defective in triggering the whole p53-dependent response to DNA damage. Since p53 binds to DNA in a tetrameric form, the mutation of a single p53 allele in a cell has been proposed to abrogate p53 functions with a dominant-negative mechanism, by forming heteromeric complexes with the wild-type counterpart.

Even if many genes that have been described to be activated by p53 contain a binding site similar to the consensus sequence, there is some variability both in the nucleotidic sequence and in the length of the segment separating the two decamers. Moreover, several p53-regulated genes contain responsive elements with four or more decamers. The binding site has been found either in the promoter regulatory region or in introns of induced genes[31]. Thus, it appears that p53 can recognize diverse sequences and that not all the binding sites are the same. Recent studies revealed how different post-translational modifications or interactions with cellular proteins can modulate p53 affinity for a particular responsive element[7]. Moreover, chromatin configuration in the promoter region seems also to be an important determinant of sequence recognition and transcriptional activation[32].

X-ray crystallography analysis revealed the structure of p53 bound to DNA. The core domain consist of a β-sandwich mediating head-to-tail dimerization of the protein and acting as a scaffold for three loops that contact directly DNA both in the major and in the minor groove and that are coordinated by a zinc ion[30]. Interestingly, two of the residues that are more often mutated in cancers, Arg 248 and Arg 273, are responsible of contacting the phosphate backbone of DNA, therefore providing a clear explanation for the loss of function of the mutant protein. Other mutations instead involve residues that are crucial for maintaining the overall conformation of the domain.

4. The tetramerization domain

p53 has been shown to form tetramer in solution via an oligomerization domain comprised between amino acids 323 and 356 and connected to the DNA binding domain by a flexible linker.

This segment can be divided into two functional sites, the first necessary for association of two p53 monomers into a dimer and the second required for assembly of two dimers into a tetramer. Studies with p53 deletion mutants demonstrated that removal of 38 C-terminal residues generates a protein that cannot tetramerize but forms stable homodimers. This protein is still able to bind to DNA and to activate transcription of p53 responsive promoters in *in vitro* assays. Instead, removal of 17 more residues generates a monomeric protein unable to recognize its specific target site[33].

In agreement with these observations, X-ray crystallography and NMR studies demonstrated that the p53 tetramer can be described as a "dimer of dimers": two monomeric peptides interacts in an antiparallel orientation and then the two dimers are held together by α-helical regions to form a four-helix bundle[34,35]. A recent work, moreover, established that formation of the p53 oligomers is due to co-translational dimerization of p53 monomers followed by post-translational tetramerization of the dimers (P.W.K. Lee, 10th p53 Workshop, 2000).

Regardless of the evidences from *in vitro* approaches, *in vivo* experiments indicated that tetramerization is required for efficient transactivation and for p53-mediated suppression of growth of carcinoma cell lines[36].

Within the tetramerization domain is comprised the most important p53 nuclear localization signal (NLS), spanning residues 316-325 (Figure 1). Mutagenesis in this region induces the synthesis of a p53 protein, which is almost completely cytoplasmic[37,38].

More recently, a nuclear export signal (NES) has also been mapped in this domain (residues 340-351, see Figure 1). This NES has been shown to be exposed and functional in the dimeric protein, but to be buried in the oligomerization domain when the tetramer is formed. Therefore a model has been proposed in which p53 is able to shuttle in and out form the nucleus when not in the tetrameric form, while the tetramer requires interaction with other export factors to exit the nucleus[39].

5. The C-terminal regulatory domain

The last 30 amino acids of p53 have recently been demonstrated to play an important regulatory role on the functions of the protein. Several lines of evidence indicate that stabilization of the protein is not enough to activate p53 functions but that also a conformational shift between a latent and an active form is required[40] (see Figure 2). This result can be obtained by deleting the C-terminal region, by binding of short peptides or antibodies, by interaction with single-stranded DNA or RNA fragments or through post-translational modification like phosphorylation or acetylation[41]. Accordingly to the "allosteric model" for regulation of p53 activity, the most C-terminal residues of the protein act as negative regulator on its DNA-binding capability, probably by interacting with some sequences in the core domain and keeping the protein in a "locked" conformation[40,42].

Alternatively, the "reciprocal interference" model proposes that the non-specific DNA binding function of the carboxyl terminus can compete with sequence-specific recognition of the p53 target promoters[43].

Modifications in this C-terminal segment cause an allosteric conformational change in the structure of the protein or prevent its non-specific association with DNA, rendering it competent for sequence-specific DNA binding and transactivation.

The ability of this region to bind to DNA ends and internal deletion loops generated by replication errors led to the hypothesis that it may act by directing p53 to the sites of DNA damage and consequently mediating the functional activation of the protein[32].

Given its important regulatory functions, this domain is target of several post-translational modifications and has been described to interact with various cellular and viral proteins[4] (see Figures 1 and 4).

Moreover, two accessory NLS has been mapped between residues 369-375 and 379-384[37,38] (Figure 1).

Finally, it should be noted that two alternatively spliced forms of p53, differing in the last amino acids and constitutively activated for DNA-binding, have been described in mouse [44].

BIOLOGICAL RESPONSES INDUCED BY p53 ACTIVATION

Eukaryotic cells have developed a network of highly conserved mechanisms (checkpoints) which ensure that damaged chromosomes are repaired before being duplicated or segregated. The tumor suppressor protein p53 was shown to be a key element of these checkpoints, acting at different levels of control during cell cycle[45]. Although being involved in differentiation and development, as well as in senescence and angiogenesis, p53 has no clear role in normal growth of most cell types. The evidence that transgenic mice lacking both p53 alleles mature normally *in utero*, but are more prone to tumor development, underlines the central role of this protein in controlling genomic integrity and cell viability[41]. After being activated by different kind of DNA damage or other cellular stresses, p53 mediates growth arrest to allow repair of the lesion or alternatively, depending on the intensity of the damage as well as on the cell type, triggers the programmed cell death pathway (Figure 2).

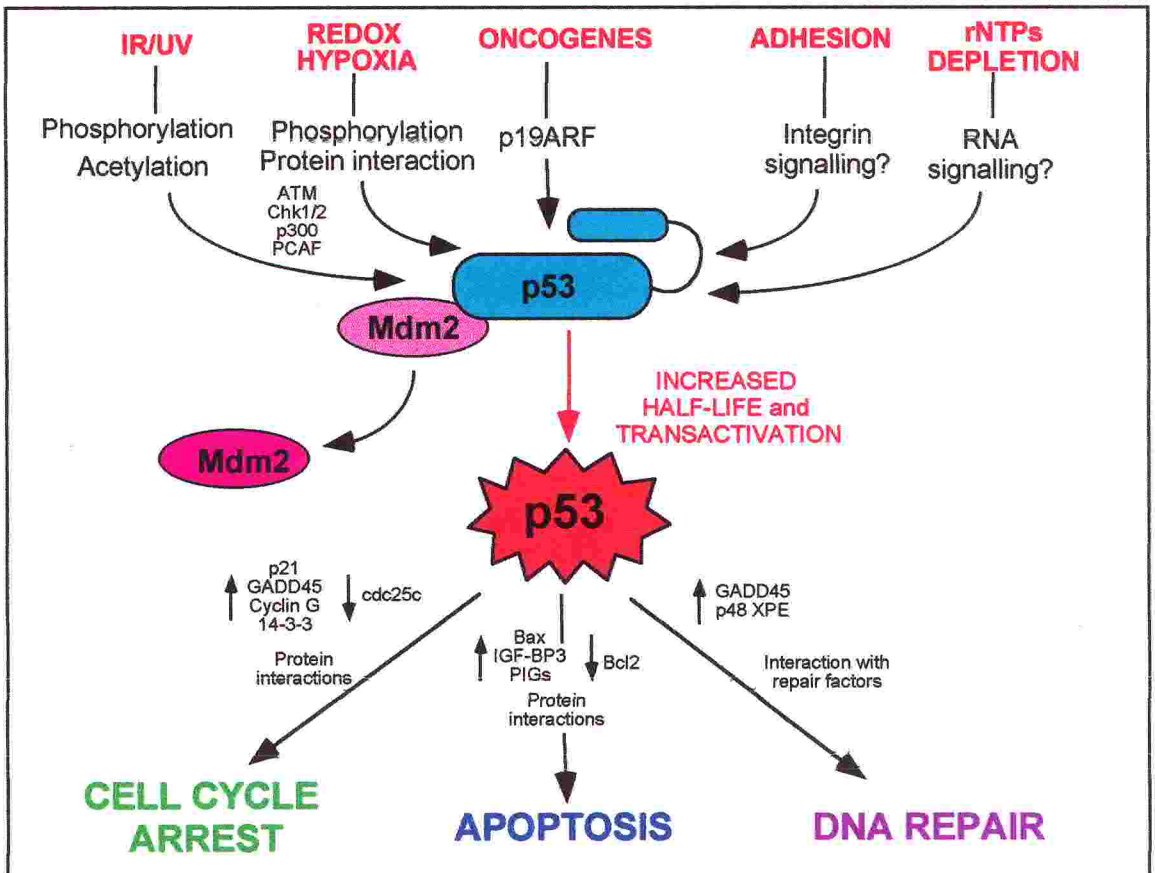


Figure 2: p53 activation pathways.

Different kinds of cellular stress target p53 with post-translational modifications resulting in activation and stabilization of the protein. Activated p53 acts as a transcription factor and interacts with several cellular partners for modulating its downstream responses.

1. Cell cycle arrest

The response to DNA damaging agents, like ionizing radiation (IR) or UV light, ending with cell cycle arrest at the G1/S border is perhaps the best characterized consequence of p53 activation. The fact that p53 is required for executing the G1/S checkpoint is demonstrated by the observation that cells lacking p53 activity, as well as fibroblasts derived from p53-null mice, are no longer able to arrest cell cycle following IR or UV exposure[46,47].

The p53-dependent G1 arrest results mainly from transactivation of the p21/Waf1 gene, whose product is a potent inhibitor of the activity of the CDK/cyclin kinase complexes[48-50]. It has been demonstrated that p21 binds and inactivates CDK4-6/cyclin D and CDK2/cyclin E resulting in accumulation of hypophosphorylated pRb. Hypophosphorylated pRb is tightly bound to the E2F transcription factor and blocks its activity, therefore preventing transcription of E2F-regulated genes that are required for S-phase entry and cell cycle progression[51].

Through p21, p53 is also able to interfere with DNA replication, instead promoting DNA repair. p21, in fact, binds to PCNA, a cofactor of polymerase δ (polδ), which is required for the elongation step in DNA synthesis. PCNA has a dual role both in replicative DNA synthesis and in repair and p21 specifically inhibits its replication functions by mediating the dissociation of the PCNA/polδ from the replication forks[52].

Moreover, p53 can inhibit DNA replication also by associating with the single-stranded DNA binding protein RP-A that is required for DNA unwinding[15].

Although being important,p21 is not the only effector of the p53-mediated G1 arrest. Embryonic fibroblasts from the p21 -/- mice are only partially defective in the ability to undergo G1 arrest following exposure to DNA damaging agents, suggesting that other mechanisms are required to obtain a complete response[53].

Other p53-induced genes have been related to cell cycle arrest, among them GADD45, whose expression is induced in response to various DNA damages. GADD45 has been shown to associate to PCNA and is able to induce growth arrest with a mechanism that is still unclear[46].

Finally, p53 can arrest cells at the G1/S transition through transcription-independent mechanisms. Recently it has been observed that p53 binds cyclin H, which is a component of the CDK activating kinase (CAK). CAK is promoting cell proliferation

by phosphorylating and activating CDK2 and by enhancing the activity of RNA polymerase II. Binding to p53, strongly impairs CAK activity, both towards CDK2 and RNA pol II[54].

Another non-transcriptional function of p53 is involved in mediating cell cycle arrest upon overexpression of the growth arrest specific gene Gas1. It has been demonstrated that Gas1-dependent growth arrest requires the presence of wt p53 but not its transactivation capabilities. Instead, it appears that this function is dependent on the presence of the Pro-rich domain, may be through the interaction with an SH3 domain-containing protein, responsible of transducing a signal from the membrane-bound Gas1 to nuclear p53[25].

A role for p53 also in the G2/M checkpoint has been postulated.

Normal mouse embryo fibroblasts (MEFs) treated with spindle inhibitors arrest in mitosis without re-entering S phase. On the contrary, p53-null MEFs treated in the same way continue to cycle and reduplicate their DNA before completion of cell division, becoming tetraploid or octaploid. The same cells, even if untreated, spontaneously develop tetraploidy, indicating that p53 is a component of the spindle checkpoint, required during normal cell division[55].

In humans, instead, DNA reduplication occurs only in cells expressing p53 missense mutations, suggesting that loss of p53 is not sufficient to induce polyploidization[56].

The G2/M checkpoint depends mainly on inhibition of the activity of the cdc2 kinase, which binds to the mitotic cyclin A and activates substrates required for cell cycle progression. When phosphorylated, cdc2 is inactive and dephosphorylation by the cdc25c phosphatase is required for entry into mitosis.

A possible mechanism mediating p53-dependent G2 arrest is again acting through p21, that has been shown to interact with cyclin A and B kinase complexes and to prevent their activity[32]. The evidence that p21 mRNA accumulates not only in G1 but also in late G2 strengthened the hypothesis of a role for this protein at the onset of mitosis[57]. However, p53-mediated increase in p21 levels is mainly required for G1 arrest and p21 does not seem to be essential for the G2/M checkpoint[32].

Other p53-induced genes have been implicated in G2 arrest, like cyclin G[58] and GADD45 that was shown to destabilize cdc2/cyclin B complexes[59,60].

p53 induces also the expression of the 14-3-3 σ protein[61], which interacts with cdc25c, an essential regulator of the G2/M transition. In response to DNA damage, cdc25c is phosphorylated by the Chk1 kinase and this phosphorylation promotes the binding of 14-3-3 proteins that inactivate cdc25c by sequestering it in the cytoplasm[62,63]. Moreover, a recent report indicates that p53 is also able to directly repress the transcription of cdc25c (J. Manfredi, 10th p53 Workshop).

An alternative model of the role of p53 in the spindle inhibitors-induced cell cycle arrest has been proposed. There are evidences indicating that cells exposed to microtubule destabilizing agents do not sustain a prolonged mitotic arrest. Instead they undergo "mitotic slippage" bypassing the block in M phase and entering a stage biochemically resembling G1. In this situation p53 can act to prevent reduplication with mechanisms similar to the ones responsible of mediating G1 arrest[5].

2. Apoptosis

p53 activation following DNA damage or oncogenes expression can also induce apoptosis. Functional p53 is necessary for apoptosis in several cell types, including hematopoietic progenitors, quiescent B and T lymphocytes, keratinocytes and small intestine cells[64].
p53-mediated cell death has been demonstrated to be crucial for suppression of transformation in oncogene-expressing cells and loss of p53 causes a dramatic enhancement of malignant transformation in cultured cells[65]. Therefore, the ability of stimulating p53-dependent apoptotic response is important for the efficacy of many cancer therapies[66,67].

Both transcriptional-dependent and -independent mechanisms have been proposed to be involved in p53-mediated apoptosis, since p53 mutants which fails to activate transcription can still induce cell death[68]. In particular, the Pro-rich region of p53 has been shown to be required for apoptosis, probably through the interaction with specific cellular factors[23].

p53 can trigger apoptosis by affecting the Bax/Bcl-2 equilibrium. In fact, it has been shown to induce the death effector Bax[69] and also to repress the anti-apoptotic gene Bcl-2[70].

Other p53-regulated genes that have been correlated with induction of apoptosis include the death receptor Fas/APO1[71], IGF-BP3[72] that inhibits the mitogenic and survival activities of IGF receptor, the cathepsin-D protease[73] and PAG608[74], a zinc-finger containing nuclear protein.

p53 has also been reported to increase the expression of a set of genes (PIGs, p53-induced genes) involved in the generation and in the response to oxidative stress[75]. Therefore, p53 may trigger the apoptotic cascade by increasing the production of reactive oxygen species that in turn result in damage to mitochondria and activation of caspases. Accordingly, generation of reactive oxygen species has been detected in several models of p53-dependent cell death.

Finally, a number of recent reports described new p53-induced genes encoding for pro-apoptotic proteins. Between them AIP1 (Y. Taya, 10th p53 Workshop, 2000), a mitochondrial protein, whose induction has been shown to be directly dependent on p53 phosphorylation on Ser 46; PIDD (S. Benchimol, 10th p53 Workshop, 2000), a novel death domain-containing protein; PERP (L. Attardi, 10th p53 Workshop, 2000), a member of the Gas3/PMP22 family of tetraspan membrane proteins and Scotin (J.-C. Bourdon, 10th p53 Workshop, 2000), an endoplasmic reticulum-localized protein.

Much less understood are the events governing the decision of a cell to undergo apoptosis rather than cycle arrest. The outcome of p53 activation is clearly cell-type specific and depends on the severity of the damage[41,76].

Several experimental evidences indicate that G1 arrest and apoptosis represent divergent biological pathways and it has been proposed that the choice between arrest and death can be mediated by the presence of survival factors in the external environment and by the cooperation between the p53 and pRb pathways.

The expression of a p53 temperature-sensitive mutant in the murine myeloid cell line M1 result in massive apoptosis. But if p53 is activated in the presence of cytokines (EPO, KIT-L, IL-3), these cells block in G1, without cell death. Moreover, the same p53 mutant induces cell cycle arrest in fibroblasts[77].

Inactivation of pRb results in loss of G1 arrest and induction of apoptosis after DNA damage, probably through the activation of the E2F transcription factor[41].

Since both cell cycle arrest and apoptosis are largely dependent on p53 transactivation ability, it is conceivable that the mechanism leading to the appropriate final response is somehow due to the activation of specific subsets of target genes. In line with this hypothesis, it has been demonstrated that, upon deletion of the p21 gene, cells that would otherwise undergo p53-dependent cell cycle arrest, instead undergo apoptosis[41].

This "selectivity" of transcriptional regulation by p53 can be obtained by different post-translational modification or through the interaction with different co-activators. For example, the Wilm's tumor protein, WT-1, has been shown to bind to p53 and to increase p53 SST, resulting in inhibition of apoptosis without affecting p53-dependent growth arrest[78].

3. DNA repair

Even if a definitive proof of a role for p53 in DNA repair is still missing, there are several experimental evidences indicating that p53 can actually be involved in mediating repair of the lesions after DNA damage-induced cell cycle arrest.

Cells that are homozygous for a mutated p53, like Li-Fraumeni syndrome fibroblasts, are defective in nucleotide-excision repair (NER), but proficient for transcription-coupled repair, indicating the existence of p53-dependent and independent repair pathways[79].

p53 carboxy-terminal region has been shown to bind with high affinity to DNA lesions[32] and recent findings demonstrated a role for p53 in base excision repair (BER) in an *in vitro* system (V. Rotter, 10th p53 Workshop). This activity is independent of transcription, but requires p53 DNA binding domain and carboxy-terminal region. Another recent report showed that p53 colocalizes with sites of active NER and that after UV irradiation a first peak of NER activity is directly dependent on the presence of p53 (J. Milner, 10th p53 Workshop).

Finally, an involvement of p53 has been postulated also in double-strand breaks (DSB) repair, which can be achieved through homologous recombination or through non-

homologous end-joining. Upon the introduction of DSBs into DNA p53 become activated[80] and binds to the lesions, suppressing homologous recombination, while increasing end-joining[81,82].

The mechanisms by which p53 stimulates repair are not yet fully understood and, despite some p53-induced genes, like p21 and Gadd45 and the p48 XPE helicase have been implicated in this process, it seems probable that biochemical function other than transactivation are required.

Beside its ability to bind aspecifically to mismatched DNA, p53 possesses exonuclease and DNA reanelling activities and has been reported to interact with several proteins involved in repair, like members of the recQ helicase family (XPB, XPD, WNR, BLM), the Cockaine Syndrome protein B, the single stranded DNA-binding protein RP-A and the hRAD51/BRCA1 complex[32].

Based on the evidences that exonuclease activity is harbored by p53 core domain[83] and is mutually exclusive with sequence-specific DNA binding[84], a "dual role" model for p53 has been proposed: in normal unstressed cells, p53 is believed not to be an inactive protein. Instead, in this latent state it may be able to perform several repair functions, therefore preventing mutations arising from endogenous DNA damage. It is possible to hypothesize that, under stress conditions, only a fraction of p53 become activated for SST and mediates growth arrest or apoptosis, while another subclass of the protein remains in the non-induced, repair-competent state[32].

REGULATION OF p53 FUNCTION

p53 growth-suppressing abilities must be maintained under a very stringent control during normal cell growth. For this reason, p53 levels in growing cells are kept extremely low by a rapid turnover rate and moreover the protein is synthesized in a latent form that is transcriptionally inactive.

Several upstream stress signals are converging on p53 and lead to functional activation of the protein (see Figure 2). Among these events, the best characterized is surely DNA damage: p53 has been shown to be activated in response to a variety of damages, including DSBs induced by gamma irradiation or radiomimetic drugs and repair intermediates caused by UV light exposure. Moreover, p53 induction can be mediated by several other stress conditions, like hypoxia, disruption of microtubules, alteration in the redox potential of the cell, unbalance in the cellular nucleotide pools, heat shock and oncogenes activation[3].

The activation of p53 response is a very rapid event that is believed to involve mainly post-translational modifications of the protein. These modifications, by directly changing the conformation of p53 or indirectly by affecting its ability to interact with other factors, have two distinct, but usually contemporary, consequences[9]:

- increase in p53 half-life, by N-terminal phosphorylations that weakens the binding with Mdm2, a protein responsible for p53 degradation;
- conversion of p53 from a latent to an active state, capable of DNA binding, by phosphorylations and other modifications both in the amino and carboxyl termini of the protein.

These two events can be mechanistically separated, as demonstrated by the evidence that increase in p53 transactivation with no relevant changes in the protein level can be observed in cells treated with low doses of UV light[42]. Similarly, sodium salicilate, which blocks protein kinases, inhibits the activation of p53 with no significant effects on the accumulation of the protein[85].

Even if post-translational modifications, and specially phosphorylation, have been clearly demonstrated to be essential in mediating p53 response, their importance in maintaining protein stability versus activation of p53 as a transcription factor remains a still debated question[5].

1. Regulation of p53 stability

Regulation of protein stability is a common mechanism by which the function of cell growth regulatory proteins is controlled. p53 has been shown to be targeted for degradation by the proteasome, although a role for other proteolytic enzymes, such as calpain, has also been implied[9].

The proteasome-mediated degradation pathway requires a complex enzymatic cascade that promotes the conjugation of multiple ubiquitin chains to internal lysine residues in the target protein, that is then recognized and degraded by the 26S proteasome. This cascade involves the sequential action of three enzymes, the E1 ubiquitin-activating enzyme that activates ubiquitin molecules prior to conjugation, the E2 ubiquitin-conjugating enzyme and the E3 ubiquitin-ligase, responsible for the specificity of substrate recognition[86] (Figure 3).

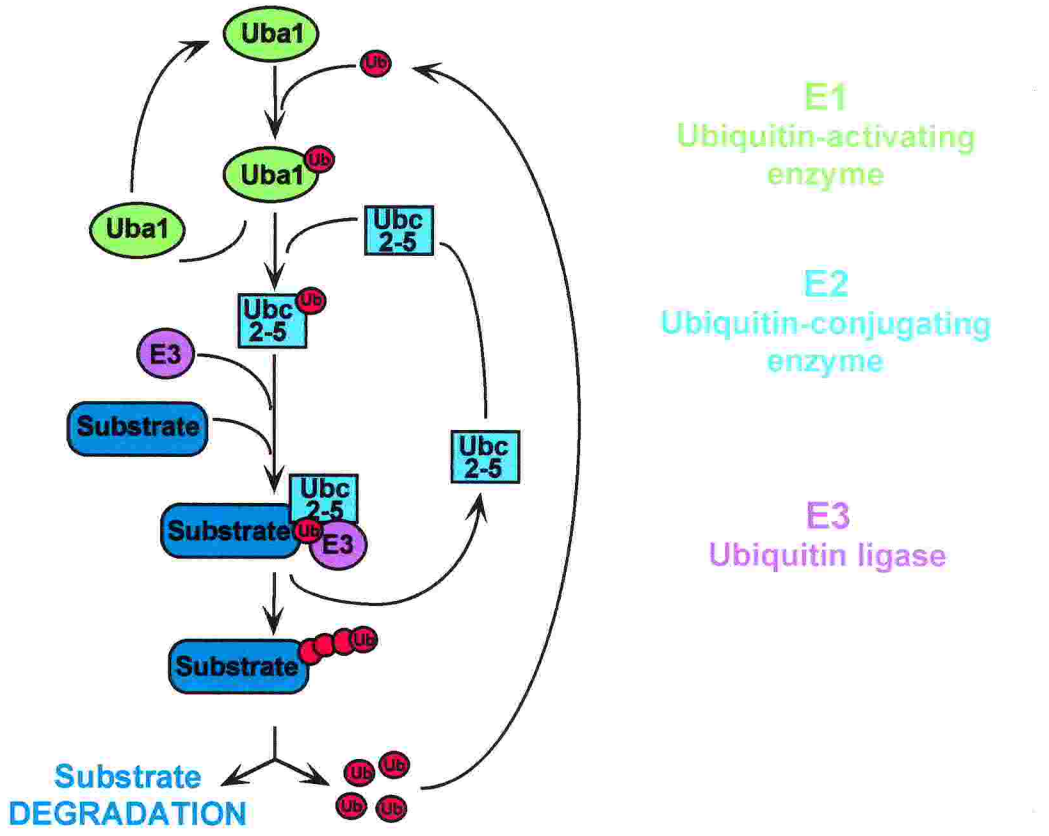


Figure 3: Ubiquitin modification pathway.

Modification by ubiquitin requires the enzymes E1, E2 and E3 and results in formation of an isopeptide bond between ubiquitin (Ub) and the substrate protein. Subsequent ubiquitin molecules are then added on ubiquitin itself; the resulting polyubiquitinated protein is then degraded, while ubiquitin is recycled.

Adapted from Hodges et al., 1998

A key role in regulation of p53 stability is exerted by Mdm2 that has been shown to function *in vitro* as a p53-specific E3 ubiquitin-ligase and has been therefore proposed to promote p53 degradation by facilitating its ubiquitination[87-89]. *In vivo*, overexpression of Mdm2 was shown to inhibit IR-induced p53-dependent G1 arrest[90] and to downregulate p53 levels[88,89]. The observation that Mdm2-null mice show early embryonic lethality[91], which can be rescued by the simultaneous deletion of p53, strongly supports the model in which Mdm2 keeps p53 activity under control. In line with this hypothesis, Mdm2 was found overexpressed in tumors bearing a wt p53 allele[19].
Interestingly, Mdm2 is also a transcriptional target of p53[92], suggesting the existence of a negative feedback loop, probably required for the termination of p53 response and recovery form G1 arrest. Mutant p53 proteins that are unable to, activate expression of Mdm2 are more stable than the wt counterpart[93].
The ubiquitin-ligase activity of Mdm2 requires its direct binding to p53 N-terminal sequences, but this event is not sufficient to obtain protein degradation, since a C-terminally truncated p53 still competent for Mdm2 binding is no longer degraded[94]. It is therefore likely that binding to other factors is also necessary for normal p53 degradation.
Moreover, recent findings indicated a possible role for Mdm2 in mediating p53 nucleo-cytoplasmic shuttling, adding an extra level of complexity in regulation of p53 degradation. Mdm2 contains a NES and has been proposed to facilitate p53 export from the nucleus and degradation[39]. Even if there are some controversial data regarding this hypothesis, it is clear that Mdm2 nucleo-cytoplasmic shuttling is required to activate its ubiquitin-ligase function[95,96] (see below).

The induction of p53 following DNA damage is related to a rapid stabilization of the protein due to abrogation of Mdm2-mediated degradation. Post-translational modifications on p53 have been proposed to be responsible of Mdm2/p53 complex dissociation and indeed several possible sites of phosphorylation have been mapped within the Mdm2 binding, including serine 15, 20, 33 and 37 and threonine 18 (see Figure 4). Different kinases acting on p53 within this region *in vitro* have been identified and phosphorylation on some of these residues has been shown to affect Mdm2 binding region[97,98]. *In vivo*, Ser 15, 20 and 33 are all phosphorylated after DNA damage, even if they show different induction kinetics in response to different types of stress[99]. However, the exact contribution of each individual modification to p53 stabilization *in*

vivo remains unclear and there are evidences indicating that phosphorylation is not essential for all the forms of stress-induced p53 stabilization.

Much excitement was generated by the finding that DNA-PK, a kinase that is directly activated by damage since it is recruited to aberrant DNA structures, was able to modify *in vitro* p53 on serine 15 and 37. However, no final evidences indicating a role for this kinase *in vivo* have been provided and p53 is efficiently activated by DSBs in cells lacking functional DNA-PK[100].

More interesting candidates as upstream regulators of p53 are the ATM and ATR kinases, members of the PI3-kinase family. The ATM gene has been found mutated in Ataxia-Teleangiectasia (AT), an autosomal recessive disorder characterized by increased incidence of cancer, chromosomal instability and radiosensitivity[101]. AT cells show delayed p53 accumulation after ionizing radiation (IR) and concomitant reduction in serine 15 phosphorylation[102,103].

Several data indicate that ATM is responsible for increase of p53 half-life after IR, while the related kinase ATR is required for response to UV irradiation and can also partially rescue ATM defects in AT cells[98]. ATM and ATR are able to phosphorylate p53 on Ser 15 and this modification relieves the interaction with Mdm2 *in vitro*, therefore providing an easy explanation for the observed effect on p53 stability[97,98] (Figure 5).

The picture, however, became more complex after recent data demonstrating that phosphorylation of Ser 20, rather than 15, is crucial to inhibit Mdm2 interaction and that substitution of Ser 20 with alanine is sufficient to abolish p53 stabilization in response to IR and UV light *in vivo*[104,105]. This residue is phosphorylated in response to IR and UV by the Chk1 and Chk2 kinases, homologues to yeast proteins involved in DNA damage checkpoints[106,107]. This phosphorylation has been correlated with increased p53 stability and with the induction of p53-dependent G1 arrest[106]. Since Chk2 is activated by gamma irradiation in the presence of functional ATM, it is possible that the effect of ATM and ATR on p53 half-life is not direct, but requires the activation of Chk1 and Chk2. These kinases in turn phosphorylate p53 on Ser 20 and destabilize the complex with Mdm2 (Figures 4 and 5).

Finally *in vitro* data indicated also an involvement of threonine 18 in mediating Mdm2 interaction. Interestingly, phosphorylation on Thr 18 requires prior modification on Ser 15 by ATM (E. Appella, 10th p53 Workshop, 2000), therefore pointing to the existence of a complex network of modifications that are influencing each other.

Despite some controversial data, probably arising also from the use of different experimental systems, the model that is emerging is that different upstream signals (i. e. different types of damage) are able to activate different transducers (kinases), resulting in different pattern of phosphorylation on p53.

The ability of Mdm2 to promote p53 ubiquitination can be modulated not only by covalent modifications but also by the binding to other regulatory proteins and, in fact, insults such as heat shock, oncogene activation or treatment with actinomycin D stabilize p53 without significant changes in N-terminal[9] phosphorylation.
The best characterized alternative pathway for p53 stabilization involves the ARF protein, encoded by an alternative reading frame in the INK4a locus[108]. ARF is able to bind to Mdm2 and this binding does not interfere with p53/Mdm2 interaction but prevents p53 proteolysis, apparently by blocking the E3 ubiquitin-ligase activity of Mdm2[109] and by sequestering it in the nucleolus[110]. Overexpression of oncoproteins, like E1A, Myc or Ras, leads to massive induction of ARF, through increase in its transcription rates, mediated at least in part by the E2F transcription factor[111,112].
In addition, the non-receptor tyrosine kinase c-Abl has been shown to bind to p53 and to protect it from Mdm2-mediated degradation, without disrupting p53/Mdm2 complex[113].

Finally, it should be noted that p53 stabilization may be obtained by modulating Mdm2 itself and, in fact, several putative phosphorylation sites have been identified in this protein. Moreover, some activating signals have been shown to specifically inhibit Mdm2 transcription, therefore resulting in reduced Mdm2 protein levels and enhanced p53 stability. Expression of alternatively spliced version of Mdm2 has also been correlated with stabilization of p53[9].

Other mechanisms for p53 ubiquitination and degradation also exist. Of particular interest is the possible role of the c-Jun N-terminal Kinase (JNK), member of the stress-activated family of protein kinases. JNK binds to p53 between residues 97 and 116 and has been shown to target p53 for degradation in non-stimulated cells. Upon cellular stress, activated JNK phosphorylates p53 (probably on serine 37), resulting in stabilization of the protein not only by disrupting the JNK/p53 complex but also by preventing Mdm2 binding[114].

2. Activation of p53 sequence-specific transactivation

Although regulation of p53 levels in response to DNA damage is clearly important, other control mechanisms also appear to exist, mediating the conversion of the protein from a latent to an active form. The sequence-specific DNA binding activity of p53 is in fact subjected to constitutive negative regulation, mostly through its inhibitory C-terminal domain. Relief of this inhibition upon exposure to stress results in increased DNA binding and SST. The transcriptional activity of p53 may also be induced by changes in other regions, e. g. modifications within its N-terminal transactivation domain, enabling a more efficient recruitment of components of the transcription machinery.

Again, stress-induced post-translational modifications play an important role in p53 activation, allowing for stabilization and functional activation to be time-coordinated.

N TERMINUS

The N terminus of p53 contains the transactivation domain and is responsible of the interaction with components of the basal transcription machinery. DNA damage-induced phosphorylation of N-terminal residues has been proposed to contribute to p53 regulation by affecting the binding of positive or negative transcriptional regulators[98].

Mdm2 controls p53 activity not only by stimulating its degradation but also by inhibiting its transcriptional activator functions. The Mdm2 binding site has in fact been mapped within the transactivation domain of p53, in correspondence of the conserved box I and binding to Mdm2 is preventing p53 association with the transcriptional machinery[16,17]. Therefore, the stress responses mediating p53 phosphorylation on serine 15 or 20, which result in abrogation of Mdm2 interaction, not only have the effect of increasing p53 half-life but also stimulate its transactivation capability[104,105].

Interestingly, phosphorylation on Ser 15, 33 and/or 37 has been implicated also in modulating the association of p53 with the transcriptional co-activator p300/CBP, which possesses histone acetyl-transferase (HAT) activity[115] (Figure 4).

Acetylation of hystones has long been implicated in regulation of transcription and a link between HAT enzymes and p53 has been proposed following the observation that the adenovirus E1A protein can bind to p300 and interfere with p53 transcriptional activity[98]. p300/CBP has been demonstrated to interact with both p53 N- and C-terminal regions[20,21] and this interaction increases the ability of p53 to stimulate

transcription of its target genes. In addition, of potentially playing a role in acetylating hystones at p53-responsive promoters, p300 has also been reported to directly acetylate p53 in the carboxyl terminus[22].

Several other amino-terminal residues have been shown to be phosphorylated *in vitro* (Figure 4): Ser 6 and 9 by casein kinase I (CKI), Ser 15 and 37 by DNA-PK, Ser 33 by CAK, Ser 37 by JNK and Thr 18 by a still unidentified kinase. However, the role of these modifications *in vivo* has not yet been addressed[99].

C TERMINUS

The C-terminal domain is an important regulator of p53 DNA activity, as demonstrated by the evidence that a p53 protein lacking the last 30 residues is constitutively active for DNA binding[116]. Similarly, binding of oligonucleotides or antibodies, like Pab421, to this domain in the absence of other modifications, has a stimulatory effect on p53 transcriptional activity. Of more physiological relevance is the observation that several post-translational modifications occur within p53 C terminus following exposure to DNA damage.

Three phosphorylation sites have been identified in p53 C terminus (Figure 4). Serine 315 is phosphorylated by the G2/M specific kinases cdc2/cyclin B and Cdk2/cyclin A and this modification increases p53 sequence-specific DNA binding in a promoter-dependent manner[117]. Phosphorylation of serine 378, which lays in the Pab421 epitope, is mediated *in vitro* by protein kinase C (PKC) and activates p53 probably by relieving the inhibitory action of the C terminus, an effect mimicked by Pab421 binding to the same region[97]. Serine 392 is phosphorylated *in vitro* by casein kinase II (CKII) and this event enhances DNA binding by facilitating p53 tetramerization[41,98,99].

There are also evidences for activation of p53 through dephosphorylation: serine 376 is normally phosphorylated in non-stimulated cells, but becomes rapidly dephosphorylated upon ionizing radiation. This modification creates a binding site for 14-3-3 adapter proteins and this interaction results in p53 activation[118] (Figure 5). In this case, therefore, conformational activation is mediated by interaction with specific factors, which in turn is modulated by post-translational modifications.

Similarly, binding of other proteins, like WT-1[78], a zinc-finger transcription factor, or BRCA-1[119,120], a tumor suppressor linked to hereditary predisposition for breast and ovarian cancers, to p53 C-terminal region has been shown to increase its SST activity.

Recently, several reports pointed to the role of acetylation in p53 activation[22,121].

In vitro experiments showed that p53 is acetylated by p300/CBP on lysine 373 and 382[22], while PCAF targets another C-terminal residue, Lys 320[115] (Figure 4). These modifications have been found to enhance p53 sequence-specific DNA binding and transcriptional ability. Moreover, DNA damage and N-terminal phosphorylation has been reported to facilitate p53 C-terminal acetylation[115,122].

On the other hand, the C-terminal domain has been shown to play a role in regulating phosphorylation events on N-terminal sites: serine 15, 20 and 33 *in vivo* cannot be modified without the presence of the tetramerization domain, probably because tetramerization provide a preferable conformation for substrate recognition[105] .

The link between N- and C-terminal modifications suggests that DNA damage-induced modifications on p53 are a carefully coordinated and sequential series of events which favor dissociation of the p53/Mdm2 complex, recruitment of key transcriptional components and activation of site-specific DNA binding functions of p53.

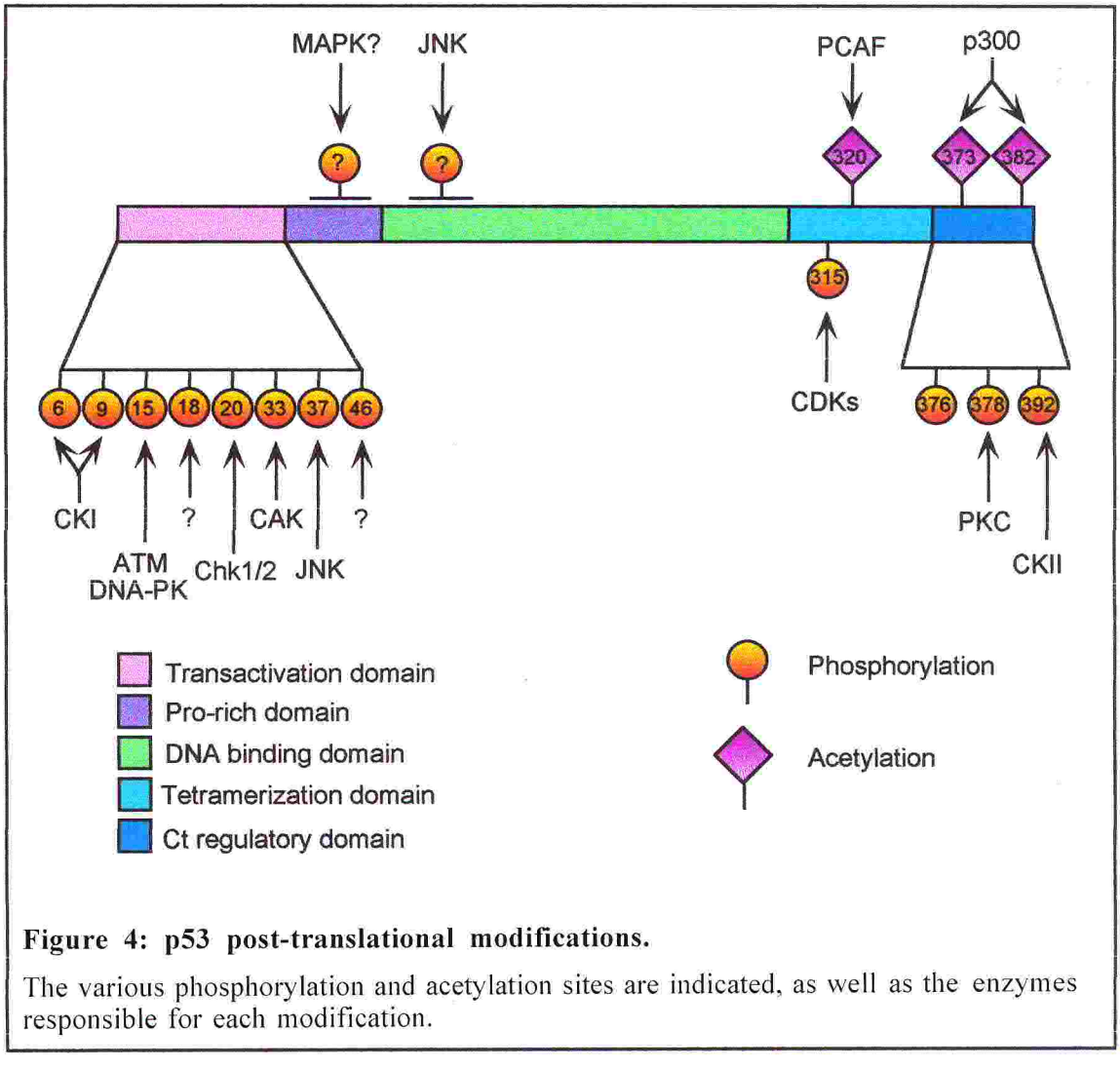


Figure 4: p53 post-translational modifications.

The various phosphorylation and acetylation sites are indicated, as well as the enzymes responsible for each modification.

3. Determination of the selectivity of p53 response

Another important issue that only recently started to be addressed is how the cell type and DNA damage specificity of p53 response is obtained. As the outcome of the cellular response upon p53 activation is largely dependent on transactivation of appropriate target genes, the question arises how selectivity of transactivation is secured.

It has been proposed that different types of stress may determine different post-translational modifications of the p53 protein, that will lead to the preferential activation of specific downstream genes or, alternatively, to the modulation of the interaction with other cellular factors[7]. In agreement with this hypothesis, several reports demonstrated that different signals are responsible of alternative p53 modifications both in the amino and in the carboxyl terminus. Moreover, these differential modifications have been linked to promoter and cell-type specificity of response.

In the amino terminus, serine 15 has been showed to be involved in modulating the interaction with TFIID: phosphorylation on the sole Ser 15 by ATM (induced by IR) destabilizes the interaction, while phosphorylation on both Ser 15 and 37 by ATR (induced by UV light exposure) is increasing the p53/TFIID complex formation[123].

Moreover, mutations of various serine residues in the N-terminal domain have a differential effect on the ability of p53 to bind to and to induce transcription from various promoters[124], suggesting that specific patterns of phosphorylation are likely to occur in response to different types of stress, leading to stress-specific patterns of gene expression.

Recently, phosphorylation on serine 46 by a still unknown kinase (Figure 4) has been reported to specifically mediate the activation of AIP1, a p53-induced pro-apoptotic gene, therefore resulting in decreased cell survival (Y. Taya, 10th p53 Workshop, 2000).

In the C-terminal region, phosphorylation at serine 392 is induced by UV but not by IR[125], while dephosphorylation at serine 376 is promoted only by gamma-irradiation, in an ATM dependent manner, probably through activation of a downstream phosphatase[118] (Figure 5). Since these two kinds of DNA lesions are repaired by alternative pathways (UV-induced pyrimidine dimers by excision repair, while IR-generated DSBs by homologous recombination and ligation) it is possible to speculate

that fine-tuning of the p53 response by specific modifications may direct the cellular choice of the appropriate means of damage repair.

The evidence that cell cycle regulated kinases target p53 carboxyl terminus and modulate promoter selectivity suggest that also a cell cycle-specificity of transcription is possible[117]. Different phosphorylation events in different phases of the cell cycle may mediate preferential activation of certain target genes with roles either at the G1 or at the G2/M checkpoints.

Not only phosphorylation, but also acetylation can be regulated in a stress-specific way, as demonstrated by the use of specific antibodies: lysine 382 is acetylated after UV and IR, along with phosphorylation on serine 33 and 37, while lysine 320 is modified only after UV[115] (Figure 5).

Apart from post-translational modifications, another way to confer selectivity to p53 response is by modulating its interaction with co-activator molecules.

For example, association of p33ING with p53 seems to be required for efficient transactivation of the p21 promoter[126].

Similarly, binding of WT-1 to p53 C-terminal tail has been shown to result in inhibition of its apoptotic functions without affecting its ability to induce cell-cycle arrest[78].

Recently, an additional mechanism providing specificity to SST by p53 has been proposed, involving remodeling of chromatin configuration at the promoter level.

The p53 consensus, bearing high internal symmetry, can assume a non-B-DNA conformation upon topological stress and p53 has been demonstrated to be able to recognize alternative conformations of the same target sequences with different affinities.

p53, moreover, can bind to proteins that affect chromatin architecture, like HMG1 and p300, therefore suggesting that also interaction with auxiliary proteins can allow conformational shifts at the promoter region and influence the binding of p53 and/or other factors[32].

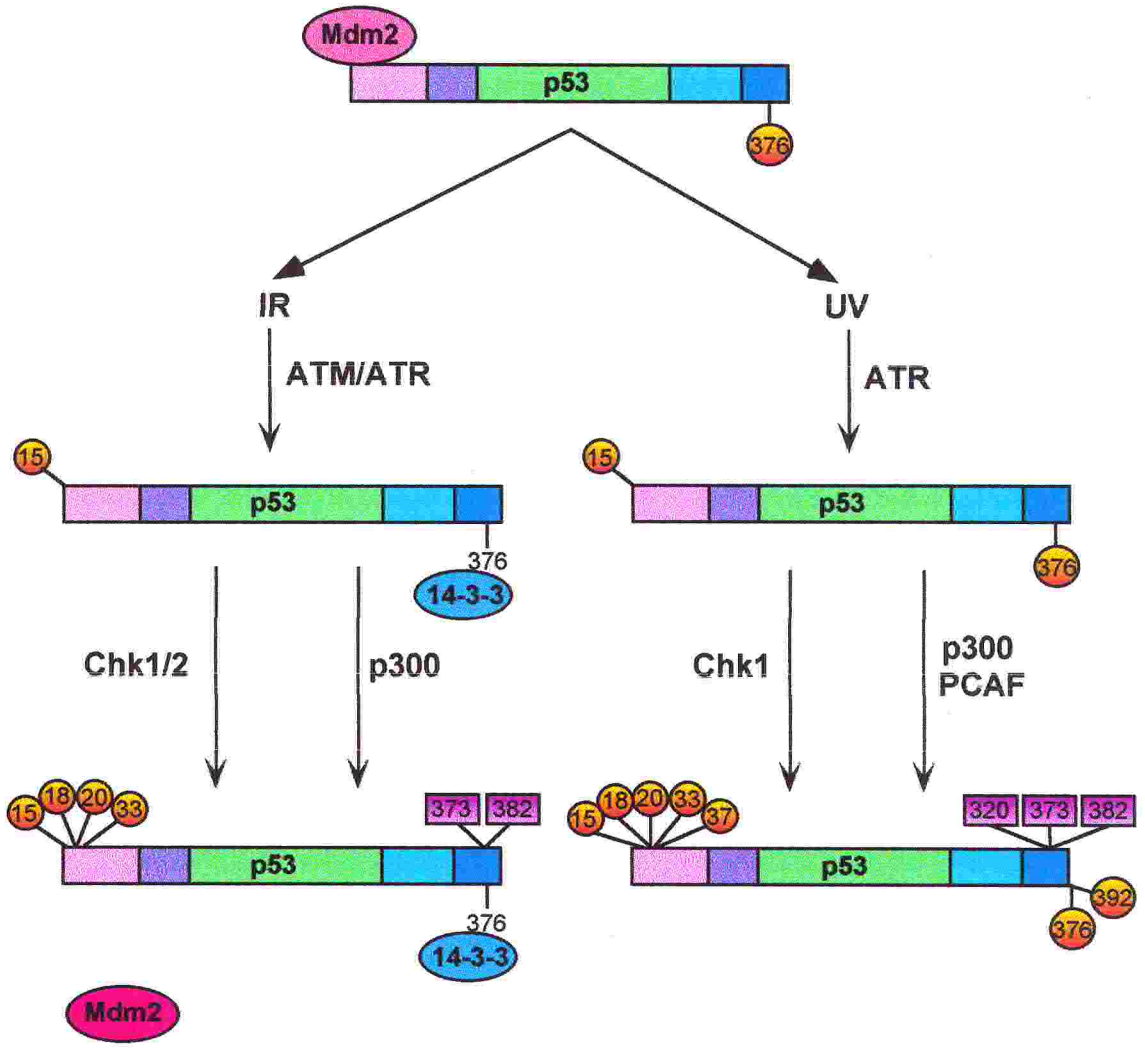


Figure 5. Model for post-translational modification of p53 in response to DNA damage *in vivo*.

In undamaged cells p53 is targeted to degradation by interaction with Mdm2.
Serine 15 is phosphorylated in an ATM/ATR-dependent manner in response to IR and an ATR-dependent manner in response to UV. Furthermore, after IR, serine 376 is dephosphorylated in an ATM-dependent manner, resulting in binding to 14-3-3 and p53 activation. It remains to be established whether a similar effect occurs in response of other types of damage.
Further phosphorylation events occur at serines 20, 33, and 37; at least some of these modifications may be mediated by Chk1/Chk2 kinases. Moreover, N-terminal phosphorylations displace Mdm2 from p53, resulting in an increase in the half-life of p53, and favor p300 binding and C-terminal acetylation, which in turn stimulates p53 DNA binding.

Adapted from Lakin and Jackson, 1999

4. Regulation of p53 activity by changes in subcellular distribution

A key step in the activation of p53 is necessary represented by its relocalization to the nucleus, where it can exert its effect as a transcription factor.

The finding that latent p53 may often be cytoplasmic, whereas exposure to stress results in its accumulation in the nucleus[127] indicates that, in addition to stabilization and functional activation of the protein, a proper subcellular localization can be necessary to trigger a full response. Indeed, many tumors have been reported to contain wild-type p53 and to inactivate the function of the protein by sequestering it in the cytoplasm. Similarly, certain types of virus can induce uncontrolled cell proliferation by keeping p53 out from the nucleus[5].

p53 cytoplasmic retention may result either from anchorage to a cytoplasmic tether or from imbalances in nucleo-cytoplasmic shuttling. Despite p53 has been shown to bind to multiple cytoplasmic proteins, the biological relevance of these interactions remains to be determined.

Alternatively, it has recently been reported that neuroblastoma cells have alterations in the mechanisms controlling p53 shuttling and that hyperactive nuclear export results in cytoplasmic accumulation of the protein.

Differences in the intracellular distribution of wt p53 during the cell cycle under normal growth conditions suggest that such a control mechanism could be cell cycle regulated[5].

Nuclear import of p53 is mediated by three lysine-rich nuclear localization signals in its C-terminal region, but little is known about regulation of this function.

The evidence that Ser 315 phosphorylation and Lys 320 acetylation sites lay within the major NLS, allows to speculate that these modifications may modulate interaction with nuclear import receptors. Indeed, phosphorylation has been reported to influence the import of other proteins, such as v-Jun or Lamin B[128,129]; however a similar effect on p53 remains to be established.

The study of p53 nuclear export has become of interest after the finding that both p53 and Mdm2 contain a leucine-rich, Rev-like NES that can be recognized by the cellular export machinery[39,96].

Since Mdm2 binding is necessary for p53 degradation, it has been proposed that Mdm2 may promote both p53 ubiquitination and export to the cytoplasm, where p53 is cleaved by the proteasome[96]. However, in conflict with this idea, p53 was demonstrated to be fully capable to exit the nucleus independently of Mdm2 [39] and there is currently no evidence supporting a role for this protein in p53 nuclear export.

To solve this contradiction, it has been hypothesized that p53 NES, which lays within the tetramerization domain, is buried at the interface between two dimers and that therefore the active tetrameric protein is no longer able to be recognized by the export receptors, but requires Mdm239 (Figure 6). This model raises the possibility of a coordinate regulation of p53 stability and transactivation functions by tetramerization.

Despite structural data in support of this hypothesis, recent findings indicate another possibility: it has in fact been shown that p53 with mutation in its NES is no longer able to localize in the cytoplasm even in the presence of functional Mdm2. It is therefore possible that Mdm2 is necessary only for p53 ubiquitination, that happens preferentially in the nucleus, and that this event, by exposing p53 NES, allows the protein to be exported to the cytoplasm and degraded (C. Maki, 10th p53 Workshop, 2000).

Independently of the hypothesis which will turn out to be true, there are clear evidences that Mdm2 nucleo-cytoplasmic shuttling is required for p53 degradation[95,96], thus indicating that Mdm2 needs to receive a modification in the nucleus to become active as ubiquitin-ligase. Regulation of Mdm2 localization therefore represents another possible mechanism for modulation of p53 activity.

In addition, Mdm2 subcellular distribution can be influenced by ARF. ARF is a nucleolar protein and has been reported to relocalize Mdm2 in the nucleolus, therefore preventing its shuttling and resulting in stabilization of p53 and cell cycle arrest[110]. A simple model of ARF action can therefore be that this protein is sequestering Mdm2 in the nucleolus, releasing p53 in the nucleoplasm. Another interpretation is that Mdm2/p53 complexes exit the nucleus via the nucleolus, and that ARF binding to Mdm2 interferes with this transport.

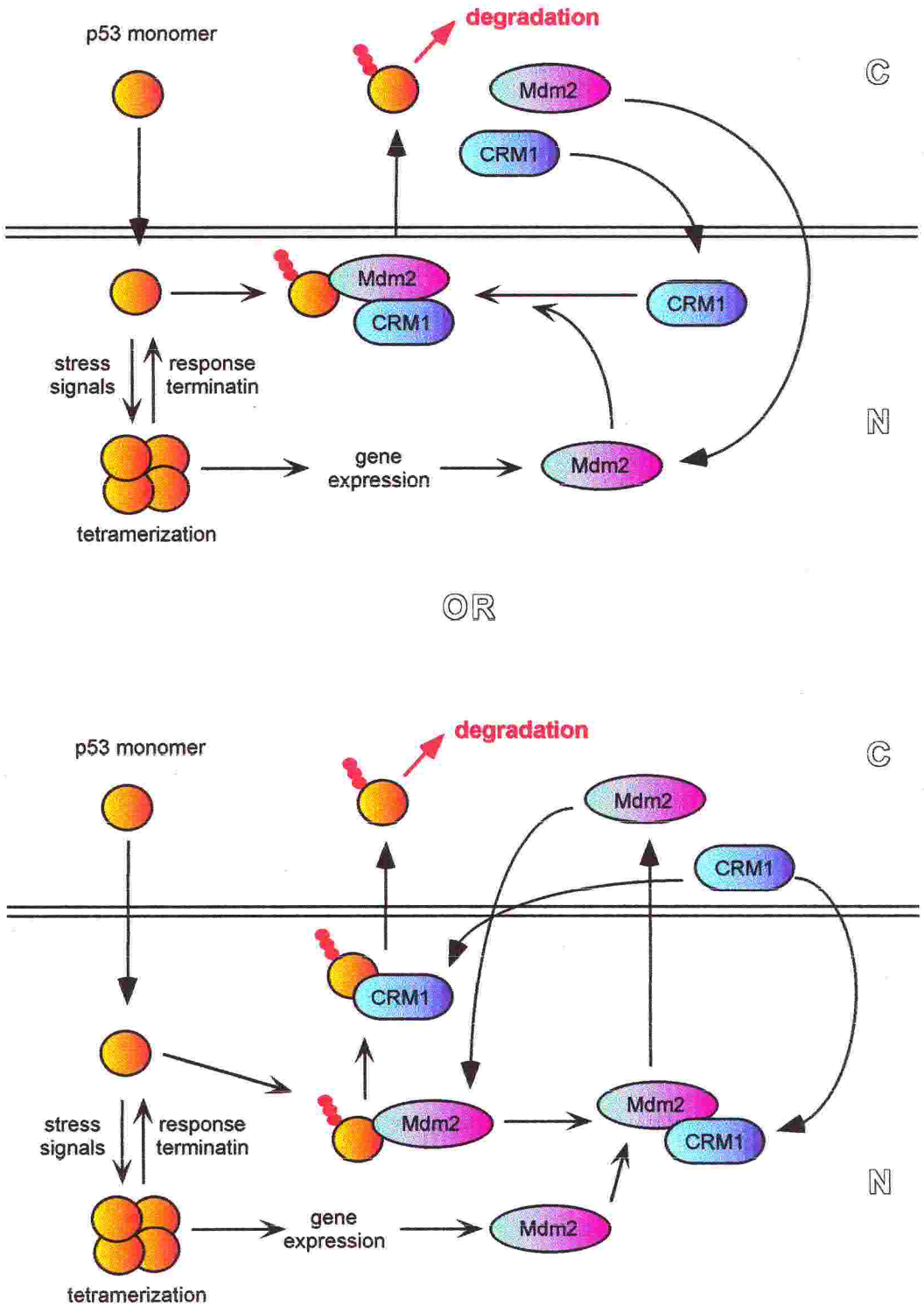


Figure 6. Models of p53 nucleocytoplasmic transport.

Two models for export of p53 from the nucleus are possible.
Inactive, monomeric p53 is bound by Mdm2, which promotes p53 ubiquitination and at the same time mediates recruitment of the export factor CRM1 and exit from the nucleus.
Alternatively, Mdm2 may be responsible only of p53 ubiquitination; this event unmasks p53 NES and allow it to directly bind to CRM1. Mdm2 shuttling is necessary to activate its E3 function.

5. Inhibition of p53 functions by viral proteins

p53 is an obstacle to unscheduled induction of cell proliferation that viruses have to overcome to execute their life cycle; therefore they evolved oncoproteins that binds and functionally inactivate this tumor suppressor.

The SV40 large T antigen can prevent the transcription of p53 target genes by interacting to its core domain and blocking its DNA binding activity[130], while the adenovirus E1B-55K protein obtains the same effect by directly associating with p53 N-terminal transactivation domain[18].

Another viral strategy for p53 inactivation is cytoplasmic sequestration: the hepatitis B protein X (HBX) keeps p53 out of the nucleus, probably by interfering with nucleo-cytoplasmic shuttling, resulting in inhibition of both its SST and its ability to induce apoptosis[131]. The adenovirus E1B-55K itself has been instead reported to anchor p53 to cytoplasmic structures[132].

In other cases, p53 inactivation by viral proteins can be obtained decreasing its cellular levels. Here the best known example is represented by the HPV E6 protein, which has been shown to increase p53 degradation by the ubiquitin-proteasome pathway[133]. E6 from high risk HPV, like HPV 16 and 18, binds to p53 central region and mediates the simultaneous recruitment of a cellular E3 ubiquitin-ligase, E6AP[134]. While E6AP alone is unable to associate with p53, the E6/E6AP complex specifically interact with the tumor suppressor, resulting in its effective ubiquitination and degradation. Moreover, E6 can also bind to p53 C-terminus, but this interaction does not induce p53 degradation, rather has been proposed to affect its transcriptional ability or nuclear localization[135].

Recently, another adenoviral protein, E4orf6, has been demonstrated to be able to induce p53 degradation, in association with the major p53-binding protein E1B-55K[136].

RESULTS

PART 1.
ACTIVATION OF p53 BY CONJUGATION TO THE UBIQUITIN-LIKE PROTEIN SUMO-1

Isolation of SUMO-1 as a candidate p53-interacting protein

I employed the yeast two-hybrid system[137] to identify new p53 associating proteins. The bait plasmid was constructed by cloning the human p53 wt, lacking the first 74 amino acids, in frame with the LexA DNA binding domain (LexAp53wtΔ74).
The cDNA library was obtained from human fetal brain and was cloned in frame with the B42 activation domain into pJG4-5.
p53-interacting clones were screened based on their ability to induce the expression of two different reporters (Leu2 and β-galactosidase) under the control of the LexA operator. Screening of about 4 million colonies able to grow in the absence of leucine resulted in the isolation of 106 putative positive clones.
pJG4-5 plasmids from these colonies were rescued and compared by Southern dot blot and restriction analysis. After classification and secondary screening, I obtained 21 individual clones showing strong and specific interaction with the bait.
Sequence analysis of one of the more representative clones (isolated 20 times out of 106 clones) revealed that it encodes for SUMO-1, a small ubiquitin-like protein.

The small ubiquitin-related modifier SUMO-1 belongs to a growing family of ubiquitin-related proteins[86] and has been reported to be covalently linked to several substrates such as RanGAP1, IκBα, Sp100 and PML[138-140].

The general mechanism mediating SUMO-1 conjugation to its substrates is very similar to the ubiquitin one, but utilizes different enzymes[86] that have only recently been characterized. In humans, these are Sua1 and hUba2, which form a dimer and, in analogy to the ubiquitin-conjugation system, represent the SUMO-activating enzyme E1[141,142], and hUbc9, which represents the SUMO-conjugating enzyme E2[143,144]. Up to now, there are no evidences of the existence of E3-like activities in the SUMO-conjugation pathway (Figure 7A).

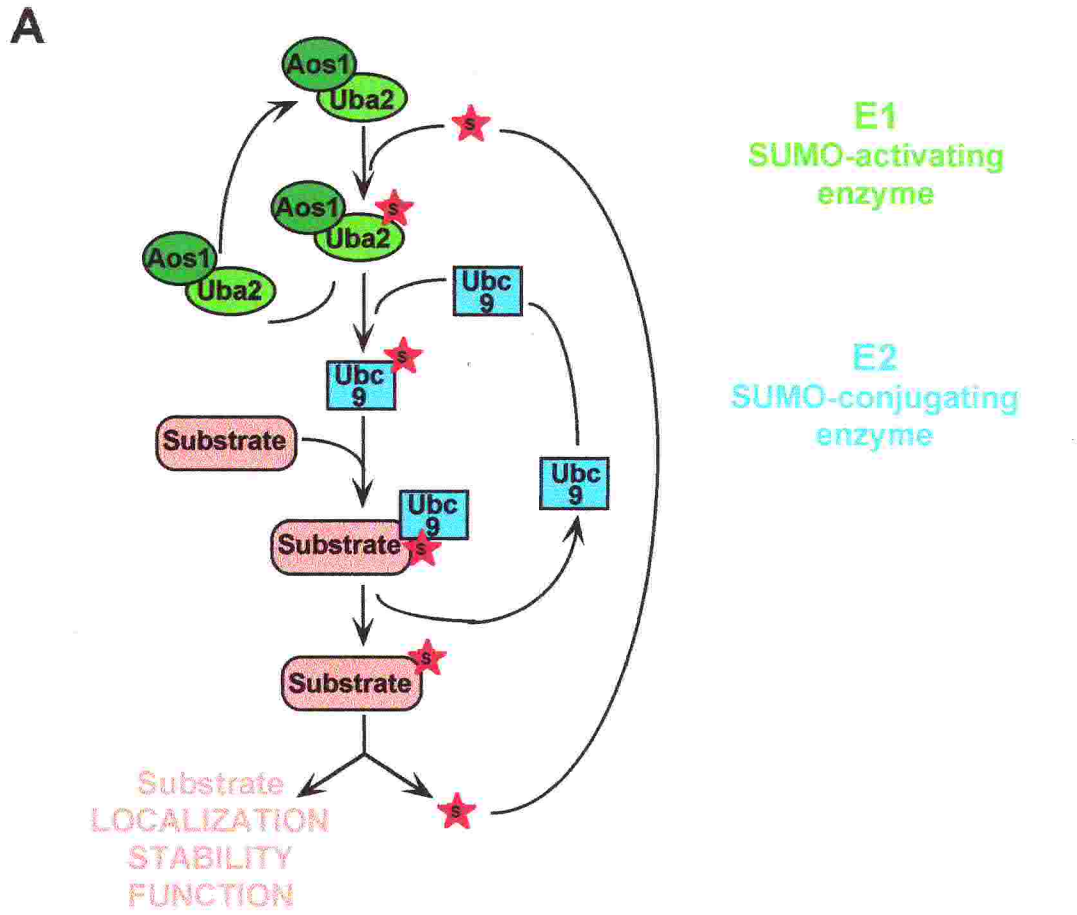


Figure 7: SUMO-1 modification pathway.

(A) Modification by SUMO-1 requires the enzymes E1 and E2 and results in formation of an isopeptide bond between SUMO-1 (S) and the substrate protein. For comparison with the ubiquitin pathway, see Figure 3.
(B) Sequence alignment between SUMO-1 and ubiquitin. The conserved glycine residues necessary for conjugation are shown in green. The ubiquitin internal lysine required for auto-conjugation, that is missing in SUMO-1, is shown in red.

Despite these homologies, the sequence similarity between ubiquitin and SUMO-1 is low[145] (Figure 7B); the two carboxy-terminal glycine residues that are required for the conjugation are conserved, while the internal lysine responsible of self-ubiquitination is missing in SUMO-1 and consequently, poly-SUMO-1 chains cannot be formed. Moreover, in contrast to ubiquitination, "sumolation" of a protein does not appear to target it for rapid degradation but rather affects the ability of the modified protein to interact with other cellular factors. For example, covalent modification of RanGAP1 by SUMO-1 affects its subcellular localization and contributes to nuclear pore complex formation[139,146]. In the case of IκBα[147], a well established substrate of the ubiquitin/proteasome system, it has been reported that SUMO-1 competes for the lysine residue involved in ubiquitin coupling, providing a possible model for regulation of IκBα degradation[138]. PML and Sp100 are important SUMO-1 conjugated components of the so-called nuclear dots or nuclear bodies[140,148], whose still unclear function has been related to cell proliferation control.

Interestingly, from the yeast two-hybrid screening I obtained not only SUMO-1 but also two enzymes of its conjugation pathway, hUbc9 and hUba2. This fact, together with the evidence that hUbc9 have been previously reported to associate with p53 in yeast, convinced me of the possible biologial relevance of the interaction and prompted me to further characterize it.

As a first step, I decided to map the domain of p53 required for the association with SUMO-1 and hUbc9. Different LexAp53wtΔ74 deletions were constructed, lacking either N-terminal (LexAp53wtΔ294) or C-terminal (LexAp53wtΔ74-298, -338, -355) sequences and interaction assays were performed in yeast.
Moreover, the same clones were tested also for their ability to bind to a tumor-derived mutant of p53 (LexAp53wtΔ74H175), which has a disrupted DNA binding domain conformation.
The results of these experiments, schematically represented in Figure 8, revealed that a wt-like conformation is not necessary for p53 to interact with hUbc9 and SUMO-1 and that instead the binding requires the C-terminal 55 amino acids of p53.

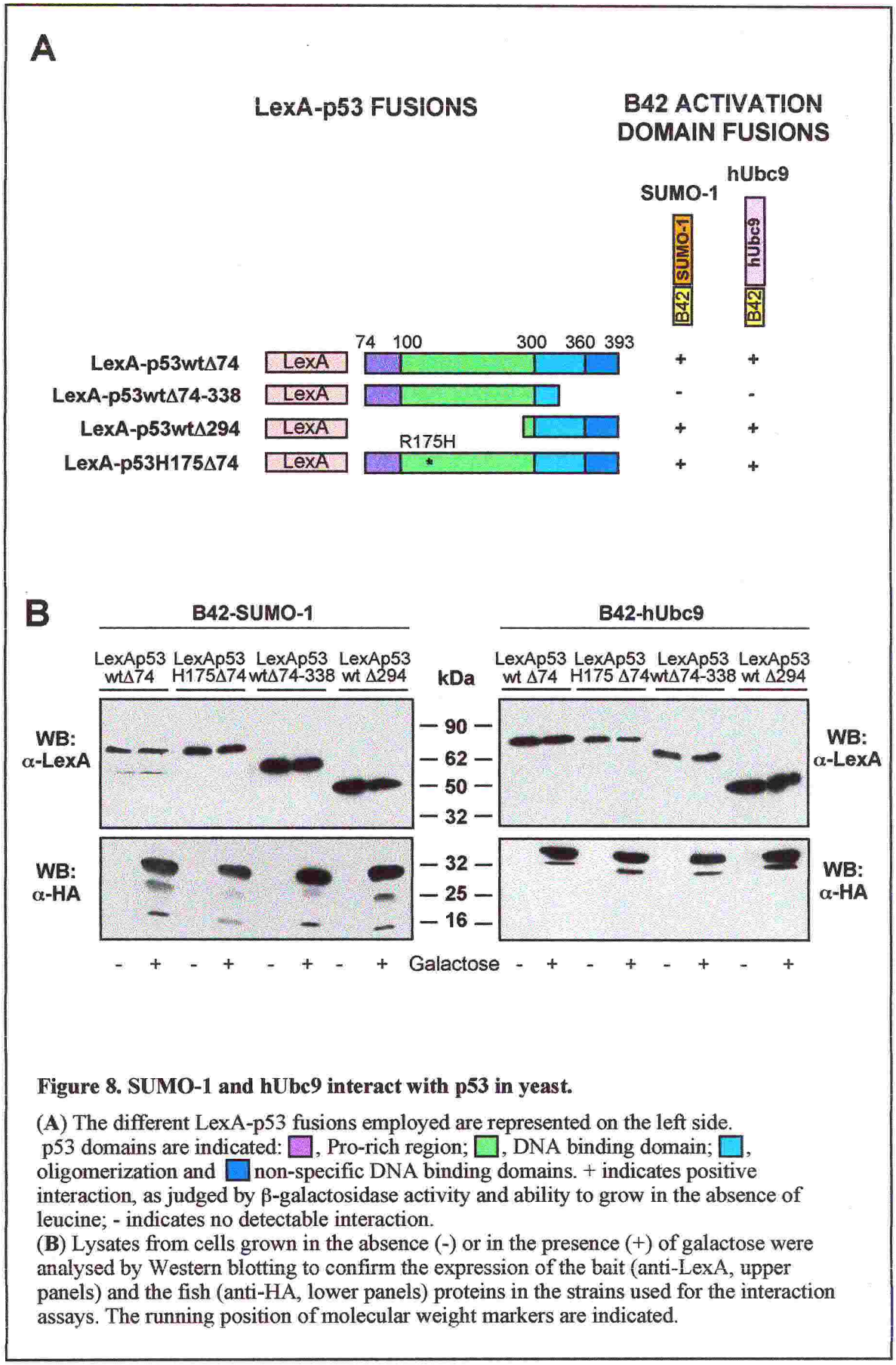


Figure 8. SUMO-1 and hUbc9 interact with p53 in yeast.

(**A**) The different LexA-p53 fusions employed are represented on the left side. p53 domains are indicated: ■, Pro-rich region; ■, DNA binding domain; ■, oligomerization and ■ non-specific DNA binding domains. + indicates positive interaction, as judged by β-galactosidase activity and ability to grow in the absence of leucine; - indicates no detectable interaction.
(**B**) Lysates from cells grown in the absence (-) or in the presence (+) of galactose were analysed by Western blotting to confirm the expression of the bait (anti-LexA, upper panels) and the fish (anti-HA, lower panels) proteins in the strains used for the interaction assays. The running position of molecular weight markers are indicated.

SUMO-1 is conjugated to p53 *in vitro*

Since other known targets of SUMO-1 conjugation have been reported to interact with both SUMO-1 and hUbc9 in the two-hybrid system[138,148], I wanted to investigate whether p53 may also be a substrate of this modification pathway.

^{35}S-methionine labeled p53 (p53 wt) was generated in rabbit reticulocyte lysate and incubated in the presence or absence of recombinant mouse Ubc9 (mUbc9, identical to human Ubc9 at the amino acid sequence level), a partially purified protein fraction containing the SUMO-activating enzyme E1, and a GST-SUMO-1 fusion protein[144].

Under the reaction conditions used, a slower migrating form of p53 was observed (Figure 9A, left panel). Since the appearance of this form was dependent on the presence of E1, mUbc9, and GST-SUMO-1, it can be concluded that it represents p53 molecules that are modified by the covalent attachment of one moiety of GST-SUMO-1.

Furthermore, the conjugation of SUMO-1 appeared to be specific for p53 in that HHR23a, a protein that is not related to p53[149], was not modified under the same assay conditions (Figure 9A, right panel).

The lysine residue at position 386 of p53 is required for SUMO-1 modification

Based on the results obtained in the yeast two-hybrid system (see Figure 8), it seemed possible that the C-terminal 55 amino acids of p53 contain all the informations that are required for p53 to be recognized by the SUMO-1 conjugation machinery.

In order to precisely map the region of p53 necessary for the conjugation, various p53 deletions were generated and tested in the *in vitro* assay as described.

N-terminal deletion mutants of p53 served as substrates for SUMO-1 conjugation with an efficiency similar to p53 wt, while deletion of the C-terminal 10 amino acids resulted in a protein that was not modified by SUMO-1 (data not shown).

The most C-terminal lysine residues of p53 are located at position 381, 382, and 386 (Figure 9B). Therefore, p53 mutants were constructed, in which these three residues were changed to arginine in various combinations, and tested for their ability to serve as a substrate for SUMO-1 conjugation. As shown in Figure 9B, mutation of lysine residue 386 results in a protein that cannot be modified by SUMO-1 *in vitro*. In contrast,

mutation of Lys 381 and 382, which have been shown to be preferential sites for acetylation[22], did not affect the ability of the respective p53 mutant to be modified.

Taken together, these results show that p53 is a substrate for SUMO-1 conjugation *in vitro*. Furthermore, the lysine at position 386 of p53 is required for this modification indicating that this residue serves as the major attachment site for SUMO-1.

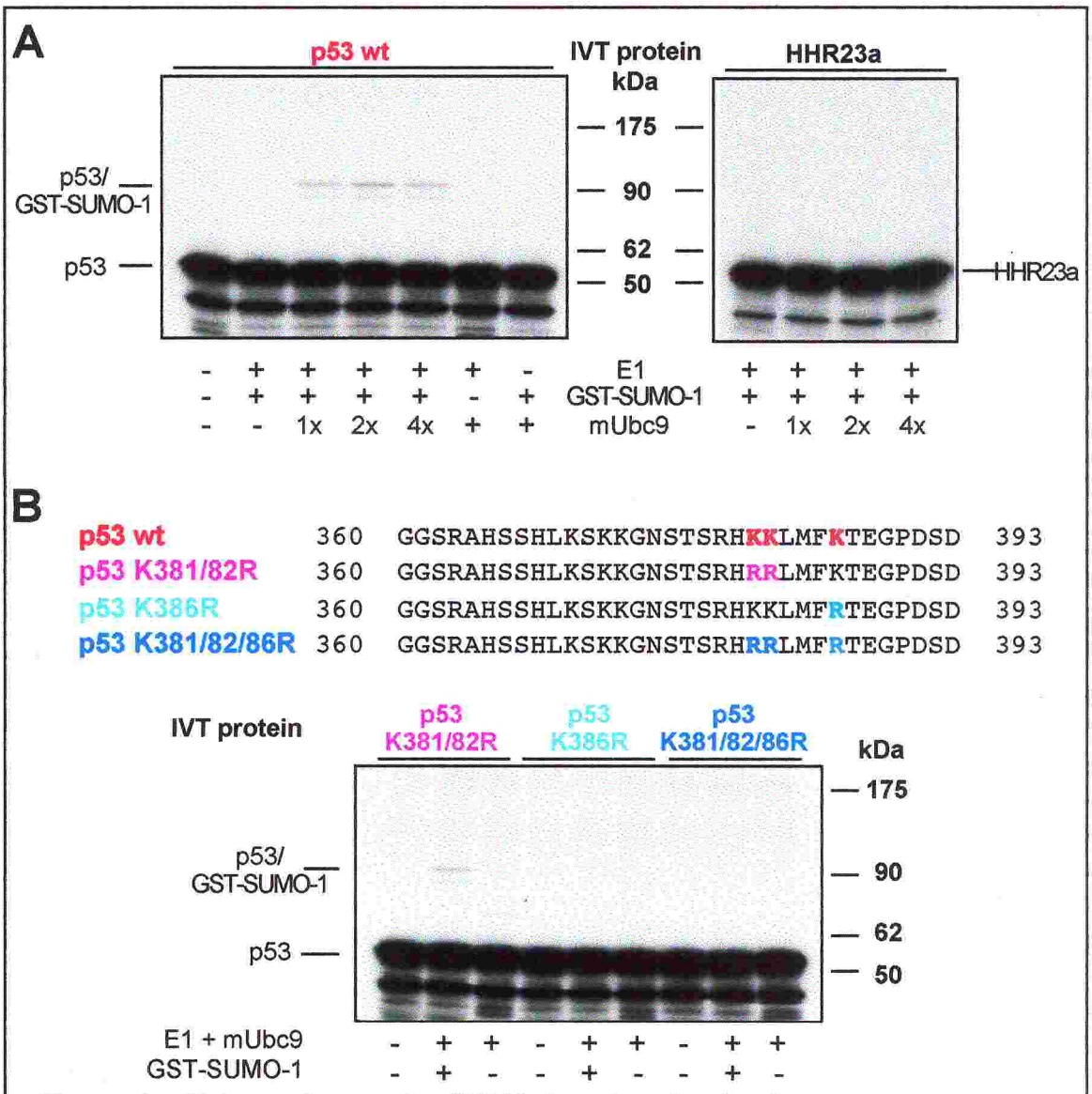


Figure 9. p53 is a substrate for SUMO-1 conjugation *in vitro*.

(**A**) *In vitro* translated ^{35}S-labeled p53 was incubated in the presence (+) or in the absence (-) of the indicated components. After 2 hours at 30°C, reactions were stopped and the whole reaction mixtures were separated by SDS-PAGE followed by fluorography. The bands corresponding to free and GST-SUMO-conjugated p53 are indicated (left panel). As a negative control, the p53 unrelated protein HHR23a was used (right panel).
(**B**) *In vitro* translated ^{35}S-labeled p53 mutants, with lysine to arginine substitutions at the indicated positions (p53K381/82R, p53K386R, p53K381/82/86R) were tested for SUMO-1 conjugation *in vitro* as described in (A). The amino acidic sequence of the C-terminal region of the various mutants is shown above.

p53 is covalently modified by SUMO-1 *in vivo*

To obtain evidences that p53 is also modified by SUMO-1 in human cells, I performed a Western blot analysis on total lysates from 293 cells. As shown in Figure 10A, a slower migrating p53 form was recognized by the anti-p53 monoclonal antibody DO-1, suggesting that this protein may represent p53 covalently linked to SUMO-1.

To confirm this hypothesis, lysates from the same cell line were first immunoprecipitated with a polyclonal antibody against p53 or with preimmune serum as a negative control. Subsequently, the immunocomplexes were analyzed by Western blotting with an anti-SUMO-1 monoclonal antibody. This revealed that a protein of the expected molecular weight (~70 kDa) was specifically recognized in the anti-p53 immunoprecipitate (Figure 10B, left panel). Finally, the same membrane was stripped and reprobed with the DO-1 antibody to demonstrate that the SUMO-1 crossreactive protein was indeed a modified form of p53 (Figure 10B, right panel).

The apparent molecular weight of the immunoprecipitated protein is consistent with the addition of a single SUMO-1 molecule to one p53 molecule. This finding is in agreement with the *in vitro* data presented above, as well as with previous observations that SUMO-1 modification usually takes place on specific single acceptor sites[138,150] and that, unlike ubiquitin, poly-SUMO-1 chains are not, or only very inefficiently, formed[86].

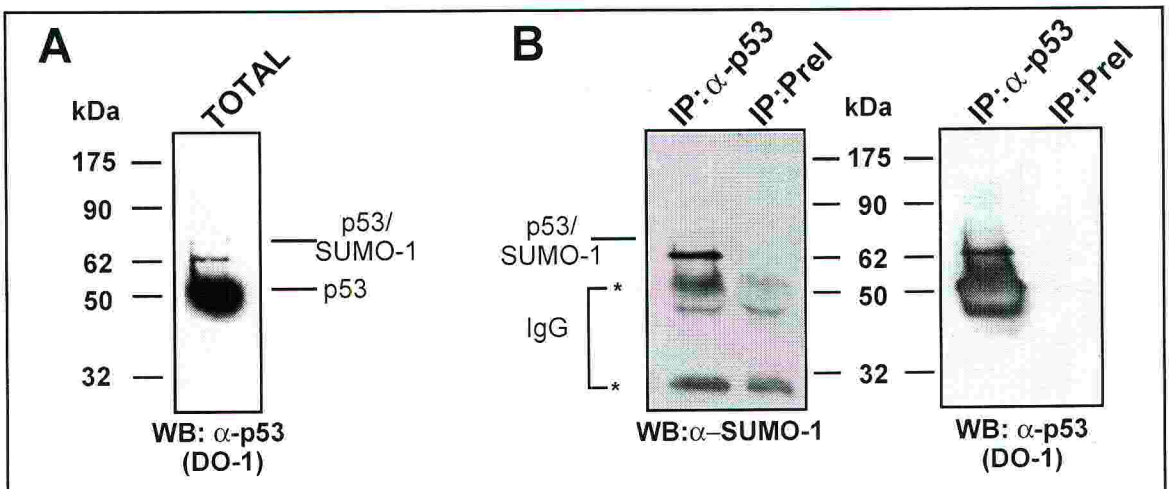


Figure 10. Endogenous p53 is modified by SUMO-1 *in vivo*.

(**A**) 293 cells were directly lysed in SDS-containing sample buffer and samples were analyzed by SDS-PAGE followed by Western blotting using the anti-p53 monoclonal antibody DO-1. The positions of free and putative SUMO-1 conjugated p53 are indicated.

(**B**) Lysates from 293 cells were immunoprecipitated with a polyclonal antiserum raised against human p53 (α-p53) or with pre-immune serum (PreI) as a negative control. Subsequently, immunoprecipitates were analyzed by Western blot with an anti-SUMO-1 monoclonal antibody (left panel). The same membrane was then stripped and reprobed with DO-1 (right panel). Running position of SUMO-1 conjugated p53 is indicated.

SUMO-1 conjugation to p53 *in vivo* requires lysine 386

To investigate if the p53 lysine residue at position 386 is also essential for *in vivo* SUMO-1 conjugation, I generated constructs encoding tagged SUMO-1 proteins by cloning the entire SUMO-1 ORF fused to either the Green Fluorescent Protein (GFP-SUMO-1) or the HA epitope (HA-SUMO-1).

These fusion proteins were then tested for their ability to be efficiently conjugated to previously reported SUMO-1 substrates. To do so, I performed transient transfections and colocalization experiments in the human osteosarcoma cell line U2OS. GFP-SUMO-1 clearly colocalized with both overexpressed and endogenous Sp100 and PML (not shown), two proteins that are modified by SUMO-1 and that have been shown to localize with SUMO-1 in subnuclear structures called nuclear bodies (NBs)[140]. Similar results were also obtained with the HA-tagged protein (not shown). In addition, the constructs were transiently transfected in U2OS cells and Western blot analysis was subsequently performed on total cell extracts using antibodies against SUMO-1, the HA-tag or the GFP-tag of the respective fusion protein. Consistent with previous reports[144], a high molecular weight smear was observed (not shown), which indicates that several cellular proteins were covalently modified by conjugation to the tagged SUMO-1.

Having shown that GFP-SUMO-1 can be conjugated to cellular proteins, pGFPSUMO-1 was transiently transfected into the p53-null cell line SaOS-2, together with a vector expressing p53 wt (pcDNA3p53wt) or the K386R mutant (pRcCMVp53K386R).

36 hours after transfection, the cells were lysed in SDS sample buffer and analyzed by Western blotting using the p53 specific antibody DO-1. When p53 wt was coexpressed with GFP-SUMO-1 (Figure 11A, lane 4), a more slowly migrating band was visible and its molecular weight (~100 kDa) was consistent with a form of p53 that is covalently modified by GFP-SUMO-1. In contrast, when the p53 mutant (p53 K386R), which is not conjugated *in vitro*, was used, no shifted p53-crossreactive band was observed (Figure 11A, lane 6).

Similar results were obtained also in Balb/c (10)1 fibroblasts (not shown).

To directly demonstrate that the higher band observed in the above experiment corresponds to p53 covalently linked to GFP-SUMO-1, SaOS-2 cells were transiently transfected with p53 wt together with GFP-SUMO-1 or with GFP. Cell lysates were

immunoprecipitated with an anti-GFP polyclonal antibody and then blotted with DO-1. As shown in Figure 11B (upper panel), a 100 kDa p53-reactive band was specifically immunoprecipitated by the anti-GFP antibody but only when GFP-SUMO-1 was coexpressed (lane 2). In contrast, no GFP-SUMO-1 linked p53 was detected when the conjugation-deficient mutant K386R was employed (lane 4).

Taken together, these results demonstrate that p53 can be covalently modified by SUMO-1 *in vitro* and *in vivo*. Furthermore, the lysine 386 residue identified by *in vitro* experiments is also required for SUMO-1 modification *in vivo*.

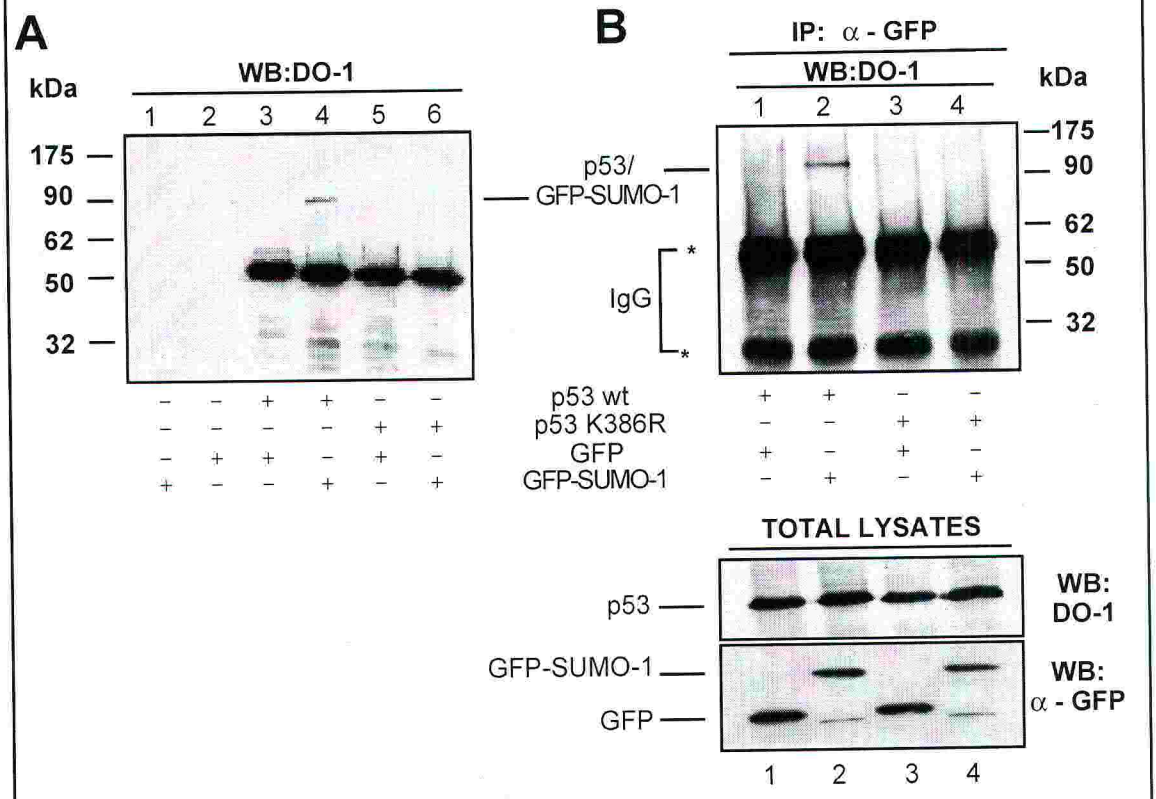


Figure 11. Lysine 386 is required for SUMO-1 conjugation to p53 in vivo.

(**A**) SaOS-2 cells were seeded in 6 cm diameter petri dishes and transfected with 1 µg of p53 expression plasmids and 2 µg of pGFPSUMO-1 or empty pGFP. Total extracts were prepared by direct lysis in SDS sample buffer, resolved by SDS-PAGE and analyzed by Western blotting using the anti-p53 specific antibody DO-1. Positions of free p53 and putative p53/GFP-SUMO-1 conjugates are indicated.

(**B**) Lysates from SaOS-2 cells transfected with 4 µg of p53 expression plasmids and 10 µg of pGFP or pGFP-SUMO-1 as indicated were immunoprecipitated with an anti-GFP polyclonal antibody and immunoprecipitates were subjected to Western blot analysis using the DO-1 antibody (upper panel). An aliquot of each lysate was checked for the expression of the transfected plasmids by staining with DO-1 (middle panel) or anti-GFP (lower panel) antibodies.

The C terminus of p53 is sufficient to be targeted by SUMO-1 conjugation *in vivo*

The results from the yeast two-hybrid and the *in vitro* assay indicated that the C terminus of p53 may be sufficient for recognition as a substrate for SUMO-1 modification.

To test if it is also sufficient in mediating the conjugation *in vivo*, I ectopically expressed a construct encoding the last 100 amino acids of p53, in fusion with the HA epitope (pcDNA3HAp53294-393), together with HA-SUMO-1. The p53 294-393 protein contains p53 NLS and is efficiently localized to the nucleus (see Figure 20). Cell lysates were immunoprecipitated with an anti-p53 polyclonal antibody and analyzed by Western blotting with anti-HA and anti-SUMO-1 antibodies. As shown in Figure 12, in total lysates (upper panel, lane 2) as well as in immunoprecipitates (upper panel, lane 4) from cells expressing HA-SUMO-1, a slower migrating HA-crossreactive band was present. When the membrane was stripped and reprobed, the same band was recognized by the anti-SUMO-1 specific antibody (lower panel, lanes 2 and 4). No protein was detected with either of the antibodies in control transfected cells (lanes 1 and 3).

Thus, the C-terminal region of p53 is likely to be sufficient for recognition as a substrate by the SUMO-1 conjugation pathway. However, a role of other domains of the protein in modulating the efficiency of the modification cannot be excluded.

Finally, it should be noted that a wt-like conformation of the full-length p53 does not appear to be required for *in vivo* sumolation, since the tumor derived mutant p53 R175H was also linked to GFP-SUMO-1 in cells (data not shown).

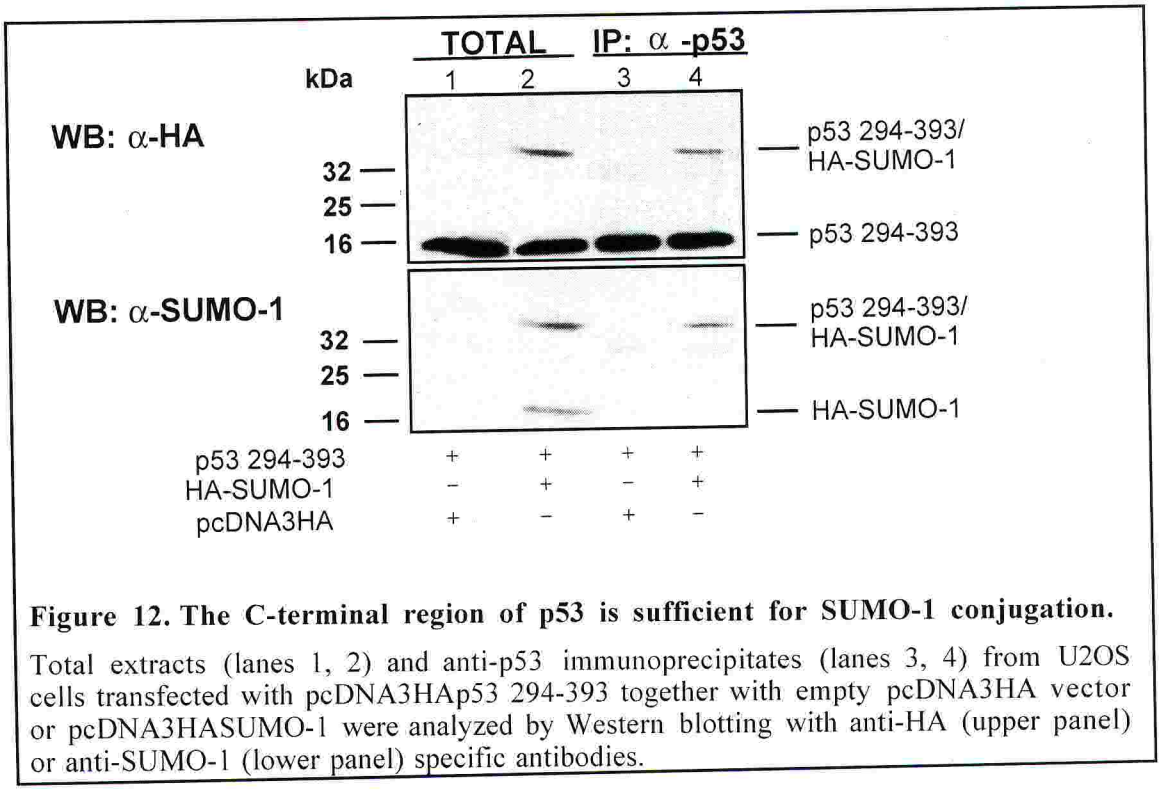


Figure 12. The C-terminal region of p53 is sufficient for SUMO-1 conjugation.

Total extracts (lanes 1, 2) and anti-p53 immunoprecipitates (lanes 3, 4) from U2OS cells transfected with pcDNA3HAp53 294-393 together with empty pcDNA3HA vector or pcDNA3HASUMO-1 were analyzed by Western blotting with anti-HA (upper panel) or anti-SUMO-1 (lower panel) specific antibodies.

SUMO-1 modification enhances p53-dependent transactivation

Since p53 C terminus plays an important role in regulating the activity of the protein and several post-translational modification taking place in this domain have been shown to affect p53 SST (see introduction), I decided to investigate whether the conjugation of SUMO-1 to the C-terminal region of p53 may affect its transactivation capacity.

To test this possibility, a luciferase reporter construct containing the p21 promoter (p21-Luc[48]) was transfected into U2OS cells, which contain endogenous wild-type p53, together with the empty pcDNA3HA vector or with increasing amounts of pcDNA3HASUMO-1. As shown in Figure 13A, HA-SUMO-1 overexpression enhanced luciferase activity from the p21 reporter up to 3 fold and this increase correlated with the amount of overexpressed protein, as judged by Western blot analysis (Figure 13A, lower panel). Efficiency of transfection was monitored by cotransfecting limiting amounts of pGFP as a marker and analyzing the lysates by Western blot with an anti-GFP polyclonal antibody (Figure 13A, lower panel).

Reporter activity from a plasmid lacking p53 binding sites was not affected by HA-SUMO-1 overexpression (data not shown). Similar results were obtained with GFP-SUMO-1 (not shown).

This indicates that sumolation of p53 increases its transcriptional activity.

To obtain further evidence that the observed increase of p21-Luc activity was due to SUMO-1 modification of p53, p21-Luc was transfected into p53-null Balb/c (10)1 fibroblasts together with vectors expressing either p53 wt or the conjugation-deficient mutant p53 K386R. The basal levels of luciferase activity obtained with the two proteins were comparable (Figure 13B, bars 1 and 3). However, when HA-SUMO-1 was coexpressed, a significant increase in the activation of the reporter was observed only in cells expressing p53 wt (Figure 13B, bars 1 and 2), with the activity of the mutant protein being unaffected by HA-SUMO-1 (Figure 13B, bars 3 and 4). As a control, the basal activity of the p21-Luc reporter in the absence of p53 (Figure 13B, bars 5 and 6) did not significantly change following HA-SUMO-1 overexpression.

To exclude the possibility that the different activities observed for p53 wt and the K386R mutant were not due to variations in expression levels, an aliquot of the lysates was subjected to Western blot analysis using the monoclonal antibody DO-1. As shown in Figure 13B (lower panel), p53 expression levels were comparable under the conditions used.

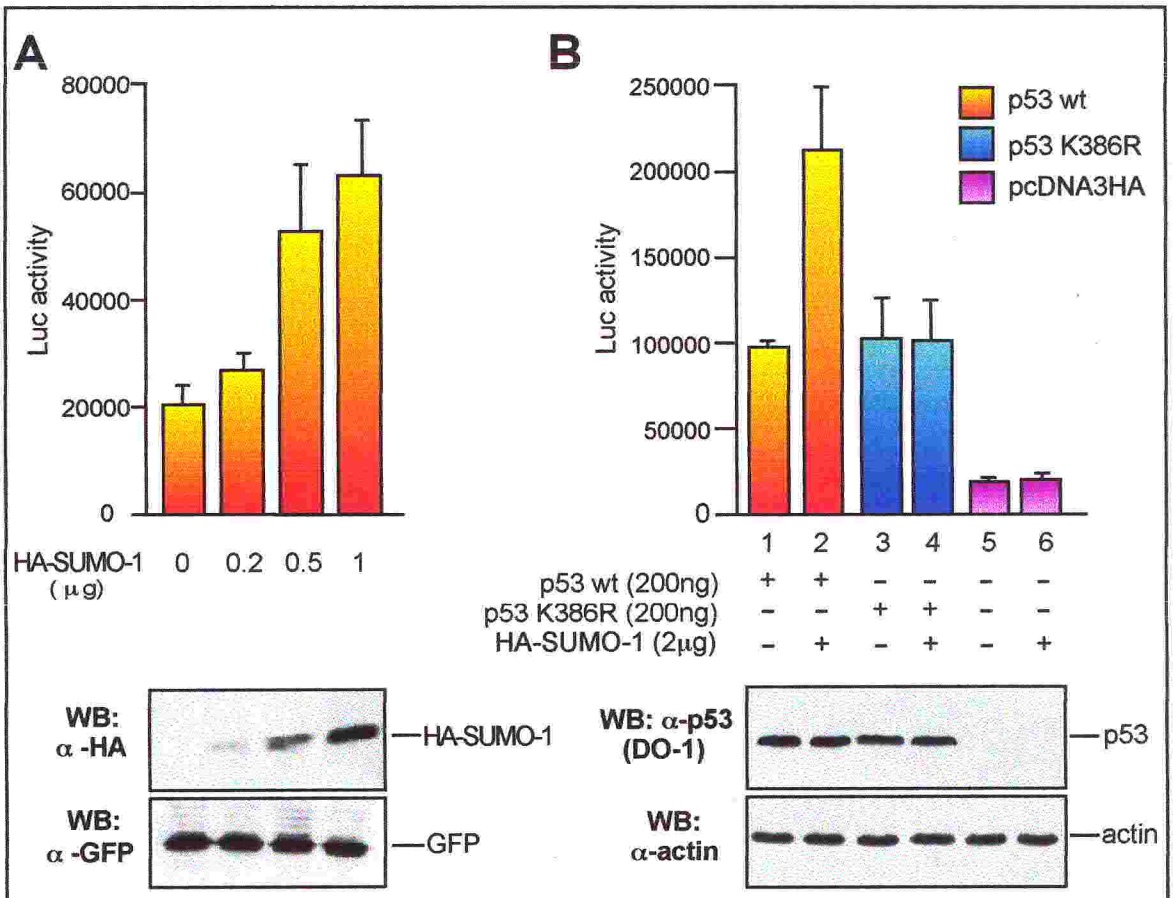


Figure 13. SUMO-1 conjugation enhances p53 transcriptional activity.

(**A**) Luciferase assays were performed on lysates from U2OS cells transfected with a p21-Luc reporter plasmid together with empty vector or increasing amounts of pcDNA3HASUMO-1, as indicated. 100 ng of pGFP were cotransfected to monitor the efficiency of transfection. An aliquot of the lysates was analyzed by Western blotting with anti-HA antibody to confirm the expression of the transfected HA-SUMO-1 and with anti-GFP antibody to evaluate the efficiency of transfection (lower panels).
(**B**) Balb/c (10)1 fibroblasts were transfected with p21-Luc, pcDNA3p53wt or pRcCMVp53 K386R or empty pcDNA3HA vector, either with or without HA-SUMO-1. Luciferase assay was performed as in (A). An aliquot of each of the lysates was analyzed by Western blotting using the DO-1 antibody to demonstrate comparable levels of expression of p53 in all the samples. The same membrane was subsequently probed with an anti-actin antibody to estimate the total amount of protein loaded in each lane (lower panels). Graphs represent the mean of at least four independent experiments.

I also analyzed the effect of SUMO-conjugation on p53 activity toward different promoters (Mdm2-Luc, PIG3-Luc, Bax-Luc and CyclinG-Luc), with the same approach described above. Figure 14 reports the obtained results: while the conjugation-deficient mutant p53 K386R showed in all the cases no differences in activity after SUMO-1 overexpression, the transactivation ability of the wt protein were enhanced by SUMO-1 in the case of the Mdm2, PIG3 and Bax promoters, even if to different extents. On the

contrary, no effect was observed on the cyclin G promoter. These findings indicate the possibility of a differential regulation of p53 activity toward specific responsive genes by SUMO-1 conjugation. However, this hypothesis needs to be further confirmed.

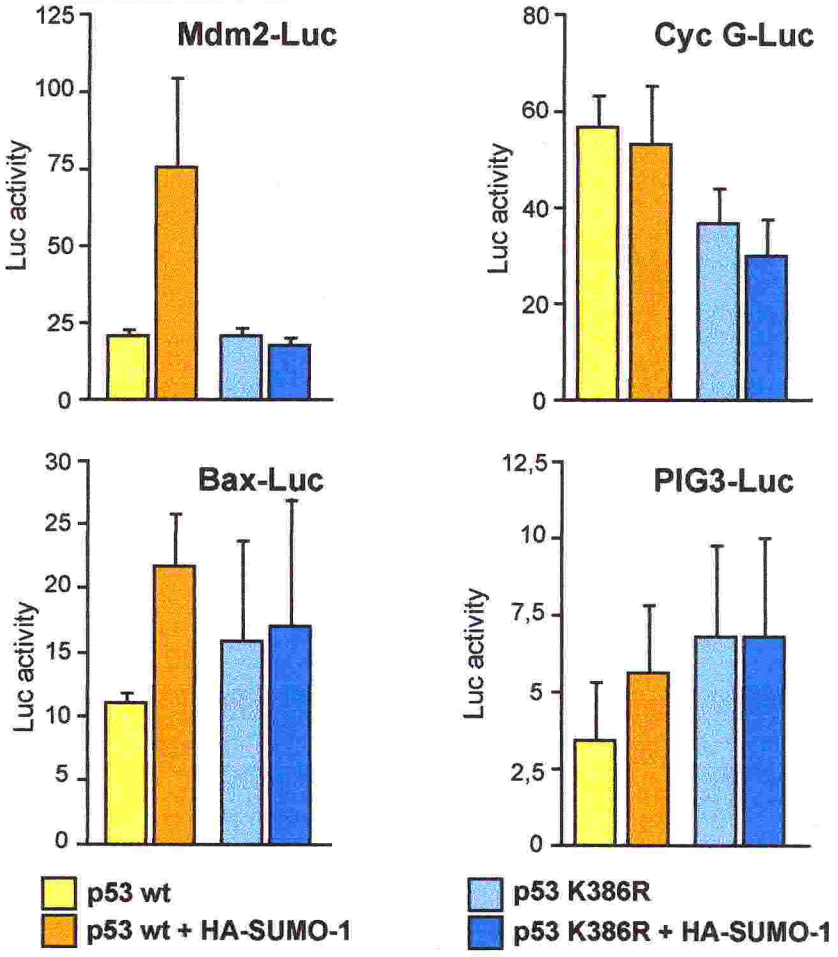


Figure 14. Effect of SUMO-conjugation on p53 transcriptional activity toward different promoters.

Luciferase assays were performed as in Figure 13 on lysates from SaOS-2 cells transfected with the indicated reporter plasmids together with pcDNA3p53wt or pRcCMVp53K386R, either with or without HA-SUMO-1

Finally, since Ubc9 is the E2 enzyme that mediates SUMO-1 conjugation, I tested the possibility that hUbc9 overexpression enhances the transactivation capacity of p53 by increasing the fraction of p53 modified by endogenous SUMO-1. Therefore, luciferase assays were performed with lysates from U2OS cells transfected with p21-Luc and increasing amounts of pcDNA3HAhUbc9. As for HA-SUMO-1, HA-hUbc9 overexpression resulted in enhanced reporter activity (Figure 15A).

Also in this case, the observed effect was most likely directly dependent on the presence of conjugation-competent p53, since no increase in luciferase activity was obtained when HA-hUbc9 was coexpressed in Balb/c (10)1 fibroblasts together with p53 K386R (Figure 15B).

These results suggest that both endogenous hUbc9 and endogenous SUMO-1 are rate-limiting factors in the conjugation of SUMO-1 to p53.

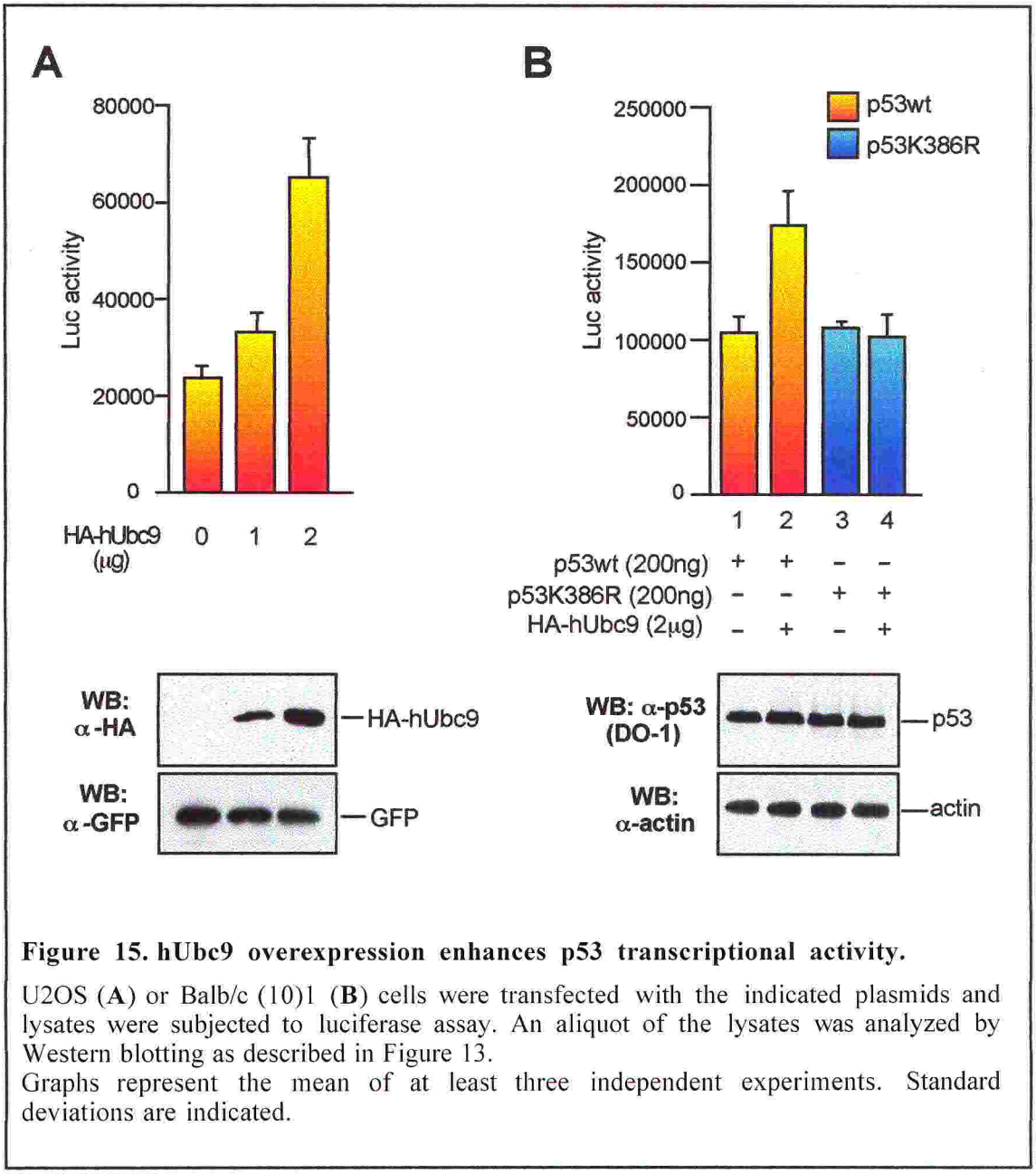


Figure 15. hUbc9 overexpression enhances p53 transcriptional activity.

U2OS (**A**) or Balb/c (10)1 (**B**) cells were transfected with the indicated plasmids and lysates were subjected to luciferase assay. An aliquot of the lysates was analyzed by Western blotting as described in Figure 13.

Graphs represent the mean of at least three independent experiments. Standard deviations are indicated.

SUMO-1 and hUbc9 act in a cooperative way in modifying p53

The results presented above suggested that both hUbc9 and SUMO-1 are rate-limiting factors in the conjugation of SUMO-1 to p53 and, thus, coexpression of SUMO-1 and hUbc9 may have a cooperative effect on the transactivation activity of p53.

To test this hypothesis, I transfected U2OS cells with p21-Luc together with GFP-SUMO-1 and HA-hUbc9, either separately or together and determined luciferase activity as described above.

As can be observed in Figure 16A, when both proteins were expressed (bar 4) the increase in the p21-Luc reporter activity was 2-fold higher than in cells expressing only GFP-SUMO-1 (bar 2) or HA-hUbc9 (bar 3). In all the samples, expression of the different fusion proteins was controlled by Western blotting (Figure 16B, central panels).

Furthermore, I tested whether conjugation of SUMO-1 to p53 also resulted in enhanced expression of the endogenous p21 protein. The lysates used for the luciferase assay were analyzed by Western blotting with an anti-p21 polyclonal antibody, revealing that the expression of p21 was increased in cells expressing either SUMO-1 or hUbc9 or both (Figure 16B, upper panel).

Finally, to obtain evidence that the observed increase in the transactivation ability if p53 was indeed linked to an increase in the amount of SUMO-conjugated p53, SaOS-2 cells were transfected with p53 wt and increasing amounts of GFP-SUMO-1 in the presence or in the absence of overexpressed HA-hUbc9 and total lysates were subjected to Western blot analysis with the p53-specific antibody DO-1 (Figure 16C).

As expected, in the absence of exogenous hUbc9, elevating the amount of transfected GFP-SUMO-1 resulted in a simultaneous increase of sumolated p53 (Figure 16C, compare lanes 1 and 3). Coexpression of HA-hUbc9 resulted in a further increase of the modified form of p53 (lanes 2 and 4), demonstrating that indeed both SUMO-1 and hUbc9 are limiting factors in the conjugation process.

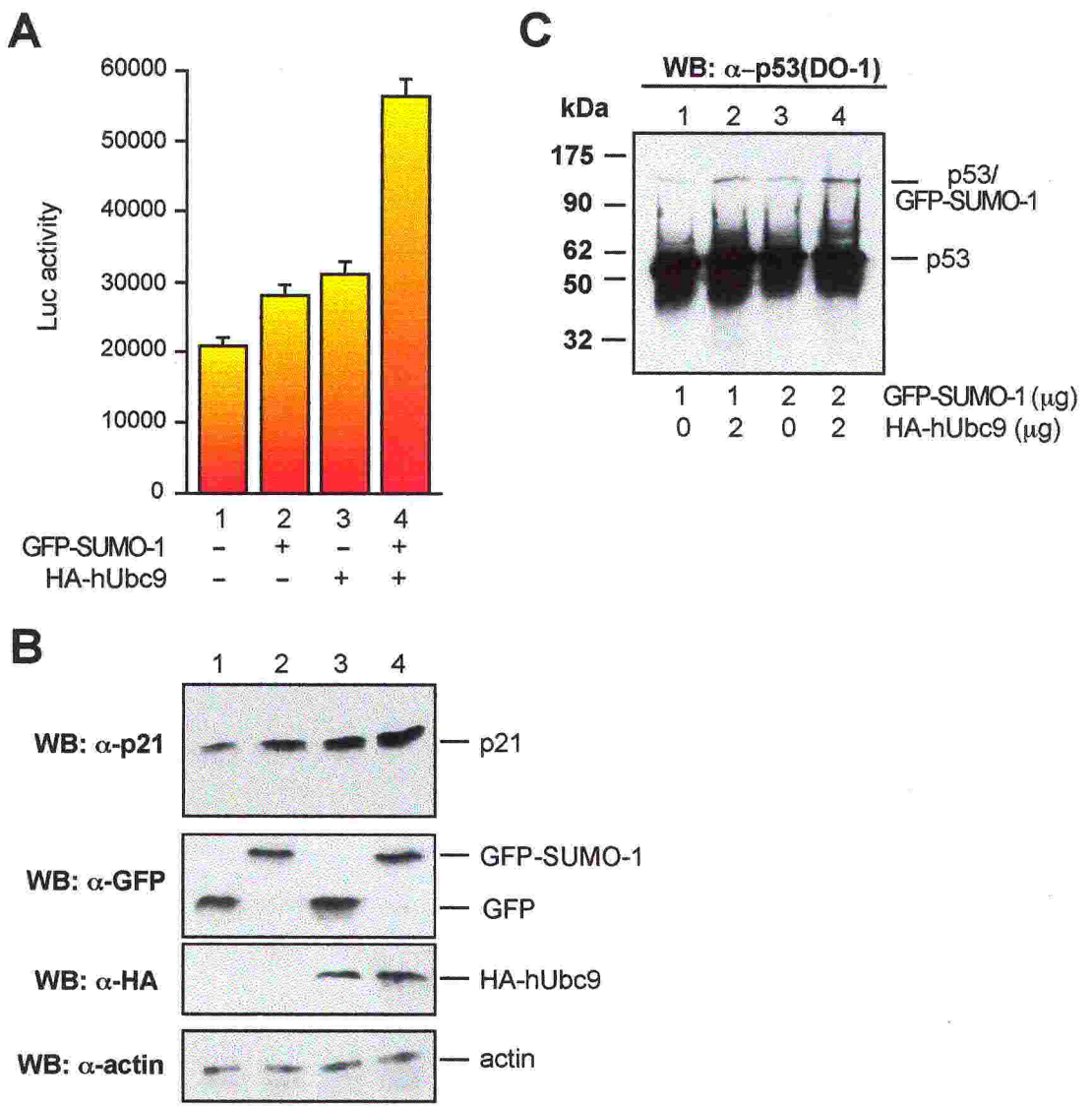


Figure 16. SUMO-1 and hUbc9 have a cooperative effect on p53 sumolation and transcriptional activation.

(**A**) Luciferase assays were performed on extracts from U2OS cells transfected with p21-Luc and with either pGFPSUMO-1 or pcDNA3HAhUbc9 or both plasmids, as indicated. Graph represents the mean of three independent experiments. Standard deviations are indicated.

(**B**) An aliquot of each lysate used for the luciferase assay was subjected to Western blot analysis with an anti-p21 polyclonal antibody (upper panel) and with anti-HA or anti-GFP antibodies to check the expression of the transfected proteins (central panels). Comparable amounts of proteins were loaded in each lane, as estimated by probing the same membrane with anti-actin antibody (lower panel)

(**C**) SaOS-2 cells were transfected with 1 μg of pcDNA3p53wt together with the indicated amounts of plasmids encoding GFP-SUMO-1 and HA-hUbc9. Total lysates were then analyzed by Western blot with anti-p53 monoclonal antibody DO-1. Positions of free and GFP-SUMO-1 conjugated p53 and molecular weight markers are shown.

PART 2.
RECRUITMENT OF p53 INTO NUCLEAR BODIES BY A SPECIFIC PML ISOFORM MODULATES ITS TRANSCRIPTIONAL ACTIVITY AND AFFECTS CELL SURVIVAL

While performing the experiments described in part 1, I realized that upon coexpression of SUMO-1 and hUbc9, p53 localization in the nucleus was somehow altered and that p53 staining assumed a punctate pattern, reminiscent of subnuclear structures defined as nuclear bodies (NBs)[151].

A similar peculiar localization of p53 has been described after coexpression of p53 with Mdm2 and ARF and has been correlated with ARF-mediated inhibition of Mdm2-p53 nuclear export[152]. Punctate nuclear distribution for p53 and Mdm2 has been also observed in human primary cells treated with Leptomycin B, a drug that specifically blocks nuclear export[153]. These dots have been shown to contain the snRNP U1A and to be partially colocalizing with Sp100-containing nuclear bodies.

Recently, several proteins involved in transcriptional and growth control have been reported to localize to nuclear bodies and they have been proposed to play a role in regulating cell proliferation and differentiation; however their exact function remains unclear[151].

PML, the promyelocytic leukemia protein, is the most prominent component of the NBs (that are also referred as PML oncogenic domains, PODs) and was first identified in acute promyelocytic leukemia (APL) patients where, as a result of a reciprocal translocation event, it is fused to the retinoic acid alpha receptor (RARα)[154,155]. PML belongs to a family of proteins characterized by a tripartite motif, named RBCC (RING B-box coiled coil), consisting of a zinc-finger domain and two additional cysteine-rich zinc-binding regions, followed by a leucine coiled coil[156] (Figure 17). The RBCC is required for PML homodimerization, for interaction with other factors and for the assembly of NBs. Several alternatively spliced forms of PML have been isolated[154,155,157], with differences mostly in the C-terminal region (Figure 17).

PML has a fundamental role in directing the complex protein-protein associations that mediates PODs formation, as underlined by the finding that the organization of several NB-associated components is impaired in PML-/- cells[158].

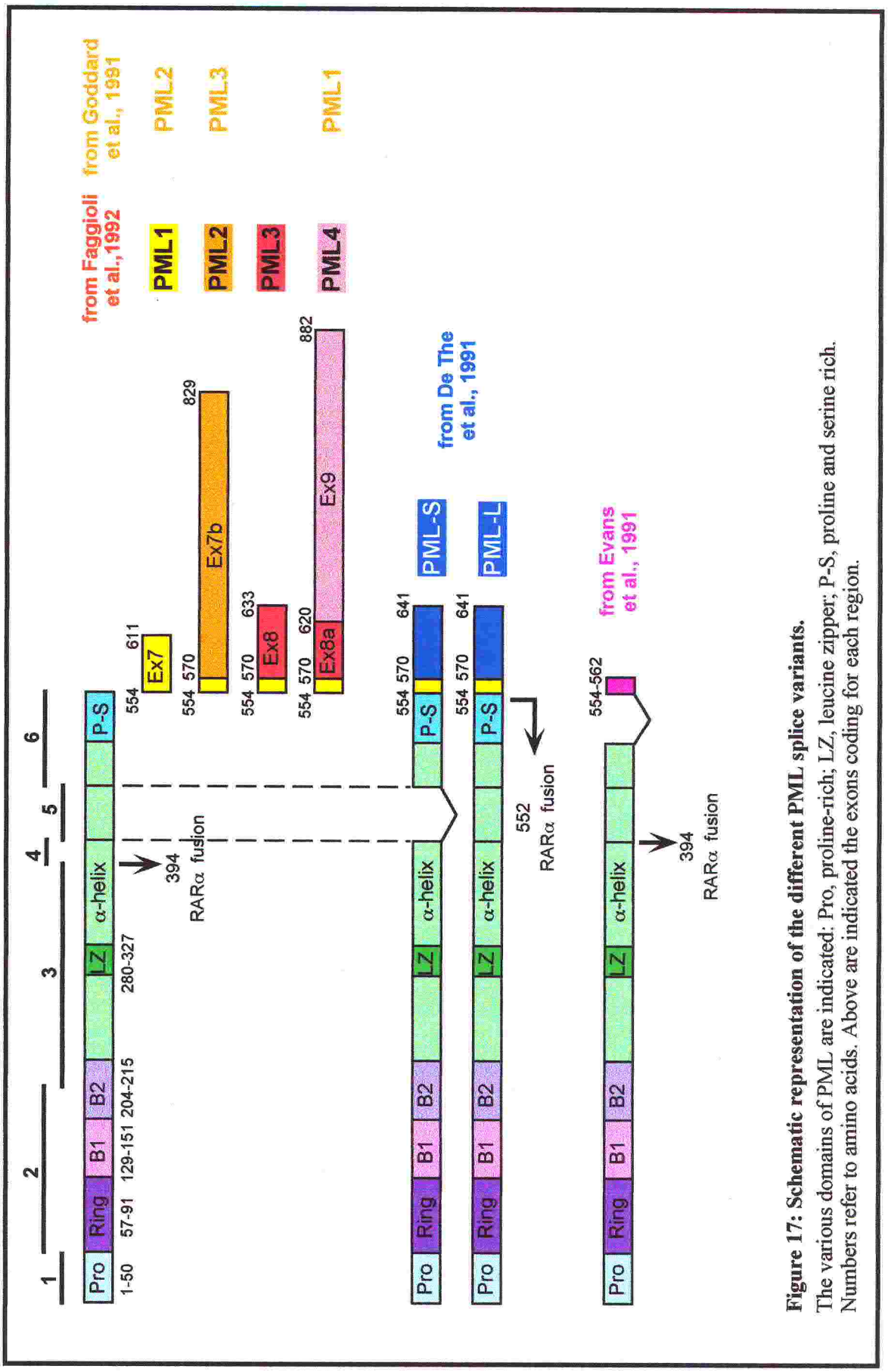


Figure 17: Schematic representation of the different PML splice variants.
The various domains of PML are indicated: Pro, proline-rich; LZ, leucine zipper; P-S, proline and serine rich. Numbers refer to amino acids. Above are indicated the exons coding for each region.

PML appears to function as a tumor suppressor and control cell growth at different levels. Fibroblasts derived from PML -/- mice show an increased proportion of cells in S phase[159]. Most strikingly, these knockout mice are resistant to lethal doses of gamma irradiation or Fas antibody-treatment, proposing a pro-apoptotic role of PML[159]. Recently, hDaxx, a protein involved in Fas-mediated apoptosis, was found to bind to and colocalize with PML and to exert its functions in NBs[160,161].

The importance of the structural integrity of NBs for normal cell growth and development is well documented in some human diseases, like APL and spinocerebellar ataxia type I (SCA1), where disruption of NBs leads to malignancy or a neurodegenerative disorder, respectively[162]. In APL cells, the expression of the PML-RARα fusion compromises the integrity of PODs, which appear as so-called "microspeckles", dispersed in the nucleus. Treatment of these cells with all-trans retinoic acid or As_2O_3 mediates the degradation of PML-RARα and the normalization of the NBs pattern and this effect is related with differentiation of malignant cells and remission of the disease[163]. Similarly, treatment with IFNs, that increase PML expression and PODs size, has been shown to induce apoptosis in APL cells[164].

Other evidences indicate that nuclear bodies are involved in the regulation of gene expression [156]. The reported interaction with Rb[165] that is recruiting histone deacetylases on one side and the direct binding to the histone acetyltransferase p300/CBP[166] on the other, suggest a relevant role for PML in the regulation of transcription. Sp100, another structural element of NBs, was demonstrated to behave as a transcriptional repressor and to interact with non-histone-type chromosomal proteins, strongly supporting the implication of NBs in transcriptional control at the chromatin level[167].

Interestingly, a common feature of two of the more representative proteins found in NBs, PML and Sp100, is the fact that both of them are post-translationally modified by SUMO-1[140]. Moreover, a recent report demonstrated that SUMO-conjugation of PML is absolutely required for NBs assembly[158].

For this reason, I decided to analyze in more detail the peculiar p53 distribution observed after SUMO-1 and hUbc9 overexpression.

This part of the work was done in collaboration with Valentina Fogal, who was performing all the microinjection experiments.

PML mediates the relocalization of p53 into Nuclear Bodies

To investigate whether, in addition to modulate p53 transcriptional activity, SUMO-1 may also assist its relocalization into NBs, I performed colocalization experiments in the human p53-null SaOS-2 cell line.

First, I analyzed the cellular localization of overexpressed GFP-SUMO-1 and HA-hUbc9. Immunofluorescence staining and confocal laser microscopy analysis revealed that GFP-SUMO-1, when expressed alone, exhibited the previously described nuclear diffuse localization, with a fraction of the protein concentrating in small dots (Figure 18, a). HA-hUbc9 alone, instead, showed a nuclear and cytoplasmic diffuse staining (Figure 18, b). When expressed together the two proteins were found to accumulate and colocalize in much larger nuclear bodies (Figure 18, c, d, e).

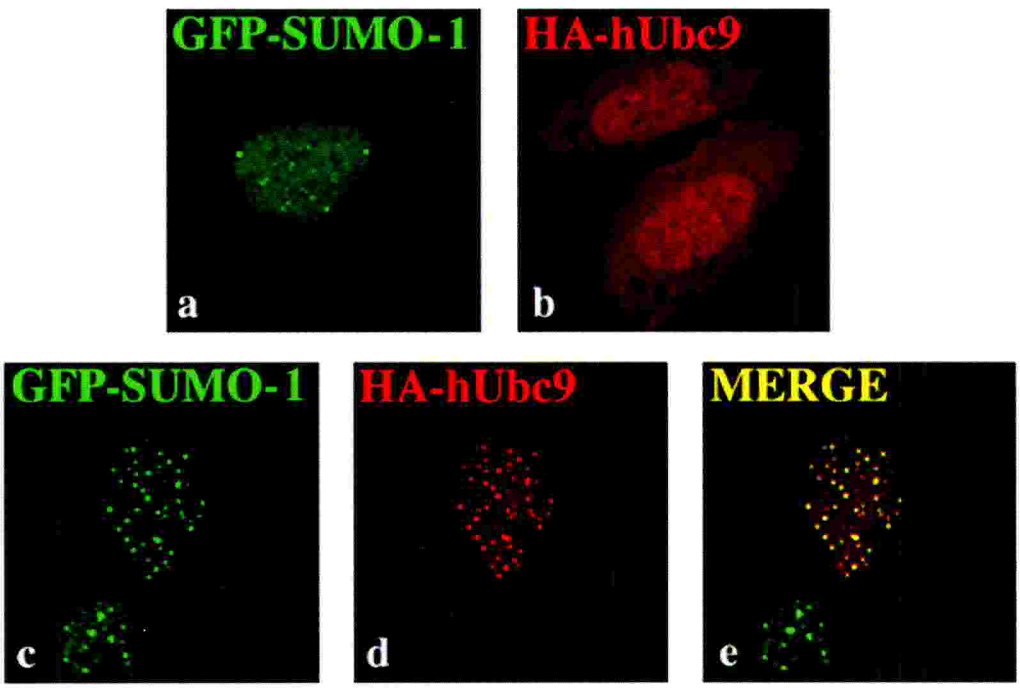


Figure 18. Analysis of GFP-SUMO-1 and HA-hUbc9 localization.

SaOS-2 cells were microinjected with plasmids encoding GFP-SUMO-1 and HA-hUbc9 either separately (a and b) or together (c-e). GFP-SUMO-1 distribution was revealed by means of the intrinsic green fluorescence of the GFP, while HA-hUbc9 was stained with anti-HA monoclonal antibody followed by TRITC-conjugated secondary antibody. The staining pattern was analyzed by confocal laser microscopy. Merging of the two colors results in a yellow signal, corresponding to colocalized proteins.

To test whether p53 could be relocalized into these subnuclear structures, SaOS-2 cells were microinjected with plasmids encoding p53 wt, GFP-SUMO-1 and HA-hUbc9 and analyzed by immunofluorescence and confocal laser microscopy. In a fraction of the injected cells (40%) the typical nucleoplasmic staining of p53 became organized in distinct GFP-SUMO-1 positive NBs (Figure 19A). This relocalization was dependent on the simultaneous expression of both GFP-SUMO-1 and HA-hUbc9, since microinjection of GFP-SUMO-1 or HA-hUbc9 alone, did not affect the nuclear diffuse distribution of p53 (not shown).

Surprisingly, the conjugation-deficient mutant p53 K386R, when microinjected in the same conditions, was relocalized to NBs to a similar extent than the wt protein (Figure 19B), thus demonstrating that sumolation of p53 is dispensable for its delivery to these structures. Of note, a similar independence of NBs localization from SUMO-1 conjugation has also been described for Sp100, another NBs resident protein that is modified by SUMO-1[168]. Nuclear dots targeting was p53-specific since, under the same conditions, a construct encoding β-galactosidase fused to a nuclear localization signal (βgal-NLS) was entirely excluded from these structures (Figure 19C).

This result suggested that another protein that can be modified by SUMO-1 is responsible to recruit p53 into NBs. Given its importance for NBs assembly, PML appeared to be the best candidate for such a factor. Moreover, SUMO-1 conjugation of PML was shown to be necessary for formation of these structures[158].

I therefore decided to test if PML could be responsible for the observed relocalization of p53. Immunofluorescence analysis of SaOS-2 cells microinjected with p53 wt together with PML3[157] revealed that a fraction of p53 was segregated into PML3-positive structures in almost all the microinjected cells (Figure 19D). A similar result was obtained also for endogenous p53 either when PML3 (Figure 19E) or GFP-SUMO-1 and HA-hUbc9 (not shown) were microinjected in U2OS cells.

From these findings it is possible to conclude that PML is the factor required for targeting p53 to nuclear bodies and that the same effect can be observed upon overexpression of SUMO-1 and hUbc9, suggesting that these two factors mediates sumolation of endogenous PML therefore favoring NBs formation.

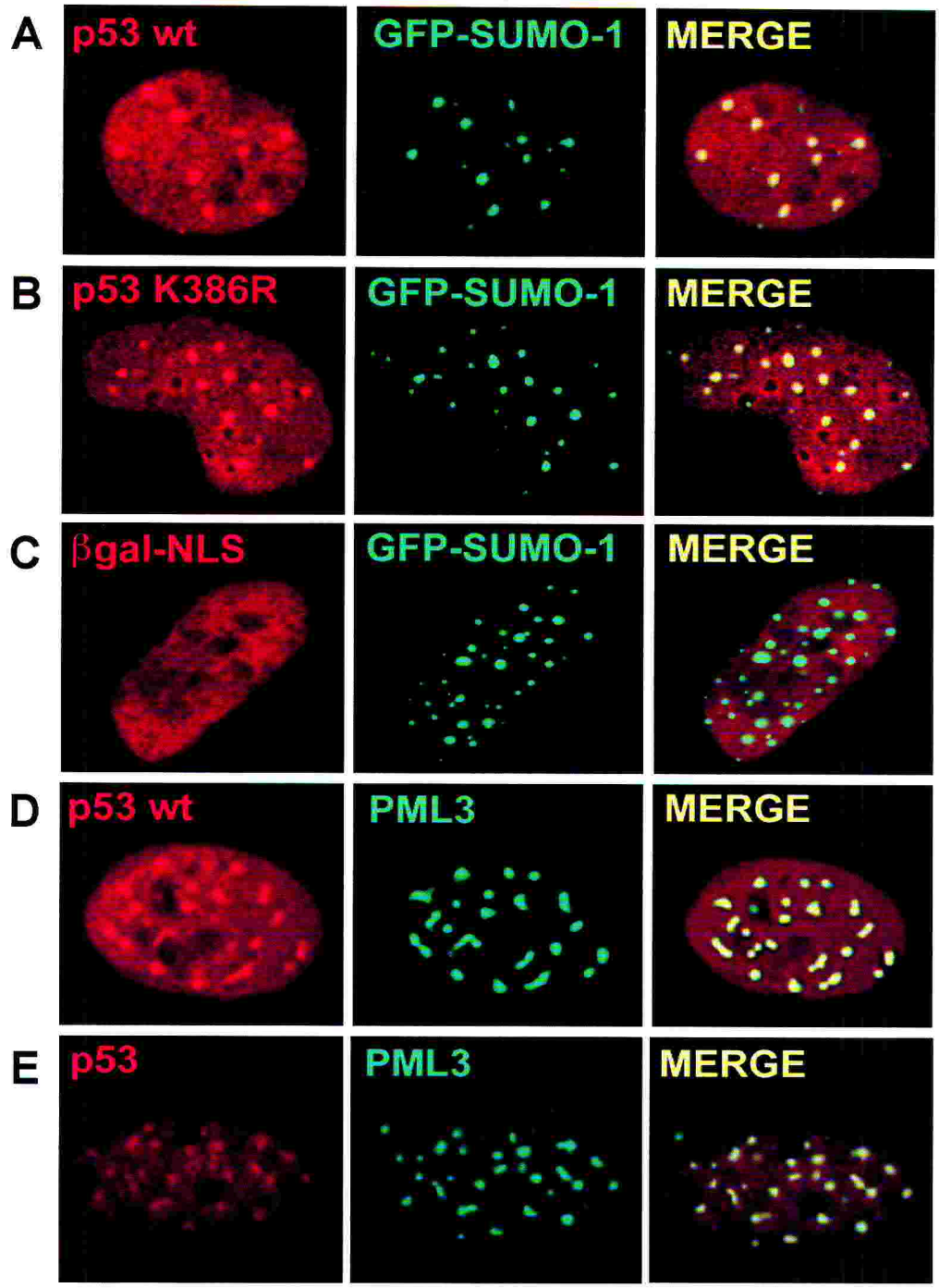


Figure 19. p53 relocalizes into NBs.

(**A-C**) SaOS-2 cells were microinjected with either 5 ng/μl of pcDNA3p53wt (A), pRcCMVp53K386R (B) or pNLS-βgal (C) together with 30 ng/μl of pGFPSUMO-1 and 50 ng/μl of pcDNA3HAhUbc9. Microinjected cells were analyzed for p53 staining by using a rabbit polyclonal anti-p53 serum followed by incubation with TRITC-conjugated secondary antibody. GFP-SUMO-1 was revealed by means of the intrinsic green fluorescence of GFP. As a negative control, the localization pattern of βgal-NLS was detected by a monoclonal anti-β-galactosidase antibody, followed by TRITC-conjugated secondary antibody.
(**D and E**) SaOS-2 cells (D) microinjected with 5 ng/μl of pcDNA3p53wt and 30 ng/μl pcDNA3PML3 were analysed for p53 expression as above and for PML staining using the anti-PML monoclonal antibody PG-M3 followed by incubation with FITC-conjugated secondary antibody. U2OS cells (E) were microinjected with pcDNA3PML3 and localization of endogenous p53 and overexpressed PML3 was examined as in (C).

Dissection of p53 region required for NBs localization

Next I wanted to identify the region of p53 required for NBs targeting. For this purpose, I generated several p53 deletion constructs that are schematically represented in Figure 20A. The levels of expression of the different proteins was judged to be comparable by Western blot analysis after transient transfection (Figure 20B) and all the deletions showed the typical diffuse nuclear staining (not shown).

All these constructs were microinjected into SaOS-2 cells together with PML3 and their localization pattern was analyzed. I observed that the ability to localize in NBs was maintained by p53 N-terminal deletions lacking the transactivation domain (p53Δ12-69, Figure 20C, a-c) or the Pro-rich region (p53Δ63-91, Figure 20C, d-f) as well as by progressive C-terminal deletions to amino acid 298 (Figure 20C, g-o).

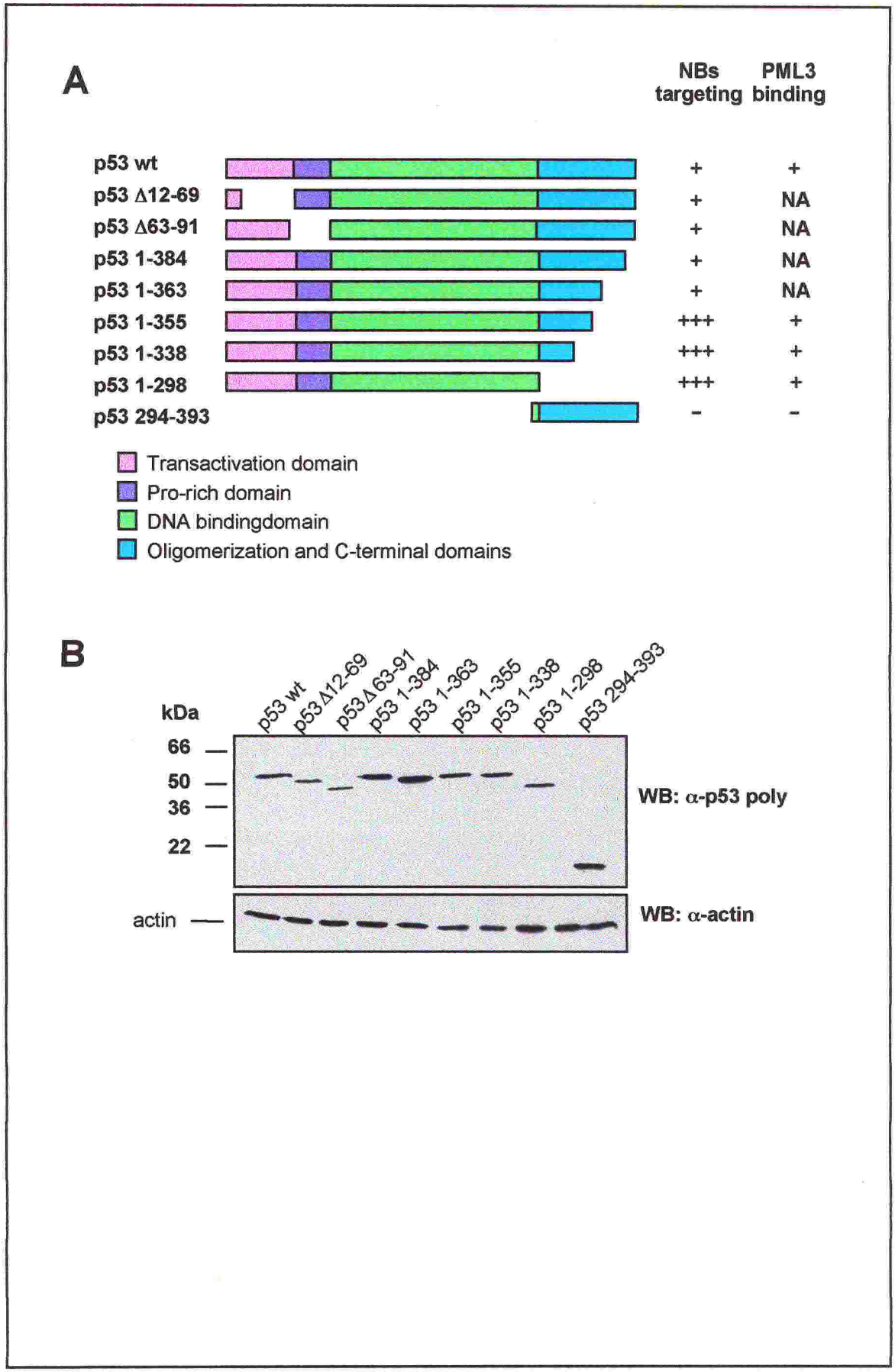
Remarkably, while p53 1-363 (Figure 20C, g-i) showed, as p53 wt (Figure 19D), both nuclear diffuse staining and NBs localization, removal of 8 more residues (p53 1-355) resulted in a very peculiar staining with the protein totally relocalized into NBs (Figure 20C, j-l).

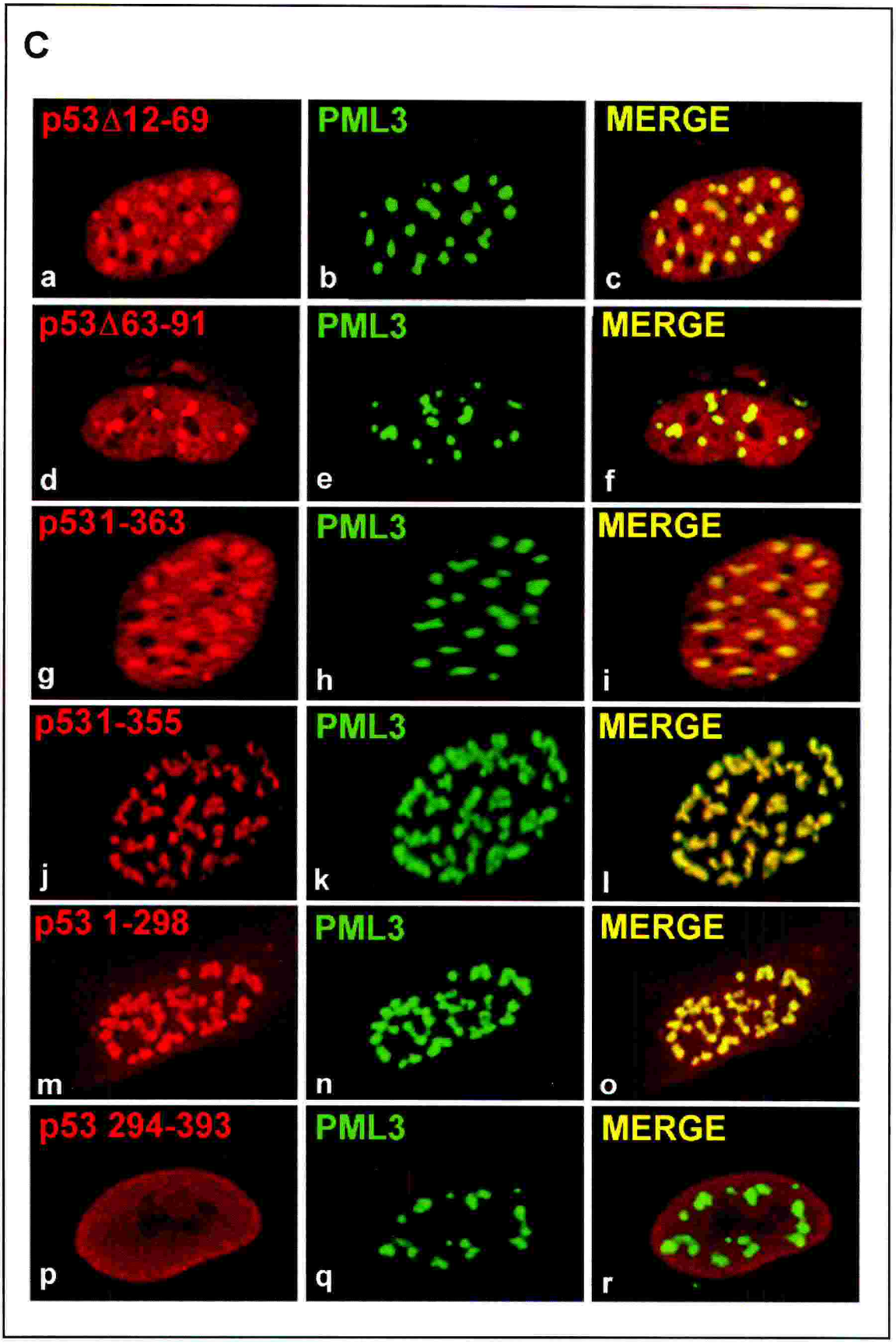
To confirm the role of p53 central region in NB-targeting, p53 294-393, which lacks the entire N-terminal and core domains, did not change its homogeneous nucleoplasmic localization upon coexpression of PML3 (Figure 20C, p-r).

Similar results were obtained in another p53-null cell line, MG63, and also when the forementioned p53 deletions were coexpressed with SUMO-1 and hUbc9 (not shown).

From these data it is possible to conclude that the core domain (amino acids 90-298) of p53 is required for NB-targeting. However, the evidence that the conformational mutant p53 H175 is also localized to NBs (not shown) demonstrated that the wt conformation of the protein is not necessary.

Furthermore, the massive PML3-induced relocalization of p53 1-355 as compared to p53 1-363 suggested that eight residues within 356 and 363 may exert a regulatory function on this process.


A
NBs targeting
PML3 binding
p53 wt
p53 Δ12-69
p53 Δ63-91
p53 1-384
p53 1-363
p53 1-355
p53 1-338
p53 1-298
p53 294-393
+
+
+
NA
+
NA
+
NA
+++
+
+++
+
+++
+
-
-
Transactivation domain
Pro-rich domain
DNA bindingdomain
Oligomerization and C-terminal domains
B
kDa
66
50
36
22
actin
p53 wt
p53Δ12-69
p53Δ63-91
p53 1-384
p53 1-363
p53 1-355
p53 1-338
p53 1-298
p53 294-393
WB: α-p53 poly
WB: α-actin


C
p53Δ12-69
PML3
MERGE
a
b
c
p53Δ63-91
PML3
MERGE
d
e
f
p531-363
PML3
MERGE
g
h
i
p531-355
PML3
MERGE
j
k
l
p53 1-298
PML3
MERGE
m
n
o
p53 294-393
PML3
MERGE
p
q
r

p53 binds to PML3 with the domain required for NBs targeting

Having established that the core domain of p53 is required to target the protein to NBs, I investigated if PML3-dependent changes in p53 subcellular distribution required the binding between the two proteins. SaOS-2 cells were transfected with plasmids encoding p53 wt together with PML3 or pcDNA3 as a negative control and cell lysates were immunoprecipitated with the anti-PML monoclonal antibody PG-M3. Immunoprecipitates were resolved on SDS-PAGE and analyzed by Western blot with the anti-p53 monoclonal antibody DO-1. As shown in Figure 21 (lanes 1 and 2), p53 can be specifically immunoprecipitated only from cells expressing PML3.

In order to demonstrate that the same domain of p53 required for NBs localization was also responsible for binding to PML, I performed immunoprecipitation experiments on lysates from cells expressing the p53 deletions 1-355, 1-298 or 294-393. Western blot analysis with DO-1 (Figure 21, lanes 3-6) or with anti-HA monoclonal antibody (Figure 21, lanes 7 and 8) demonstrated that, while p53 deletion mutants containing the DNA binding domain were able to bind to and coprecipitate with PML3, the HA-tagged p53 294-393 protein, which no longer relocalized to NBs, was impaired in the interaction.

These results therefore clearly indicate that p53 is able to bind to PML3 with its core domain and that this binding mediates p53 targeting to NBs in cells overexpressing PML3.

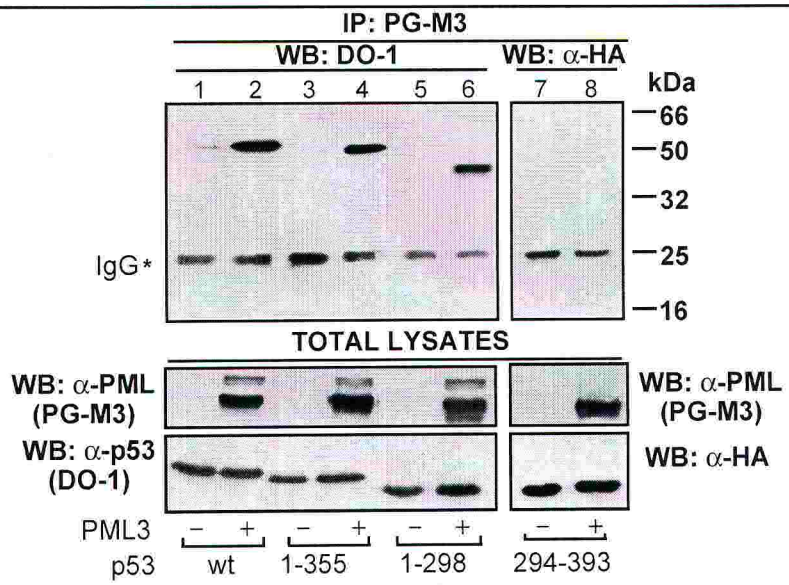


Figure 21. p53 binds to PML3 with its core domain.

Lysates from SaOS-2 cells overexpressing p53 wt (lanes 1 and 2), p53 1-355 (lanes 3 and 4), p53 1-298 (lanes 5 and 6) and p53 294-393 (lanes 7 and 8) together with PML3 or empty vector, were immunoprecipitated with anti-PML monoclonal antibody PG-M3 and immunoblot was performed with the anti-p53 monoclonal antibody DO-1 (lanes 1-6) or with anti-HA monoclonal antibody (lanes 7 and 8). Lower panels show Western blot analysis on total lysates to compare the expression levels of the various p53 proteins (low) and of PML3 (up) in the different samples.

Targeting of p53 to NBs depends on a specific PML isoform

The evidence that integrity of NB structures is disrupted in APL cells by expression of the PML-RARα fusion protein prompted me to assess whether p53 recruitment into NBs was specific for PML3 and not for the oncogenic PML-RARα product.

SaOS-2 cells were microinjected with plasmids encoding PML-RARα and p53 wt and analyzed by immunofluorescence and confocal microscopy. The injected cells showed the typical microspeckled pattern for PML-RARα (Figure 22B b, c), but the homogeneous nuclear diffuse staining of p53 was not affected (Figure 22B a, c).

Since the PML-RARα fusion protein is lacking the PML C-terminal region (Figure 22A), I hypothesize that this domain may be required to target p53 into nuclear bodies. Interestingly, PML is expressed in several alternatively spliced forms that differ mostly in their C-terminal part (Figure 17 and 22A) and, although all of them are able to organize the NB structures, it is conceivable that the carboxyl terminus may be critical for the function of the different PML proteins.

I therefore investigated the behavior of another PML splice variant in binding and targeting p53 into NBs. PML-L^{155}, which differs only in its short C-terminal tail from the PML3 protein employed so far (Figure 22A), was coinjected with p53 wt into SaOS-2 cells and the immunostaining pattern was analyzed as above. Although, as expected, PML-L formed NBs, where other resident protein, like Sp100 and SUMO-1, were found (not shown), the distribution of p53 remained diffuse in almost all the injected cells and the protein did not significantly relocalize into these structures (Figure 22B, d-f). Parallel experiments performed with p53 1-355 gave similar results (not shown).

Since colocalization between PML and p53 is lost in cells overexpressing the oncogenic PML-RARα or the alternative splice variant PML-L, it was expected that in these cases also the binding of the two proteins was abrogated. *In vitro* binding experiments with GST-p53 on lysates from cells expressing PML3, PML-L or PML-RARα demonstrated that p53 wt binds efficiently only to PML3, while the interaction with PML-L and PML-RARα was severely impaired (Figure 22C).

A GST-fusion containing a 61 residues long C-terminal region specific for PML3 (Figure 22A), efficiently bound to *in vitro* translated p53 wt, p53 1-355 and p53 1-298 (Figure 22D), indicating that a specific region present in a single PML isoform, PML3, is necessary and sufficient to mediate p53 binding and relocalization to NBs.

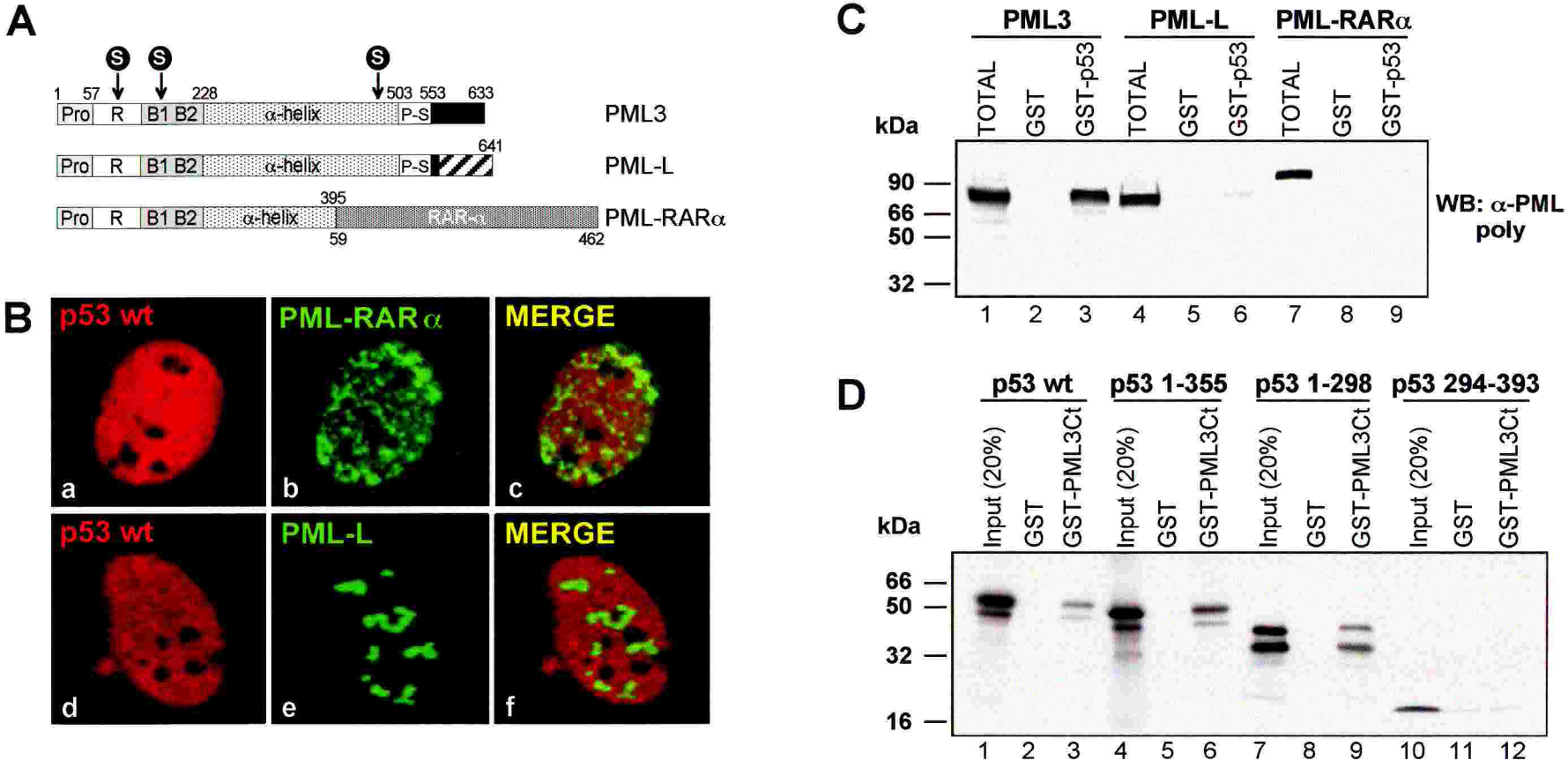


Figure 22. Recruitment of p53 to NBs depends on its interaction with a specific PML isoform.

(**A**) Schematic representation of the various PML proteins showing their functional domains. Upper numbers refer to PML-specific amino acids, lower numbers corresponds to residues in RARα. SUMO-conjugation sites (S) are indicated.
(**B**) p53 fails to colocalize with PML-RARα and PML-L. SaOS-2 cells microinjected with p53 wt (5 ng/μl) and 30 ng/μl of PML-RARα (a, b, c) or PML-L (d, e, f) were analysed by immunofluorescence as in Figure 19D.
(**C**) p53 binds specifically to PML3. Lysates from SaOS-2 cells transfected with PML3, PML-L or PML-RARα and precipitated with GST-p53 or GST, as indicated, were analysed by Western blotting with an anti-PML polyclonal antibody.
(**D**) Interaction with p53 is mediated by a 61 amino acids C-terminal segment of PML3. Pull down experiment was performed with *in vitro* translated p53 wt or different deletions, as indicated, and GST-PML3Ct or GST. Complexes were resolved by SDS-PAGE and visualized by autoradiography.

Analysis of p53-containing nuclear bodies

Having observed that p53 can be localized into NBs by overexpression of PML3 or of SUMO-1 and hUbc9, I decided to analyze more in detail the composition of these structures.

First I wanted to confirm whether the p53 NBs observed in the two different experimental conditions were the same structures and not simply two similar, though unrelated, ones. Therefore I decided to transiently transfect SaOS-2 cells with vectors expressing p53 1-355 (the protein that completely relocalized into NBs) and GFP-SUMO-1, HA-hUbc9 or PML3 in different combinations and to determine the localization of the various overexpressed proteins, as well as the one of the endogenous counterparts.

By overexpressing p53 1-355 with GFP-SUMO-1 and HA-hUbc9 and analyzing the distribution of endogenous PML, I observed that indeed all the GFP-SUMO-1 positive nuclear bodies also contained PML (Figure 24A). A similar result was obtained also for endogenous Sp100 (not shown). On the other hand, when PML3 was overexpressed, either with or without p53 1-355, the subcellular distribution of endogenous SUMO-1 was markedly changed and the protein completely concentrated in PML-containing bodies (Figure 24B).

These observations, that are also in line with previous evidences, allowed me to conclude that p53-containing bodies obtained after overexpression of PML3 or of SUMO-1 and hUbc9 are the same structures and that the latter ones are bona-fide NBs, since they contain the two major NB-resident proteins, PML and Sp100.

It is therefore possible to hypothesize that SUMO-1 and hUbc9 act upstream of PML3 and that, upon overexpression of the two factors, sumolation of endogenous PML3 specifically enhances the assembly of p53-containing NBs. This can be achieved either by a direct increase in binding of PML3 to p53 or, more probably, since the interaction with p53 requires a PML3 region that is not SUMO-modified, by facilitating PML3 nuclear bodies formation.

To provide experimental support for this hypothesis, I checked whether PML3 is actually expressed in U2OS, SaOS-2 and MG-63 cells. RT-PCR analysis using either oligonucleotides specific for the PML3 mRNA or oligonucleotides selected in a region

present in all the PML splice variants demonstrated that indeed PML3 is expressed in all the cell lines (Figure 23).

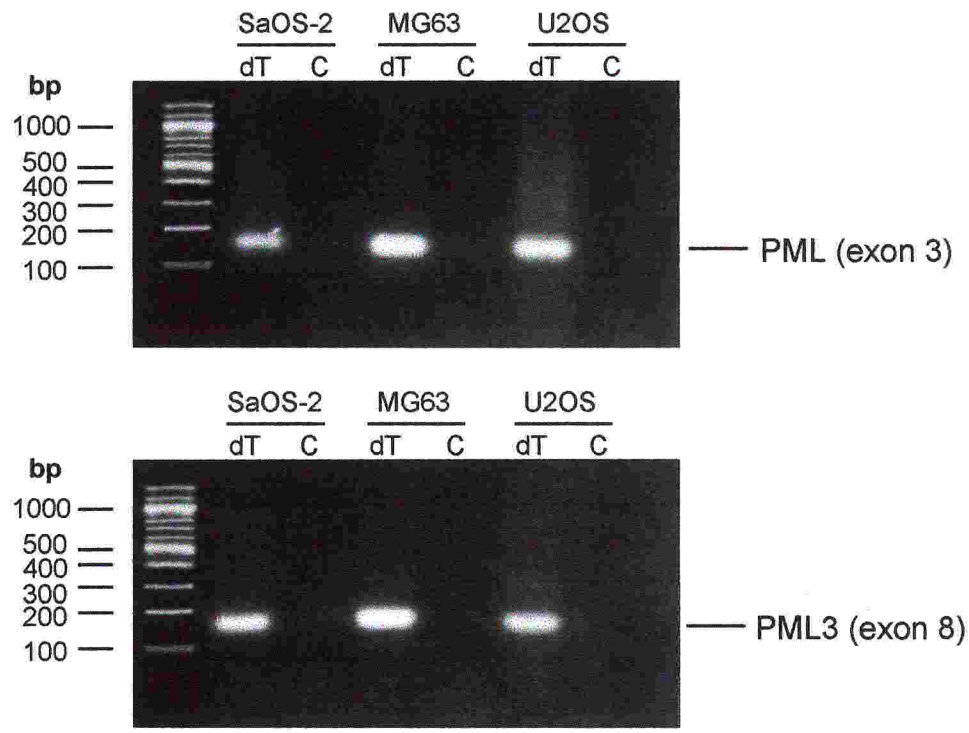


Figure 23: Analysis of PML3 expression in human osteosarcoma cell lines.

RT-PCR was performed on total RNA from the different human osteosarcoma cell lines used in this study, SaOS-2, MG63 and U2OS. dT: reverse transcription reaction performed with oligodT; C: control reaction with no RNA added. Two different primer pairs were used for amplification: the first one selected in the exon 3, which is common to all the various PML splice variant (upper panel) and the second one specific only for PML3 (lower panel). Molecular size markers are shown on the left.

Finally, I wanted to test a possible role of Sp100, another major component of NBs, in mediating p53 relocalization. I examined the subcellular distribution of p53 wt after overexpression of Sp100. In contrast with the massive relocalization observed upon introduction of PML3, p53 staining was not affected by Sp100 (Figure 24C a-c). However, when the three proteins were expressed together, Sp100 was found into PML3 nuclear bodies and colocalized also with p53 (24C d-e). These findings underline the critical role of PML3 in NBs assembly and indicates that Sp100 and p53 may interact in the nuclear body only when both of them are targeted there by PML.

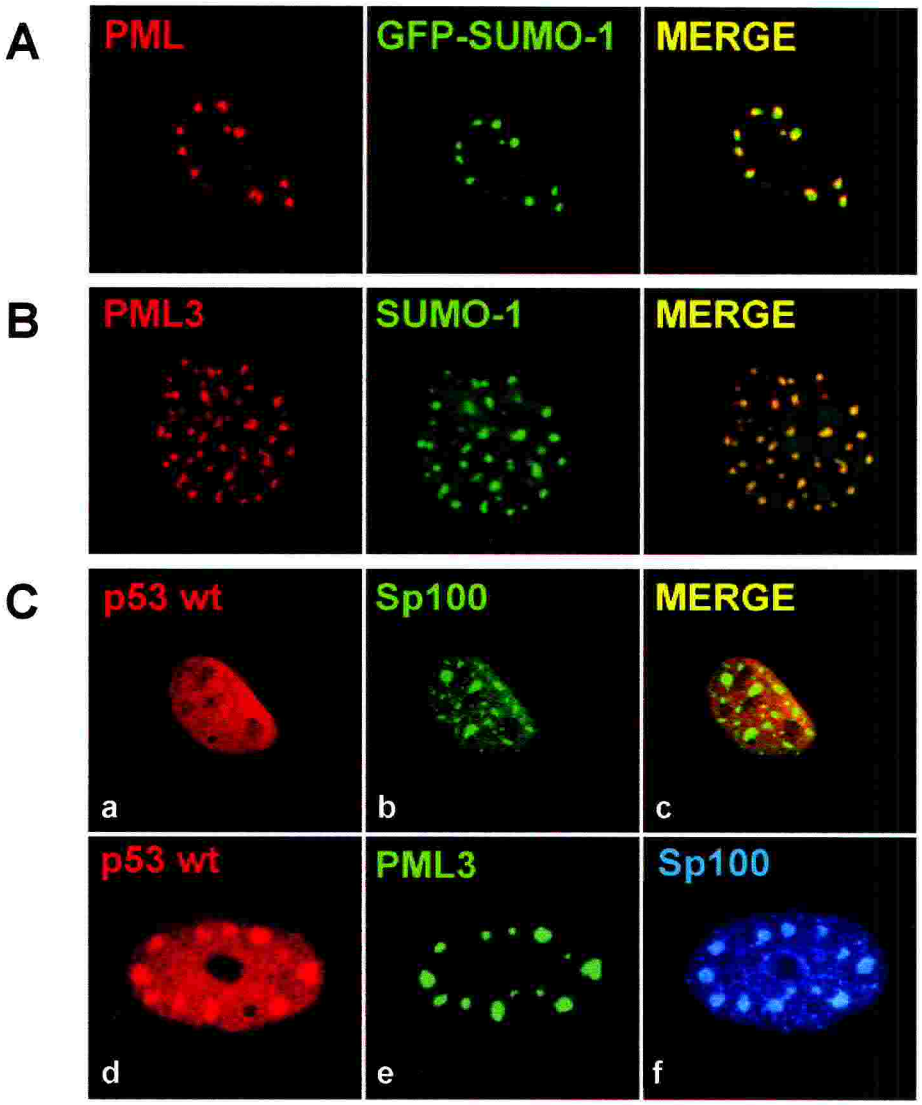


Figure 24. Analysis of the composition of p53-containing nuclear bodies.

(**A**) SaOS-2 cells were transfected with p53 1-355, GFP-SUMO-1 and HA-hUbc9 as described in Figure 19. Endogenous PML staining was analysed with the PG-M3 antibody followed by TRITC secondary antibody. GFP-SUMO-1 was revealed by the intrinsic green fluorescence of GFP.
(**B**) SaOS-2 cells were transfected with p53 1-355 and PML3. PML3 localization was detected by a rat polyclonal specific antibody followed by incubation with anti-rat biotin-conjugated secondary antibody and TRITC-streptavidin. Endogenous SUMO-1 was stained with anti-SUMO-1 monoclonal antibody and FITC-conjugated anti-mouse secondary antibody.
(**C**) SaOS-2 cells were transfected with p53 wt and Sp100, in the absence (a-c) or in the presence (d-f) of overexressed PML3. Staining of p53 and PML was revealed by using the respective monoclonal primary antibodies DO-1 and PG-M3 and heavy chain-specific secondary antibodies. Sp100 was detected with a polyclonal antiserum followed by Alexa350-conjugated secondary antibody.

PML3-dependent recruitment of p53 into NBs increases p53 transcriptional activity and affects cell survival

Since NBs and PML have been linked to regulation of cell growth and differentiation and p53 is a well established tumor suppressor, I wanted to examine whether recruitment of p53 into NBs can modulate cell survival. Plasmids encoding PML3 or human placental alkaline phosphatase (PLAP) as a negative control were microinjected into human U2OS and MG63 cells together with a GFP expression vector as marker. Twenty hours later cell survival was scored as the number of recovered cells positive for the intrinsic green fluorescence of GFP. U2OS cells express wt p53 that, upon introduction of PML3, was efficiently recruited into NBs (Figure 19E), while MG63 cells lack endogenous p53.
Upon overexpression of PML3, we consistently observed a significant reduction of survival in U2OS cells (Figure 25A, left panel), while no considerable effect was detected in MG63 cells (Figure 25A, right panel).

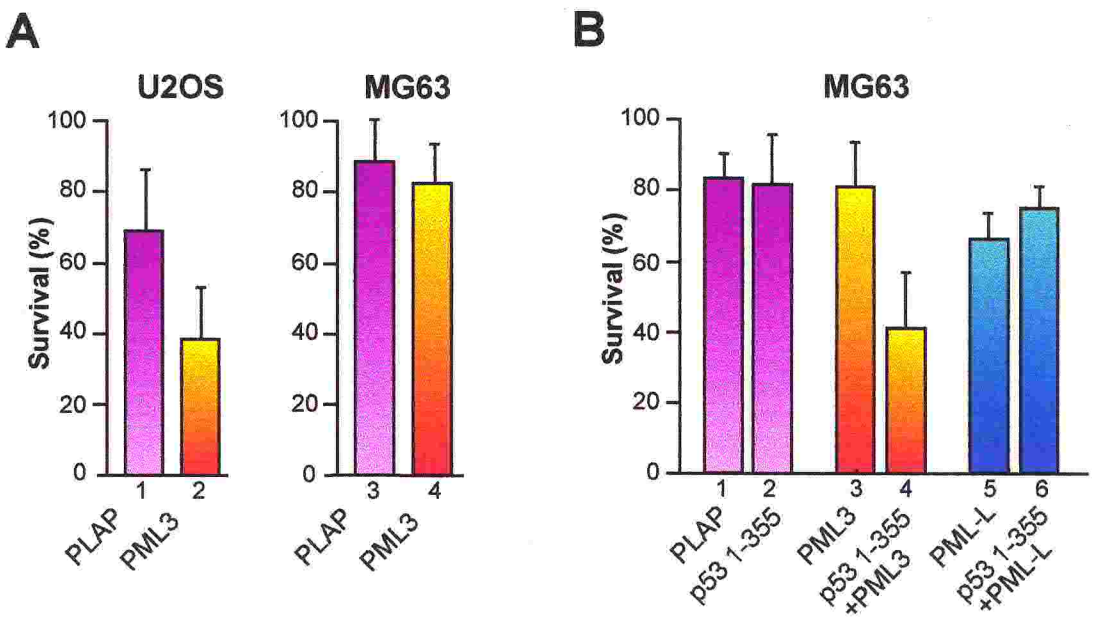


Figure 25. PML3 affects cell survival in a p53-dependent manner.

(**A**) U2OS (left panel) and MG63 (right panel) cells were microinjected with the indicated plasmids (30 ng/µl) together with pGFP (15 ng/µl) as marker. 20 hours later, cell survival was scored as the percentage of recovered cells expressing GFP.
(**B**) Cell survival assay was performed in MG63 cells after ectopic expression of p53 1-355 (20 ng/µl), PML3 (30 ng/µl) and PML-L (30 ng/µl), either alone, or in combinations, as indicated. PLAP was used as negative control and to adjust total DNA amount in all the samples. Graphs represent the mean of at least seven independent experiments.

To provide experimental evidence that PML3-mediated recruitment of p53 into NBs contributes to the observed phenotype, I analyzed whether overexpression of p53 1-355 (the C-terminal deletion that totally relocalized to NBs, see Figure 20C) could restore the PML3-dependent effect on MG63 cells. Cell survival was analyzed by microinjecting MG63 cells either with PML3 and p53 1-355 alone or with a combination of the two plasmids, and was scored as above. As plotted in Figure 25B, recovery of GFP-positive cells was severely impaired when both PML3 and p53 1-355 were simultaneously expressed (bar 4), while no effect was observed when the two proteins were individually expressed (bars 2 and 3). Under the same conditions, PML-L, the isoform impaired in binding and relocalizing p53 into NBs, did not affect cell survival when coexpressed with p53 1-355 (Figure 25B, bars 5 and 6). It appears therefore that recruitment of p53 into nuclear bodies by a specific PML isoform modulates the survival functions linked to these structures.

The evidence that PML associates with several transcription factors and coactivators, like p300/CBP, and recruit them in NBs suggests a relevant role for PML and the whole NB structure in transcriptional control[156]. I therefore analyzed whether the observed effect on cell survival upon PML3-dependent relocalization of p53 into NBs was linked to changes in p53 transactivation ability. Transient transfection assays with constructs containing two well established p53 responsive promoters, PIG3[75] and p21[48], cloned upstream of the luciferase reporter, were performed in MG63 cells with combinations of p53 wt, p53 1-355, PML3 and PML-L expression vectors. Coexpression of p53 wt with PML3 strongly increased the transcriptional activity of p53 toward the PIG3 promoter (Figure 26A, bars 6 and 8), while under the same conditions PML-L overexpression showed no significant effect (Figure 26A, bars 6 and 10). Consistent with previous reports[33], p53 1-355 alone transactivated the p53 responsive promoters, although to a lesser extent as compared to p53 wt (Figure 26B, bars 5 and 6). However, upon coexpression of PML3, the transcriptional activity of p53 1-355 was increased to values comparable to the ones obtained with p53 wt under the same conditions (Figure 26B, bar 8). This effect was likewise dependent on the ability of p53 1-355 to be recruited into NBs by PML3, since a significantly reduced effect was consistently observed upon coexpression of p53 1-355 and PML-L (Figure 26B, bar 10). Interestingly, the PML3-mediated enhancement of p53 transactivation ability was much

less evident when tested on the p21 promoter. As shown in Figure 26A and B, only a slight increase in p21 luciferase activity was detected when PML3 was coexpressed either with p53 wt or p53 1-355 (compare bars 5 and 7).

The obtained results were not due to differences in the level of expression of the various p53 proteins, as judged by Western blot analysis (Figure 26, lower panels).

These results therefore demonstrate that PML3 is able to enhance p53-dependent transactivation in a promoter–specific manner. Of note, PIG3 belongs to a group of p53-regulated genes with the potential to induce oxidative stress and apoptosis[75], thus providing a possible link between the observed decrease in cell survival and the specific activation of the PIG3 promoter following PML3-mediated recruitment of p53 into NBs.

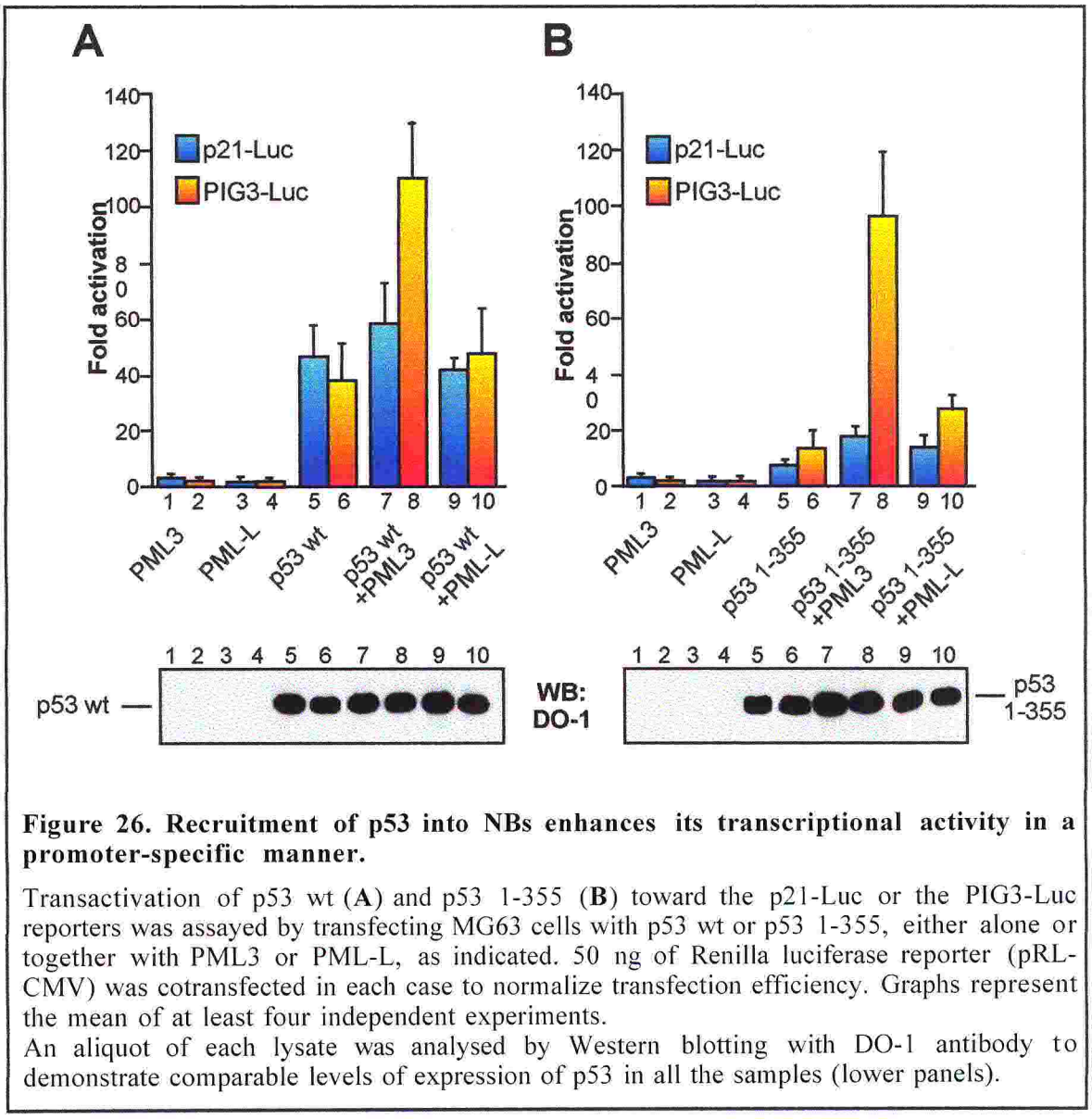


Figure 26. Recruitment of p53 into NBs enhances its transcriptional activity in a promoter-specific manner.

Transactivation of p53 wt (**A**) and p53 1-355 (**B**) toward the p21-Luc or the PIG3-Luc reporters was assayed by transfecting MG63 cells with p53 wt or p53 1-355, either alone or together with PML3 or PML-L, as indicated. 50 ng of Renilla luciferase reporter (pRL-CMV) was cotransfected in each case to normalize transfection efficiency. Graphs represent the mean of at least four independent experiments.

An aliquot of each lysate was analysed by Western blotting with DO-1 antibody to demonstrate comparable levels of expression of p53 in all the samples (lower panels).

DISCUSSION

The growth-suppressive properties of p53 appear to be regulated by at least two general mechanisms that are not mutually exclusive. Under normal growth conditions, p53 is expressed at low levels, which are at least in part due to the short half-life of the protein. In response to a variety of stress signals, however, p53 has a significantly extended half-life resulting in its accumulation. Furthermore, p53 is subject to post-translational changes that may affect either its overall structure, and thus its biochemical properties, or its turnover rate, or both[4].

In the first part of the work I demonstrated that p53 can be covalently modified by conjugation to the ubiquitin-like protein SUMO-1 and that this post-translational modification enhances the transactivation ability of p53. Thus, conjugation of SUMO-1 to p53 provides a previously undescribed modification that probably contributes to control the growth-suppressive properties of p53.

Similar to other known substrates of the SUMO-1 conjugation system, modification of p53 by SUMO-1 requires the activity of the SUMO-activating enzyme E1 and the SUMO-conjugating enzyme Ubc9[86]. Accordingly, I showed that p53 transcriptional activity is enhanced by SUMO-1 or hUbc9 overexpression, indicating that both factors are rate limiting in the conjugation process. This hypothesis is supported by the observation that overexpression of SUMO-1 together with hUbc9 increases the amount of sumolated p53.

In this context, it should be noted that only a small percentage of the total p53 appears to be modified by SUMO-1 *in vitro* and *in vivo*. This observation may be explained by the possibility that an additional factor(s) is required to target hUbc9 to p53 for SUMO-1 conjugation. Similarly, it is possible that only a subfraction of p53 is recognized as a substrate by the SUMO-conjugation system since, for example, recognition may require prior modification of p53 by phosphorylation or acetylation. In line with this hypothesis, a very recent report demonstrated that hyperphosphorylation of p53 prevents its conjugation to SUMO-1[169]. The wt conformation of the p53, however, is dispensable since both p53 wt and the tumor-derived H175 mutant are sumolated with similar efficiencies.

Alternatively, SUMO-conjugated p53 may be subject to the action of SUMO-specific proteases that revert SUMO-conjugated p53 to its non-modified form. Interestingly, a

SUMO-specific protease has recently been described that is required for cell cycle progression in yeast[170]. Thus, it is tempting to speculate that conjugation of SUMO-1 to p53 may directly affect its growth-suppressive properties. However, further studies will be required to address this issue.

How is p53 activated by conjugation to SUMO-1? Based on previous results as well as the results I presented, several mechanisms can be envisioned, including allosteric regulation, interference with ubiquitin/proteasome-mediated degradation, and changes in subcellular distribution. With respect to allosteric regulation, it has been proposed that the C terminus of p53 interacts with the core domain, thereby keeping the protein in an inactive form. Indeed, removal of the C terminus of p53 or modification of this region by phosphorylation or acetylation, activate the sequence-specific DNA binding activity, probably by changing the overall conformation of the protein[22,40]. Since sumolation occurs within the C-terminal 30 amino acids of p53, a similar mechanism may account for the observed increase in the transactivation activity of p53 upon SUMO-1 conjugation. Alternatively, several cellular proteins have been shown to interact with the carboxyl terminus of p53 and, thus, the presence of SUMO-1 on lysine 386 may alter the capacity of p53 to interact with these proteins. Along these lines, it will be interesting to determine if sumolation of p53 affects the phosphorylation and/or acetylation status of its C-terminal region.

It has recently been demonstrated that binding of Mdm2 induces the rapid degradation of p53 via the ubiquitin/proteasome system[88,89]. Interestingly, a deletion mutant of p53 in which the C-terminal 30 amino acids were removed was still bound by Mdm2 but was not targeted for degradation[94]. Thus, similarly to the allosteric model discussed above, sumolation may prevent Mdm2-mediated degradation of p53 which, in turn, would be expected to result in enhanced transactivation activity. Alternatively, in analogy to IκBα[138], sumolation of p53 may inhibit its degradation, by competing for the same lysine residue that is required for p53 ubiquitination or by interfering with conjugation of ubiquitin molecules to neighbouring sites. Although these models may prove to be correct for endogenous p53, I never observed a significant increase in p53 levels upon expression of SUMO-1 and/or hUbc9. This fact can be however explained by the evidence that overexpressed p53 shows an extended half-life as compared to the endogenous protein and therefore the SUMO-mediated stabilizing effect may be

masked. More detailed and precisely designed experiments will be required to rule out this possibility.

Finally, another hypothesis that I took into account is that SUMO-1 modification may affect the subcellular distribution of p53. Therefore, the observed increase in transactivation could be due to physical recruitment of p53 in sites of active transcription or in specific chromatin domains. This idea was sustained by the fact that other SUMO-1 conjugated proteins, as PML and Sp100, were reported to localize to a particular kind of subnuclear structures defined as nuclear bodies or PML-oncogenic domains. NBs are distinct subnuclear domains that have been found disrupted in some human diseases and hypothesized to serve as three-dimensional surfaces for the convergence of several biochemical pathways involved in growth and differentiation control[151,162,171].

Interestingly, it has been already reported that LMB treatment or coexpression of p53 together with Mdm2 and ARF can induce the localization of p53 into structures reminiscent of the PML/Sp100 nuclear bodies[152,153] and indeed some preliminary results suggested me that SUMO-1 could be related to a particular p53 subnuclear distribution.

In the second part of the work presented herein, I demonstrated that the simultaneous overexpression of SUMO-1 and hUbc9 mediates the assembly of large nuclear dots, where p53 is localizing. These structures are likely to be NBs, as proven by the fact that the two major NB-resident factors, PML and Sp100, are also localized there. In contrast to my starting hypothesis, however, this p53 relocalization was not dependent on the direct sumolation of the protein, since the conjugation-deficient mutant K386R was efficiently targeted to NBs as well. Instead, I was able to demonstrate that recruitment of p53 into NBs is mediated by overexpression of PML3, a specific isoform of the promyelocytic leukemia protein.

A recent report demonstrating that conjugation of SUMO-1 to PML is prerequisite for its ability to form NBs and consequently to recruit other proteins into these structures[156] let me hypothesize that enforced expression of SUMO-1 and hUbc9 results in the augmented assembly of NBs where also p53 is targeted (see the model presented in Figure 27).

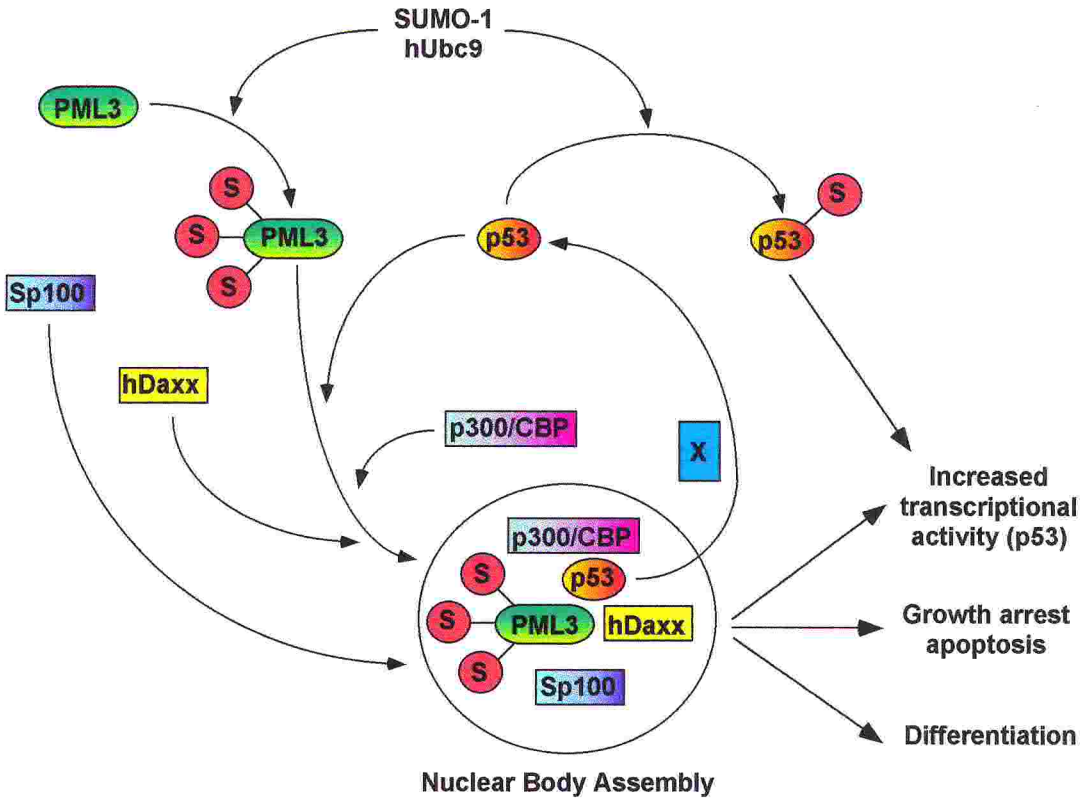


Figure 27: Model of two possible p53 activation pathways mediated by SUMO-1.

Direct conjugation of SUMO-1 on p53 can convert the protein to an active form with increased DNA binding. On the other way, SUMO-1 modification of PML mediates p53 recruitment in NBs, where the protein speficifically transactivates pro-apoptotic promoters, resulting in impaired cell survival. Interaction with other resident proteins can confer specificity to the outcome of the action of NB-targeted p53. Still unidentified factors (X) can be involved in modulating this relocalization and its biological consequences.

The recruitment of p53 into NBs by association with PML3 is reminiscent of that of hDaxx[160,161] and pRb[165], which also depends on PML protein interaction, but it is so far unclear whether also in these cases a specific isoform of PML is required.

Interestingly, amino acids from 355 to 363 of p53 play a negative role in NB-targeting, since while only a fraction of the full length protein or the deletion mutant p53 1-363 was localized to these structures, p53 1-355, as well as longer C-terminal deletions, showed a complete relocalization to NBs upon PML3 coexpression. As already noticed, the C terminus of p53 is target of various post-translational modifications and serves as a surface for intense protein-protein interactions that may modulate p53 functions[4,6]. Therefore, it is tempting to speculate that the segment between residues 355 and 363 is a binding site for a factor that removes or, more probably, keeps p53 out from the NBs

(Figure 27). Alternatively, a post-translational modification may have the same outcome, as suggested by the presence at position 362 of a serine residue, which is a potential phosphorylation site.

PML exists in numerous alternatively spliced variants that mostly differ in their C-terminal sequences[154,155,157]. However, very few data are available regarding the regulation of expression and possible roles of the different isoforms. All of them described so far contain the RING finger, B-box and coiled coil motifs (RBCC) and a nuclear localization signal, which together have been shown to be required and sufficient to target PML into NBs[156]. Nevertheless, one can speculate that the different PML proteins may interact with diverse cellular partners, affecting NB-composition and functions. Supporting this interpretation, I provided evidence that the interaction between p53 and PML is specific for the PML3 variant and is mediated by a short C-terminal segment (61 amino acids) that is present only in PML3 and in a longer, related splice variant, PML4 (see Figure 17). No significant binding was instead detected with the PML-L isoform and consequently p53 was never found in PML-L NBs.

These findings are, to my knowledge, the first data on functional differences between the various PML proteins and raises the possibility that the complex splicing pattern of PML represents a cellular mechanism generating alternative binding interfaces for a variety of cellular factors. In this context, SUMO-1 modification on PML may influence recruitment of other factors either by directly affecting PML association with these proteins or by enhancing its ability to form nuclear bodies. With respect to p53, the latter hypothesis is more likely to be true, since I demonstrated that the region of PML required for binding p53 is outside the central domain, where SUMO-conjugation takes place.

Of note, while coexpression of SUMO-1 and hUbc9 could induce the relocalization of p53 into NBs only in 30-40% of the cells, overexpression of PML3 led to change p53 staining in more than 90% of the cells. This can be explained by supposing that although overexpression of SUMO-1 and hUbc9 may facilitate, in general, the assembly of NBs by the various PML isoforms, recruitment of p53 into these structures is PML3-specific. Indeed, the presence of the endogenous PML3 transcript was detected in all the employed cell lines.

The function of the NBs is not yet fully understood, however their possible involvement in growth suppression has been postulated since the discovery that

leukemia cells from APL patients have aberrant nuclear dot organization[162]. Several correlative evidences suggested that PML is a critical component for triggering the programmed cell death pathway, probably due to its activity to recruit apoptotic proteins into NBs[172].

Since the role of p53 in apoptosis induction and growth suppression is well established[173], I hypothesized that recruitment of p53 into NBs may contribute to PML3-dependent growth inhibition. In line with this idea, a significant reduction of cell survival was consistently observed when PML3 was introduced into the p53-expressing U2OS cells but not in the p53-null MG63 cell line. By using the p53 1-355 deletion mutant, that shows complete relocalization into NBs upon coexpression with PML-3, we were able to demonstrate that recruitment of p53 into PML-containing dots is indeed necessary for the promotion of PML-mediated cell death.

The apoptotic function of p53 has been shown to involve transcriptional-dependent activities as well as its capability to associate with other cellular factors[173]. Here I demonstrated that PML3 selectively increased the p53-dependent activation of the PIG3 promoter. PIG3 was originally isolated as one of several p53-regulated genes with the potential to induce or mimic oxidative stress[75]. The evidence that reactive oxygen species are involved in apoptosis and in cell aging[174], suggests that PML3-dependent relocalization of transcriptionally active p53 into NBs could contribute to these physiological processes. In line with this hypothesis, PML3-mediated increase in transactivation of the PIG3 promoter was more pronounced with the p53 1-355 protein, underlying that relocalization of p53 into NBs under certain conditions may contribute to an enhanced apoptotic effect that most likely involves p53 transcriptional activity toward a specific pro-apoptotic promoters.

Which mechanisms control this promoter-specificity? The interaction of p53 with PML3 and other NB-targeted factors involved in transcriptional control, like p300/CBP[166] or pRb[165], may regulate the recognition of p53 target genes. In addition, specific post-translational modifications taking place in NBs could also contribute to activate p53 transcriptional functions in a promoter-specific manner. Phosphorylation or acetylation of particular residues in the p53 C terminus may neutralize its inhibitory effect on NB-targeting and sequence-specific DNA binding by a conformational change of the protein. This may allow the favored interaction with a subset of factors that, in turn, would provide the promoter-specificity. p53 1-355, that totally relocalizes to

PML3 NBs and selectively activates the PIG3 promoter, may mimic such a conformational state. p300/CBP is not only acting as a coactivator due to its histone acetyltransferase (HAT) activity, but also acetylates p53 in several C-terminal residues[22,121]. Therefore, the possible effect of acetylation on NB-targeted p53 transcriptional activity should be well considered (P.G. Pelicci, personal communication). It has been suggested that NBs are also involved in chromatin remodelling[151], therefore access to a particular promoter region may depend on the association between transcription factors and a non-histone chromosomal proteins.

Of note, hDaxx, a protein involved in Fas-mediated apoptosis, has been found recently to bind to PML and exert its apoptotic function in NBs[160,161]. The physical interaction between p53 and other NBs resident factors may add another level of complexity to the role of these structures in cell death control.

About 5-15% of p53 mutations occurs in the carboxyl terminal domain, resulting in truncated proteins that, although still transcriptionally active, are defective in apoptosis induction[175]. Based on our results, it is tempting to speculate that in cells containing such p53 mutants, apoptosis can still be induced by stimulating the relocalization of p53 proteins into PML-containing nuclear bodies following treatment with agents that modulate the expression (or the sumolation) of PML3, such as interferons, all-trans retinoic acid, or arsenic trioxide.

CONCLUSIONS

In this thesis I presented how a novel post-translational modification can activate the sequence-specific transactivation function of p53 by two apparently independent pathways.

In the first part, I demonstrated that p53 itself serves as a substrate for conjugation to the small ubiquitin-like protein SUMO-1 and I showed that this modification increases the transcriptional activity of p53. Although the exact mechanism responsible for p53 activation remains elusive, I hypothesized that sumolation may affect the stability of the protein, increase its DNA binding ability or lead to a change in its subcellular distribution.

In the second part of the work I analyzed in detail the third possibility and demonstrated that upon overexpression of SUMO-1 and its conjugating enzyme, hUbc9, p53 relocalizes into punctate subnuclear structures, called Nuclear Bodies. However, I provided strong experimental evidences, that this effect depends on the direct physical interaction of p53 with a specific PML isoform and the conjugation of SUMO-1 to p53 is dispensable for the process. I supposed that enforced sumolation of PML results in the augmented assembly of these structures where p53 relocalizes. Moreover, I showed that recruitment of p53 into NBs led to an increase in p53 transcriptional activity in a promoter-specific manner and I suggested a model in which well-controlled protein-protein interactions are regulating this selectivity.

Several questions remain to be answered: in which cellular compartment conjugation of SUMO-1 to p53 occurs? What are the exact molecular mechanisms that lead to SST of sumolated p53? What are the proteins or post-translational modifications that regulate p53 entry and exit to/from NBs? What kinds of mechanisms are responsible for the observed promoter-specific increase in the transcriptional activity of NB-targeted p53?

Nevertheless, my observations clearly established the importance of sumolation of p53 and of its PML3-dependent recruitment in NBs in control of cell growth and differentiation. Moreover, the discovery of cross talks between the PML and the p53 pathways could represent a platform to develop new methods for blocking the transformation process and to design novel therapeutic strategies.

MATERIALS AND METHODS

Cell lines

All the cell lines used were routinely cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 µg/ml). U2OS, MG63 and SaOS-2 cells are human osteosarcoma cell lines, respectively wt for p53 and pRb, wt for pRb but null for p53 and null for both p53 and pRb. The Balb/c (10)1 fibroblast cell line is a murine cell line which does not contain endogenous p53. 293 is a human embryonic kidney cell line.

Plasmids

To generate the LexA-fusion constructs, human p53 wt and p53 H175 cDNAs, as well as p53 wt deletions, were PCR-amplified and cloned in frame into pLexA202[137]. pcDNA3p53wt contains the full-length human wt p53 cDNA cloned by EcoRI into pcDNA3 (Invitrogen). cDNAs encoding the p53 mutants K386R, K381/82R, and K381/82/86R were generated by PCR-directed mutagenesis and the respective cDNAs were cloned by HindIII and XbaI restriction into pRcCMV (Invitrogen). pCMVp53Δ63-91[23] and pCMVp53Δ12-69[33] contain human p53 cDNA lacking the Pro-rich region and the transactivation domain respectively. To construct pcDNA3HAp53294-393, a cDNA fragment of human p53 encoding aminoacids 294 to 393 was PCR-amplified and cloned by EcoRI/XhoI downstream of the HA epitope into pcDNA3 (pcDNA3HA). The other p53 deletions (1-363, 1-355, 1-338, 1-298) were generated by PCR and cloned in pcDNA3 by EcoRI/XhoI. pcDNA3PML3 contains the whole PML3 cDNA[157] and was a kind gift by P.G.Pelicci; pSG5PML-L and pSG5PML-RARα were provided by H. Will[155]. pGEX-PML3Ct were constructed by PCR. To generate the different SUMO-1 fusions, a cDNA encoding SUMO-1 was PCR amplified and cloned by BamHI/XhoI into pcDNA3HA or by BamHI/XbaI into pGFPC1 (Clontech). hUbc9 was subcloned from pJG4-5 into pcDNA3HA (Invitrogen) by EcoRI/XhoI. The Sp100 expression plasmid[140] as well as the p53-reporter plasmid employed for luciferase assays, p21-Luc[48] and PIG3-Luc[75], have been described previously. pNLS-βgal was kindly provided by C. Kuhne. All PCR amplified products were fully sequenced to exclude the possibility of second site mutations.

Yeast two-hybrid screening

p5wt3Δ74 fused to the LexA DNA-binding domain was introduced into the EGY48 (MATa trp1 ura3 his3 LEU2::pLEXAop6LEU2) yeast strain, previously transformed with the pSH1834 β-galactosidase reporter plasmid[137]. The resulting strain was then transformed with a human fetal brain cDNA library cloned into the pJG4-5 plasmid[137,176]. Approximately 3 x 10^6 primary yeast transformants were selected on Ura- His- Trp- plates and then pooled together. The interaction screening was performed by plating about 10 x 10^6 clones from the pooled library onto Ura- His- Trp- Leu- plates containing galactose.

106 clones that grew and turned blue when subjected to β-galactosidase expression assay were purified and further processed for isolation of the pJG4-5 plasmid by selection in the B290 *E. coli* strain[137]. Rescued clones were classified by restriction analysis and Southern dot blot; single clones were subjected to secondary screening to confirm the specificity of interaction.

***In vitro* conjugation assay**

The various forms of p53 and the p53-unrelated protein HHR23a[149] were generated in the TNT rabbit reticulocyte lysate system in the presence of ^{35}S-methionine according to the manufacturer's instructions (Promega). Murine Ubc9 and GST-SUMO-1 were expressed in *E. coli* BL21(DE3) and E. coli DH5α, respectively, as described[144]. Crude bacterial extracts were used as a source of mUbc9. GST-SUMO-1 was purified by affinity chromatography using glutathione-Sepharose (Amersham Pharmacia). As a source of SUMO-activating enzyme activity, protein extracts were prepared from confluent NIH 3T3 cells and fractionated by anion exchange chromatography on a 1 ml Mono Q column as described previously[144].

SUMO-conjugation assays were performed in reaction mixtures containing 10 μg of the Mono Q fraction of the NIH 3T3 cell extracts, 100-300 ng of mUbc9, 4 μg GST-SUMO-1, 1 μl of radiolabeled p53 or HHR23a in 25 mM Tris-HCl pH 7.5, 2 mM ATP, 4 mM $MgCl_2$, 1 mM dithiothreitol. After 2 hours at 30°C, reactions were terminated by boiling the mixtures in SDS-containing buffer. Reaction mixtures were separated on 10% SDS-polyacrylamide gels and radioactively labeled bands were visualized by fluorography.

Transfections and luciferase assays

Transfections were performed by the standard calcium phosphate precipitate method. Cells were seeded 8 hours before transfection and further processed 24 hours after removal of the precipitate. For luciferase assay, 6 cm petri dishes were transfected with 500 ng of the reporter construct and the indicated amount of other plasmids. The assay was performed with the luciferase kit from Promega. The luciferase activity was determined in a Turner Design luminometer (Promega). For the experiments presented in part 1, the values obtained were normalized for protein concentration in each sample, as determined by a colorimetric assay (Biorad Protein Assay). For the experiments presented in part 2, in each transfection, 50 ng of pRL-CMV, expressing the Renilla luciferase, were added and luciferase activity was measured and normalized using the Dual Luciferase Reporter Assay System (Promega).

Immunoprecipitation, *in vitro* binding assays and Western blot analysis

Subconfluent cells seeded on 10 cm diameter Petri dishes were transfected with the indicated expression vectors and further processed 36 hours after transfection.

For SUMO-1/p53 coimmunoprecipitations, cells were washed with ice-cold phosphate buffered saline (PBS), then harvested in 1 ml of ice-cold RIPA buffer containing 10 mM N-ethylmaleimide, 1 mM PMSF, 10 mg/ml each of chymostatin, leupeptin, antipain and pepstatin. Lysis was performed at 4°C for 20 minutes. Then, the lysates were clarified by centrifugation, precleared with 200 µl of Immunoprecipitin (Gibco) and finally incubated for 4 hours at 4°C with the respective antibodies, prebound to 20 µl of Protein A-Sepharose CL-4B (Amersham Pharmacia). The beads were then washed three times in 1 ml of ice-cold lysis buffer and the bound proteins were solubilized by addition of 20 µl of 2x Laemmli sample buffer. In the case of GFP-SUMO-1 immunoprecipitations, 2 µg of anti-GFP polyclonal antibody (Invitrogen) were used, while for p53 immunoprecipitations, 5 µl of polyclonal antiserum raised against human p53, covalently crosslinked to Protein A-Sepharose, were used.

PML/p53 coimmunoprecipitations were performed in 150 mM NaCl-containing buffer[177] supplemented with protease inhibitors as above, with 1 µg of anti-PML monoclonal antibody PG-M3 (Santa Cruz), covalently crosslinked to 20 µl of Protein A-Sepharose CL-4B (Amersham Pharmacia). Immunoprecipitation and washing conditions were as above.

For *in vitro* binding assays, the indicated GST-fusion proteins were produced in BL21 *E. coli* cells and purified by conventional procedures[177]. For the pull-down assays, 4 µg of GST-fusion protein or GST were incubated with ^{35}S-labeled *in vitro* translated protein (TNT-coupled Reticulocyte Lysate System, Promega) in 200 µl of binding buffer (150 mM NaCl, 20 mM HEPES pH 7.5, 10% glycerol, 0.05% NP40). After washing, the bound complexes were resolved on SDS-PAGE and the bands were visualized by autoradiography. For GST-binding assays using cell lysates, transfected cells were lysed in 300 mM NaCl-containing lysis buffer (300 mM NaCl, 50 mM Tris-HCl pH 7.5, 0.5% NP40, 10% glycerol). The lysates were then diluted with the same volume of buffer without NaCl and incubated with 4 µg of GST-fusions or GST alone. Complexes were resolved on SDS-PAGE and processed for Western blotting.

Western blot analysis was performed according to standard procedures using the following primary antibodies: 12CA5 (monoclonal anti-HA, Roche Molecular Biochemicals), DO-1 (monoclonal anti-p53), rabbit polyclonal antiserum raised against GST-hp53[178], 21C7 (monoclonal anti-SUMO, Zymed), polyclonal anti-GFP (Invitrogen), polyclonal anti-actin (Sigma), rat polyclonal anti-PML[179], PG-M3 (monoclonal anti PML, Santa Cruz). Bound primary antibodies were visualized by enhanced chemiluminescence (Amersham).

Microinjection , Immunofluorescence Analysis and Survival Assay

For microinjection assays, cells were grown on coverslips in 35-mm Petri dishes containing 12x10^4 cells per dishes seeded in DMEM containing 10% FCS. After a 24-h incubation at 37°C in a 5% CO_2 atmosphere, nuclear microinjection was performed using the Automated Injection System (Zeiss, Oberkochen, Germany). Each cell was injected with the different expression vectors for 0,5 s at the constant pressure of 150 hectopascal. After injection, cells were incubated at 37°C in a 5% CO_2 atmosphere.

For indirect immunofluorescence analysis, microinjected cells were washed in PBS and fixed with 3% paraformaldehyde in PBS at room temperature. After 20 min, the coverslips were washed three times in PBS and incubated for 5 min in 0,1M glycine-PBS. Permeabilization was performed with 0,1 % Triton X-100 in PBS for 5 min. The coverslips were incubated for 1 hour at 37°C with the different primary antibodies and then primary antibodies were revealed by a 30 min incubation with the respective secondary antibodies. The following antibody combinations were used: DO-1

(monoclonal IgG2a anti-p53, Santa Cruz) + goat anti-mouse IgG2a TRITC-conjugated (Southern Biotechnology Associates, Inc); polyclonal antiserum raised against human p53[178] + goat anti-rabbit TRITC-conjugated (Dako, Glostrup, Denmark); polyclonal anti-Sp100 antiserum[179] + goat anti-rabbit FITC-conjugated (Sigma); PG-M3 (monoclonal IgG1 anti-PML, Santa Cruz) + goat anti-mouse IgG1 FITC-conjugated (Southern Biotechnology Associates, Inc) or goat anti-mouse FITC-conjugated (Sigma), 12CA5 (monoclonal IgG2b anti-HA, Roche Molecular Biochemicals) + goat anti-mouse IgG2b FITC-conjugated (Southern Biotechnology Associates, Inc); 21C7 (monoclonal anti-SUMO, Zymed) + anti-mouse FITC-conjugated; monoclonal anti-β-galactosidase (Promega) + anti-mouse TRITC-conjugated; rat polyclonal anti-PML3 + anti-rat biotinylated secondary antibody (Pierce) and TRITC-conjugated streptavidin (Southern Biotechnology Associates, Inc). For the triple immunofluorescence the goat anti-rabbit Alexa350-conjugated antibody was used. GFP-SUMO-1 and GFP staining was revealed by means of the intrinsic green fluorescence of the GFP.

Cells were examined by epifluorescence with a Zeiss Axiovert 35 microscope or a Zeiss laser scan microscope (LSM 410) equipped with a 488 nm argon laser and a 543 nm helium neon laser.

The effect on cell survival of different genes was analyzed using an automated injection system. For each experiment an established number of cells (200) was microinjected with the gene of interest and a reporter gene. Cell survival was calculated as the number of recovered cells expressing the reporter gene as previously described[180].

RT-PCR

Total RNA was extracted from U2OS and SaOS-2 cells with the standard guanidinium-thiocyanate method. 3.5 μg of total RNA was retrotranscribed with an oligo-dT primer using the SuperScript II Reverse Transcriptase kit (Gibco) in the final volume of 20 μl and the reaction products were then treated with RNaseA. PCR amplifications were performed on 4 μl of the RT reactions using the Taq Gold polymerase (Perkin Elmer), with the following parameters: 10 min. at 94° C; 30 cycles of 45 sec. at 94° C, 1 min. at 48° C and 1 min. at 72° C; 5 min. at 72° C.

The following oligonucleotide pairs were used: 5'ATGAAGTGCTACGCCTCGGAC3' and 5'TCCCCTGGGTGATGCAAGAGCT3' for all the various PML splice variants; 5'CGAGAGCTGGATGACAGCAGC3' and 5'CTAAATTAGAAAGGGGTGGGG3' specific for PML3.

Amplifications were carried out in a MJ Research Minicycler.

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ACKNOWLEDGEMENTS

I would like to acknowledge professor Claudio Schneider, who gave me the opportunity to work in the L.N.C.I.B. and with whom I started my PhD studies.

I'm extremely grateful to professor Gianni Del Sal, under whose supervision I carried out all the work presented in this thesis. Even though sometimes he was overwhelming me with his enthusiasm and plenty of ideas, I found working with him very stimulating and I learned a lot from him.

I thank all the people of the "GDS group", specially Valentina Fogal, who was performing most of the microinjection and immunofluorescence work, and Peter Sandy, for his friendship and continuous support, for providing chocolate and Hungarian cakes and for couple of "over-nights" during the preparation of this thesis.

My gratitude goes also to all the colleagues of the L.N.C.I.B., particularly to Roberta Benetti, Martin Monte, Licio Collavin and Stefania Marzinotto, as well as to my friend Fiamma Mantovani, for helpful discussion and for the many nice days shared.

Last but not least, I really want to thank Cristiano Covre, for patiently sustaining me in all of these years and for the nights spent at home waiting for me to finish working.