Identification of three novel p53-interacting proteins.

Péter Sandy

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Laboratorio Nazionale, Consortio Interuniversitario per le Biotecnologie, Trieste, Italy

and

Institute of Biochemistry, Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary

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"There is a theory which states that if ever anyone discovers exactly what the Universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable."

"There is another theory which states that this has already happened."

Douglas Adams: The Hitchhikers Guide to the Galaxy

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ABBREVIATIONS

APC	Adenomatous Polyposis Coli protein
APL	Acute Promyelocytic Leukaemia
ARF	Alternative Reading Frame protein
ATM	protein Mutated in Ataxia Teleangiectasia (AT)
ATR	ATM Related Kinase
Bax	Bcl2 Associated protein
Bcl2	B Cell Lymphoma protein 2
BER	Base Excision Repair
BLM	Bloom Syndrome protein
BRCA1/2	Breast Cancer Associated protein 1/2
BrdU	Bromodeoxyuridine
CAK	CDK Associated Kinase
CAT	Chloramphenicol Acetyltransferase
CBP	cAMP Response Element Binding protein (CREB) Binding Protein
CDK	Cyclin-dependent Kinase
Chk1/2	Checkpoint associated kinase 1/2
CK	Casein Kinase
DMEM	Dulbecco's Modified Eagle's Medium
DNA-PK	DNA damage-dependent Protein Kinase
DSB	Double Strand Break
E6AP	HPV E6 Associated Protein
FCS	Fetal Calf Serum
FITC	Fluorescein Isothyocyanate
Gas1	Growth arrest-specific protein 1
GFP	Green Fluorescent Protein
GST	Glutathione S-Transferase
HA	epitope from the Influenzavirus Hemagglutinin protein
HAT	Histone Acetyltransferase
hDaxx	Human Death Domain Associated Protein
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HHR23a	Human Homologue of yeast repair protein RAD23
HMG-1	High Mobility Group protein 1
HPV	Human Papillomavirus
HRPO	Horse Radish Peroxidase
IgG	Immunoglobulin G
INK4a	Inhibitor of CDK4
IP	Immunoprecipitation
IR	Ionizing Radiation
IκBα	Inhibitor of Nuclear Factor (NF) κB
JNK	c-jun N terminal Kinase
JPS	Juvenile Polyposis Syndrome
Luc	Firefly Luciferase enzyme
MAPK	Mitogen Activated Protein Kinase
Mdm2	Mouse Doube Minute protein 2
MEFs	Mouse Embryo Fibroblasts
NBs	Nuclear Bodies
NER	Nucleotid Excision Repair
NES	Nuclear Export Signal
NLS	Nuclear Localization Signal

NMR	Nuclear Magnetic Resonance
PBS	Phosphate Buffered Saline
PCAF	p300/CBP Associated Factor
PIGs	p53 Induced Genes
PKC	Protein Kinase C
PLAP	Placental Alkaline Phosphatase
PML	Promyelocytic Leukaemia protein
PMSF	Phenylmethylsulfonyl fluoride
PODs	PML Oncogenic Domains
pRb	Retinoblastoma protein
RARα	Retinoic Acid Receptor alpha
ROS	Reactive Oxigen Species
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SCA1	Spinocerebellar Ataxia type 1
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SST	Sequence Specific Transactivation
SUMO-1	Small Ubiquitin-related Modifier 1
SV 40 LT	Simian virus 40 large T antigen
TAF	TBP Associated Factor
TBP	TATA Binding Protein
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
TRITC	Tetramethylrhodamine B Isothyocyanate
Ubc9	Ubiquitin conjugating protein 9
UV	Ultraviolet light
VHL	Von Hippel Lindau protein
WB	Western Blot
WNR	Werner syndrome protein
wt	wild-type
WT-1	Wilms Tumour protein 1
XPB, XPD	Xeroderma Pigmentosum protein B and D, respectively

INTRODUCTION

Development of neoplasia is a multistep process that involves the accumulation of genetic alterations along the way [1]. If, in imagination, we travel in a car on a road that is finishing in cancer, we can illustrate these mutations like a jammed gas pedal, that is in reality the activation of oncogenes; a never-ending source of fuel, that represents the activated telomerase enzyme; and a loss of control on the brakes, which depicts the inactivation of tumour suppressor genes [2]. These three events are sufficient for reaching the end of the road, regardless how far is it, and, in fact, normal human cells have been converted into tumours in *in vitro* systems, by acquiring these genetic modifications, recently [3].

Oncogenes were first identified in the genomes of transforming viruses and subsequently their cellular counterparts, the so-called proto-oncogenes, have been isolated by sequence similarities [4,5]. Proto-oncogenes gain an oncogenic function by activating mutations of one allele of the two copies of the relevant gene after exposure to carcinogenic agents or other mutagenic stimuli [6,7]. Unlike normal cells, the ones transformed by oncogenes can survive without growth factors and without a solid support. The best characterized and most studied oncogenes are Myc, Ras, Jun and Fos. Although to transform normal rodent cells to a cancerous state delivery of two oncogenes is sufficient [8,9], development of tumours in humans is more complex.

In contrast to their mouse counterparts, normal human tissues have very low levels of telomerase [10], an enzyme that is responsible for maintaining the ends of the chromosomes [11]. These so-called telomeres are shortening with each replication event [12] and normal cells reach a non-dividing, senescent state before the critical loss of telomeric repeats would lead to a "genetic catastrophe", a point where genomic instability results in death [13]. Upregulated telomerase activity in nearly all the human tumours [14,15], therefore, explains how immortalization allows other genetic alterations to occur without the elimination of cancerous cells.

Tumour suppressor genes (previously termed as antioncogenes) were initially defined by the observation that germline mutation of one allele predisposes to tumour formation and somatic mutation of the second allele will result in tumour progression [16]. The 24 known tumour suppressors can be divided in three groups with not strict boundaries between them [17]. The traditional "gatekeepers" act directly to prevent tumour growth and their loss of function is rate limiting for a particular step of tumourigenesis [18]. Restoration of the missing gatekeeper function leads to suppression of neoplasia. The pRb, VHL, APC and p53 proteins belong to this group. The "caretakers" are cancer-susceptibility genes that indirectly

suppress growth by ensuring the fidelity of the genetic code through effective DNA-repair and prevention of genomic instability [18]. As these genes are never required for neoplasia, restoration of caretaker function to a cancer cell will not affect its growth. The best examples of this group are the ATM protein kinase, the XPB helicase and the DNA-repair genes MSH2 and MLH1. The BRCA1/2 and the p53 tumour suppressors have also significant caretaker activities [17]. The so-called "landscapers" act by modulating the microenvironment in which tumour cells grow, perhaps by regulation of extracellular matrix proteins, cell surface markers, adhesion molecules or secreted growth factors [19]. The genes defective in patients with juvenile polyposis syndrome (JPS) can be classified as landscapers.

Proto-oncogenes and tumour suppressors join in finely balanced signalling pathways where deregulated expression of any of the components may have serious effects on cell proliferation. Moreover, it is becoming increasingly clear that these pathways intersect at common points, forming a highly sensitive molecular network where the final outcome of stochastic events is hardly predictable [17].

Loss of function of the p53 tumour suppressor is generally one of the last steps in tumourigenesis [1] and occurs in more than half of the human cancers [20], underlining that elimination of this checkpoint has a crucial impact on the development of neoplasia.

p53 is a transcription factor that activates genes involved in cell cycle, apoptosis and DNA-repair [21,22]. Under various stress conditions, like DNA damage, hypoxia, ribonucleotide depletion, oncogene activation or changes in the redox potential, p53 becomes activated by post-translational modifications that affect its conformation and binding to several proteins, resulting in its stabilisation and increased DNA-binding potential [23].

Given its central role in control of cell growth and transformation, p53 became one of the best characterized molecules in cancer research and although more than twenty years have passed since its discovery, the identification of its target genes and its interacting partners is still in progress [24]. The better understanding of how other proteins affect its checkpoint functions will definitely augment the design and implementation of novel anticancer therapies.

STRUCTURAL AND FUNCTIONAL DOMAINS OF THE p53 PROTEIN

The human p53 protein consists of 393 amino acids (aa) and can be divided from a structural and functional point of view into 5 domains [21]. In addition, sequence comparison analysis of different organisms led to the identification of five regions that has been conserved during evolution (boxes 1-5; aa 13-23, 117-142, 171-181, 234-250 and 270-286) and represent the most frequent sites of inactivating point mutations (Figure 1).

The transactivation domain (aa 1-42)

The acidic N terminus of p53 is responsible for the recruitment of the basal transcriptional machinery, by directly associating with the TATA-binding protein (TBP) and its associated factors (TAFs) [25,26]. Moreover, the transcriptional co-activator p300/CBP also binds this region and increase the transcriptional ability of p53 [27,28]. Two highly conserved residues, Leu22 and Trp23, have been demonstrated to directly contact TAFII40 and TAFII60 and to be required for the sequence-specific transactivation (SST) function of p53 [29]. Importantly, the same amino acids are targeted by competitive interaction with viral (adenovirus E1B 55K, human papillomavirus E6) and cellular (Mdm2) oncogenes that efficiently abrogate SST [30-32] and, in addition, mediate the degradation of the protein by the ubiquitin-proteasome pathway [33-35].

The proline-rich domain (aa 60-94)

This region contains five PXXP repeats, a motif that is a docking site for SH3 domain-containing proteins involved in signal transduction pathways [36]. Although no protein interacting with this region of p53 has been identified yet, the importance of this domain in the Gas1-mediated growth arrest [37] and in the specific activation of the PIG3 gene [38] is well established. Interestingly, a sequence polymorphism at amino acid position 72 has been correlated with the risk of development of human papillomavirus (HPV)-associated cervical cancers [39].

The DNA binding domain (aa 100-300)

The core domain of p53 has been shown to bind DNA in a sequence-specific manner [40,41]. The consensus binding site for p53 is composed by two copies of the palyndromic 10 bp motif: 5'PuPuPuC(A/T)(T/A)GPyPyPy3', separated by 0-13 nucleotides [40]. The internal symmetry of the four half-sites suggests that p53 binds DNA as a tetramer and this evidence is confirmed by several experimental data and consistent with the known crystal structure of

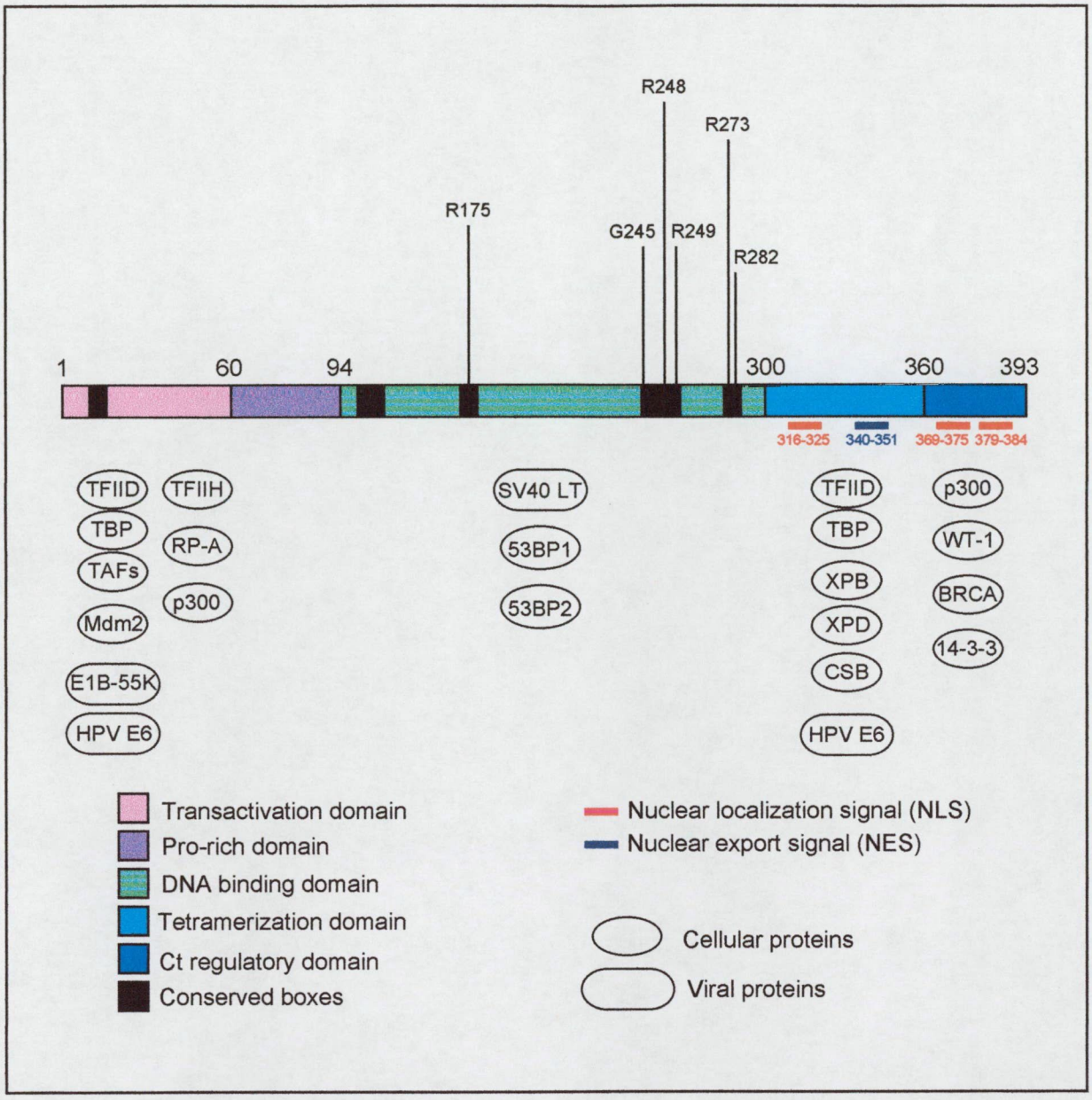


Figure 1. Structure and interacting partners of p53

Schematic representation of the structural and functional domains of the p53 protein. The evolutionarly conserved boxes and the "hot-spot" residues frequently targeted by mutations are also shown. Cellular and viral interacting proteins as well as the nuclear localization and export signals are indicated. Numbers refer to amino acids.

the protein [42]. 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 [20]. In particular, the observed mutations in p53 more frequently involve only some residues (so-called "hot-spots", aa 175, 245, 248, 273 and 282) that are believed to be crucial for either contacting DNA or maintaining the overall structure of the domain [42]. Strikingly, the majority of mutant p53 proteins isolated from cancers are impaired in binding to DNA and, as a consequence, in activating transcription of target genes [20]. Similarly, the large T antigen of the oncogenic SV40 virus interacts with the core domain of p53 and blocks its DNA binding ability [43]. Since p53 binds DNA in a tetrameric form, mutation of a single allele has been proposed to abrogate p53 functions in a dominant-negative manner, by forming heteromeric complexes with the wild-type counterpart [22].

The tetramerization domain (aa 323-356)

The oligomerization domain of p53 is connected to the core domain by a flexible linker. 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 [44,45]. Within this region is comprised the main nuclear localization signal (NLS), spanning residues 316-325 [46]. More recently, a nuclear export signal (NES) has also been mapped in this domain (aa 340-351) [47]. This NES was shown to be exposed and functional in the dimeric protein, but to be buried in the oligomerization domain when the tetramer is formed, suggesting that p53 enters and exits the nucleus in its dimeric form [47].

The C-terminal regulatory domain (aa 364-393)

According to the "allosteric model", the last 30 amino acids act as negative regulators of p53 DNA-binding ability by interacting with sequences in the core domain and keeping the protein in a "locked" conformation [48]. Post-translational modifications, binding of proteins or single-stranded DNA fragments to this region can convert p53 from this inactive, latent form to an active state [49]. Two accessory NLSs have also been mapped in this domain (aa 369-375, 379-384) [46].

BIOLOGICAL FUNCTIONS OF THE p53 PROTEIN

The evidence that transgenic mice lacking both p53 alleles mature normally *in utero*, but are more prone to tumor development, underline the central role of this protein in controlling genomic integrity and cell viability [50]. After being activated by different kind of DNA damages or other cellular stresses, p53 mediates growth arrest to allow repair of the lesions or alternatively, depending on the intensity of the damage as well as on the cell type, triggers the programmed cell death pathway (Figure 2).

Cell cycle arrest

The best characterized consequence of p53 activation in response to ionizing radiation (IR) or ultraviolet (UV) light is the cell cycle arrest at the G1/S border [51]. This is mainly achieved through activation of the p21/Waf1/Cip1 gene that encodes a cyclin-dependent kinase (CDK) inhibitor [52-54]. The p21 protein binds and inactivates the CDK4-6/cyclin D and CDK2/cyclin E complexes that are essential for the hyperphosphorylation of pRb. The resulting hypophosphorylated form of pRb is tightly associated with the E2F transcription factor and efficiently blocks its activity on the induction of genes required for S phase entry [55].

It has been shown that p53 can also block G1/S transition in a way that is independent of its transcriptional activity. p53 binds to cyclin H, a component of the CDK activating kinase (CAK) [56]. 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 biological activities. Similarly, the Gas1-dependent growth arrest requires the presence of p53 but not its transactivation function [37].

A possible role for p53 in the G2/M checkpoint has also been postulated. GADD45, a p53-induced gene, was shown to destabilize the cdc2/cyclin B complex that is involved in the activation of substrates required for the entry into mitosis [57,58]. Similarly, another p53-regulated gene product, the 14-3-3 σ protein binds and sequesters cdc25c in the cytoplasm, restraining its ability to activate cdc2 [59]. Moreover, it has been shown, that p53-null mouse embryo fibroblasts (MEFs) treated with spindle-inhibitors fail to arrest in mitosis and, consequently, continue to cycle and reduplicate their DNA before completing cell division. These cells develop tetraploidy, indicating that p53 is a component of the mitotic spindle checkpoint [60].

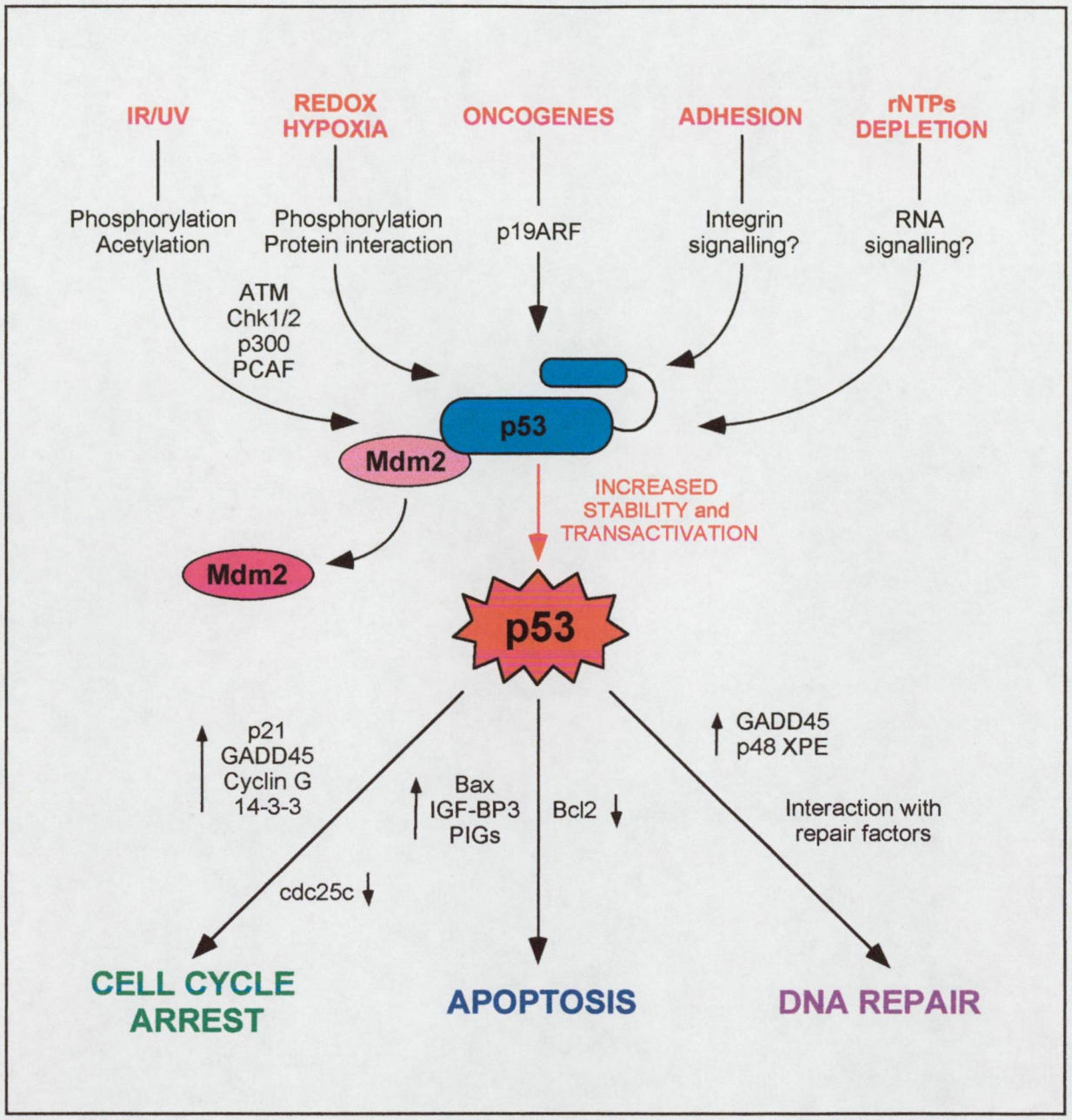


Figure 2. Functional activation of p53

Various environmental and cellular stress stimuli activate p53, mainly by post-translational modifications, transforming the protein from a latent to a stabilized form with enhanced DNA binding and transactivating capacities. p53 exerts its biological functions either by sequence-specific transactivation of target genes involved in growth arrest, apoptosis and DNA-repair or by interacting with other proteins implicated in these processes.

Apoptosis

Several experimental evidences indicate that cell cycle arrest and apoptosis represent divergent biological pathways and it has been proposed that the choice between them depends on the presence of survival factors in the external environment and is mediated by the activation of specific subsets of target genes [61].

p53 can trigger apoptosis by directly affecting the Bax/Bcl-2 equilibrium: p53-mediated transcriptional activation of the death effector Bax [62] and repression of the anti-apoptotic gene Bcl-2 [63] have been reported. Moreover, p53 was shown to increase the expression of a set of genes (PIGs, p53-induced genes) involved in the generation of and in the response to oxidative stress [64]. Therefore, p53 may initiate the apoptotic cascade by increasing the production of reactive oxygen species that in turn result in damage to mitochondria and activation of caspases. Numerous other p53-regulated proteins have been implicated in apoptosis, like Fas [65], IGF-BP3 [66], PIDD [67], PERP [68] and most recently, p53AIP1 [69].

It has been demonstrated that p53 mutants, which fail to activate transcription, can still induce death [70]. In particular, the Pro-rich region of p53 has been shown to be required for apoptosis, probably through the interaction with specific cellular factors [36].

DNA repair

Since the demonstration that p53 possesses a 3' to 5' exonuclease activity [71] that is mutually exclusive with its sequence-specific DNA biding capability [72], it was thought that p53 may perform some DNA repair functions. In fact, several evidences proved the role of p53 both in nucleotide-excision repair (NER) [73] and in base-excision repair (BER) [74]. Involvement of p53 in repair of double-strand breaks (DSB) has also been postulated [75].

The mechanisms by which p53 stimulates repair are not yet fully understood, but it seems that is mostly achieved in a transactivation-independent manner. Beside its ability to aspecifically bind to mismatched DNA, p53 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 [76].

REGULATION OF THE p53 PROTEIN

The growth-suppressing and apoptotic functions of p53 have to be maintained under a very stringent control during normal cell growth and differentiation. For this reason, p53 is synthesized in a transcriptionally inactive, latent form and its protein level is kept extremely low by a rapid turnover rate. Several upstream stress signals are converging on p53 and lead to functional activation of the protein (Figure 2). Among these events, the best characterized is surely DNA damage, but other stimuli, like hypoxia, disruption of microtubules, alteration in the redox potential of the cell, unbalance in the cellular nucleotide pools and heat shock are all resulting in a stabilized and transcriptionally active protein [24]. This activation is a very rapid event and involves mainly post-translational modifications of p53 [23] (Figure 3A). Another way to modulate p53 biological functions is to prevent it to reach its target genes by cytoplasmic sequestration.

Regulation of stability

p53 has been shown to be targeted for degradation by the 26S proteasome [77]. Regulation of protein stability by the ubiquitin/proteasome pathway is a common mechanism and requires a complex enzymatic cascade that promotes the conjugation of multiple ubiquitin chains to lysine residues in the target protein, that is then recognized and degraded by the proteasome [78]. 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 (see Figure 9A).

Mdm2 has been shown to function as a p53-specific E3 ubiquitin-ligase *in vitro* [79] and proposed to promote p53 degradation by facilitating its ubiquitination *in vivo* [33,34]. In line with this idea, overexpressed Mdm2 downregulates p53 levels in experimental conditions [33,34] and in tumours bearing a wt p53 allele [80]. Moreover, Mdm2-null mice show early embryonic lethality that can be rescued by simultaneous deletion of p53 [81]. Interestingly, Mdm2 is a transcriptional target of p53, suggesting the existence of a negative feedback loop, probably required for the termination of p53 response and for the recovery from G1 arrest [82]. Mutant p53 proteins unable to activate Mdm2 are more stable than their wt counterpart [83].

To exert its ubiquitin-ligase activity, Mdm2 must associate with p53 (Figure 1) and induction of p53 following DNA damage is mediated by phosphorylation of specific amino acids of both proteins resulting in the dissociation of the complex. Several serine and

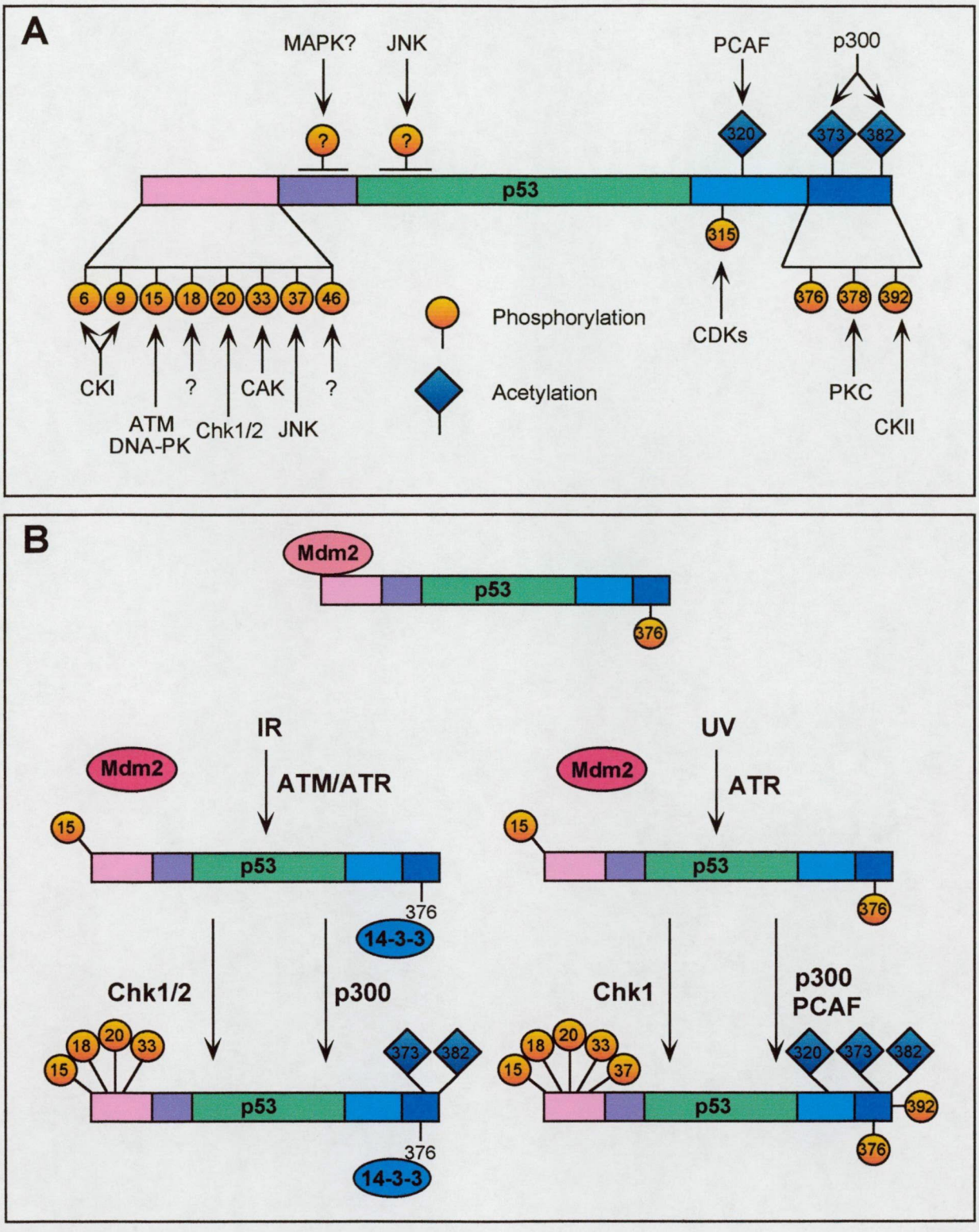


Figure 3. Post-translational modifications of p53

(**A**) p53 can be phosphorylated and acetylated at several residues by various kinases and acetyltransferases, respectively. (**B**) Schematic representation of the distinct phosphorylation and acetylation patterns of p53 that occurs in response to DNA damage induced either by ionizing radiation (IR) or by ultraviolet light (UV). The functional domains of p53 are represented as in Figure 1. Numbers refer to amino acids.

threonine residues in the Mdm2 binding region have been suggested to serve as potential phosphorylation sites of various protein kinases (Figure 3A) and, among them, Ser15 and Ser20 seem to be the best candidates in the regulation of the p53/Mdm2 complex stability *in vivo* [23,84].

The possible kinases mediating the DNA damage response on Ser15 are ATM and ATR, members of the PI3-kinase family. Several data indicate that ATM is responsible for increase of p53 half-life after IR, while ATR is required for response to UV [85] (Figure 3B). ATM and ATR are able to phosphorylate p53 on Ser15 and this modification relieves the interaction with Mdm2 *in vitro*, therefore providing an easy explanation for the observed effect on p53 stability [85].

Recently it has been shown that substitution of Ser 20 with alanine is sufficient to abolish p53 stabilization in response to IR and UV light *in vivo* [86,87]. This residue is phosphorylated by the Chk1 and Chk2 kinases (Figure 3A), homologues to yeast proteins involved in DNA damage checkpoints. Phosphorylation of Ser20 has been correlated with increased p53 stability and with the induction of p53-dependent G1 arrest [87-89]. Since Chk2 is activated by IR 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 (Figure 3B). These kinases, in turn, phosphorylate p53 on Ser20 and destabilize the complex with Mdm2.

The ability of Mdm2 to promote p53 ubiquitination can be modulated not only by covalent modifications but also by the interaction with other regulatory proteins. The best characterized alternative pathway for p53 stabilization involves the ARF protein, encoded by an alternative reading frame in the INK4a locus [90,91]. ARF binds to the Mdm2/p53 complex and prevents p53 proteolysis by blocking the E3 ubiquitin-ligase activity of Mdm2 [92] possibly by sequestering it in the nucleolus [93]. Overexpression of several 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, providing a link between the p53 and pRb pathways [94,95].

Other mechanisms for p53 ubiquitination and degradation also exist. The c-Jun N-terminal Kinase (JNK), member of the stress-activated family of protein kinases, binds to p53 and target it for degradation in non-stimulated cells [96]. Upon cellular stress, JNK become activated and phosphorylates p53 (Figure 3A), resulting in stabilization of the protein not only by disrupting the JNK/p53 complex but by also by preventing Mdm2 binding [97]. Viral oncoproteins are also involved in the destabilization of p53 by decreasing its cellular levels. E6 from high risk HPV strains binds to p53 (Figure 1) and mediates the simultaneous

recruitment of a cellular E3 ubiquitin-ligase, E6AP. While E6AP alone is unable to associate with p53, the E6/E6AP complex specifically interacts with the tumor suppressor, resulting in its effective ubiquitination and degradation [35,98]. In adenovirus-infected cells, E4orf6 has been shown to induce the degradation of p53, in association with the major p53-binding viral protein E1B 55K [99] (Figure 1).

Regulation of transcriptional activity

Beside the regulation of p53 levels in response to DNA damage or other stress stimuli, another control mechanism should be discussed that mediates the conversion of the protein from a latent to a transcriptionally active form. This is, again, mainly achieved by post-translational modifications of specific residues located in the N and C termini of p53 [100].

Mdm2 regulates p53 activity not only by mediating its degradation but also by preventing p53 association with the transcriptional machinery. Therefore, the dissociation of the p53/Mdm2 complex by phosphorylation of Ser15 and Ser20 after exposure to DNA damage not only increases p53 half-life but also simultaneously stimulates its transactivation capability [86,101].

Phosphorylation of serine 15, 33 and/or 37 has been also implicated in modulating the association of p53 with the p300/CBP transcriptional co-activator [102]. p300/CBP interacts both with the N- and C-terminal regions of p53 (Figure 1) and this interaction increases the ability of p53 to stimulate transcription of its target genes by two possible mechanisms [27,28]. First, due to its intrinsic histone acetyltransferase (HAT) activity, p300/CBP acetylates histones at p53-responsive promoters allowing the access of these DNA sequences by the transcriptional machinery [103]. Second, p300/CBP acetylates p53 on lysines 373 and 382 [104] (Figure 3B) leading to a conformational switch of the molecule that is required to relieve the inhibitory effect of the carboxyl terminus (see below). It should be noted that E1A, the immediate early viral protein of Adenovirus, binds to p300/CBP and efficiently interferes with the transcriptional activity of p53 in this way [105].

The sequence-specific DNA binding ability of p53 is subject to a constitutive negative regulation through its C-terminal domain, as underlined by the evidence that a protein lacking the last 30 amino acids is constantly active for DNA binding [106]. Relief of this inhibition by phosphorylation and acetylation of relevant residues upon exposure to stress results in increased DNA binding.

Three phosphorylation sites has been identified in this region (Figure 3A): Ser315 is phosphorylated by the cdc2/cyclin B and CDK2/cyclin A complexes and this modification increases the sequence-specific DNA binding activity of p53 in a promoter-dependent manner

[107]. Phosphorylation of Ser378 by protein kinase C (PKC) activates p53 in a similar fashion [84]. Ser392 is modified *in vitro* by casein kinase II (CKII) and this event enhances DNA binding by facilitating p53 tetramerization.

There are also evidences for activation of p53 through dephosphorylation: serine 376 is normally phosphorylated in unstimulated cells, but becomes rapidly dephosphorylated following IR (Figure 3B). This modification creates a binding site for the 14-3-3 adapter proteins and this interaction results in p53 activation [108]. In this case, therefore, conformational activation is mediated by interaction with specific factors, which in turn is modulated by post-translational modifications.

Several experimental evidences suggest that the stress-induced modifications of p53 on its N and C termini are carefully coordinated, sequential series of events that are also determining the specificity of the p53 response. For example, phosphorylation of Ser392 is induced by UV but not by IR [109], while dephosphorylation at serine 376 is promoted only by IR [108] (Figure 3B). Since the two kinds of DNA lesions are repaired by different mechanisms, it is possible to speculate that fine-tuning of the p53 response by specific post-translational modifications may direct the cellular choice for the appropriate damage repair.

Regulation of subcellular distribution

In addition to stabilization and functional activation of the protein, the proper subcellular localization of p53 is essential to trigger a full biological response [110]. Indeed, certain types of tumours (for example neuroblastomas) have been reported to contain wild-type p53 that, however, is inactivated by cytoplasmic sequestration [111]. Similarly, the X protein of the Hepatitis B virus keeps p53 out from the nucleus, probably by interfering with its nucleo-cytoplasmic shuttling [112].

The nuclear import of p53 is mediated by its three lysine-rich NLSs (Figure 1) and post-translational modifications of Ser315 and Lys320 may regulate this function.

Previous findings that nucleo-cytoplasmic shuttling of Mdm2 is required for the degradation of p53 by cytoplasmic proteasomes suggested that Mdm2 might be involved not only in the ubiquitination but also in the nuclear export of p53 [113]. Both Mdm2 and p53 contain a leucine-rich, Rev-like NES (Figure 1), that is recognized by the nuclear export machinery [47,113]. The current model, based on very recent experimental data, is that Mdm2-mediated ubiquitination of p53 in the nucleus results in a conformational change of the tetramerization domain, and leading to the exposure of the previously buried NES of p53 [47,114,115]. In addition, ARF has been shown to bind and sequester Mdm2 in the nucleolus, allowing p53 to be released from the Mdm2/p53 complex to the nucleoplasm [93].

AIM OF THE STUDY

The tumour suppressor protein p53 plays an essential role in maintaining genomic stability, by allowing repair of damaged DNA or by triggering the programmed death of cells that escape the normal cell cycle. This is clearly underlined by the fact that in more than half of human cancers p53 is functionally inactive. p53 is a transcription factor that is activated by various post-translational modifications following exposure to diverse stimuli. These modifications lead to a conformational change of the protein allowing its enhanced sequence-specific DNA binding and simultaneously result in its association with a set of interacting partners that modify its biological functions. Identification of new p53-interacting proteins would help to better understand the molecular mechanisms through which p53 exerts its growth suppressive activities and may aid the development of novel anticancer therapies.

Our group aimed to reach the following scientific goals:

1. Identification of new p53-interacting proteins by using a powerful molecular tool, the yeast two-hybrid system.
2. Demonstration of the association between p53 and the isolated protein in human cells and mapping of the domains required for complex formation on both proteins.
3. Understanding the biological relevance of the observed *in vivo* interaction by studying the changes in p53 transcriptional activity, apoptotic and growth inhibitory functions and subcellular distribution.

The presented works were done in collaboration with Dr. Monica Gostissa and Valentina Fogal (LNCIB, Trieste, Italy) and are published in the following scientific articles:

Sandy, P., Gostissa, M., Fogal, V., De Cecco, L., Szalay, K., Rooney, R.J., Schneider, C. and Del Sal, G. (2000) p53 is involved in the p120E4F-mediated growth arrest. *Oncogene* **19**, 188-199.

Gostissa M., Hengstermann, A., Fogal, V., Sandy, P., Schwarz, S., Scheffner, M. and Del Sal, G. (1999) Activation of p53 by conjugation to the ubiquitin-like protein SUMO-1. *EMBO J.* **18**, 6462-6471.

Fogal, V., Gostissa, M., Sandy, P., Zacchi, P., Jensen, K., Sternsdorf, T., Pandolfi, P.P., Will, H., Schneider, C. and Del Sal, G. (2000) Regulation of p53 activity in Nuclear Bodies by a specific PML isoform. *EMBO J.* (in press)

p53 IS INVOLVED IN THE p120E4F-MEDIATED GROWTH ARREST

Isolation of p120E4F as a candidate p53-interacting protein

We employed the yeast two-hybrid screening to identify proteins from quiescent human WI38 fibroblasts that are able to associate with the p53H175 hot spot mutant. Approximately 3.5 million independent transformants were screened and twenty seven single clones were isolated and analysed for their ability to bind also wt p53. Sequence analysis revealed that one of the clones, which interacted both with mutant and wt p53, corresponds to the carboxy-terminal half (starting from aa 350) of the E1A-regulated transcription factor p120E4F.

Originally identified in HeLa cells as DNA-binding activities that recognize the adenovirus E4 promoter in an E1A-dependent manner [116], E4F factors are low abundance zinc-finger proteins that are expressed in a wide range of tissues [117]. Although their cellular target genes are unknown, current evidence suggests that transcriptional regulation by E4F proteins may be somewhat complex. Within cells, two forms of E4F are present with distinct transcriptional activities. The full-length p120E4F protein behaves as a transcriptional repressor of the E4 promoter in the absence of E1A, and introduction of the viral protein relieves this repression [118]. In contrast, p50E4F, a proteolytic fragment of p120E4F that contains the amino terminal zinc-finger domain required for E4 promoter recognition, is able to activate that promoter, but only in the presence of E1A [118]. E1A regulates the transcriptional activities of both forms of E4F by inducing their phosphorylation at critical residues involved in DNA-binding [118-120].

Interestingly, ΦAP3, the murine homologue of E4F, is processed in a similar fashion and was originally isolated based upon its ability to bind to a completely different DNA sequence; a negative regulatory element within the adenovirus E1A promoter/enhancer [119]. When overexpressed, ΦAP3 specifically repressed the transcription of a reporter gene controlled by that promoter. Moreover, whereas binding to the E4F consensus sequence requires only the amino terminal zinc-finger domain, recognition of the ΦAP3 site may occur through the central domain [118,119,121]. Thus, depending upon their proteolytic form and phosphorylation state, E4F proteins may participate in a number of independent transcriptional and physiological responses.

Structurally, p120E4F contains six zinc-finger motifs which are clustered in two separate regions: two motifs are contained within an amino terminal domain that is also present in p50E4F, while the remaining four are grouped within its central region (aa 436-568) [118]. The isolated clone (E4FΔ350) encodes the central zinc-finger domain and all further carboxy-terminal residues that are specific for p120E4F (Figure 4A).

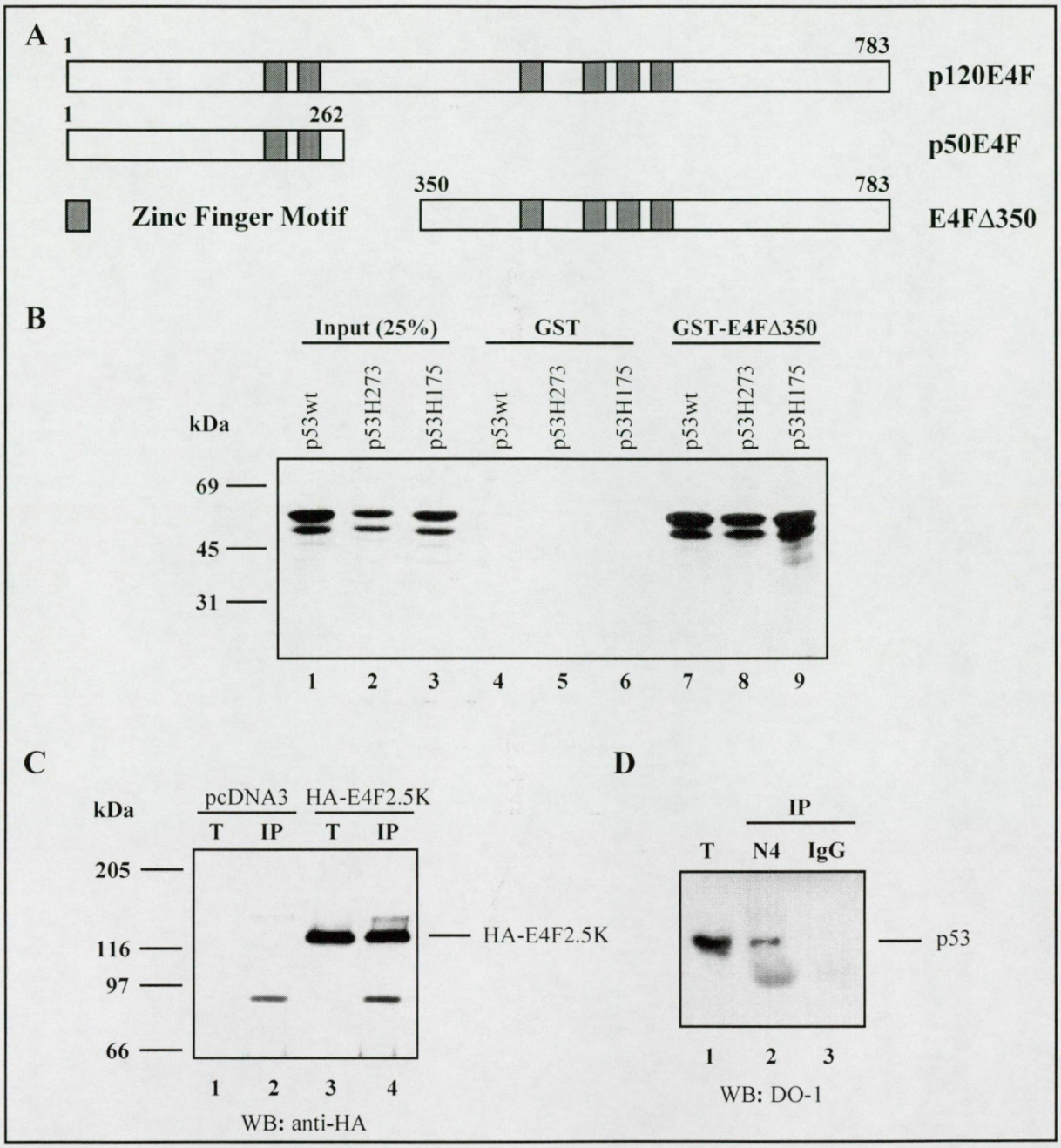


Figure 4. p120E4F is a p53-interacting protein

(A) Schematic representation of p120E4F and p50E4F proteins and the relative position of E4FΔ350 isolated in the yeast two-hybrid screen. Numbers refer to amino acids. **(B)** E4FΔ350 binds to p53 *in vitro*. Pull down experiment with GST-E4FΔ350 and radiolabelled wt or mutant (H273 and H175) p53 proteins were performed as described in Materials and Methods. **(C)** p120E4F associates with p53 *in vivo*. U2OS cells overexpressing HA-tagged p120E4F (HA-E4F2.5K) were immunoprecipitated (IP) with the p53-specific antibody DO-1, then probed with anti-HA antibody in Western blot. **(D)** Endogenous E4F was immunoprecipitated from U2OS cells with the E4F-specific N4 antibody, or with preimmune sera (IgG) as control and immunocomplexes were analysed in Western blot by using the DO-1 antibody. T, total lysates.

To verify the interaction *in vitro*, the isolated cDNA was expressed in bacteria as GST-fusion protein (GST-E4FΔ350) and tested in a pull down experiment with *in vitro* translated p53H175, p53H273 and wt p53 proteins (Figure 4B). In agreement with the data obtained in the yeast two-hybrid system, wt p53 and p53H175, as well as p53H273 bound to GST-E4FΔ350 (lane 7, 8 and 9). A specific interaction was also observed with *in vitro* translated mouse wt p53 protein (not shown). These data therefore demonstrate that p120E4F can directly associate with p53.

p120E4F associates with p53 *in vivo*

To confirm the association in human cells, the full-length p120E4F cDNA, cloned in pcDNA3HA, was transiently overexpressed in U2OS, a human osteosarcoma cell line expressing wt p53. Cell lysates were immunoprecipitated with the anti-p53 antibody DO-1, then blotted with an anti-HA antibody. As shown in Figure 4C, endogenous wt p53 associated with ectopically expressed p120E4F. Complex formation was also detected in cells transfected with a construct expressing E4FΔ350 (not shown). Similar results were obtained with ectopically expressed p120E4F and ΦAP3 in murine fibroblasts harbouring wt or mutant murine p53 alleles (not shown).

Finally, to establish the existence of a complex of endogenous proteins, cellular extracts from growing U2OS cells were immunoprecipitated with the E4F-specific antibody N4 or with preimmune serum as control and immunocomplexes were probed with the DO-1 antibody. As shown in Figure 4D, endogenous p53 was immunoprecipitated from U2OS cell lysates in complex with endogenous p120E4F.

Binding to p120E4F requires amino acids 256 to 294 of p53

To identify the region of p53 involved in the interaction with p120E4F, we produced various p53 deletions by *in vitro* translation and tested them for binding to GST-E4FΔ350 in a pull down experiment. As summarized in Figure 5B, E4FΔ350 bound to all the C-terminally truncated p53 proteins used, however, we observed significant differences in the efficiency of interaction. While the full length protein, capable of tetramerization, bound strongly to GST-E4FΔ350 (Figure 5A, lane 3), p53 1-355, that forms dimers in solution exhibited a weaker interaction (lane 6), suggesting that the quaternary structure of p53 may influence the association. Supporting this hypothesis, the two monomers, p53 1-338 and p53 1-298, are more impaired in their ability to bind to GST-E4FΔ350 (lane 9 and 12, respectively). The interaction with p53 1-298 was very weak, suggesting that the p120E4F-binding region is located near to the end of the core domain. In agreement with this assumption, p53 294-393

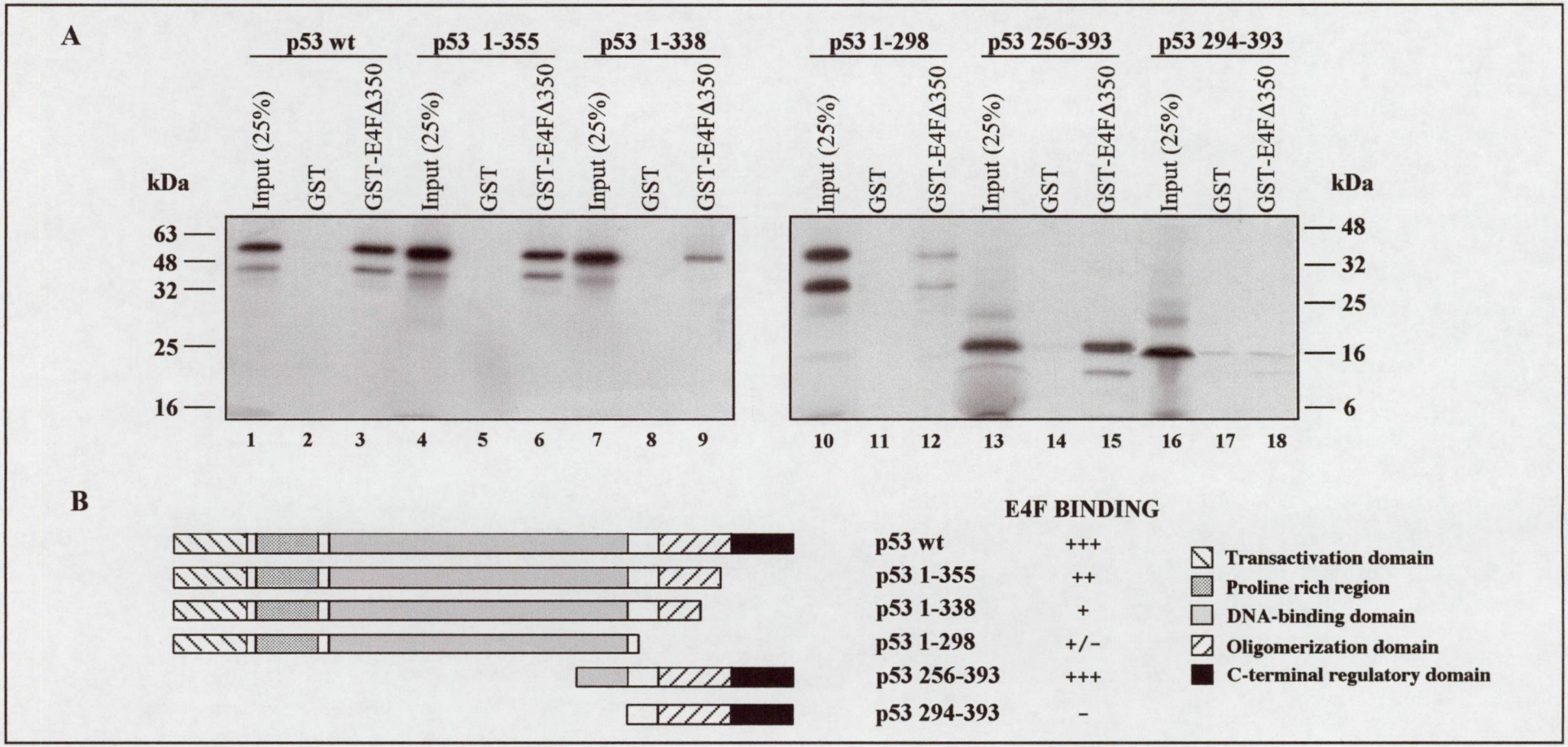


Figure 5. p120E4F binds to the core domain of p53

(**A**) Pull down experiment of radiolabelled p53 proteins with GST-E4FΔ350. (**B**) Schematic representation of the various p53 constructs used in the pull down assay and a summary of their binding properties to p120E4F. All the numbers refer to amino acids. The functional domains of p53 are indicated.

that lacks the DNA-binding domain was unable to interact (lane 18). p53 256-393, that overlaps 42 amino acids with p53 1-298 and contains the entire tetramerization domain and further C-terminal residues, interacted strongly (lane 15), demonstrating that binding to p120E4F requires amino acids 256 to 294 of human p53.

p120E4F does not affect the transactivation ability of p53

To test whether the transactivation ability of p53 was affected by the interaction with p120E4F, p53-null SaOS-2 cells were transfected with the pG13CAT reporter plasmid, that contains the consensus DNA binding site for p53, together with vectors encoding for human wild-type p53 and HA-tagged full-length or amino terminally truncated p120E4F (HA-E4F2.5K and HA-E4FΔ350, respectively). An HA-tagged, carboxy terminal fragment of human p53 (p53 294-393) was used as a positive control, since a corresponding fragment derived from the murine protein has been shown to affect wt p53 functions in a dominant negative manner. As shown in Figure 6, neither HA-E4F2.5K nor HA-E4FΔ350 did significantly affect the transactivation ability of p53 (lane 4 and 5), whereas p53 294-393 almost completely inhibited that function (lane 3). Of note, a slight repression of other reporters containing the SV40 or the thymidine kinase promoters by overexpressed p120E4F were consistently observed regardless the presence of p53 (not shown). The expression levels of each protein were comparable as judged by Western blotting. These findings therefore suggest that the interaction between p120E4F and p53 does not affect the transactivation ability of p53.

Binding to p53 requires amino acids 521 to 580 of p120E4F

The previous assays demonstrated that residues specific to p120E4F are required for p53 binding. To better define this region, several E4FΔ350 deletions were expressed as GST-fusions and used for pull down assay with *in vitro* translated wt p53 (Figure 7). Deletion of the last 89 amino acids (GST-E4FΔ350/NcoI) had no effect on p53 binding (lane 4), while removal of residues 428-783, including the entire central zinc-finger domain (GST-E4FΔ350/SfiI), completely abolished the interaction (lane 6). A construct lacking amino acids from 552 to 783 (GST-E4FΔ350/PstI) was severely impaired in its ability to associate with *in vitro* translated p53 (lane 5), suggesting that residues in the last zinc-finger motif (comprising amino acids 548 to 568) might be involved in mediating the interaction. Indeed, two fusions that contain the intact last zinc-finger motif, GST-E4FΔ350-580 and GST-E4FΔ521 strongly bound to p53 (lane 7 and 9), while no interaction was detected with an E4F deletion lacking the entire zinc-finger domain and sequences upstream of it (GST-E4FΔ581, lane 8). Similar

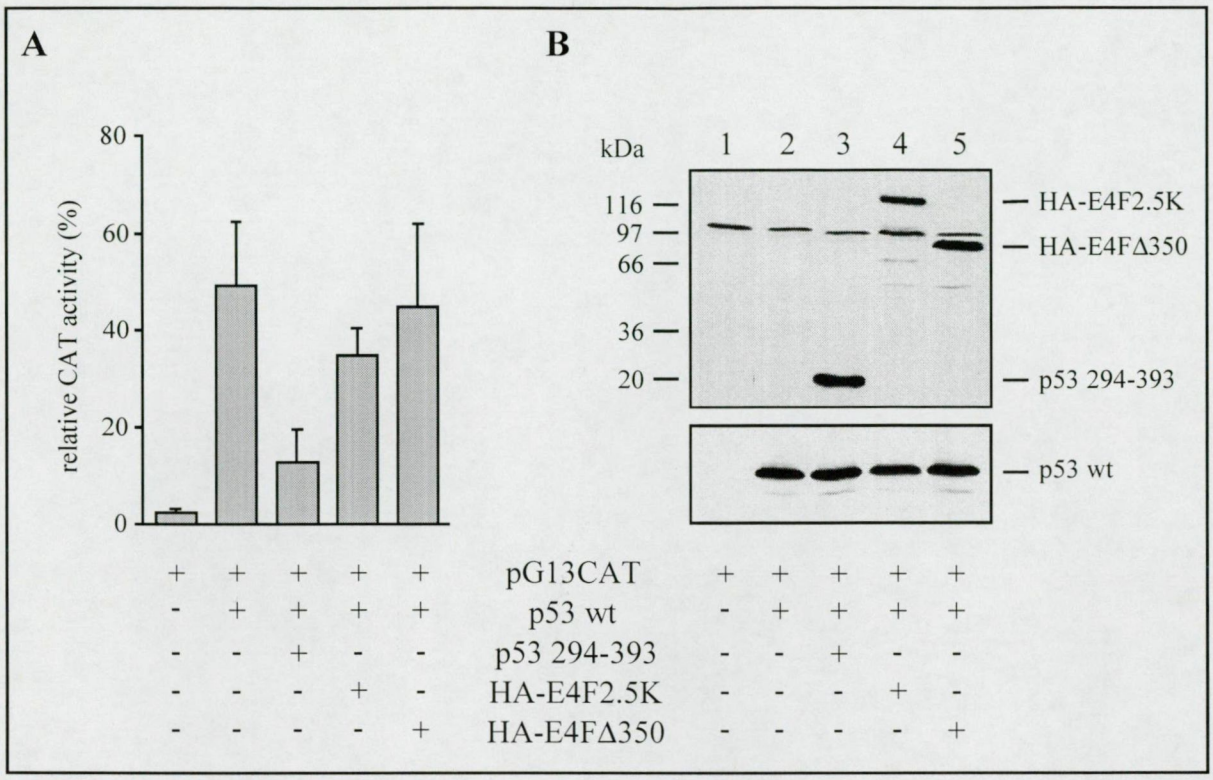


Figure 6. p120E4F does not affect p53 transcriptional activity

(A) CAT reporter assay in SaOS-2 cells transfected as indicated. Bars represent the mean of three independent experiments. **(B)** Western blot analysis of an aliquot of the lysates used for the CAT reporter assay. Upper panel: membrane was probed with the anti-HA antibody; lower panel: membrane was probed with the DO-1 antibody that recognizes N-terminal residues of p53. Note that p53 294-393 was expressed as an HA-tagged protein.

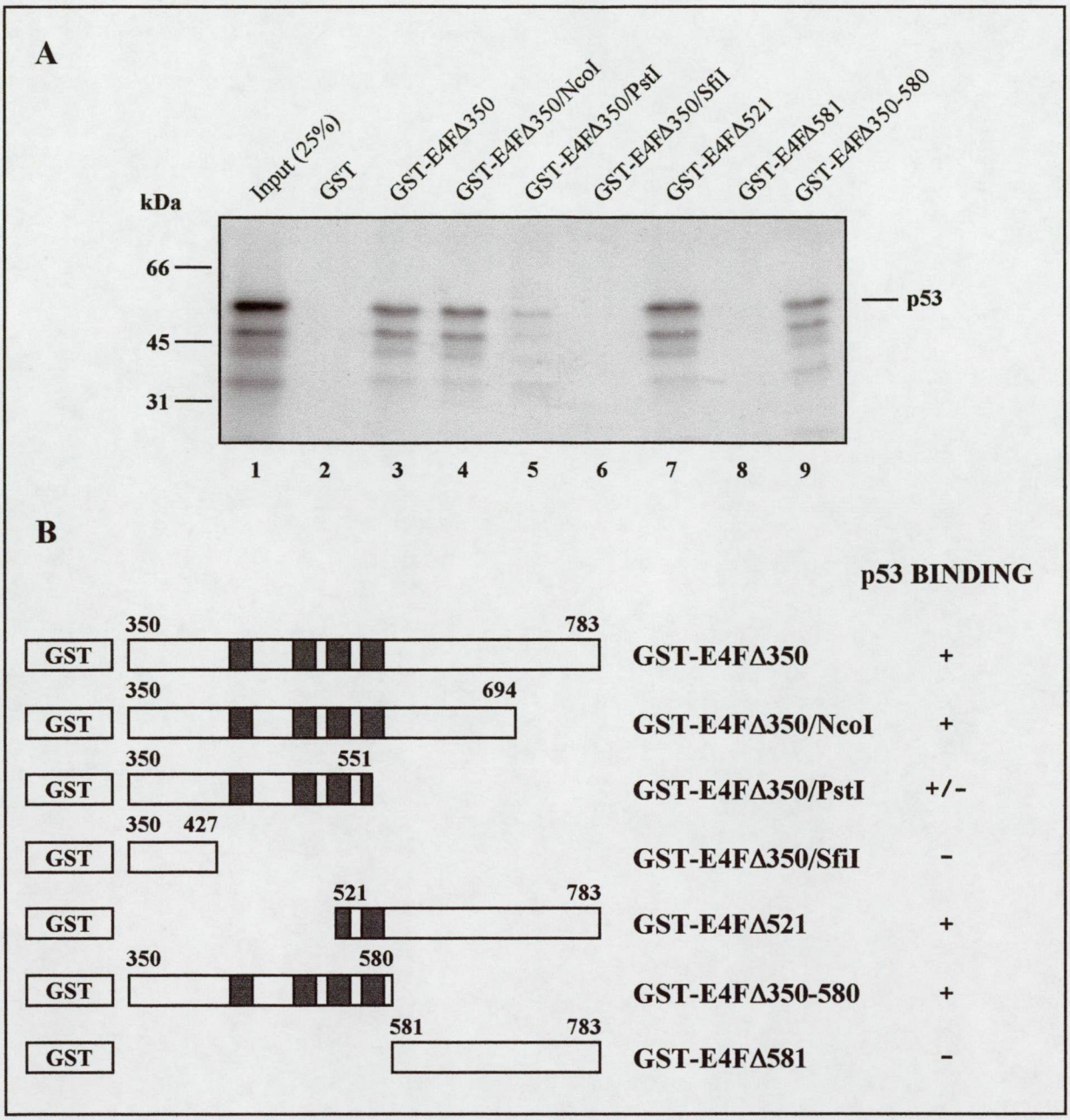


Figure 7. The last zinc finger motif of p120E4F is required for the interaction with p53
(**A**) Pull down experiment with radiolabelled p53 and various p120E4F deletion constructs expressed as GST-fusion proteins. (**B**) Schematic representation of the p120E4F proteins used in the pull down experiment and a summary of their binding properties to p53. Numbers refer to amino acids.

results were obtained when the different deletions were assayed in the yeast two-hybrid system (not shown). These data indicate that p120E4F contacts p53 through a carboxy-terminal region located between amino acids 521 and 580.

Ectopic expression of p120E4F requires wt p53 for growth suppression

Although cellular genes specifically regulated by E4F proteins are unknown, it was recently shown that p120E4F can mediate a growth arrest in NIH 3T3 cells, suggesting an important role for this protein in cell growth control [122].

In order to test the biological relevance of the complex formation between p53 and p120E4F, human cell lines expressing wt p53 (U2OS) or lacking p53 (SaOS-2) were transfected with plasmids expressing HA-E4F2.5K, HA-E4FΔ350 or empty pcDNA3HA vector and tested for colony formation ability after two weeks of selection in G418-containing medium. The results of three independent experiments are shown in Figure 8B. A similar reduction (~50%) in colony numbers was observed both with HA-E4F2.5K and HA-E4FΔ350 in SaOS-2 cells as compared to the positive control empty vector (100%). In contrast, a much stronger growth suppression effect was detected when the E4F constructs were transfected into U2OS cells, thus suggesting the involvement of wt p53.

To more firmly establish the potential role of wt p53 in the p120E4F-mediated growth suppression, we microinjected a panel of murine cell lines expressing wt p53 (NIH 3T3) mutant p53 (Val 5, maintained at 37°C) or in which both p53 alleles are deleted (Balb/c (10)1) and tested the inhibition of S phase entry by bromodeoxyuridine (BrdU) incorporation assay. Asynchronously growing cells were microinjected with various p120E4F deletion constructs (Figure 8A) together with a plasmid expressing the green fluorescent protein (GFP) as a marker gene. Growth suppression in microinjected cells (GFP-positive) was measured by scoring the relative fraction of cells in S phase (anti-BrdU-positive) with respect to uninjected cells in adjacent fields.

The results of four independent experiments are reported in Figure 8C. Enforced expression of HA-E4F2.5K blocked S phase entry in NIH 3T3 cells but not in Balb/c (10)1 or in Val5 cells. Similar results were observed with HA-E4F/NcoI and HA-E4FΔ350, both of them being able to associate with p53. In contrast, overexpression of the carboxy terminally truncated construct HA-E4F/PstI, which is deficient in binding to p53, did not inhibit S phase entry in any of the cell lines tested.

To further prove the involvement of wt p53 in the p120E4F-mediated growth suppression, Balb/c (10)1 cells were coinjected with HA-E4F2.5K or HA-E4F/PstI and limiting amount of wt p53 together with pGFP, as marker, and processed as described above. Overexpression of

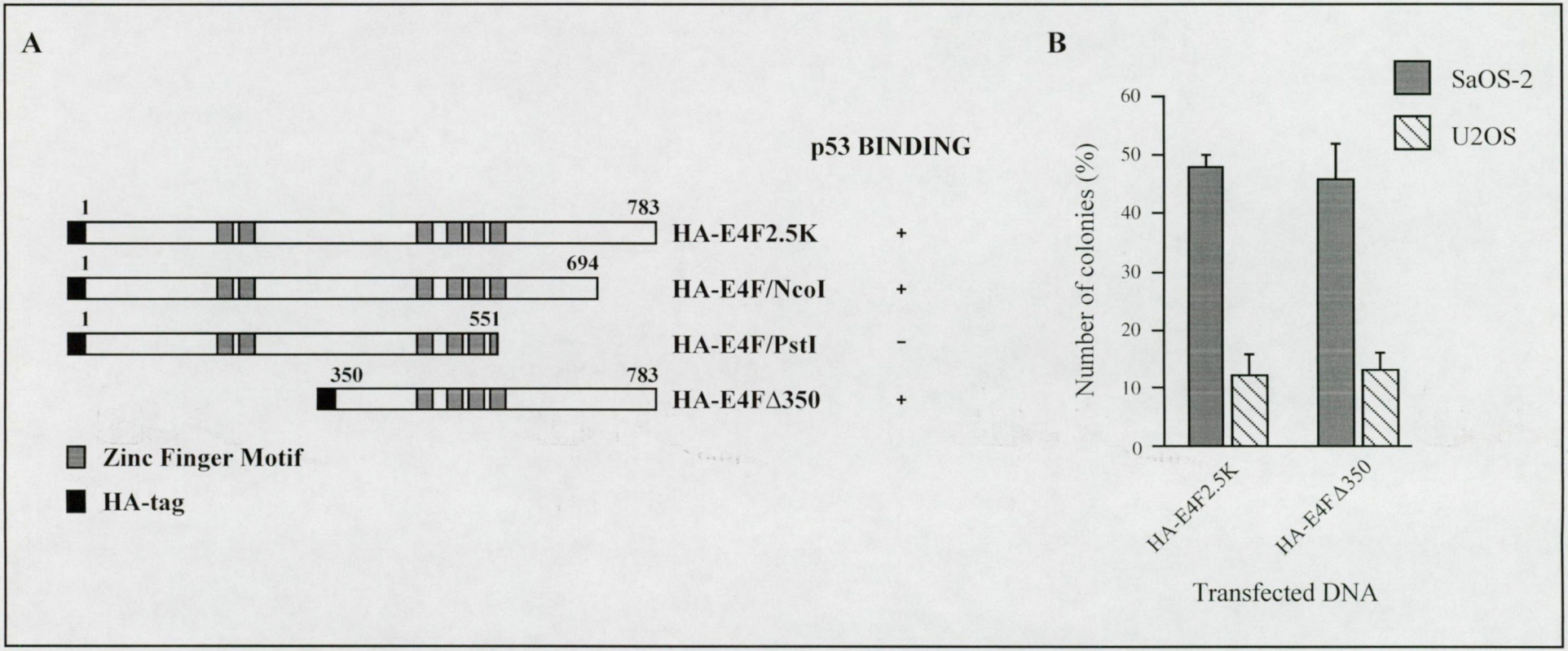


Figure 8. p120E4F inhibits S phase entry in a p53-dependent manner

(A) Schematic representation of the various p120E4F deletions used for colony formation and growth inhibition assays. The ability of each protein to interact with p53 is indicated. **(B)** Colony formation assay in human cell lines overexpressing p120E4F and E4FΔ350. U2OS cells harbour endogenous wt p53, while SaOS-2 cells lack functional p53 protein. Colony forming ability was measured and calculated as described in Materials and Methods. The bars represent the mean of three independent experiments.

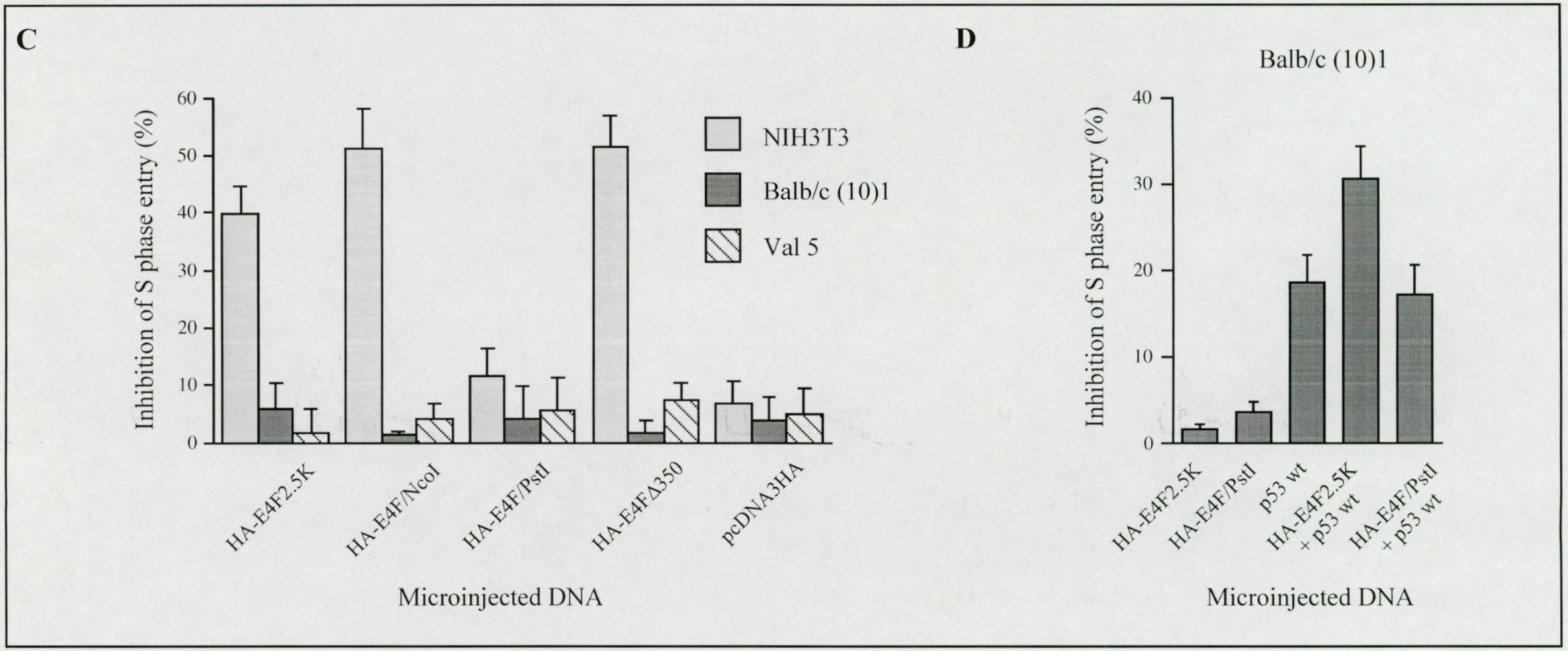


Figure 8. p120E4F inhibits S phase entry in a p53-dependent manner

(C) Growth inhibition assay in murine cell lines microinjected with various p120E4F deletion constructs. NIH 3T3 cells harbour endogenous wt p53, while Val5 cells express a mutant form of murine p53 under the experimental conditions used. Balb/c (10)1 cells lack functional p53 protein. **(D)** Growth inhibition assay in Balb/c (10)1 cells coinjected with either HA-E4F2.5K (50 ng/µl) or HA-E4F/PstI (50 ng/µl) and limiting amount (5 ng/µl) of wt p53. Inhibition of S phase entry was measured and calculated as described in Materials and Methods. The bars represent the mean of four independent experiments.

wt p53 alone resulted in a moderate growth inhibition (18%, Figure 8D) that was not affected by the coexpression of HA-E4F/PstI. Microinjection of HA-E4F2.5K alone had no any effect on cell growth, while coinjection of wt p53 and HA-E4F2.5K resulted in a 30% inhibition of S phase entry.

The results obtained by colony formation assays in human cell lines, together with the data from microinjection experiments in mouse cells, demonstrate that ectopic expression of p120E4F causes a growth suppression and cell cycle arrest that depends upon the presence of wt p53 and the ability of p120E4F to associate with it.

Discussion

In this part of the thesis, I demonstrated that the E1A-regulated transcription factor p120E4F associates with p53 and depends upon wild-type p53 to exert its growth inhibitory effect in human and murine cells.

One apparent discrepancy with previous results that should be noted, is that the role of p53 in mediating p120E4F growth suppression was initially excluded based upon the observation that cotransfection of a dominant negative p53 mutant with p120E4F had no effect on colony reduction in NIH 3T3 cells [122]. However, the design of those experiments did not take into account the ability of p120E4F to physically interact with wt and mutant p53. Thus, given the relative amounts of transfected p120E4F and mutant p53 constructs used by Fernandes *et al.*, it is likely that the excess levels of p120E4F were sufficiently high to titre out the mutant p53 protein and prevent its abrogation of wt p53 function.

Our evidence, that p120E4F can induce a p53-dependent block of cell proliferation without having an obvious effect on p53 transactivation function is consistent with the previous observation that elevated p21 protein levels in p120E4F-arrested cells occurred independently of an increase in p21 gene transcription [122]. This suggests that the p120E4F-mediated growth suppression may involve a number of target genes that are not directly regulated through consensus p53-binding sequences, rather through E4F or ΦAP3 sites.

I should note that in both the colony formation and the microinjection assays, the truncated E4FΔ350 protein, which lacks the amino-terminal zinc-finger domain and therefore fails to bind the E4F consensus sequence, was sufficient to induce growth inhibition both in human and murine cells. This suggests that cellular target genes of p120E4F responsible for the growth arrest effect may contain the ΦAP3 site [119]. However, it is possible that p120E4F mediates this effect solely by bridging other proteins and may not require any DNA-binding function.

How p53 fits into this scenario is not yet clear. Control of gene expression generally requires the interaction of multiple factors, including several activators, coactivators, repressors and basal factors to stabilise the transcriptional machinery and determine its specificity. It is conceivable that p53 may act as a p120E4F-cofactor, to regulate the differential recognition of alternative DNA-binding sites for E4F proteins or the association of p120E4F with other factors in the transcriptional complex. Alternatively, p120E4F may be involved in modulating the transcriptional repression function of p53. Notably, although mutant p53 can still associate with p120E4F, the complex is not functional in terms of growth suppression. This indicates that the conformation of p53 in the complex is important for the function of p120E4F, and may reflect a requirement to bridge p120E4F to other factors that recognize only wt p53. Several wt p53-binding proteins are also able to interact with mutant forms of p53, but, as with p120E4F, the mutant proteins lack the ability to regulate the activity of these partners. The potential role of p53 as a p120E4F-cofactor may be particularly relevant when cells are exposed to stimuli, which normally induce p53, since cells lacking the p53 checkpoint are seriously deficient in the p120E4F-dependent response.

Truncations at the carboxy-terminal end of p120E4F, which impair the binding of p53, nullify its growth inhibitory effect. Thus, it would be of interest to look for the presence of genetic alterations in the E4F gene (E4F1), mapped on chromosome 16p13.3 [117,123], within tumour cells that express wt p53.

A better understanding of the role of the p53/p120E4F complex requires characterization of other factors that associate with p120E4F and most of all, the identification of p120E4F cellular target genes. A very recent report demonstrating the physical and functional interaction between pRb and p120E4F [124] suggests an important role for p120E4F in cell cycle control and raises the possibility that p120E4F is a key component of the two major tumour suppressor pathways.

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

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

Another yeast two-hybrid interaction trap was performed on proteins expressed in human foetal brain to identify new p53 associating partners. Screening of about 4 million colonies resulted in the isolation of 21 individual clones showing strong and specific interaction with the bait. Sequence analysis of one of the more representative 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 and has been reported to be covalently linked to several substrates such as RanGAP1, IκBα, Sp100 and PML [125]. The general mechanism mediating SUMO-1 conjugation to its substrates is very similar to the ubiquitin one, but utilises different enzymes [126,127] (Figure 9A). 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 [128] and hUbc9, which is the SUMO-conjugating enzyme, E2 [129,130]. Up to now, there are no evidences of the existence of E3-like activities in the SUMO-1 conjugation pathway. Despite these homologies, the sequence similarity between ubiquitin and SUMO-1 is low (Figure 9B); the two carboxy-terminal glycine residues that are required for the conjugation are highly 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 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 [131]. In the case of IκBα, 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 [132]. PML and Sp100 are important SUMO-1 conjugated components of the so-called nuclear bodies [133], multiprotein complexes involved in transcriptional and growth control [134].

Interestingly, from this yeast two-hybrid screening we also obtained two enzymes of the SUMO-1 conjugation pathway: hUbc9 and hUba2 (not shown). This fact prompted us to further characterize the biological relevance of the interaction between p53 and SUMO-1.

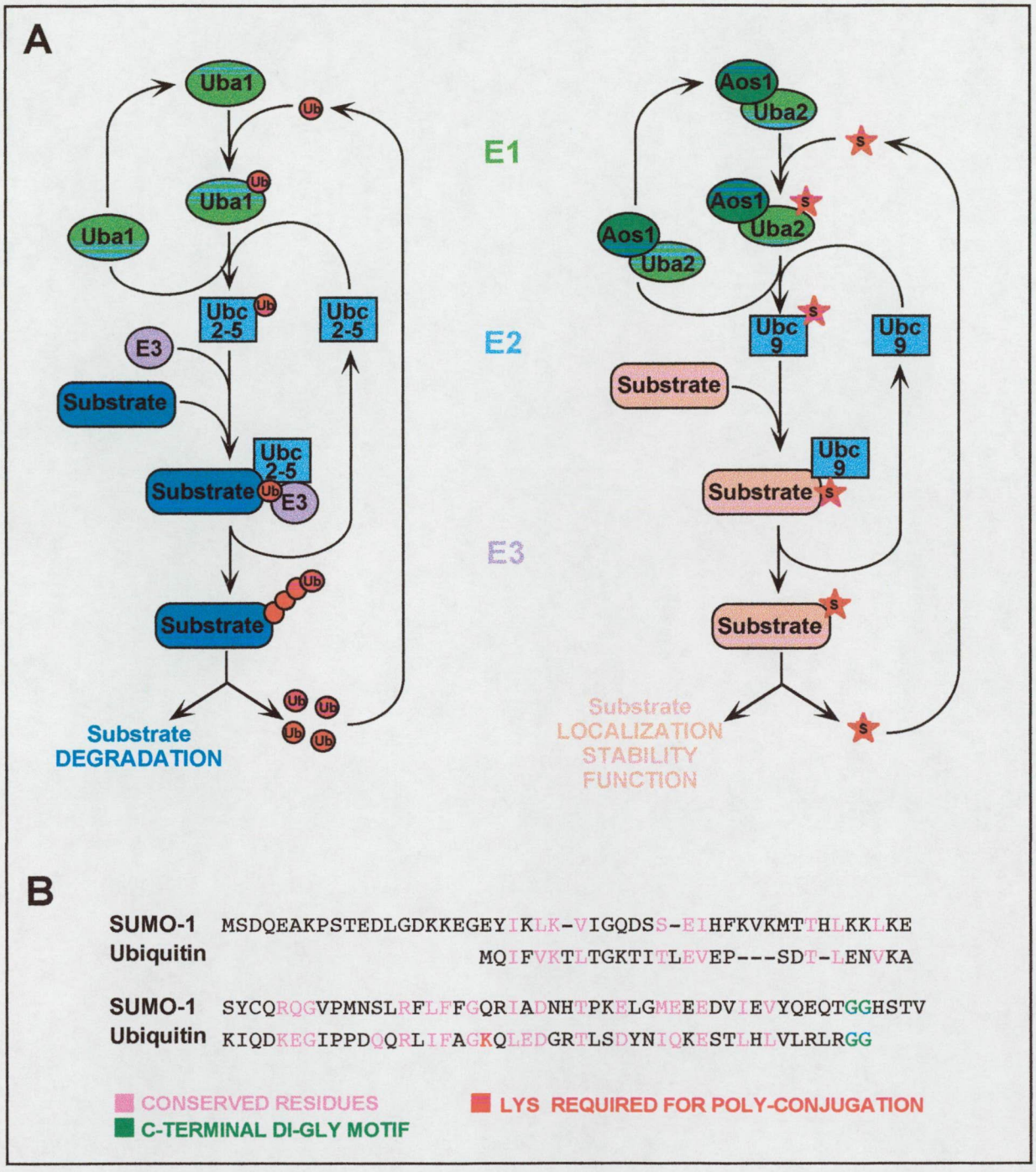
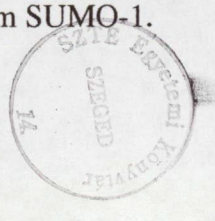


Figure 9. The ubiquitin and SUMO-1 conjugation pathways

(**A**) Schematic representation of the ubiquitin (left) and SUMO-1 (right) conjugation pathways. Ubiquitination requires three enzymatic steps and results in the formation of poly-ubiquitinated substrates that are degraded by the 26S proteasome. Sumolation is mediated by only two enzymatic steps, poly-SUMO-1 chains are not formed and the conjugation affects the biological functions of the substrate protein rather than its stability. (**B**) Sequence alignement of ubiquitin and SUMO-1. The two conserved glycine (G) residues required for conjugation are shown in green. Note that the internal lysine (K, shown in red) in ubiquitin, which serves as an acceptor site for poly-conjugation, is missing from SUMO-1.



SUMO-1 is conjugated to p53 *in vitro*

As a first step, we tested whether p53 is a substrate of the SUMO-1 conjugation pathway. ^{35}S-labelled p53 was generated in rabbit reticulocyte lysate and incubated in the presence or absence of recombinant mouse Ubc9 (mUbc9), a partially purified protein fraction containing the SUMO-activating enzyme E1, and a GST-SUMO-1 fusion protein [130]. Under the reaction conditions used, a slower migrating form of p53 was observed (Figure 10A, left panel). Since the appearance of this band was dependent on the presence of E1, mUbc9, and GST-SUMO-1, it can be concluded that this form 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 since HHR23a, another protein used as a control [130], was not modified under the same assay conditions (Figure 10A, right panel).

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

To map the lysine residues of p53 that serve as potential attachment sites for SUMO-1, we utilised several C-terminal deletion mutants of p53 in conjugation assays as described above. Removal of the C-terminal 10 amino acids was sufficient to render p53 incompetent for conjugation (data not shown) indicating that a lysine residue within this region, or close to it, may serve as the major attachment site for SUMO-1. Therefore, p53 mutants were constructed, in which the most C-terminal lysine residues (K381, K382 and K386) 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 10B, substitution of lysine to arginine at position 386 resulted 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, did not affect the ability of the respective p53 mutant to be modified.

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

To obtain evidences that p53 is also modified by SUMO-1 *in vivo*, total lysates from 293 cells were analysed by Western blot using the anti-p53 antibody, DO-1. As shown in Figure 11A, beside the 53 kDa molecular weight protein, a slower migrating band was also recognized, suggesting that this approximately 65-70 kDa protein is the SUMO-1 conjugated form of p53. To confirm this hypothesis, lysates from the same cell line were immunoprecipitated with a polyclonal antibody raised against p53 or with preimmune serum as a negative control and the immunocomplexes were analysed by Western blotting with an anti-SUMO-1 monoclonal antibody. This revealed that a protein with the expected molecular weight was specifically recognized in the anti-p53 immunoprecipitate (Figure 11B, left

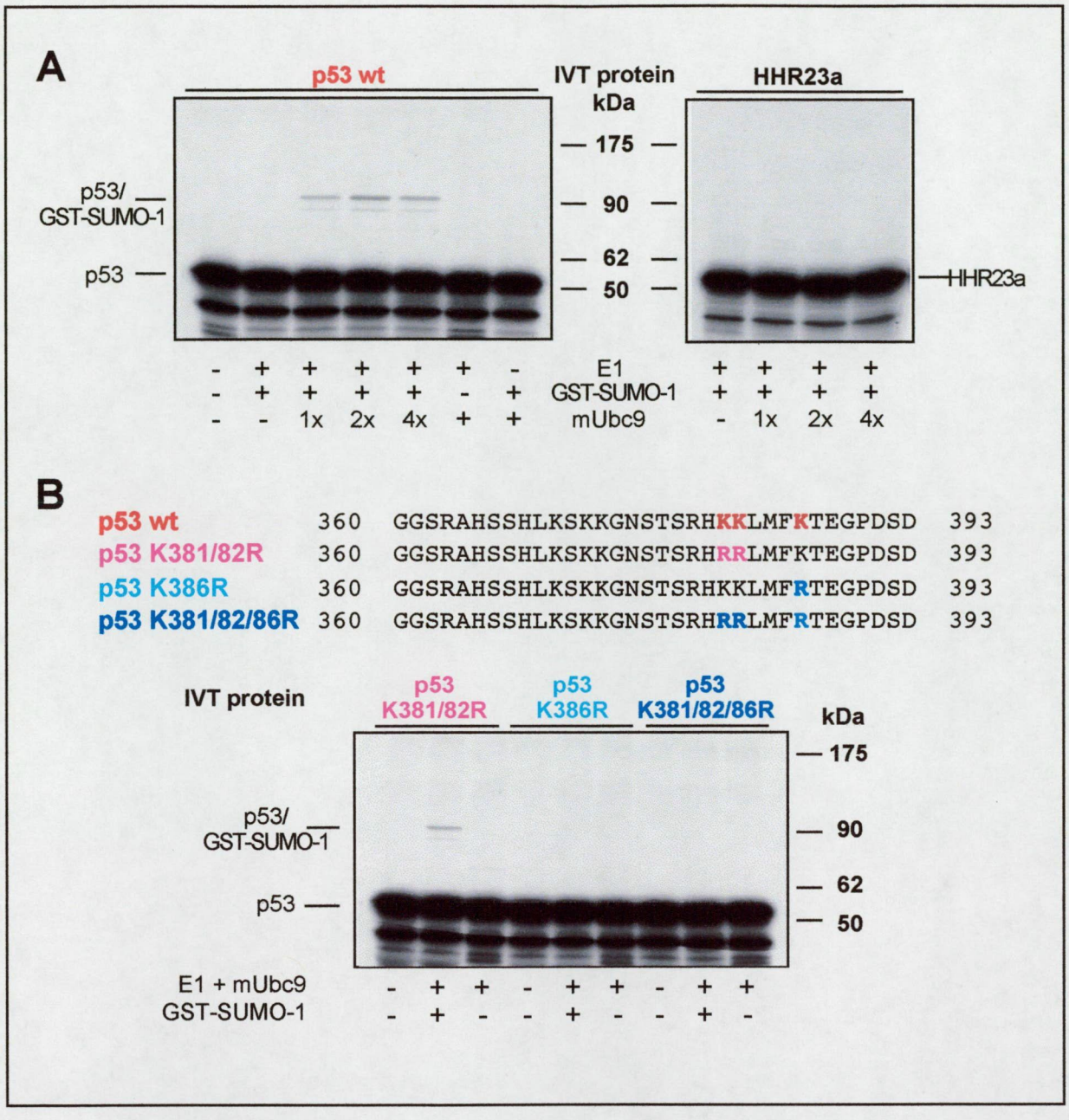


Figure 10. SUMO-1 is conjugated to p53 at position K386 *in vitro*

(**A**) *In vitro* conjugation assays with radiolabelled p53 (left panel) or with HHR23a, used as negativ control (right panel), were performed as described in Materials and Methods in the presence (+) or absence (-) of the indicated components. (**B**) *In vitro* conjugation assays with radiolabelled mutant p53 proteins, in which lysines were substituted to arginines as indicated. The amino acidic sequence of the C-terminal region of the mutant proteins is shown above.

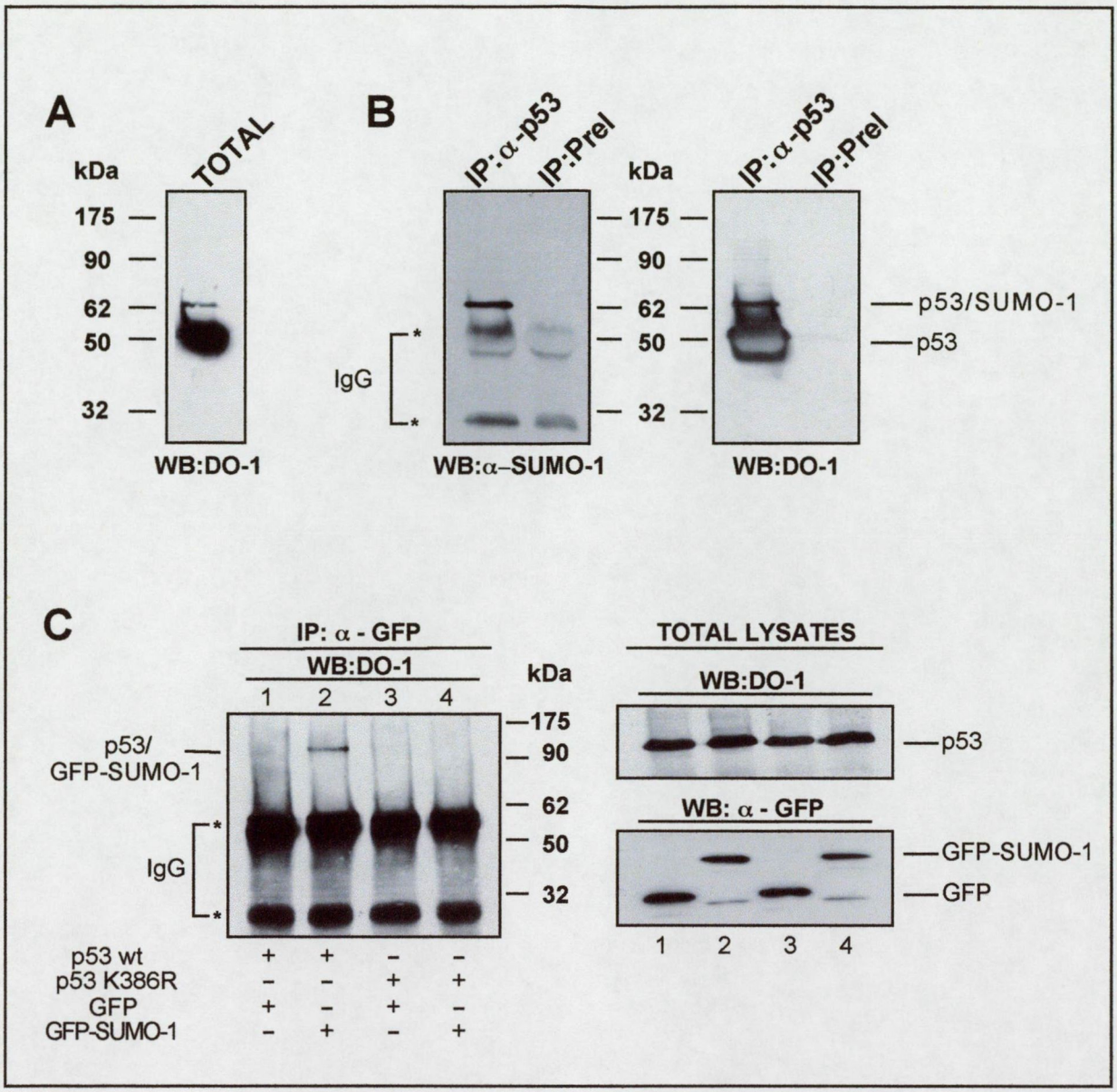


Figure 11. p53 is modified by SUMO-1 *in vivo*

(**A**) Western blot analysis of 293 cell lysate with the DO-1 antibody. (**B**) 293 cells were immunoprecipitated with an anti-p53 serum or with pre-immune serum (PreI), as control, and subsequently analyzed by Western blot with the anti-SUMO-1 antibody (left panel). The same membrane was stripped then reprobed with DO-1 (right panel). (**C**) SaOS-2 cells co-expressing p53 wt or K386R and either GFP-SUMO-1 or GFP, as indicated, were immunoprecipitated with an anti-GFP antibody and then analyzed by Western blot using the DO-1 antibody (left panel). An aliquot of each lysate was checked for the expression of p53 and GFP-SUMO-1 or GFP, respectively, by Western blot (right panels). p53 and its modified forms are indicated.

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 11B, 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 and that, unlike ubiquitin, p53 proteins conjugated to multiple SUMO-1 molecules are not formed.

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, first tagged SUMO-1 proteins were generated by cloning in frame the entire SUMO-1 ORF downstream of either the Green Fluorescent Protein (GFP-SUMO-1) or the HA epitope (HA-SUMO-1). Next, pGFPSUMO-1 was transiently transfected into the p53-null cell line SaOS-2, together with a vector expressing either p53 wt or the K386R mutant and cell lysates were immunoprecipitated with an anti-GFP polyclonal antibody and then blotted with DO-1. As shown in Figure 11C, a 100 kDa p53-reactive band was specifically immunoprecipitated with the anti-GFP antibody 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 clearly 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*.

SUMO-1 modification enhances p53-dependent transactivation

As several post-translational modifications of the p53 C terminus have been shown to affect the transcriptional activity of the protein, we investigated whether the conjugation of SUMO-1 may also affect this function of the tumour suppressor. A luciferase reporter construct containing the p21 promoter (p21-Luc,) was transfected into U2OS cells, which contain endogenous p53 wt, together with increasing amounts of vector expressing HA-SUMO-1. As shown in Figure 12A, 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 detected by Western blot analysis (middle panel). Efficiency of transfection was monitored by cotransfecting limiting amounts of pGFP as a marker and analysing the lysates by Western blot with an anti-GFP antibody (lower panel). Reporter

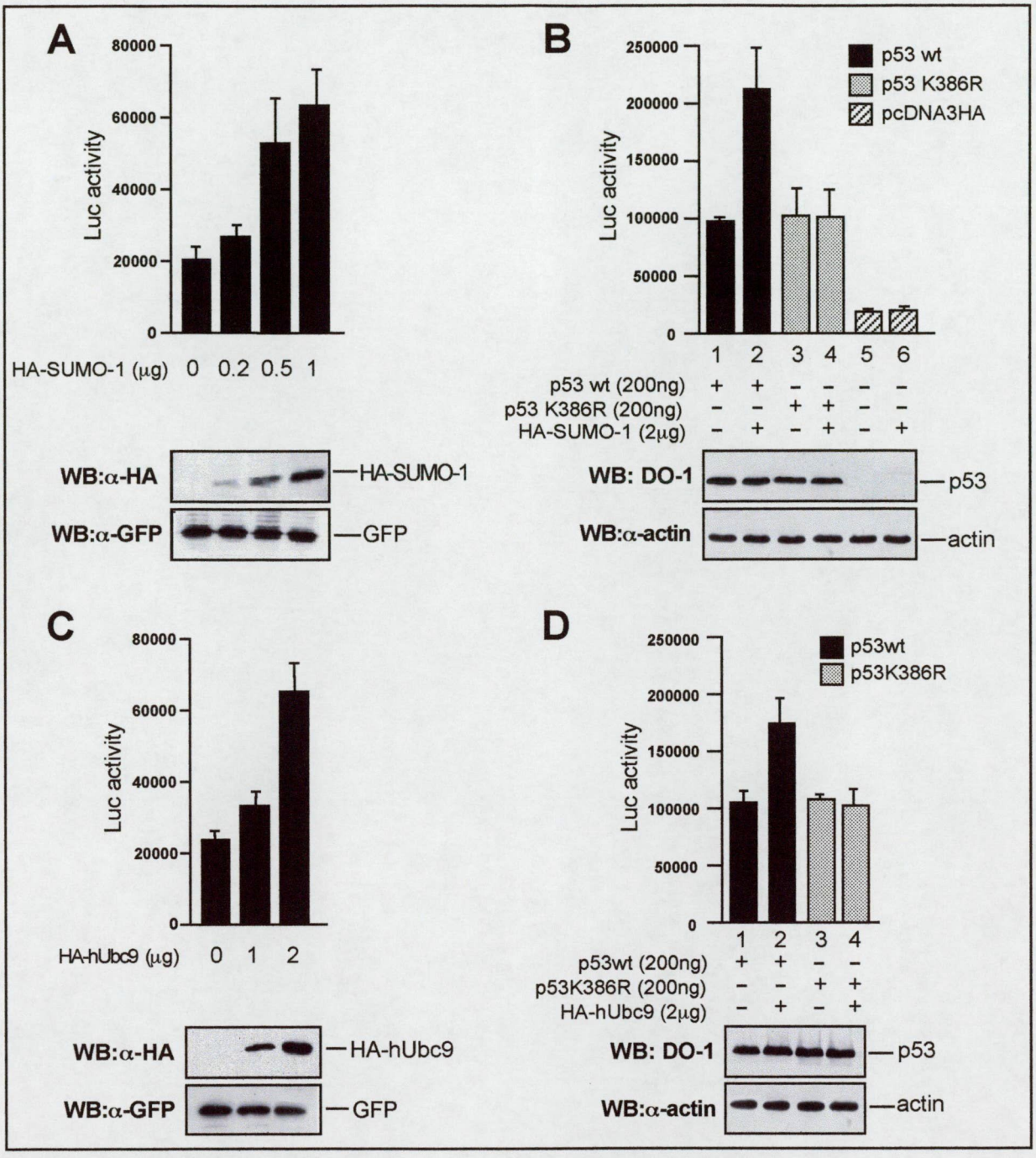


Figure 12. SUMO-1 conjugation enhances p53 transcriptional activity

(**A**) Luciferase assay in U2OS cells expressing the p21-Luc reporter and increasing amounts of HA-SUMO-1, as judged by Western blot. The efficiency of transfection was monitored by Western blot analysis of coexpressed GFP. (**B**) Luciferase assay in Balb/c (10)1 cells coexpressing the p21-Luc reporter and p53 or p53 K386R, either with or without HA-SUMO-1. Expression of p53 was monitored by Western blot and equal sample loading was confirmed by anti-actin immunoblot. (**C**) U2OS or (**D**) Balb/c (10)1 cells expressing the indicated proteins were subjected to luciferase assay. An aliquot of each lysate was analyzed by Western blot to monitor protein expression and transfection efficiency (lower panels). Graphs represent the mean of three independent experiments.

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 as well (not shown).

To obtain 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 12B, 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 (bars 1 and 2).

Finally, since Ubc9 is the E2 enzyme that mediates SUMO-1 conjugation, we tested if hUbc9 overexpression was able to enhance the transactivation ability 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 a plasmid encoding HA-tagged hUbc9 (HA-hUbc9). As for HA-SUMO-1, HA-hUbc9 overexpression resulted in enhanced reporter activity (Figure 12C). 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 12D).

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, U2OS cells were transfected with p21-Luc together with GFP-SUMO-1 and HA-hUbc9, either separately or together and luciferase activity was measured. As can be seen in Figure 13A, when the proteins were expressed simultaneously (bar 4) the increase in the p21-Luc reporter activity was 2-fold higher than in cells expressing either only GFP-SUMO-1 (bar 2) or HA-hUbc9 (bar 3).

Furthermore, the lysates used for the luciferase assay were analysed by Western blotting with an anti-p21 antibody, revealing that the expression of endogenous p21 was increased in cells expressing either SUMO-1 or hUbc9 or both (Figure 13B).

Finally, to obtain evidence that the observed increase in the transactivation ability of p53 was indeed linked to an increase in the amount of SUMO-1 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

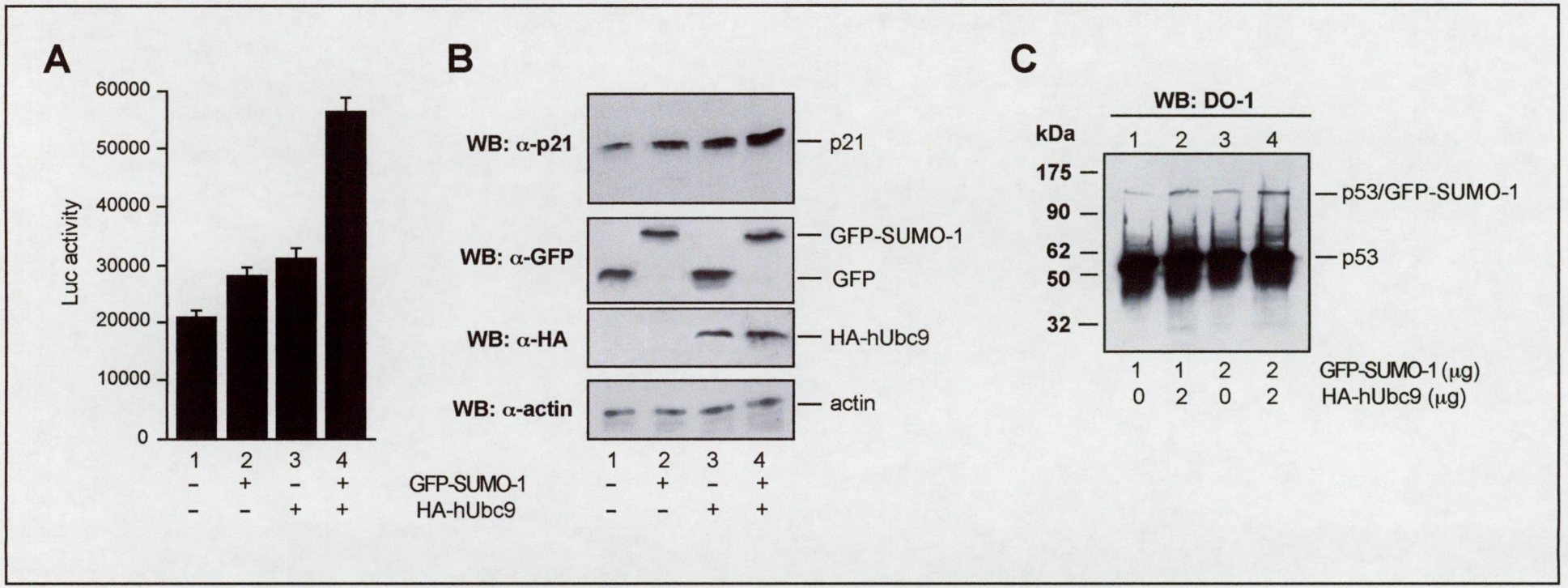


Figure 13. SUMO-1 and hUbc9 have a cooperative effect on the sumolation and the transcriptional activation of p53

(**A**) Luciferase assay on U2OS cells coexpressing the p21-Luc reporter and GFP-SUMO-1 or HA-hUbc9, either alone or together, as indicated. Graph represents the mean of three independent experiments. (**B**) An aliquot of each lysate used for the luciferase assay was subjected to Western blot analysis with an anti-p21 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 anti-actin staining (lower panel). (**C**) Western blot analysis of SaOS-2 cells expressing p53 and increasing amounts of GFP-SUMO-1 and HA-hUbc9, as indicated, with the DO-1 antibody. Positions of free and GFP-SUMO-1 conjugated p53 are shown.

analysis with DO-1 (Figure 13C). As expected, in the absence of exogenous hUbc9, elevating the amount of transfected GFP-SUMO-1 resulted in a simultaneous increase of sumolated p53 (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.

Discussion

In this part of the thesis 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 represents a previously undescribed modification that probably contributes to control the growth-suppressive properties of p53.

It should be noted, however, that only a small percentage of the total p53 appears to be modified by SUMO-1 both *in vitro* and *in vivo*. This observation may be explained by supposing 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-1 conjugation system and recognition may require prior modification of p53 by phosphorylation or acetylation. In line with this idea, a very recent report demonstrated that hyperphosphorylation of p53 prevents its conjugation to SUMO-1 [135]. Alternatively, sumolated p53 may be subject to the action of specific proteases that revert SUMO-1 conjugated p53 to its non-modified form. Interestingly, a SUMO-1 specific protease has been described recently that is required for cell cycle progression in yeast [136]. Thus, it is tempting to speculate that conjugation of SUMO-1 to p53 may directly affect its growth-suppressive properties.

How is p53 activated by conjugation to SUMO-1? Based on previous results as well as the data presented here, 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 [106]. Indeed, removal or post-translational modifications of this region activate the sequence-specific DNA binding activity, probably by changing the overall conformation of the protein [48]. 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 been demonstrated that binding of Mdm2 induces the rapid degradation of p53 via the ubiquitin/proteasome system [33,34]. Interestingly, a deletion mutant of p53 in which the C-terminal 30 amino acids were removed was still able to bind to Mdm2 but was not targeted for degradation [137]. 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, 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.

Finally, another hypothesis that should be considered 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 to sites of active transcription or into specific chromatin domains. This idea is supported by the fact that other SUMO-1 conjugated proteins, like PML and Sp100 were reported to localize to distinct subnuclear structures known as nuclear bodies that are involved in transcriptional and growth control [134].

Although the exact mechanism remains to be determined, modification of p53 by conjugation to SUMO-1 affects the transcriptional activity of the tumour suppressor. Modulation of the kinetics of this process, for example by the inhibition of SUMO-1 specific proteases, may provide a novel platform for developing alternative strategies to activate p53 response.

REGULATION OF p53 ACTIVITY IN NUCLEAR BODIES BY A SPECIFIC PML ISOFORM

Recently, it has been shown that coexpression of p53, Mdm2 and ARF in human osteosarcoma cells results in the accumulation of p53 in dot-like structures, reminiscent of the so-called nuclear bodies (NBs) [138]. A similar peculiar localization for p53 and Mdm2 has been also reported in human primary cells treated with Leptomycin B, a drug that specifically blocks nuclear export [139]. A direct proof that the observed p53-containing speckles are indeed the known nuclear bodies and the biological relevance of this subcellular redistribution, however, remained unclear.

NBs are cell cycle-regulated, matrix-associated subnuclear domains that appear as punctuate foci in the interphase nuclei [140,141]. The structural integrity of these large multiprotein complexes seems to be important for normal cell growth and development, since in some human diseases, like acute promyelocytic leukaemia (APL) [142,143] and spinocerebellar ataxia type 1 (SCA1) [144], disruption of NBs leads to malignancy or neurodegenerative disorder, respectively. Moreover, these structures are targeted and subsequently destroyed by numerous immediate early viral proteins [141]. Several proteins involved in transcriptional and growth control have been reported to localize to nuclear bodies and the most prominent components of NBs, the promyelocytic leukaemia protein (PML) and Sp100, are well known substrates of the SUMO-1 conjugation machinery [133]. These facts therefore prompted us to test whether the covalent attachment of SUMO-1 on p53 may result in the relocalization of the modified protein into NBs.

Coexpression of SUMO-1 and hUbc9 mediates the relocalization of p53 into NBs

SaOS-2 cells were microinjected with plasmids encoding wild-type p53 (p53 wt), GFP-SUMO-1 and HA-hUbc9 and analysed by immunofluorescence and confocal laser microscopy. In a fraction of the injected cells (40%), the typical nucleoplasmic staining of p53 became organised in distinct GFP-SUMO-1 positive nuclear structures (Figure 14A). These dots appeared to be authentic NBs, since colocalization of overexpressed SUMO-1 and endogenous PML or Sp100 in structures similar in size and numbers to the ones that we observed have been reported previously [133]. Moreover, although we could not perform triple immunofluorescence analysis for technical reasons, parallel experiments with coexpressed p53 wt, GFP-SUMO-1 and HA-hUbc9 demonstrated that the p53 accumulating speckles are also positive for endogenous PML and Sp100 proteins (not shown).

To our surprise, however, 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 14B), thus demonstrating that sumolation of p53 is dispensable for its delivery to these structures. NB-targeting was p53-specific, since under the same conditions, a construct encoding β-galactosidase fused to a nuclear localization signal (βgal-NLS) was excluded from these structures (not shown). These results suggested that modification of another protein by SUMO-1 is required to drive p53 into NBs. Given its fundamental role in directing the complex protein-protein interactions that mediates NB-formation, PML appeared to be the best candidate to be such a factor [145-148]. Moreover, a recent report demonstrated that sumolation of PML is prerequisite for NBs assembly [149].

PML3 recruits p53 into NBs

Immunofluorescence analysis of SaOS-2 cells microinjected with p53 wt together with PML3 revealed that a fraction of p53 was segregated into PML3-positive NBs in almost all the microinjected cells (Figure 14C). This PML3-dependent recruitment of ectopically expressed p53 into NBs was also observed in MG63, another p53-null cell line, demonstrating that this effect was not cell line specific (not shown). A similar relocalization was obtained for endogenous p53 as well, when PML3 were microinjected in U2OS cells (not shown).

PML is the most prominent component of the NBs (that are also referred as PML oncogenic domains, PODs) and appears to function as a tumour suppressor and control cell growth at different levels [150]. The protein exists in numerous alternatively spliced variants that mostly differ in their C-terminal sequences [151]. 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. From the results presented above, we can conclude that at least one isoform of PML is required for targeting p53 into nuclear bodies. Since coexpression of SUMO-1 and hUbc9 has the same final outcome, it is likely that this enhances the sumolation of endogenous PML and thus augments assembly of NBs and recruitment of p53 into these structures. The presence of PML3 mRNA, as detected by RT-PCR analysis in the employed cell lines (not shown), and the previous evidence that the assembly of NBs depends on prior sumolation of PML corroborate this interpretation.

PML was first identified in APL patients where, as a result of a reciprocal translocation event, it is fused to the retinoic acid alpha receptor (RARα) [142,143]. 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 [152]. Treatment of these cells with arsenic

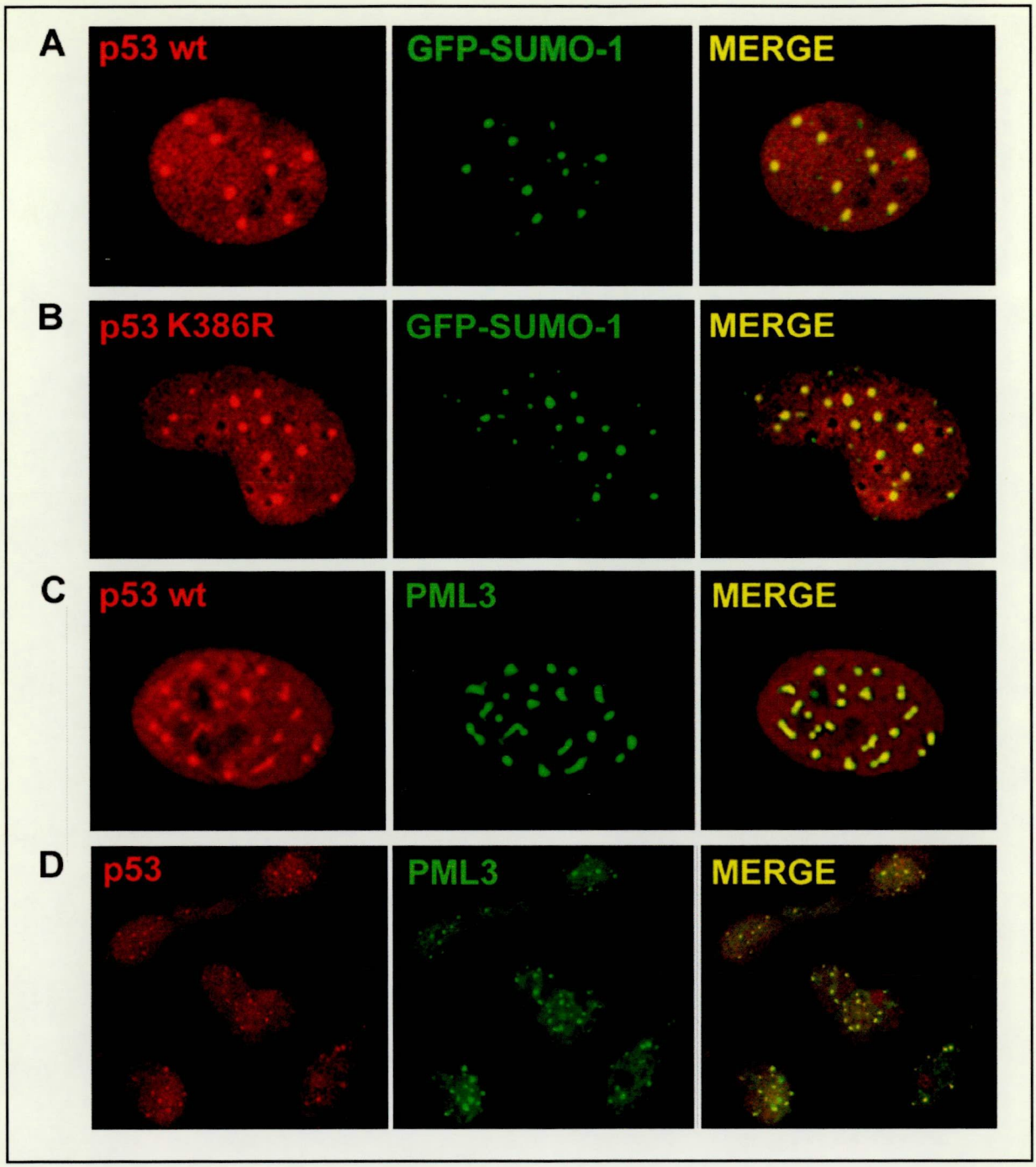


Figure 14. PML3 recruits p53 into NBs

Immunostaining pattern of GFP-SUMO-1 (green) and p53 proteins (red) in SaOS-2 cells expressing GFP-SUMO-1 and HA-hUbc9 together with either p53 wt (**A**) or p53 K386R (**B**). (**C**) Immunostaining pattern of PML3 (green) and p53 (red) in SaOS-2 cells ectopically expressing these proteins. (**D**) Immunostaining pattern of endogenous PML3 (green) and endogenous p53 (red) in LOVO cells irradiated with UV light and treated with As2O3. Microinjection, immunofluorescence analysis, UV- and As2O3-treatments were performed as described in Materials and Methods. Merging of the green and red images results in yellow signals in case of protein colocalization

trioxide (As_2O_3), a drug that specifically increases the sumolation of PML, mediates the degradation of PML-RARα and leads to the normalization of NB-pattern and, consequently, to the remission of the malignant phenotype [153,154].

To provide another proof that conjugation of SUMO-1 on PML3 facilitates the recruitment of p53 into NBs, we tested whether upon treatment with As_2O_3 endogenous p53 of the human colon-carcinoma cells LOVO could be recruited into PML3-containing NBs. Although As_2O_3-treatment, as expected, increased the number and size of PML3-containing NBs, p53 was not recruited into these structures (not shown). When cells were irradiated with UV light and treated with As_2O_3, we clearly detected colocalization between endogenous PML3 and p53 in nuclear bodies (Figure 14D). UV-treatment alone was not sufficient to change the diffuse nucleoplasmic staining of p53 (not shown). These results therefore demonstrate that colocalization of endogenous p53 and PML3 can be induced under conditions that simultaneously activate p53 and enhance the sumolation of PML3.

Dissection of p53 region required for NB-localization

To identify the region of p53 required for NB-targeting, several p53 deletion constructs were generated (Figure 15B) and microinjected into SaOS-2 cells together with PML3. The different p53 proteins were expressed at comparable levels, as judged by Western blot analysis after transient transfection and showed the typical homogeneous nuclear staining (not shown).

We found that amino-terminal deletions missing the transactivation domain (amino acids 12-69) or the Pro-rich region (amino acids 63-91) of p53 displayed both a nuclear diffuse staining and accumulation in PML3-containing NBs (not shown), as observed for the full length protein. In contrast, p53 294-393, which lacks the N-terminal and core domains did not change its homogeneous nucleoplasmic localization upon coexpression of PML3 (Figure 15A, j-l). These findings suggested that the core domain is required for the relocalization of p53 into NBs. Accordingly, p53 1-298, a protein that contains this domain as well as upstream N-terminal regions, efficiently relocalized into NBs (Figure 15A, g-i). Interestingly, unlike p53 wt and the deletions tested so far, this protein exclusively accumulated in nuclear bodies, indicating that sequences in the carboxyl terminus of p53 may possess a negative regulatory role on NB-targeting. Deletion of a C-terminal segment until amino acid 363 resulted in a protein that showed a staining pattern similar to the wt protein (Figure 15A, a-c). On the contrary, p53 1-355, a deletion lacking the last 38 residues of p53, totally relocalized into NBs upon PML3 coexpression (Figure 15A, d-f). Similar results were obtained in MG63 and also

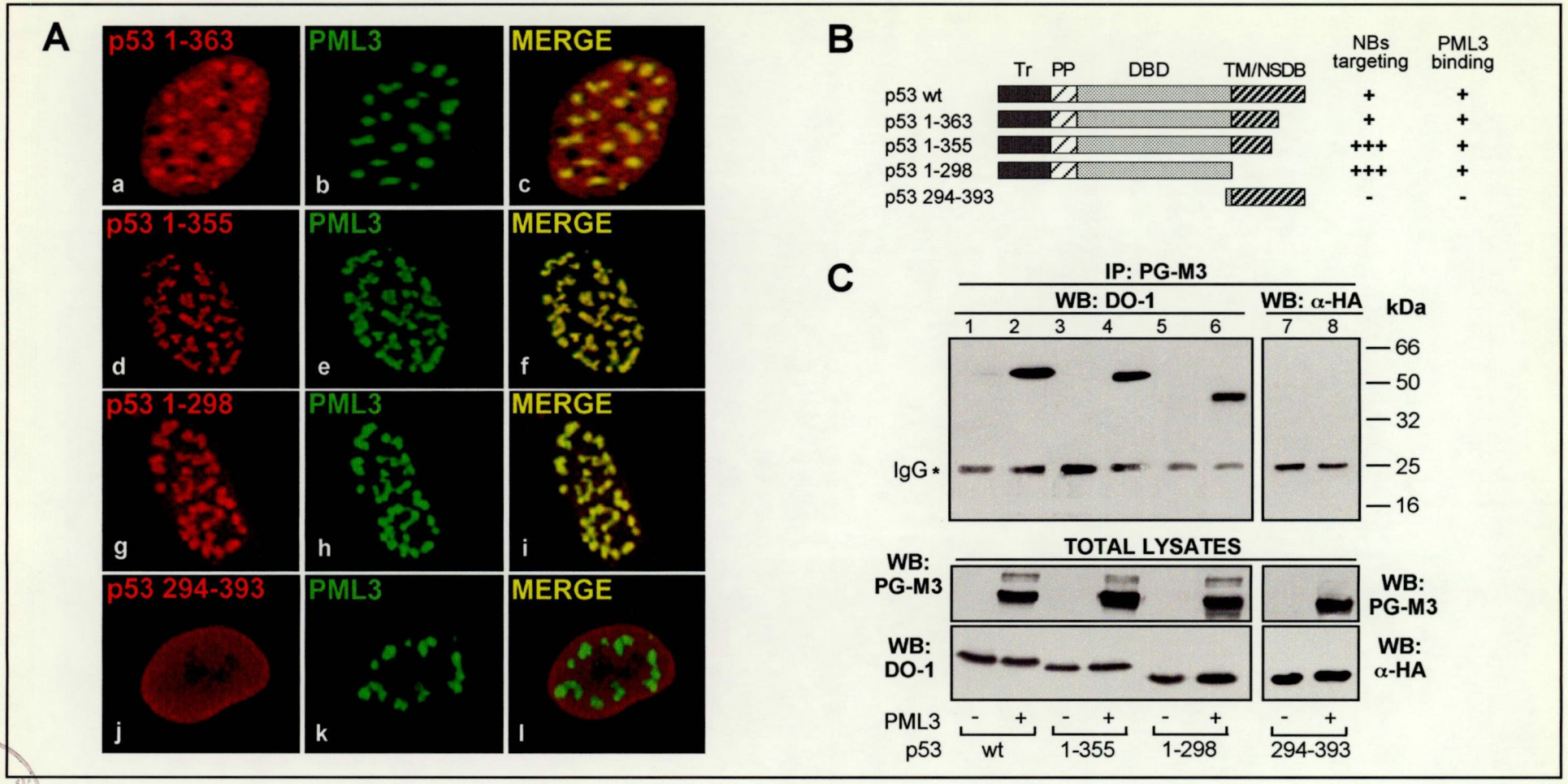


Figure 15. PML3 binds to the core domain of p53

(**A**) Immunostaining pattern of PML3 (green) and various p53 deletion mutants (red) in SaOS-2 cells expressing the indicated proteins. (**B**) Schematic representation of the p53 proteins used in (A) and in (C). Numbers refer to amino acids. (**C**) SaOS-2 cells expressing PML3 and the indicated p53 deletions were immunoprecipitated with an anti-PML antibody then probed with DO-1 (upper left panel) or anti-HA (upper right panel) antibodies in Western blot. Note that p53 294-393 was expressed as an HA-tagged protein and can not be recognized by DO-1. Comparable protein expression was judged by Western blot analysis of the total lysates (lower panels).

when the aforementioned p53 deletions were coexpressed with SUMO-1 and hUbc9 (not shown).

These results indicate that the core domain (amino acids 100-300) of p53 is required for NB-targeting. However, the evidence that the p53 H175 mutant is also localized to NBs (not shown) demonstrate that the wt conformation of the p53 DNA-binding region is dispensable. Furthermore, the massive PML3-induced relocalization of p53 1-355 as compared to p53 1-363 strongly suggests that eight residues within 356 and 363 exert a regulatory function on this process. It is tempting to speculate that this segment is a binding site for a factor that removes or, more probably, keeps p53 out from the NBs. Alternatively, the presence of a serine at position 362 raises the possibility that phosphorylation of this residue may have the same final outcome.

p53 binds to PML3 with the domain required for NB-targeting

Next we investigated whether the PML3-dependent change in p53 subcellular distribution was mediated by direct association between the two proteins. SaOS-2 cells were transfected with plasmids encoding p53 wt or various deletions together with PML3 and cell lysates were immunoprecipitated with the anti-PML antibody PG-M3. The bound protein complexes were analysed by Western blot using the DO-1 antibody for p53 wt and C-terminal deletion mutants (p53 1-355 and p53 1-298), or the anti-HA antibody for the HA-tagged p53 294-393 protein. As shown in Figure 15C, only p53 proteins able to relocalize to NBs were immunoprecipitated from cells expressing PML3. These results 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.

NB-targeting of p53 is mediated by a specific PML isoform

To assess whether p53 recruitment into NBs was specific for PML3 and not for the oncogenic PML-RARα product (Figure 16A), SaOS-2 cells were microinjected with plasmids encoding PML-RARα and p53 wt and analysed by immunofluorescence and confocal microscopy. The injected cells showed the typical microspeckled pattern for PML-RARα (Figure 16B b,c), but the homogeneous nuclear diffuse staining of p53 was not affected (a,c).

Since the PML-RARα fusion protein is lacking the PML C-terminal region, we hypothesised that this domain is required to target p53 into nuclear bodies. PML-L, another PML splice variant, which differs only in its short C-terminal tail from the PML3 protein employed so far (Figure 16A), was coinjected with p53 wt into SaOS-2 cells and the immunostaining pattern was analysed as above. Although PML-L, as expected, formed NBs

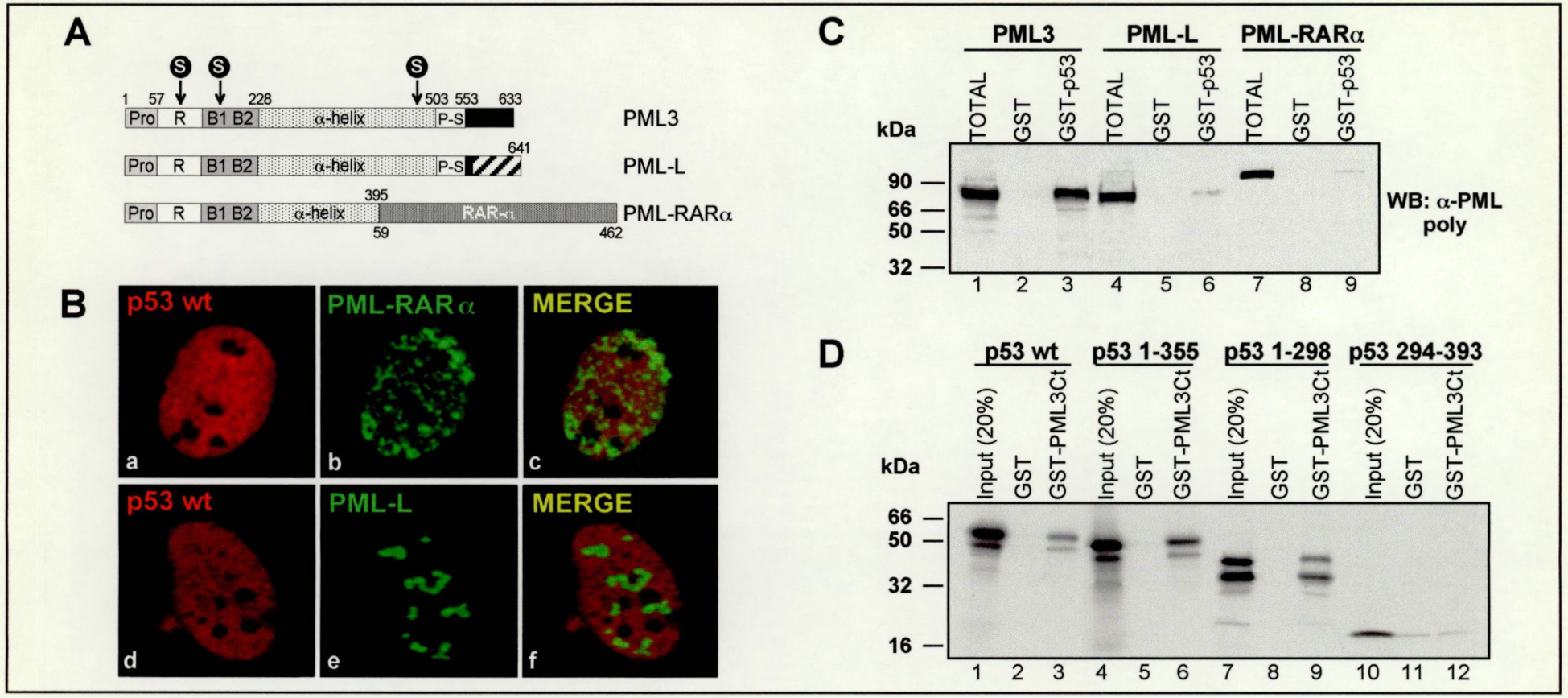


Figure 16. NB-targeting of p53 is mediated by a specific PML isoform

(**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-1 conjugation sites (S) are indicated. (**B**) p53 fails to colocalize with PML-RARα and PML-L. Immunostaining pattern of p53 (red) and PML (green) in SaOS-2 cells overexpressing p53 with PML-RARα (a-c) or with PML-L (d-f), respectively. (**C**) p53 binds specifically to PML3. *In vitro* binding assay with GST-p53 in SaOS-2 cells expressing the indicated PML proteins was performed as described in Materials and Methods. (**D**) The C-terminal region of PML3 mediates the interaction with p53. Pull down experiment with GST-PML3Ct and radiolabelled p53 proteins were performed as described in Materials and Methods.

(Figure 16B e,f) where other resident proteins, like Sp100 and SUMO-1, were found to localize (not shown), the distribution of p53 remained diffuse in the injected cells (d,f). Parallel experiments performed with p53 1-355 gave similar results (not shown).

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 with PML-RARα was severely impaired (Figure 16C). A GST-fusion containing the 61 residues long C-terminal tail specific for PML3, efficiently bound to *in vitro* translated p53 wt, p53 1-355 and p53 1-298 (Figure 16D). These results indicate that a region of the PML3 isoform, which is outside the central domain of the protein, where the three known sumolation sites have been mapped, is necessary and sufficient to mediate p53 binding.

These findings are, to our knowledge, the first data on functional differences between the various PML proteins and raise the possibility that the complex splicing pattern of PML represents a cellular mechanism generating alternative binding interfaces for a variety of factors. In addition to splicing, SUMO-1 modification on PML may provide another level of complexity either by directly affecting these interactions or by enhancing the ability of PML to form NBs.

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 [134,155] and p53 is a well established tumour suppressor, we examined whether recruitment of p53 into NBs can modulate cell survival. U2OS and MG63 cells were microinjected with plasmids encoding PML3 or human placental alkaline phosphatase (PLAP), as control, together with a GFP expression vector, as marker. Twenty-four hours later cell survival was scored as the number of recovered cells positive for the intrinsic green fluorescence of GFP [156]. U2OS cells express wt p53 that, upon introduction of PML3, was efficiently recruited into NBs, while MG63 cells lack endogenous p53. Upon overexpression of PML3, we consistently observed a significant reduction of survival in U2OS (Figure 17A, left panel), but not in MG63 cells (right panel). To provide experimental evidence that PML3-mediated recruitment of p53 into NBs contributes to the observed phenotype, we analysed whether overexpression of p53 1-355 (that totally relocalizes to NBs upon PML3 coexpression) could restore the PML3-dependent effect in MG63 cells. Cell survival was analysed 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 17B, recovery of GFP-

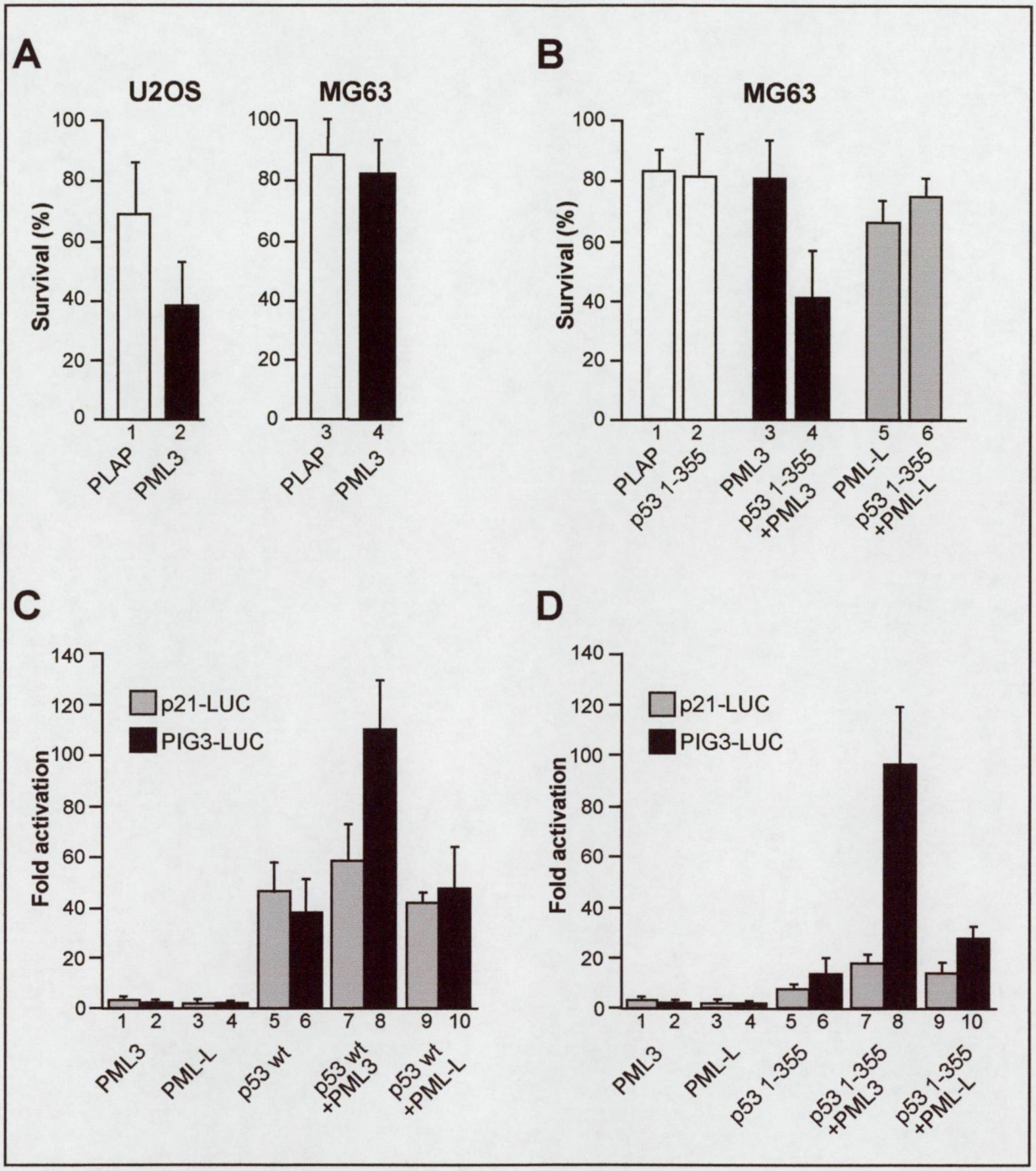


Figure 17. PML3 affects cell survival by modulating the transcriptional activity of p53 in a promoter-specific manner

(**A**) Survival assays in U2OS (p53 +/+) and in MG63 (p53 -/-) cells ectopically expressing PML3 were performed as described in Materials and Methods. PLAP was used as a negative control. (**B**) Survival assay in MG63 cells expressing p53 1-355 either alone or together with PML3 or PML-L, as indicated. (**C**) and (**D**) Luciferase assays in MG63 cells expressing the indicated proteins wih the p21-Luc (grey) or PIG3-Luc (black) reporters.

The bars represent the mean of three independent experiments.

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 expressed individually (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 (bar 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 [148] and recruits them into NBs, suggests a relevant role for PML and the whole NB structure in transcriptional control [134]. We therefore analysed 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 [64] and p21 [53], 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 17C, bars 6 and 8), while under the same conditions PML-L overexpression showed no significant effect (bars 6 and 10). Consistent with previous reports, p53 1-355 alone activated the p53 responsive promoters, although to a lesser extent as compared to p53 wt (Figure 17D, 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 (bar 8). This was likely dependent on the ability of p53 1-355 to be recruited into NBs by PML3, since a significantly reduced effect was observed upon coexpression of p53 1-355 and PML-L (bar 10).

Interestingly, the PML3-mediated enhancement of p53 transactivation ability was much less evident when tested on the p21 promoter. As shown, only a slight increase in p21 luciferase activity was detected when PML3 was coexpressed either with p53 wt or with p53 1-355 (compare bars 5 and 7).

These findings 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 [64], 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.

To rule out the possibility that the observed increase in p53 transcriptional activity is due solely to the interaction between p53 and PML3 and recruitment of p53 in NBs is only a correlative rather than a causative effect, we performed reporter assays in PML-/- MEFs [157]

with a PML3 protein containing mutations in all the three known sumolation sites [158]. The conjugation-deficiency of PML3S- was demonstrated biochemically by an *in vitro* sumolation assay (not shown) and functionally in PML -/- MEFs, where this protein could not form NBs, instead it accumulated in large protein aggregates when overexpressed (Figure 18A b,e). As expected, PML3S- efficiently bound to p53 *in vitro* (Figure 18B) and recruited both p53 wt and 1-355 into these aberrant subnuclear structures (Figure 18A). When PML-/- MEFs were transfected with the PIG3-Luc reporter plasmid and p53 1-355 together with PML3 or PML3S-, p53 transcriptional activity was significantly increased by PML3 (Figure 18C, bar 4) but not by PML3S- (Figure 18C, bar 5). Similar results were obtained also in SaOS-2 cells, which express low levels of PML3 (not shown).

These results clearly demonstrate that the interaction between p53 and PML3 *per se* is not sufficient to affect p53 transcriptional activity, but that the correct relocalization into nuclear bodies is also required for this effect.

Discussion

In this part of the thesis, I demonstrated that PML3, a specific isoform of the promyelocytic leukaemia protein, recruits p53 into NBs. I provided evidences that this relocalization is mediated by a direct protein-protein interaction involving the core domain of p53 and the C terminus of PML3. Moreover, I showed that NB-targeting of p53 results in enhanced transcriptional activation of a p53-regulated pro-apoptotic gene and affects cell survival.

Which mechanisms control this promoter-specificity? The interaction of p53 with PML3 and other NB-targeted factors involved in transcriptional control, may regulate the recognition of p53 target genes. For example, p300/CBP is a well-known co-activator of p53 and very recent works demonstrated that it is interacting with p53 and PML in the nuclear bodies [159-161]. Interestingly, while in MEFs oncogenic Ras-induced PML promotes premature senescence that depends on the presence of both p53 and p300/CBP [159,160], in transformed cells PML seems to cooperate with p53 and p300/CBP in the induction of apoptosis [161]. This strongly suggests that cell type, differentiation state and environmental conditions have a great influence on the promoter-specificity. In addition, distinct post-translational modifications taking place in NBs can also confer selectivity in p53-mediated gene expression. In line with this idea, it has been demonstrated that overexpressed PML3 induces the phosphorylation of p53 on Ser15, which in turn results in facilitated p53-p300/CBP complex formation and increased acetylation of p53 [159,160]. The accumulation of SUMO-1 and hUbc9 in NBs argues for the presence of the SUMO-1 conjugation

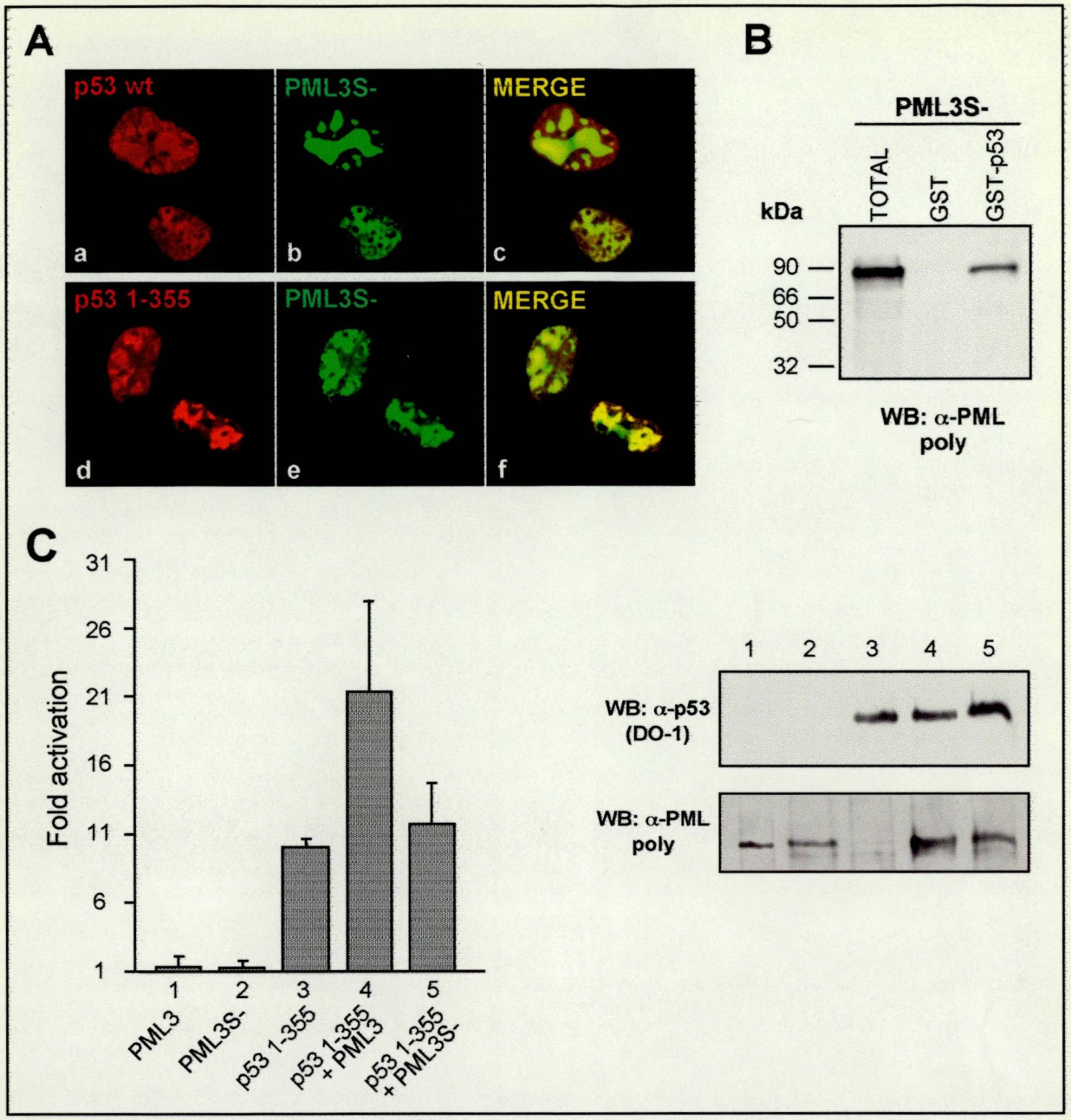


Figure 18. Sumolation of PML3 is indispensable for the recruitment of p53 into NBs and for the enhancement of p53 transcriptional activity

(A) The conjugation-deficient mutant of PML3 recruits p53 into aberrant nuclear structures. Immunostaining pattern of p53 (red) and PML3S- (green) in PML-/- MEF cells coexpressing PML3S- and either p53 wt (a-c) or 1-355 (d-f). **(B)** PML3S- binds to p53. *In vitro* binding assay with GST-p53 in SaOS-2 cells expressing PML3S- was performed under the same conditions as used for Figure 16. C. **(C)** PML3S- fails to increase p53 transcriptional activity. Luciferase assay in PML-/- MEF cells expressing the indicated proteins together with the PIG3-Luc reporter. Bars represent the mean of three independent experiments. Right panels show the expression pattern of p53 1-355 (upper) and PML3 (lower) proteins as monitored by Western blot analysis of the luciferase samples with the indicated antibodies.

machinery in these structures, although other post-translational modifications, like phosphorylations or acetylations are probably affecting the sumolation process. Hyperphosphorylation of p53, for example, has been shown to prevent its conjugation to SUMO-1 [135]. While the sumolated form of p53 efficiently induces the expression of p21, it has a moderate effect on the PIG3 promoter (Gostissa, M. and Del Sal, G., unpublished results). Post-translational modifications may also assist conformational changes that can influence the interaction between various NB-associated proteins involved in transcriptional control (see above for an example). In this context p53 1-355, which exhibits an increased PML3-dependent ability to activate the PIG3 promoter, could mimic a special conformational change of the full-length protein. Finally, it has been suggested that NBs are also involved in chromatin remodelling [162] Access to a particular promoter region, therefore, may depend on the association between transcription factors and non-histone chromosomal proteins. The reported interaction of p53 with HMG-1 [163] gains considerable interests in this scenario. 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 [145,146]. The physical interaction between hDaxx and p53 (Gostissa, M. and Del Sal, G., unpublished results) may add another level of complexity in the role of cell death control of NBs.

About 5-15% of p53 mutations occurs in the carboxy terminal domain and result in truncated proteins that, although transcriptionally active, are defective in apoptosis induction [164]. 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.

Control of cell death and differentiation may proceed through pathways involving either PML or p53 and, as it is demonstrated in this work, at least some of them are converging. Relocalization of various factors involved in transcriptional and growth control into NBs may allow the formation of specific protein-protein interactions and lead to the transactivation of particular promoters (Figure 19). The knowledge of integration and cross talks between different apoptotic pathways could therefore allow the implementation of methods for blocking the transformation process and the design of novel therapeutic strategies.

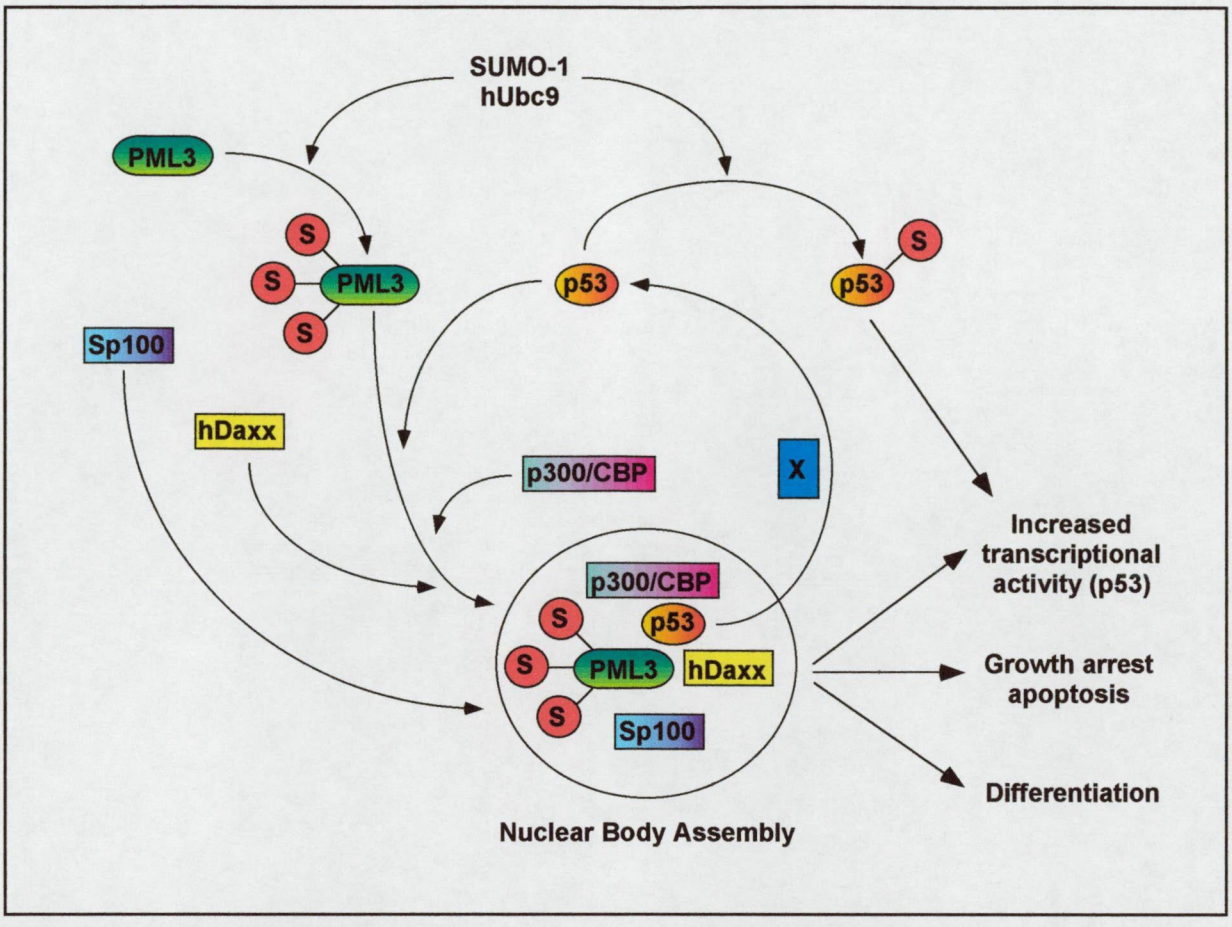


Figure 19. Model of two possible p53 activation pathways mediated by SUMO-1
Conjugation of SUMO-1 on p53 may convert the protein to an active form with increased DNA binding ability. On the other way, SUMO-1 modification of PML3 can mediate p53 recruitment into NBs, where the protein specifically transactivates pro-apoptotic genes, resulting in impaired cell survival. Interaction with other resident proteins would confer specificity to the function of NB-targeted p53. Still unidentified factors (X) can be involved in regulating this relocalization and its biological consequences.

SUMMARY AND CONCLUSIONS

The results presented in the previous parts can be summarized as follows:

1. In this thesis I reported the isolation of three p53-interacting molecules: p120E4F, an E1A-regulated zinc finger protein, involved in the control of cell growth; SUMO-1, a ubiquitin homologue that is covalently attached to various substrate proteins; and PML3, a specific isoform of the promyelocytic leukaemia protein, known to be essential for the assembly of the so-called Nuclear Bodies (NBs). p120E4F and SUMO-1 was isolated by two different yeast two-hybrid screenings, while PML3 was found to associate with p53 by other biochemical approaches.
2. I presented the existence of the endogenous p53/p120E4F complex in human cells by coimmunoprecipitation experiments and mapped the domains required for the interaction on both proteins by a GST pull-down assay *in vitro*. I showed that SUMO-1 is conjugated to p53 on lysine 386 both *in vitro* and *in vivo*. The association between PML3 and p53 is mediated by the core domain of p53 and the last 61 amino acids of PML3 and was verified by colocalization experiments *in vivo*.
3. I showed that the interaction between p120E4F and p53 is indispensable for the p120E4F-mediated growth arrest. The mechanism of action of p53, however, should be elucidated. Conjugation of SUMO-1, instead, clearly increases the transcriptional activity of p53 as measured by the activation of the p21 gene, one of the best known targets of the p53 protein. I demonstrated that PML3 recruits p53 into NBs, structures involved in transcriptional and growth control. Accordingly, NB-targeted p53 activates the promoter of a pro-apoptotic gene and triggers the PML-dependent apoptotic pathway.

Although the presented data clearly demonstrate the importance of these proteins in the checkpoint functions of p53, several questions remain to be addressed. What is the molecular mechanism by which p53 is involved in the p120E4F-mediated growth arrest? Is p120E4F an upstream or a downstream element in the p53 tumour suppressor pathway? Which is the cellular compartment where conjugation of SUMO-1 to p53 occurs? What are the proteins or post-translational modifications that regulate p53 entry and exit to and from the NBs? How promoter-specificity is regulated in the case of NB-targeted p53?

The answers to these questions will definitely augment the understanding of the complex cellular network of proteins involved in cell proliferation control and may help us to develop new strategies to fight against cancer.

MATERIALS AND METHODS

Cell lines

All the cell lines were routinely cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal 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. NIH 3T3 is a murine fibroblast cell line harbouring endogenous wt p53, while the murine Balb/c (10)1 fibroblasts are lacking endogenous p53. The Val 5 cell line is a Balb/c (10)1 derivative, stably expressing murine p53V135, which behaves as mutant at 37°C and as wt at 32°C. 293 is a human embryonic kidney cell line stably expressing the E1 region of Adenovirus 5. LOVO is a human colon cancer cell line. PML-/- mouse embryo fibroblasts have been described previously [157].

Plasmids

To generate the LexA-fusion constructs, human p53 wt and p53 H175 cDNAs , were PCR-amplified (between aa 74 and 393) and cloned in frame into pLexA202. pcDNA3p53wt contains the full-length human p53 cDNA cloned into pcDNA3 (Invitrogen). cDNAs encoding the p53 mutants K386R, K381/82R, and K381/82/86R were generated by PCR-directed mutagenesis and cloned into pRcCMV (Invitrogen). pCMVp53Δ63-91 and pCMVp53Δ12-69 express human p53 lacking the Pro-rich region and the transactivation domain, respectively. p53 deletions 1-363, 1-355, 1-338 and 1-298 were generated by PCR and cloned into pcDNA3 or pGEX4T-1 (Amersham Pharmacia Biotech). p53 256-393 and 294-393 were generated by PCR and cloned in frame in pGEX4T-1 or downstream of the HA-epitope previously inserted into pcDNA3 (pcDNA3HA). GST-fused p53wt, p53H175 and p53H273 were expressed from the appropriate pGEX plasmids.

pcDNA3HAE4F2.5K was generated by subcloning the full-length p120E4F cDNA from pCMVs-E4F2.5K into pcDNA3HA. The DNA encoding for p120E4FΔ350 (aa 350-783) was excised from pJG4-5 and cloned into pGEX4T-1 and pcDNA3HA. All the carboxy-terminal truncations of E4FΔ350 as well as E4FΔ521 and E4FΔ581 were obtained by PCR-amplification and cloned in frame into pGEX vectors. C-terminal deletions of p120E4F were generated by PCR and cloned in pcDNA3HA.

To generate the different SUMO-1 fusions, a cDNA encoding SUMO-1 was PCR amplified and cloned into pcDNA3HA or pGFPC1 (Clontech). hUbc9 was subcloned from pJG4-5 into pcDNA3HA. pcDNA3PML3 contains the entire PML3 cDNA. pcDNA3PML3S- was

generated by site-directed mutagenesis of pcDNA3PML3. pGEX-PML3Ct was constructed by PCR. pSG5PML-L and pSG5PML-RARα were described previously [143].
pG13CAT [165], p21-Luc [53] and PIG3-Luc [64] reporters have been already published.
All PCR amplified products were fully sequenced to exclude the possibility of second site mutations.

Yeast two-hybrid screenings

The "baits", LexAp53wt or LexAp53H175, respectively, were introduced into the EGY48 (MATa trp1 ura3 his3 LEU2::pLEXAop6LEU2) yeast strain, previously transformed with the pSH1834 β-galactosidase reporter construct [166]. The resulting strains were then transformed with cDNA libraries obtained from human foetal brain or arrested WI38 fibroblasts, respectively, cloned in the pJG4-5 ("fish") plasmid [166,167]. Primary yeast transformants (approx. 3.5 million colonies) were selected on Ura- His- Trp- plates and then pooled together. The interaction screening was performed by plating about 10 million clones from the pooled library onto Ura- His- Trp- Leu- plates containing galactose. The 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. Rescued clones were classified by restriction analysis and Southern dot blot and the single clones were subjected to secondary screening to confirm the specificity of interaction.

Antibodies

The following primary antibodies were used: DO-1 (monoclonal anti-p53, Santa Cruz), N4 (polyclonal anti-p120E4F), anti-GSTp53 (polyclonal), anti-GFP (polyclonal, Invitrogen), PG-M3 (monoclonal anti-PML, Santa Cruz), 12CA5 (monoclonal anti-HA, Roche Molecular Biochemicals), anti-GST (polyclonal, Amersham Pharmacia Biotech), 21C7 (monoclonal anti-SUMO-1, Zymed), anti-actin (polyclonal, Sigma), C19 (polyclonal anti-p21, Santa Cruz), anti-PML (rat polyclonal, [154]), anti-BrdU (monoclonal, Amersham Pharmacia Biotech), anti-βgalactosidase (monoclonal, Promega). HRPO-conjugated secondary antibodies were obtained from Sigma, the TRITC- and FITC-conjugated ones were purchased either from Southern Biotechnology Associates or from Dako.

Transfections, CAT and luciferase assays

Transfections were performed by the standard calcium phosphate precipitate method or by lipofection (Lipofectamin Plus Kit, Life Technologies) in the case of PML-/- MEFs. Cells

were seeded 8-12 hours before transfection and further processed 24-36 hours after removal of the precipitate.

CAT-reporter assays were performed by routine procedures in the presence of acetyl coenzyme A and ^{14}C-labelled chloramphenicol and analysed by thin layer chromatography.

Luciferase assays were done with luciferase kits from Promega according to the manufacturer's instructions. Luciferase activity was determined with a Turner Design luminometer (Promega). The obtained values were normalised for protein concentration in each sample either by a colorimetric assay (Biorad Protein Assay) or by using the Dual Luciferase Reporter Assay System (Promega) in cases when pRL-CMV was coexpressed.

***In vitro* conjugation assay**

The various forms of p53 and the p53-unrelated protein HHR23a 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 bacteria, as described [130]. Crude bacterial extracts were used as a source of mUbc9. GST-SUMO-1 was purified by affinity chromatography using Glutathione-Sepharose beads (Amersham Pharmacia Biotech). 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. SUMO-conjugation assays were performed as described previously [130]. Reaction mixtures were separated on 10% SDS-polyacrylamide gels and radioactively labelled bands were visualised by fluorography.

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

Cells were washed with ice-cold phosphate buffered saline (PBS), then harvested in 1 ml of the appropriate ice-cold lysis buffer. Cells were lysed in RIPA buffer for SUMO-1/p53 coimmunoprecipitations (CoIPs), while p120E4F/p53 and PML/p53 CoIPs were performed in previously described lysis solutions. In each experiment 10 mM N-ethylmaleimide, 1 mM PMSF, 10 mg/ml each of chymostatin, leupeptin, antipain and pepstatin was freshly added to the lysis buffers. Lysis was performed at 4°C for 20 minutes. Lysates were clarified by centrifugation and incubated for 4-6 hours at 4°C with 1-2 µg of the respective antibodies, prebound or covalently crosslinked to 20 µl of Protein A-Sepharose CL-4B beads (Amersham Pharmacia Biotech). 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 and resolved by SDS-PAGE.

For *in vitro* binding assays, the indicated GST-fusion proteins were produced in bacteria and purified on Glutathione-Sepharose beads by conventional procedures. For the pull-down assays, 4 μg of purified GST-fusion protein or GST were incubated with ^{35}S-labelled *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, loading 25% of the amounts of *in vitro* translated products used for the binding reaction as input. The bands were visualized by autoradiography. For GST-binding assays using cell lysates, transfected cells were lysed in a buffer containing 300 mM NaCl, 50 mM TrisHCl pH 7.5, 0.5% NP40, 10% glycerol. The lysates were then diluted twice for NaCl content and incubated with 4 μg of purified 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. Bound primary antibodies were revealed with the appropriate HRPO-conjugated secondary antibodies and visualised by enhanced chemiluminescence (Amersham).

Microinjection and immunofluorescence analysis

Cells grown on coverslips in 35-mm Petri dishes were microinjected by using the Automated Injection System (Zeiss). 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 UV- and/or As_2O_3-treatment cells were irradiated with 30 J/m^2 UV light and subsequently incubated for 18 hours in the presence of 1 μM As_2O_3 prior to fixation. The preextraction protocol on UV- and/or As_2O_3-treated cells was performed as previously described [168].

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 0,1 M glycine-PBS. Permeabilization was achieved with 0,1 % Triton X-100 in PBS for 5 min. The coverslips were incubated for 1 hour at 37°C with the appropriate primary antibodies and then revealed by 30 min incubation with the relative TRITC- or FITC-conjugated secondary antibodies. 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.

Colony formation, BrdU incorporation and survival assays

For colony formation assays cells were seeded at low density in 35-mm dishes and transfected with 1 μg of the indicated vectors. One day posttransfection cells were trypsinized and replated in 10 cm dishes in a medium containing 500 μg/ml geneticin (G418-sulphate, GIBCO). After two weeks of selection, the surviving colonies were fixed with 3% paraformaldehyde and stained with 10% Giemsa. The number of the empty vector-expressing colonies was scored as 100%.

For BrdU incorporation assay, cells were microinjected with the indicated expression vectors (50 ng/μl) together with 25 ng/μl pGFP-C1 (Clontech) used as marker. After microinjection, cells were grown for 20 hours then pulsed with 50 μM BrdU for 4 hours. Incorporated BrdU was revealed by indirect immunofluorescence following denaturation of DNA of the fixed cells by treatment with 50 mM NaOH for 10 seconds. Nuclei were counterstained with Hoechst 33342.

Cell survival was analysed by microinjecting 200 cells for each experiment with the gene of interest and GFP as marker and calculated as the percentage of recovered cells expressing GFP, as described previously [156].

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