



N-terminal isoforms of the p53 tumour suppressor protein : effects on p53 transcriptional activity and expression in cutaneous melanoma

Hind Hafsi

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N° d'ordre : 302-2012

Année 2012

THESE DE L'UNIVERSITE DE LYON

Délivrée par

L'UNIVERSITE CLAUDE BERNARD LYON 1

ECOLE DOCTORALE
BIOLOGIE MOLECULAIRE INTEGRATIVE ET CELLULAIRE

DIPLOME DE DOCTORAT

(arrêté du 7 août 2006)

soutenue publiquement le 20 décembre 2012

par

M^{elle} Hind HAFSI

**N-terminal isoforms of the p53 tumour suppressor protein:
effects on p53 transcriptional activity and expression in cutaneous melanoma**

JURY :

Dr Pierre ROUX, Rapporteur
Pr Klas WIMAN, Rapporteur
Dr Stéphanie COURTOIS-COX, Examineur
Pr Charles DUMONTET, Examineur
Pr Gérard ZALCMAN, Examineur
Dr Pierre HAINAUT, Directeur de thèse

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À mes parents et ma soeur,

Acknowledgements

First of all, I would like to thank Dr Stéphanie Cox-Courtois, Pr Charles Dumontet, Dr Pierre Roux, Pr Klas Wiman and Pr Gérard Zalcman for accepting to be jury members of my thesis, thus enabling my work to be evaluated by very high quality researchers. I am very grateful to Dr Pierre Roux and Pr Klas Wiman for their careful review of my thesis manuscript and their valuable comments.

I wish to warmly thank all the people who participated to my life in the lab during these four years of PhD:

***Pierre**, cette thèse n'aurait jamais vu le jour sans votre confiance et votre générosité. Un grand merci pour avoir toujours été là pour moi et pour m'avoir tant appris. Vous avez toujours cru en moi et je vous remercie énormément pour cela. Pour toutes ces discussions stimulantes, ces nombreuses heures de travail acharné, mais aussi ces moments moins sérieux et ces déjeuners « soupe et fromage », MERCI du fond du cœur !*

To all my dear friends and colleagues that I saw leaving one by one from our group so friendly, “multi-cultural”, “multi-language” and so pleasurable to work with!

***Amina, Chiara, Suna and Yayun**, it was a big pleasure to share the office with you. I will never forget the wonderful time we spent together. You have always been here for me, in good and bad moments, and I am deeply thankful for that. Yayun, thank you very much for all these nice talks, your help, your advices, and your friendship during all these years; I hope to see you soon (and your baby!).*

***Dominique**, merci pour ces fous rires, ces coups de gueule, ta franchise et ton honnêteté. Les fins de journée, lorsque tout le monde partait, étaient moins monotones grâce à toi ! Promis, je viens te voir prochainement !*

***Nathalia**, le rayon de soleil brésilien du labo ! Merci pour ta grande gentillesse, ces fous rires, ces super moments ensemble au labo et surtout le week-end génial que tu m'as fait passer à Rio !!*

***Daniela**, it was a great pleasure to collaborate with you. Thank you very much for your valuable help, the hundreds of experiments you performed and your enthusiasm doing them! You were the best trainee I have ever worked with and I am glad we achieved to finish the paper together.*

***Sandra**, mi querida amiga! Fue un gran placer discutir contigo durante horas y horas!! Je te remercie pour avoir été là, pour toujours réussir à apporter ta touche d'humour dans n'importe quelle situation, bref pour être una verdadera colombiana !!*

Eve, merci énormément pour ton aide, ton écoute, ton soutien et tes encouragements. Tu as cru en moi jusqu'au bout et je t'en suis extrêmement reconnaissante. Je ne connais personne capable de voir toujours les choses du bon côté comme toi. C'est un vrai plaisir !

Ghyslaine, tu m'as beaucoup soutenue et encouragée dans les moments difficiles. Je te remercie pour tout et surtout pour ton excellente expertise technique !

Michelle, merci pour ton indispensable soutien administratif, ta persévérance à me placer dans l'agenda de Pierre et ta spéciale "English touch" inimitable !

Sid, un grand grand merci pour m'avoir proposé ton aide tant de fois, mais aussi pour ton sourire et ta véritable gentillesse. Je ne sais pas comment j'aurais fait sans toi !

Zdenko, I would like to thank you very much for all what you have done to help me to finish my thesis in the best conditions. I would like to express you my deep gratitude for your valuable support and your enthusiastic encouragements.

Agnès, comment te remercier pour l'aide précieuse que tu m'as apportée pendant ma thèse ? C'était un véritable plaisir de travailler avec toi, toujours avec efficacité, discrétion et dans la bonne humeur. Tu as laissé une grande place après ton départ qui n'a jamais été comblée.

Reetta, one of the last survivors! Je suis ravie d'avoir été entourée par quelqu'un d'intègre, authentique et juste comme toi pendant cette "aventure". On a su rester soudés et surtout bien rigoler malgré tout ! Merci pour avoir partagé tout ça avec moi.

Andre, a real friendship is born between us thanks to your kindness, sincerity and of course your absolute "locura" who makes you so unique! Thank you so much for all these great moments in and outside of the lab. The atmosphere is always more fun when you are around!

Felipe, el mejor compañero de oficina del mundo! I will miss our nice talks about "life", your nice compliments every morning, your attitude as a real gentleman... Muchas gracias for your excellent company in the office, for being a real friend and for simply being you.

Mes années au CIRC n'auraient pas été les mêmes sans la rencontre de certaines personnes qui comptent aujourd'hui beaucoup pour moi.

Roland, mon paparazzi-conseiller-psychologue-thérapeute préféré ! Ta générosité et ta qualité d'écoute ont été sans faille depuis le jour où je t'ai rencontré. Merci pour avoir fait de moi tantôt égérie Chanel, tantôt modèle de couverture pour le volume des Monographies, tantôt top model,... Tu as inlassablement réussi à "capturer" mes moments de blues et les "développer" en moments de rigolade. Même si ce ne sera jamais suffisant, je te remercie profondément pour ta présence, ton écoute et les mots justes que tu as toujours trouvés pour me reconforter et me faire rire.

Gertrude, ta grande gentillesse, ton grand cœur et ta jovialité m'ont tout de suite touchée. Merci énormément pour ton soutien, ton écoute et tes conseils précieux. Tu aimes donner sans espérer recevoir et c'est une qualité noble et rare de nos jours.

Anouar, merci pour ton soutien, ton écoute, tes conseils, mais aussi pour m'énerver comme personne d'autre n'y arrive ! ☺ Ta générosité et ta gentillesse sont toujours sans limite pour moi, c'est pourquoi je t'en suis infiniment reconnaissante.

A ma famille et mes amies, qui sont toujours là lorsque j'en ai besoin et qui me soutiennent et m'accompagnent dans les moments heureux mais aussi moins heureux de ma vie :

***Papa, Maman,** c'est grâce à vous si je suis arrivée là aujourd'hui. Vous m'avez inlassablement poussée et encouragée pendant toutes mes études, sans jamais lésiner sur les moyens, jusqu'à la toute fin. J'espère vous rendre aujourd'hui, par ce titre de Docteur, une partie de tout ce que vous avez fait pour moi. Je vous aime fort.*

***Sarah,** la plus belle et la plus intelligente de toutes les sœurs ! ☺ Tu as connu de très près la "galère" de la thèse et c'est ce qui fait de toi la meilleure des sœurs. Pour toutes ces heures passées devant l'ordinateur mais surtout pour ce lien immuable qui nous unit, merci à toi et merci d'être là (même quand tu n'en as pas envie !).*

***Mounia,** mon amie, ma complice, ma confidente. Tu as toujours été là pour moi depuis la toute première fois où je t'ai rencontrée (je me souviendrais toujours lorsque tu m'as apporté un gilet quand j'avais froid dans le bureau de Michelle). Cinq ans après, rien n'a changé... Les amis se comptent sur les doigts de la main et ce n'est pas nécessaire d'en avoir plus lorsqu'ils ont toutes tes qualités.*

***Maïlys,** à mon tour de te dire que tu es une véritable amie sur qui je sais que je peux compter. Merci pour m'avoir réconfortée à chaque fois que j'ai douté. Ton énergie, ta joie de vivre, ta sincérité, ta sensibilité, sans parler de ton extrême gentillesse et ta générosité expliquent très bien pourquoi nos chemins ne se sont jamais séparés depuis notre rencontre. Tu es une fille brillante, battante, toujours volontaire ; merci pour ce que tu es et ce que tu donnes.*

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Foreword

Cancer is a widespread disease present in the global population. Despite the clinical and molecular heterogeneity of this pathology, there is one molecular event that is commonly observed in many cancers: p53 inactivation. Mutated in more than half of cancers or repressed by other mechanisms, p53 seems to be one of the cornerstones of tumourigenesis.

The p53 protein (encoded by the *TP53* gene) is an inducible transcription factor, which exerts widespread anti-proliferative effects through cell growth control and regulation of cell-cycle arrest, apoptosis, senescence, differentiation and basal bioenergetics metabolism. Loss of p53 function therefore eliminates a critical emergency brake that normally prevents abnormal cell growth and transformation.

During the past 10 years, evidence has accumulated that p53 was not expressed as a single protein. The discovery of multiple isoforms of p53, differing from the full-length protein by truncations in the N- and C-terminal domains, has added another layer of complexity to the many complex transcriptional and post-translational mechanisms that regulate p53 functions. So far, up to 12 p53 isoforms have been postulated and detected at mRNA level, although only a subset has been unambiguously identified at protein level. Some isoforms activate and others inhibit p53 functions, multiplying the possible biological effects. Do they play a role in basal conditions or after a stress? Are they constantly expressed in the cell or are they finely regulated? And by which mechanisms? Do they have a role in cancer?

These questions formed the basis of my PhD Thesis project. We have focused on one particular group of isoforms, the N-terminal isoforms lacking the main transactivation domain. This group includes two main members, $\Delta 40$ p53 and $\Delta 133$ p53, lacking respectively, the first 39 and the first 132 residues of the full-length protein. We have addressed three main questions:

- Do N-terminal isoforms interfere with basal p53 activity? If so, by which mechanisms? And what are the biochemical and biological consequences of this interference?

- Are the two N-terminal isoforms functionally equivalent or do they each have a specific functional and regulatory profile?
- Given the potential of these isoforms to antagonise p53 function, can their overexpression provide an alternative mechanism to inactivate p53 in tumours without *TP53* mutation?

This manuscript describes our attempts to address and answer these questions. It opens with a bibliographic survey in which we review the role of p53 as a key element of the molecular hallmarks of cancer and we describe the current, state-of-the-art knowledge on p53 isoforms. After a brief section presenting the specific aims and objectives, the Result section includes 3 manuscripts either submitted, published or in preparation, which illustrate the progress we have made in resolving the questions outlined above. In the Discussion, we have taken a step ahead to propose hypotheses on the role of N-terminal isoforms in p53 regulation. The picture emerging from our work and from the current literature is that N-terminal p53 isoform may exert essential roles in the control of p53 activity in stem cells. This general perspective invites us to a re-assessment of basal p53 activities in non-transformed cells as well as of the functional consequences of p53 inactivation in cancer cells.

1. Introduction

1 INTRODUCTION

1.1 Cancer: from society to genes

1.1.1 The global burden of cancer

The global burden of cancer has more than doubled during the past thirty years. In 2008, it is estimated that there were 12.4 million new cases diagnosed with cancer, 7.6 million deaths from the disease and over 25 million persons alive with cancer (Boyle P and Levin B, World Cancer Report 2008). The most common cancers in the world in term of incidence were lung (1.52 million cases), breast (1.29 million) and colorectal (1.15 million). Because of its poor prognosis, lung cancer was also the most common cause of death (1.31 million), followed by stomach cancer (780,000 deaths) and liver cancer (699,000 deaths).

In developed countries, the overall cancer mortality is more than twice as high as in developing countries (Figure 1). The main reasons for the greater cancer burden of affluent societies are the earlier onset of the tobacco epidemic, the earlier exposure to occupational carcinogens and the Western diet and lifestyle. In developing countries, up to one quarter of malignancies are caused by infectious agents, including the hepatitis (HBV and HCV), and human papillomaviruses (HPV). Nevertheless, today the situation has dramatically changed, with the majority of the global cancer burden found in low- and medium-resource countries. Overall, 53% of the total number of new cancer cases and 60% of the total number of deaths occur in the less developed countries. One third of cancers in high-resource countries are caused by tobacco smoking, which also causes a large proportion of deaths from other chronic disease including vascular disease and chronic obstructive pulmonary disease. Currently, the most common forms of cancer differ between high-income countries and the remainder. In high-income countries, cancers of the lung, breast, prostate and colorectum dominate, and one third of cancers are caused by tobacco use and 10% by chronic infection (Stewart BW and Kleihues P, World Cancer Report 2003). In low-resource and medium-resource countries, cancers of the stomach, liver, oral cavity, and cervix dominate (Boyle, 2006; Parkin, 2006) (Figure 2). One quarter of the cancer burden in low resource countries appears to be attributable to chronic infection, but 12% is currently caused by tobacco, and this proportion is growing (Parkin, 2006).

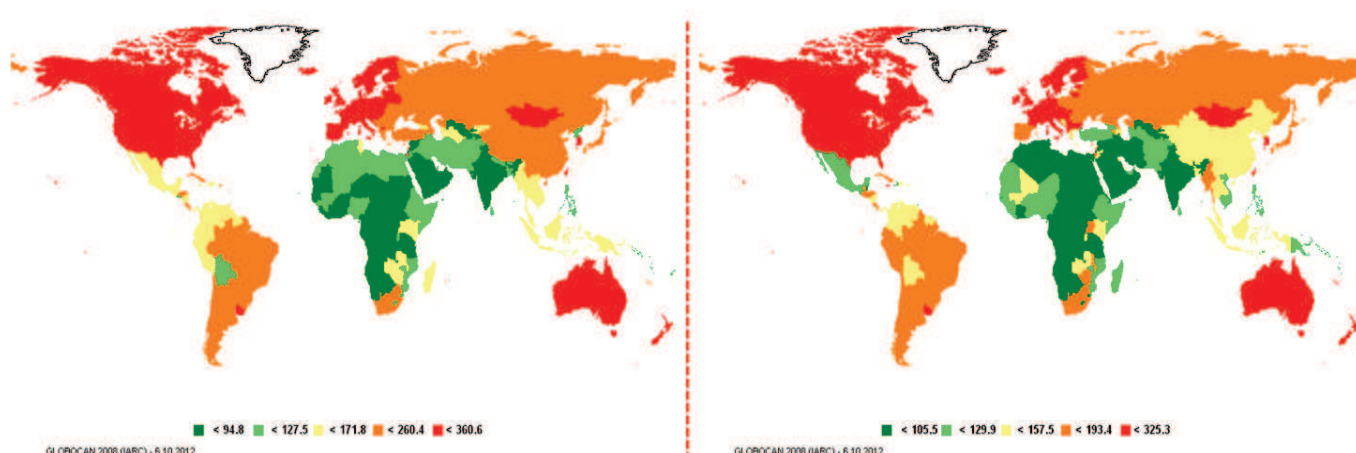


Figure 1. Global incidence of cancers

Estimated age-standardised incidence rate per 100,000 in male (left) and female (right). The highest rates are recorded in affluent countries. Non-melanoma skin cancer is not included. *Globocan 2008, IARC.*

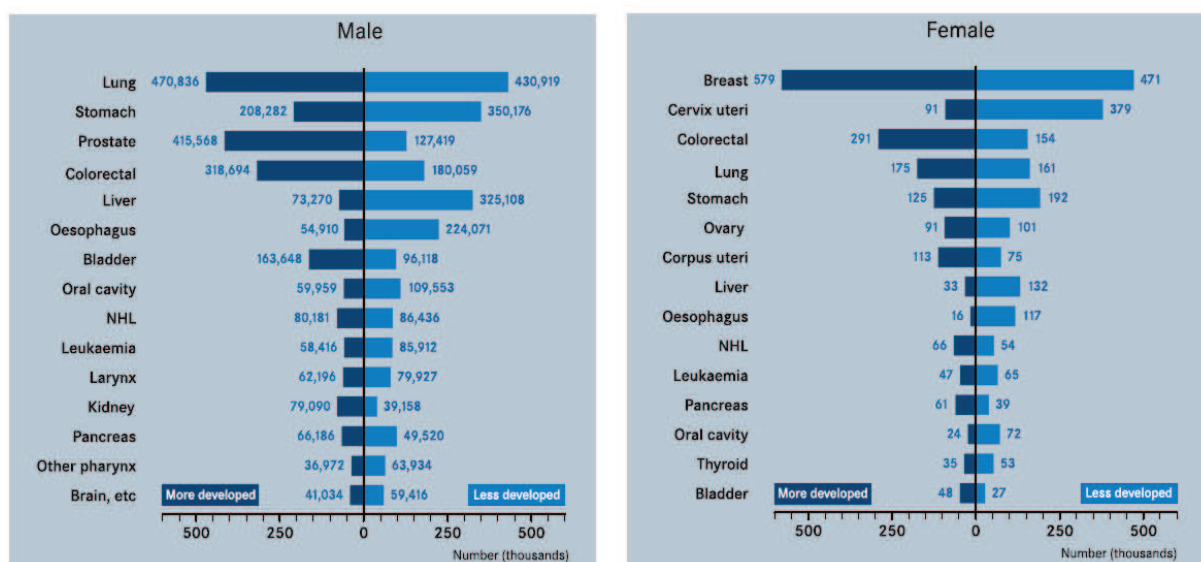


Figure 2. Comparison of the most common cancers in more and less developed countries in 2000

Left panel: male; right panel: female. NHL: Non-Hodgkin lymphoma. *Globocan 2003, IARC.*

A better clinical diagnosis has led to more cases of cancer being diagnosed, a proportion of which would have previously been missed. There has been a remarkable improvement in imaging and other diagnostic techniques, which have contributed substantially to an increased chance of diagnosis and to a more accurate diagnosis of cancer (Fass, 2008). While there are reasons for an artefactual increase in the cancer burden, there has undoubtedly been a real increase in the number of people who develop cancer due to an increased exposure to etiological agents.

Cancer is caused by both internal factors (such as inherited mutations, hormones, and immune conditions) and environmental factors (such as tobacco smoke, alcohol consumption, diet, radiation, and infectious organisms) (Table 1). The variation in incidence and progression of cancers among individuals can be attributed to interindividual variation in genetic makeup. Cancer genetic susceptibility is due to inheritance of specific sequence variants in cancer susceptibility genes that confer increased risk of cancer. The Li-Fraumeni susceptibility gene *TP53* and the breast/ovarian cancer susceptibility gene *BRCA1* are classic examples.

Some examples of carcinogenic agents		
	Some agents that are carcinogenic to humans	Some agents that are probably carcinogenic to humans
Chemicals	Benzene, 1,3-butadiene, formaldehyde, vinyl chloride	Trichloroethylene, styrene oxide
Complex mixtures	Aflatoxins, coal-tar, soots	PCBs, creosote, emissions from high-temperature frying
Occupations	Painting, chimney sweeping, coal gasification, coke production	Petroleum refining, hairdressing
Metals	Arsenic and compounds, beryllium and compounds, cadmium and compounds, chromium [VI]	Inorganic lead compounds, cobalt metal with tungsten carbide
Particles and fibres	Asbestos, crystalline silica, wood dust	Diesel engine exhaust
Pharmaceuticals	DES, estrogen-progesterone menopausal therapy, tamoxifen, phenacetin	Androgenic (anabolic) steroids, chloramphenicol
Radiation	Radon, solar radiation, X- and Gamma-radiation	
Biological agents	Hepatitis B and C, human papillomaviruses (type 16 and several others), <i>Helicobacter pylori</i>	
Lifestyle factors	Tobacco smoke (active and passive smoking), areca nut, alcoholic beverages, household combustion of coal	Shiftwork that involves circadian disruption, household combustion of biomass fuel (primarily wood)

Table 1. Some examples of carcinogenic agents

IARC Monographs, <http://monographs.iarc.fr>.

Identification of risk factors for cancers as well as delivering effective prevention will reduce the burden of cancer.

1.1.2 The concept of the Hallmarks of Cancer

Cancer is a complex disease that is very variable in its presentation, development and outcome from one patient to the other. The same heterogeneity and variability exist at the cellular and molecular level. Cancer is a multi-step process during which cells undergo profound metabolic and behavioural changes, leading them to proliferate in an excessive and untimely way, to escape surveillance by the immune system, and to invade distant tissues to form metastases (Merlo et al., 2006). Metastasis is the main cause of death by cancer.

In 2000, Hanahan and Weinberg proposed the model of “Hallmarks of cancer” describing six biological capabilities that cells need to acquire to become tumourigenic and eventually malignant (Hanahan and Weinberg, 2000) (Figure 3). These Hallmarks include sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Remarkable technical progress in cancer research in the last decade has led to a deeper understanding of these Hallmarks, as well as the emergence of two new Hallmarks: reprogramming of energy metabolism and evading immune destruction (Hanahan and Weinberg, 2011). Moreover, their acquisition is made possible by two “enabling characteristics”, namely genomic instability and tumour-promoting inflammation, which occur in some, if not all, cancer cells (Figure 4).

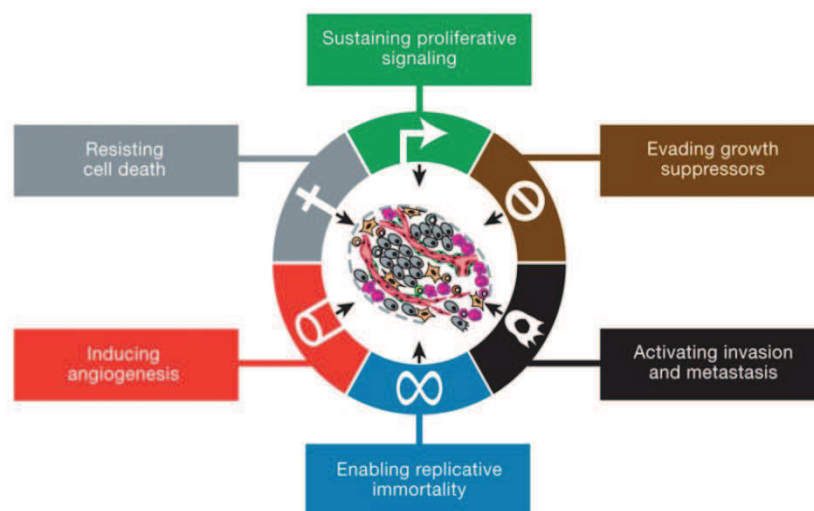


Figure 3. The Hallmarks of Cancer

Model of the 6 Hallmarks proposed by Hanahan and Weinberg in 2000 suggesting that most of the cancer cells acquire the same set of functional capabilities during their development, through various mechanistic strategies (Hanahan and Weinberg, 2000).

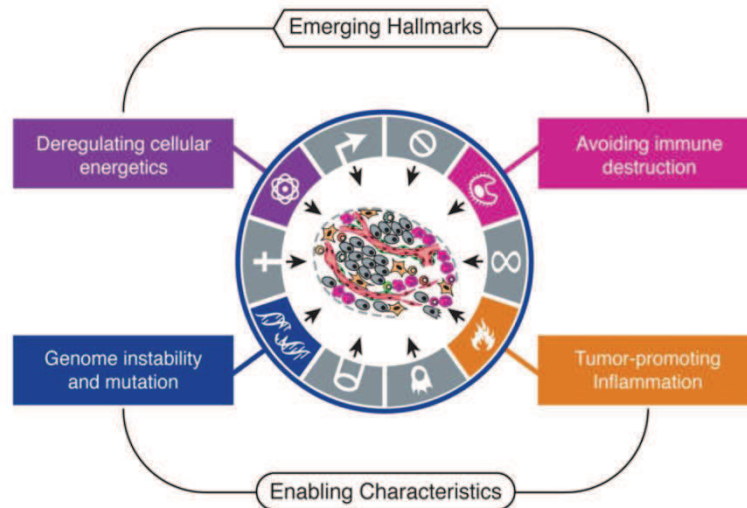


Figure 4. Emerging Hallmarks and Enabling Characteristics

In 2011, Hanahan and Weinberg updated their model of the Hallmarks of Cancer from 2000 with two additional Hallmarks, the capability to modify cellular metabolism and to evade immunological destruction. The acquisition of those Hallmarks is facilitated by two characteristics of neoplasia, genomic instability and inflammation (Hanahan and Weinberg, 2011).

Normal tissues carefully control the balance between production of growth-promoting and anti-proliferating signals, thereby ensuring a homeostasis of cell numbers and maintenance of the tissue architecture and functions. Cancer cells can acquire the capability to sustain proliferative signalling through growth factors production or growth receptor activation (Witsch et al., 2010). In addition, cancer cells must circumvent powerful programs that negatively regulate cell proliferation, mainly by inactivating tumour suppressor genes like *Retinoblastoma* (*Rb*) and *TP53*. Natural “emergency brakes” exist in normal cell lineages in the body, which enable them to pass through only a limited number of successive cell growth and division cycles. This limitation has been associated with two distinct barriers to proliferation: senescence, a typically irreversible entrance into a non proliferative but viable state, and crisis, which involves apoptosis. When cells emerge from a population in crisis and exhibit unlimited replicative potential, they circumvent these barriers and become immortalized. A major reprogramming of cellular energy metabolism then occurs in order to support continuous cell growth and proliferation. An “angiogenic switch” is almost always activated and remains on, causing normally quiescent vasculature to continually sprout new vessels that help sustain expanding neoplastic growths (Hanahan and Folkman, 1996). The capability of invasion and metastasis enables cancer cells to escape the primary tumour mass

and colonize new tissues (Talmadge and Fidler, 2010). They acquire the characteristic to evade immunological destruction by T and B lymphocytes, macrophages and natural killer cells. Acquisition of these multiple Hallmarks depends in large part on a succession of alterations in the genomes of neoplastic cells. Genomic instability and thus mutability endow cancer cells with genetic alterations that drive tumour progression.

Another dimension of complexity in tumours arises from their capacity to develop an intricate network of interactions between neoplastic and normal cells that contribute to the acquisition of hallmark characteristics by creating the “tumour microenvironment” (Hanahan and Weinberg, 2011). Such considerations suggest that drug development and the design of treatment protocols will benefit from incorporating the concept of hallmark capabilities, resulting in a more effective and durable therapies for human cancer.

1.2 *p53: the molecular gatekeeper*

1.2.1 *From oncogene to tumour suppressor gene*

Around thirty years after its discovery in 1979, the p53 protein has achieved stardom status among tumour suppressors (Hainaut and Wiman, 2009). With 63,999 papers published in September 2012, and a steady flow of new ones every day, p53 is undoubtedly one of the most extensively studied genes and proteins. However, the idea that p53 is a pivotal tumour suppressor and a mainstay of our body's natural anticancer defence did not come easily (Levine and Oren, 2009). The road leading to p53's eventual rise to prominence and its recognition as the most frequently altered gene in human cancer was long and winding, with concepts being repeatedly revised, extensively modified and sometimes even turned totally upside down (Figure 5).

The p53 protein owes its name to its apparent molecular weight of 53 kDa and was initially identified by two parallel approaches. First, p53 was shown to complex with the Large T antigen of the SV40 virus, thus representing critical cellular target for transformation induced by this virus (Tegtmeyer et al., 1977; Tegtmeyer et al., 1975). Second, it was found that the serum of mice (and later, patients) carrying tumours often contained antibodies reacting with the same 53 kDa cellular phosphoprotein (DeLeo et al., 1979). In these early years, p53 was thought of as a type of oncogene. In 1984, Jenkins and colleagues cloned the full-length mouse p53 cDNA and further experiments demonstrated that when p53 expression plasmid was transfected into rodent primary fibroblasts, it resulted in cell immortalization and transformation in presence of an activated *Ras* oncogene (Eliyahu et al., 1984; Jenkins et al., 1984a; Jenkins et al., 1984b). The notion of p53 as an oncogene received additional support from studies showing that point mutations introduced in the p53 protein sequence increased its capacity to participate in cellular transformation (Jenkins et al., 1985).

Several observations were indications of major advances to follow. First, in 1984, Milner showed that different forms of p53 could be distinguished in non-dividing and dividing cells, leading to the concept of conformational changes in p53 which would later explain the effect of mutations in cancer (Milner, 1984). In the same year, Maltzman and Czyzyk provided the first evidence that p53 was induced after UV irradiation (Maltzman and Czyzyk, 1984). One year later, Benchimol and colleagues identified *TP53* alterations in leukemia cell lines

transformed by the Friend leukaemia virus (Mowat et al., 1985). In the late 1980s, the emergence of the concept of “tumour suppressor gene”, largely based on studies of the retinoblastoma gene, paved the way for a paradigm shift that totally changed the understanding of p53 and of the molecular mechanisms of carcinogenesis. This shift was triggered by *in vitro* experiments and studies of human tumour samples. Levine and Oren

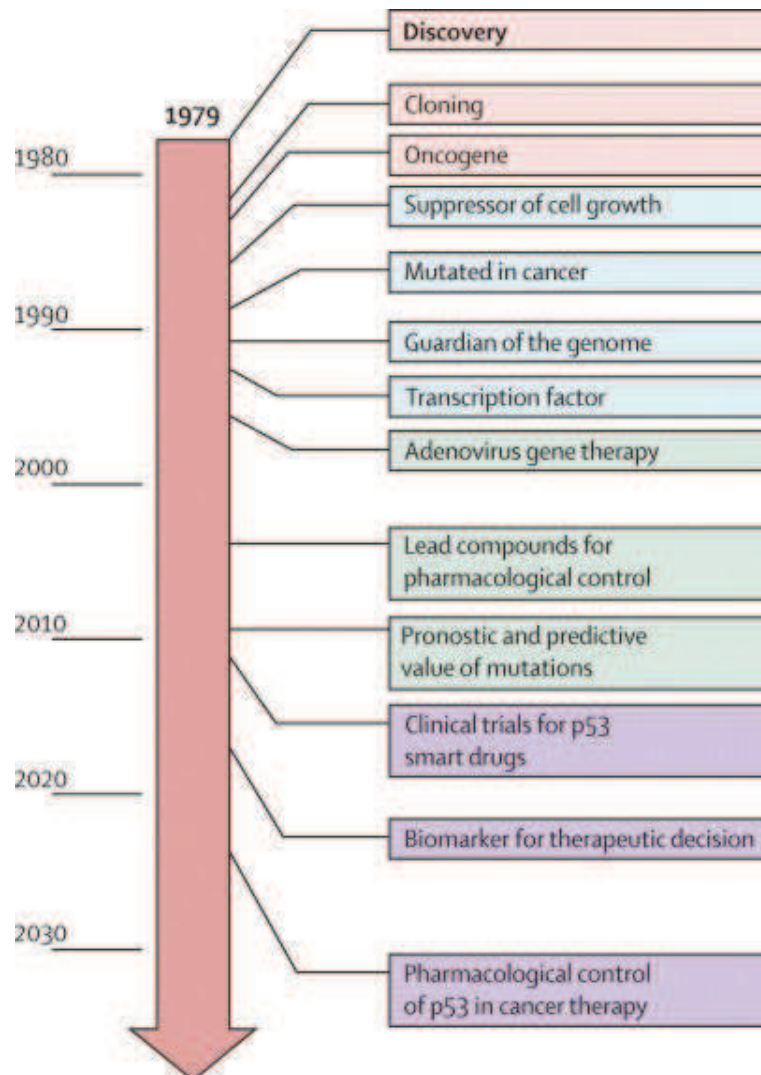


Figure 5. p53, from discovery to clinical application

Key steps in p53 research from discovery of the protein in 1979 to projected applications for 2030. Pink: early steps in the search for p53 function (1979-1988). Blue: definition of p53 as a tumour suppressor and transcription factor involved in growth suppression after DNA damage (1988-1994). Green: current developments of drugs targeting p53 and prognostic studies of *TP53* mutations in cancer (1995-present). Purple: projected applications of p53 research to cancer diagnosis and treatment (Hainaut and Wiman, 2009).

showed that wild-type p53 cDNA clones could suppress transformation of rodent cells in culture, whereas point mutant versions of p53 were transforming them (Eliyahu et al., 1989; Finlay et al., 1989). Similarly, Vogelstein and Harris, followed by many others, found that point mutations in *TP53* were common in colorectal, lung, breast, liver, and many other cancers (Baker et al., 1989; Hollstein et al., 1991). In 1990, Malkin and Srivastava showed that inherited *TP53* mutations were the underlying genetic defect of Li-Fraumeni syndrome, a familial syndrome of predisposition to multiple, early cancers (Malkin et al., 1990; Srivastava et al., 1990). All these findings led to the recognition of *TP53* as “the ultimate tumour-suppressor gene” (Oren, 1992).

In a “News and Views” article in *Nature*, Lane qualified p53 as “the guardian of the genome”, an expression that captured its main physiological function (Lane, 1992). After all of these developments, p53 eventually received the award from the Science journal as the “Molecule of the Year 1993” (Koshland, 1993).

1.2.2 Structure of human p53 gene and protein

1.2.2.1 *TP53* gene and its promoters

The human *TP53* gene (OMIM 191170) spans about 20 kb of DNA and is localized on the short arm of the chromosome 17 on position 17p13.1. This gene is composed of 11 exons, the first of which is non coding and localized 10 kb away from exon 2 (reviewed in (May and May, 1999)). Two main promoters P1 and P2 regulate the *TP53* gene. Although *TP53* gene lacks a TATA box, it has been demonstrated that a region of about 300 bp upstream of the initiation site of the non-coding exon 1 (P1 promoter) contains responsive elements (REs) for AP1, NF- κ B, PAX and members of the HOX family, among others (Hollstein and Hainaut, 2010; Reisman et al., 1988). The precise role of each is not clear; while HoxA5 induces p53 expression, AP1 and NF- κ B have been described as promoter repressors (Raman et al., 2000; Webster and Perkins, 1999). A second promoter (minimal P1’ promoter) is located within intron 1 (Reisman et al., 1988). This promoter does not appear to activate p53 transcription but to control a transcript of unknown function located within the non-coding p53 intron 1 (*Hp53int1*). A third promoter (P2 promoter), has been recently identified within *TP53* gene in

a region that spans the distal part of intron 1 to the proximal part of exon 5, covering about 1.5 kb (Bourdon et al., 2005). It has been described to contain several REs in intron 4 for TFIID, SP1, AP1, NF- κ B and p53 itself (Bourdon et al., 2005; Shamsher and Montano, 1996). This promoter directs the expression of a short form of p53 mRNA (*p53I4* mRNA). This transcript is initiated in the middle of intron 4 and generates Δ 133p53 isoform by translation from an AUG at position 133 (*see paragraph 1.5.1*). The presence of up to four p53 REs suggests an auto-regulatory loop by which p53 controls the expression of Δ 133p53 (Bourdon et al., 2005; Marcel and Hainaut, 2009). Interestingly, both P1 and P2 promoters are relatively conserved among species and also among the members of the *TP53* family.

1.2.2.2 p53 protein: domain organization and functions

The nuclear phosphoprotein p53 contains 393 amino acids and is divided in four major functional domains ([Figure 6](#)). The N-terminal region consists of an acidic transactivation domain (TAD), which is often sub-divided in two sub-domains TAD1 (residues 1-42) and TAD2 (residues 43-73), and a proline-rich region (residues 61-94). It is followed by the DNA-binding core domain that is responsible for the sequence-specific DNA binding (residues 102-292). The C-terminal region (residues 300-393) contains a flexible linker region (residues 300-318 approximately) and a tetramerization domain (residues 326-355), which contains a so called regulatory domain (Joerger and Fersht, 2007a). Here, we give a brief description of the structure and function of each p53 domain.

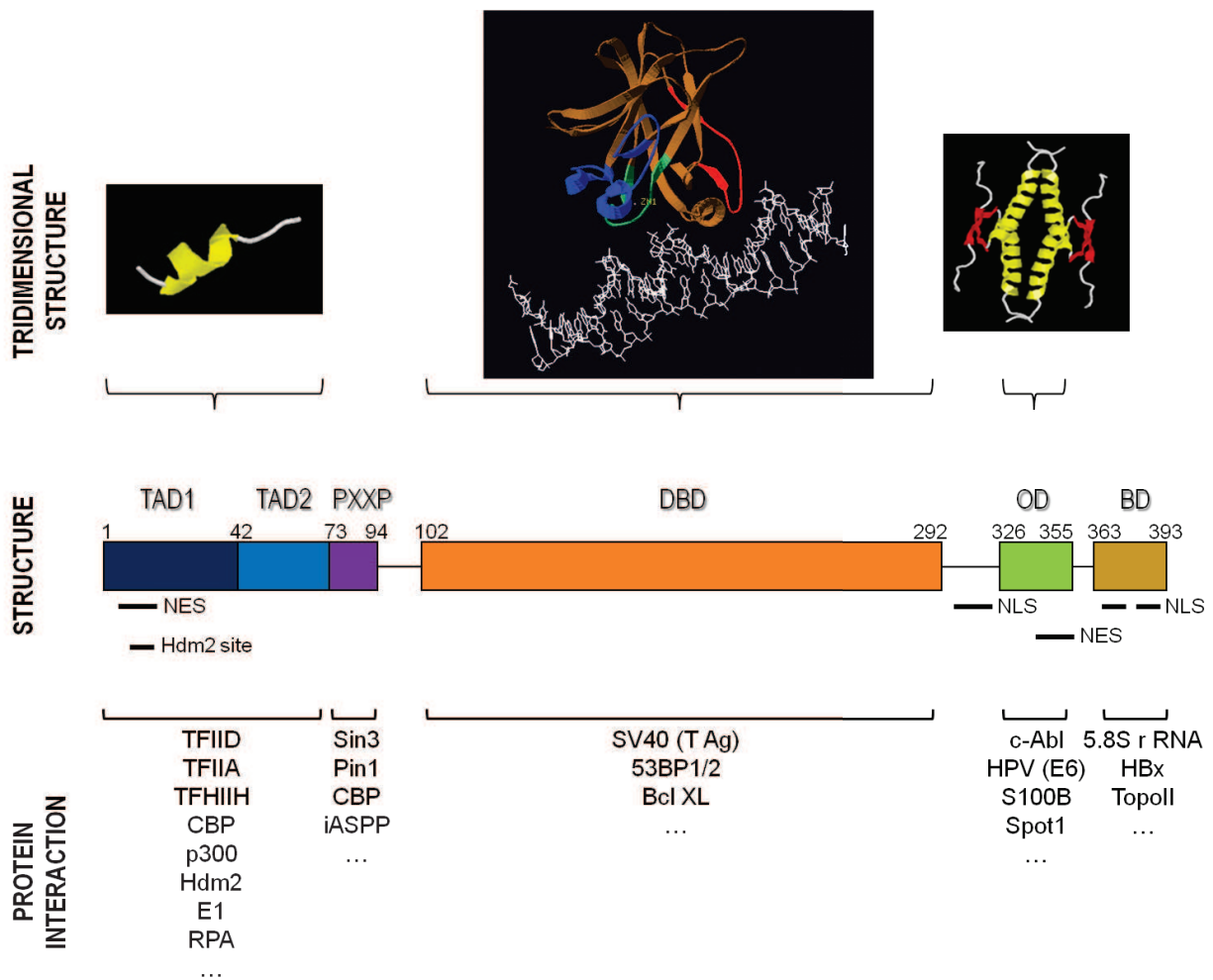


Figure 6. Structure of the full-length p53 protein and the protein-interaction associated domains

p53 contains an N-terminal transactivation domain (TAD1 and TAD2), followed by a proline-rich region (PXXP), the central DNA-binding domain (DBD), the oligomerization domain (OD), and the extreme C-terminus consisting of a basic domain (BD). Nuclear Export Signal (NES), Nuclear Localization Signal (NLS) and Hdm2-binding site are indicated by a black line. Some of the proteins described to interact with these domains are listed (lower part of the figure). Tridimensional structure of the p53 protein domains is illustrated (upper part of the figure) (*Swiss PDBViewer*) (Cho et al., 1994).

1.2.2.2.1 The amino-terminal transactivation region

This region consists of an acidic TAD subdivided into two sub-domains TAD1 (residues 1-42) and TAD2 (residues 43-73), a proline-rich region (residues 61-94) and a nuclear export signal (Figure 6). The transactivation domains are pivotal to p53 function in cells. The transcriptional activity of this domain resides in protein-protein interactions. For example, the TAD is a binding site for components of the transcription machinery, namely TFIID subunits

(TATA box Binding Protein (TBP), TFII40 and TFII60 (TBP-associated factors)) (Seto et al., 1992; Truant et al., 1993; Xing et al., 2001). p53 also interacts with transcriptional co-activators such as CBP (CREB Binding Protein)/p300, an histone deacetylase which is essential for the transcriptional function of p53 (Dornan et al., 2003; Gu et al., 1997; Liu et al., 2003) or RPA (Replication protein A) (Dutta et al., 1993). Through this domain p53 also binds viral proteins, such as E1B, which are inhibitors of its transcriptional activity (Yew and Berk, 1992).

The main transactivation domain corresponds to TAD1, whereas it is still debated whether TAD2 has an independent transactivation capacity (Attardi et al., 1996; Haupt et al., 1995). However, TAD2 appears to modulate the transactivation of specific subsets of target genes mediated by TAD1. Within TAD1, amino acids 22 and 23 have been shown to be required for transcriptional activation of *p21WAF1*, a gene encoding an inhibitor of cyclin kinase that regulate cell cycle progression in G1/S and G2/M phases (el-Deiry et al., 1993), but are dispensable for apoptosis triggering, transactivation from other p53-responsive promoters and repression of promoters by p53. However, mutation of amino acids 53 and 54 abrogates transactivation and apoptosis capacities of p53 (Venot et al., 1999).

The transactivation domain also contains the binding site of Human double-minute 2 and 4 (Hdm2/Hdm4) (residues 17-29) (Figure 6), E3-ubiquitin ligases which mediate p53 ubiquitination in the C-terminal region, its nuclear export and its cytoplasmic degradation by the proteasome (Haupt et al., 1997; Kubbutat et al., 1997; Marine and Jochemsen, 2005; Marine and Lozano, 2010).

The N-terminus of p53 is highly post-translationally modified (Appella and Anderson, 2001) (see paragraph 1.2.3.2 *Post-translational modifications*). Multiple phosphorylation events in this region have been implicated in both the stabilization of p53 and the specificity of target transactivation (Chao et al., 2003). Specifically, phosphorylation of residues such as Serine 15 and Threonine 18 are critical for the post-translational control of p53 interactions with protein regulating its stability such as the Hdm2/Hdm4 complex, which prevents p53 interaction with Hdm2/Hdm4 that results in both p53 stabilization and nuclear localization by altering the accessibility of the nuclear export signal (NES, residues 11-27) (Craig et al., 1999; Zhang and Xiong, 2001). Phosphorylation of p53 at Serine 15 has also been shown to inhibit binding of TFIID *in vitro* (Pise-Masison et al., 1998), and Ser15/Ser37 phosphorylation to correlate inversely with Hdm2 binding (Shieh et al., 1997). CBP/p300,

through its interaction with the N-terminus of p53, acts as a coactivator for p53 and increases the sequence-specific DNA-binding activity of p53 by acetylating its C-terminus (Gu et al., 1997). Phosphorylation of p53 at Serine 15 increases its ability to recruit CBP/p300 and stimulates p53-dependent transactivation (Dumaz and Meek, 1999; Lambert et al., 1998).

1.2.2.2.2 Polyproline domain

The proline-rich domain (residues 61-94) contains five repeats of the sequence PXXP, where P represents Proline and X, any other amino acid (Walker and Levine, 1996) (Figure 6). This region is structured as a SH3 (Src homology 3) binding domain, which provides a rigid link between the N-terminus and the DNA-binding domain. It has been shown to play a role in signal transduction via its SH3 domain binding activity. Indeed, it is required for p53-mediated apoptosis and transmission of anti-proliferative signals (Sakamuro et al., 1997; Walker and Levine, 1996). Of note, this domain contains a common polymorphic site at codon 72, which results in either a Proline (P) or an Arginine (R). Interestingly, the replacement of Proline with Arginine alters one of the five PXXP motifs (Matlashewski et al., 1987).

1.2.2.2.3 DNA-binding domain

The DNA-binding domain (DBD) is localized between amino acids 102 and 292 (Figure 6). Sequencing of the p53 locus from over 25,000 human tumours provided striking evidence for the importance of the sequence-specific DNA binding domain for intact tumour suppressor function (Olivier et al., 2002). This region lies in the centre of the protein and specifies a globular, folded domain made of a complex of two beta sheets interconnected by large loops and helices. The loops are kept together by the binding of a Zn^{2+} ion on a cluster of 3 cysteines and one histidine, conferring to the domain a dependence upon Zinc availability and oxidation-reduction conditions for correct folding (see *Review* in paragraph 1.2.4 *Regulation of p53 in basal conditions*). Consensus sequences for p53 binding contain two copies of the inverted pentameric sequence PuPuPuCA/TT/AGPyPy (Pu: purine, Py: Pyrimidine) separated by 0 to 13 base pairs with the 4th C and 7th G being the least variant. Sequences generally conforming to this consensus have been found in many genes that are

induced by p53. In most cases, repeats of imperfect consensus sequences are found in promoters or in introns, the invariant requirement being the conservation of the CA/TA/TG motif at the centre of the consensus. This motif is present at thousand of positions in the human genome, identifying an extremely wide potential spectrum of p53-regulated genes (Jordan et al., 2008; Wei et al., 2006).

The isolated core domain is capable of binding target sites in a cooperative manner as a tetramer, even without the aid of the C-terminal tetramerization domain (Ko and Prives, 1996) and there is some evidence that protein-protein interactions between core molecules partially mediate this phenomenon (Rippin et al., 2002). It has been found that this domain is able to interact with pro-apoptotic proteins such as Bcl-XL (Mihara et al., 2003) and also with the main regulator of p53, Hdm2 (Shimizu et al., 2002). Diverse post-translational modifications have been described in this domain such as acetylations, that regulate apoptosis induction (Sykes et al., 2006); conformation-dependent phosphorylations (Adler et al., 1997) and S-glutathionylations during oxidative stress (Velu et al., 2007).

Interestingly, it has been suggested that p53 binds with higher affinity to cell cycle control response elements than to those involved in apoptosis, suggesting a transcriptional regulation based on the nature of response elements (Weinberg et al., 2005).

1.2.2.2.4 Oligomerization domain

The oligomerization domain is located between amino acids 326-355 ([Figure 6](#)). This domain is responsible for the tetrameric state of p53. The structure of the tetramerization domain has been determined and forms a “dimer of dimers”. The domain is made of a beta-strand followed by a short alpha-helix. While beta-strands of two monomers assemble in a beta-sheet to form a dimer, the alpha-helices of two dimers form hydrogen bonds that bring two dimers into a tetramer ([Figure 6](#)). The oligomerization domain is preceded by a nuclear localization signal (NLS, residues 316-325) (Shaulsky et al., 1990). A nuclear export signal (NES, residues 340-351), which may require ubiquitination for efficient usage, also resides in the tetramerization domain (residues 340-351) (Stommel et al., 1999).

1.2.2.2.5 C-terminal basic domain

This highly basic domain is located between the C-terminal amino acids 363-393 and is itself capable of interacting with DNA in a non-specific sequence fashion (Foord et al., 1991; Wang et al., 1993) (Figure 6). Two minor nuclear localization signals (NLS) reside in this region (Shaulsky et al., 1990) that also contains multiple ubiquitination sites (Michael and Oren, 2003), the major site of sumoylation at Lysine 386 (Rodriguez et al., 1999) and many other stress-inducible modification sites including phosphorylation, acetylation and glycosylation (Appella and Anderson, 2001). In particular, phosphorylation at the Serine 392 stabilizes p53 as a tetramer (Warnock et al., 2008). This domain also interacts with proteins such as topoisomerase II (Cowell et al., 2000), c-Abl (Nie et al., 2000) and HBx (Lin et al., 1997).

It has been proposed that the C-terminus maintains p53 in a conformationally inert “latent” state which could be converted allosterically to an activated state by different post-translational modifications induced by several treatments or stresses (Hupp and Lane, 1994). As a result, binding of p53 to DNA *in vitro* requires the neutralisation of the extreme C-terminus, either by clipping out the last 30 residues, or by interaction with antibodies that bind specifically to C-terminal epitopes (Hupp et al., 1992).

1.2.3 Regulation of p53 activation and stability

p53 is a protein with a short half-life (around 20 minutes in most cells and tissues) being almost undetectable in unstressed cells. Several types of stresses induce post-translational modifications in p53 leading to its activation and accumulation. Activated p53 can, either by gene transactivation/transrepression or by protein interactions, trigger a response that either takes care of the damage by inducing cell cycle arrest and DNA repair and/or senescence, or eliminates the affected cells from the replicative pool through induction of apoptosis, thereby preventing its expansion into a large population of malignant progeny. However, despite its rapid turnover, there is evidence that p53 may regulate a number of processes even when expressed at basal levels. For example, p53 plays a role in the control of a number of genes involved in energy metabolism, downregulating glucose usage and increasing oxidative

phosphorylation by the mitochondria (see paragraph 1.2.4 *Regulation of p53 in basal conditions*).

1.2.3.1 p53/Hdm2 feedback loop

The E3-ubiquitin ligase Hdm2 protein is integral to the process of maintaining p53 at low levels by targeting p53 for degradation by ubiquitin-mediated proteolysis (Haupt et al., 1997; Kubbutat et al., 1997). Endogenous levels of Hdm2 are sufficient to regulate p53 stability, and overexpression of *Hdm2* can reduce the amount of endogenous p53. On the other hand, p53 activates the expression of the *Hdm2* gene in an auto-regulatory feedback loop (Wu et al., 1993).

The other prominent regulator of Hdm2 activity is Hdm4 (also known as Hdmx). Like Hdm2, Hdm4 is a critical negative regulator of p53. Hdm4 does not have intrinsic E3-ligase activity for p53 but instead represses p53-mediated transcriptional activation (Marine and Jochemsen, 2005). Hdm4 physically interacts with p53, forming a protein complex on target gene promoters that represses p53 function by preventing access to the general transcriptional machinery (Finch et al., 2002; Migliorini et al., 2002; Parant et al., 2001). Thus, the major role of Hdm4 is to repress but not degrade p53 (Kruse and Gu, 2009). It has also been reported that Hdm4 stimulates Hdm2-mediated ubiquitination of p53. Thus, Hdm4 acts as a stimulator, rather than as an inhibitor, of the E3 activity of Hdm2 and, at least in certain conditions, Hdm4 is actively involved in the degradation of both p53 and Hdm2 (Linares et al., 2003).

So, although the oncoprotein Hdm2 is considered to be the primary E3 ubiquitin ligase for the p53 tumour suppressor, an increasing amount of data suggests that p53 ubiquitination and degradation are more complex than once thought. The discoveries of Hdm4, HAUSP, ARF, COP1, Pirh2, and ARF-BP1 continue to uncover the multiple facets of this pathway (Figure 7). There is no question that Hdm2 plays a pivotal role in down-regulating p53 activities in numerous cellular settings. Nevertheless, growing evidence challenges the conventional view that Hdm2 is essential for p53 turnover (Brooks and Gu, 2006).

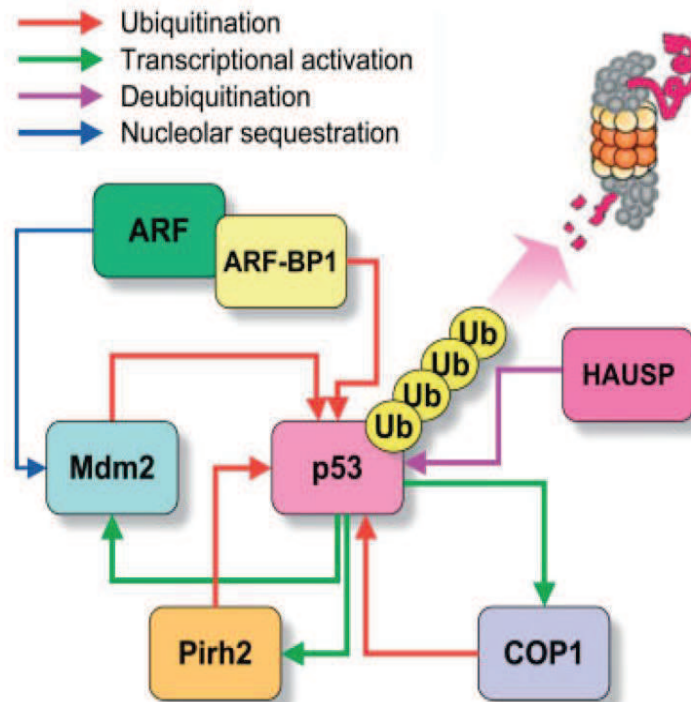


Figure 7. p53/Hdm2 feedback loop and other regulatory networks

p53 levels are tightly controlled by several negative feedback loops, involving Hdm2, Pirh2 and COP1, which regulate p53 ubiquitination and are regulated by p53 at the transcriptional level (Hollstein and Hainaut, 2010).

1.2.3.2 Post-translational modifications

Upon stress, p53 protein is stabilized due to a range of post-translational modifications. Post-translational modifications have been described to occur in all domains with different effects on protein stability and function (Figure 8). The number of post-translational modifications on p53 that have been reported appears endless. Mono- and poly-ubiquitination, sumoylation, methylation, neddylation, acetylation, as well as phosphorylation on multiple sites were found to have physiological relevance (Bode and Dong, 2004).

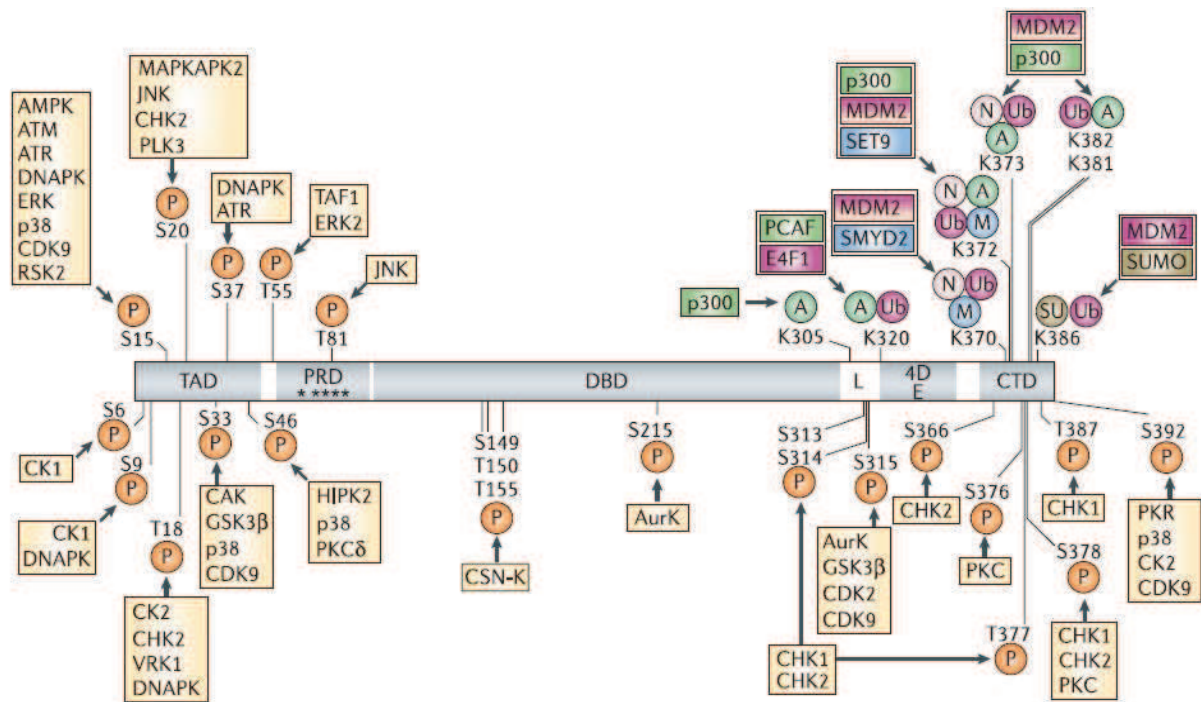


Figure 8. Post-translational modifications of p53

Specific residues are modified with phosphorylation (P) in orange, acetylation (A) in green, ubiquitination (Ub) in purple, neddylation (N) in pink, methylation (M) in blue and sumoylation (SU) in brown. Proteins responsible for these modifications are shown in matching colours. TAD: transactivation domain; PRD: proline-rich domain; DBD: DNA-binding domain; L: nuclear localization signal; 4DE: tetramerization domain; CTD: C-terminal domain (Toledo and Wahl, 2006).

Several transducer proteins are involved in these post-translational modifications which are stress-specific: ionizing radiations induce DNA-PK (Morozov et al., 1994) as well as ATM (ataxia telangiectasia mutated) (Canman et al., 1998), kinases that both phosphorylate Ser15 in the N-terminus of p53. This serine is located in the Hdm2-binding region of the protein and blocks the interaction between p53 and Hdm2, thus preventing p53 degradation. UV induce ATM and ATR, which will activate Chk2 (Matsuoka et al., 2000) and Chk1 (Guo et al., 2000) kinases, respectively, and the result in both cases is the Ser20 phosphorylation of p53 that also blocks p53-Hdm2 interaction (Chehab et al., 1999). Spindle poisons, such as nocodazole and Taxol, induce the spindle checkpoint kinase TTK/hMps1 which phosphorylates the N-terminal domain of p53 at Thr18, and this phosphorylation disrupts the interaction with Hdm2 and abrogates Hdm2-mediated p53 ubiquitination (Huang et al., 2009).

p53 activity may also be regulated through interactions with proteins such as the ASPP1/2, p300, and the two other members of the p53 family p63 and p73. ASPPs and p300 are cofactors of p53, stimulating the p53-dependent apoptosis or potentiating by acetylation transcriptional activating function of p53, respectively (Dornan et al., 2003; Samuels-Lev et al., 2001). A recent study indicates that sumoylation of p53 at K386 blocks acetylation by p300 and impairs DNA binding (Wu and Chiang, 2009).

p53 C-terminal lysines are modified by ubiquitination, acetylation, sumoylation, neddylation and methylation. Neddylation seems to inhibit transactivation, whereas sumoylation can positively or negatively affect p53 function (Bode and Dong, 2004). Unlike lysines 372, 373, 381 and 382, which are acetylated by p300 and ubiquitinated by Hdm2, lysine 320 is acetylated by the p300 and CBP associated factor (PCAF) (Di Stefano et al., 2005; Knights et al., 2006; Sakaguchi et al., 1998).

It has been also found that p53 regulates and is regulated by microRNA. For example, it was shown that miR-125b was an important negative regulator of p53 and p53-induced apoptosis during development and during the stress response (Le et al., 2009). Another study showed that miR-34a was a direct proapoptotic transcriptional target of p53 that could mediate some of p53's biological effects. They suggested that perturbation of miR-34a expression, as occurring in some human cancers, may contribute to tumourigenesis by attenuating p53-dependent apoptosis (Raver-Shapira et al., 2007).

Finally, several studies have shown that the activity of p53 and its ability to bind DNA was influenced by its redox status. Indeed, oxidation of p53 leads to the loss of the wild-type conformation and consequently to the loss of DNA binding activity, while reduction promotes wild-type p53 conformation and binding to DNA (Hainaut and Milner, 1993). Redox status of p53 influences its binding to the DNA consensus sequence, however binding to unspecific sequences remains unaffected (Parks et al., 1997).

1.2.4 Regulation of p53 in basal conditions

Beyond the indisputable importance of p53 as a tumour suppressor, an increasing number of new roles for p53 in basal conditions (i.e. in absence of stress) have recently been reported. These functions of p53 highlight an involvement in physiological processes that include the ability to regulate basal oxidative cell metabolism, stem cell maintenance, aging and senescence, and various aspects of differentiation and development.

In the following review entitled “***Redox control and interplay between p53 isoforms: Roles in the regulation of basal p53 levels, cell fate, and senescence***” and published in *Antioxidants & Redox Signaling*, we propose that there are two main levels of regulation of basal p53 through: (1) p53 sensing redox changes and (2) the expression of p53 isoforms, which act as modulators of p53 functions. Indeed, there is growing evidence that p53 is a central player in the network of redox signalling mechanisms, acting in cellular antioxidant defense systems, and in metabolic pathways involved in energy production. On the other hand, p53 isoforms and in particular $\Delta 40$ p53 and $\Delta 133$ p53 have been demonstrated to regulate senescence and aging in cellular and animal models. An increased production of reactive oxygen species (ROS) is one of the characteristics of aging process, suggesting a role for p53 isoforms in controlling levels of p53 activation in response to endogenous oxidative stress, thus linking redox regulation, p53 isoforms and organismal senescence.

Altogether, the vision of p53 as a transcription factor that promotes a tumour suppressive response to acute stress is now enlarged by its capacities at the basal state to regulate redox-dependent physiological processes.

Redox Control and Interplay Between p53 Isoforms: Roles in the Regulation of Basal p53 Levels, Cell Fate, and Senescence

Hind Hafsi and Pierre Hainaut

Abstract

The p53 tumor suppressor protein has achieved stardom in molecular oncology owing to frequent inactivation in a large range of cancers. Known as a factor activated by multiple forms of stress and causing a broad suppressive response to DNA damage, its regulation and functions in basal (non-stress) conditions has received relatively little attention. We summarize recent findings highlighting roles of p53 in physiological processes such as stem cell maintenance, development, aging and senescence, and regulation of basal oxidative cell metabolism. We suggest that these properties are regulated through two integrated biochemical systems: the redox-sensing capacity of the p53 protein (due to its structural features and its regulation by redox factors such as thioredoxin, metallothioneins, or the redox-repair enzyme APE1/ref-1), and the expression of p53 as multiple isoforms with antagonist effects. We propose that interactions between p53 and its isoforms D40p53 or D133p53 play critical roles in intracellular signaling by reactive oxygen species. We also discuss evidence that p53 controls energy production by repressing glycolysis and enhancing mitochondrial oxidative metabolism. Together, these mechanisms suggest that p53 acts not only as a “guardian of the genome” against DNA damage but also as a finely-tuned regulator of redox-dependent physiological processes. *Antioxid. Redox Signal.* 15, 1655–1667.

Introduction

Thirty years after its discovery, the p53 protein has achieved stardom status among tumor suppressors (25). This protein owes its name to its apparent molecular weight of 53 kDa and was initially identified by two parallel approaches. First, p53 was shown to complex with the Large T antigen of the SV40 virus, thus representing critical cellular target for transformation induced by this virus. Second, it was found that the serum of mice (and later, patients) carrying tumors often contained antibodies reacting with the same 53 kDa cellular phosphoprotein. Ten years later, in 1989, the TP53 gene was identified as the site of mutations or loss of alleles in almost every known type of human cancer. Today, alterations in this gene remain by far the most universal cancer-related genetic defect, with high mutation prevalence (over 50%) in common cancers such as lung, ovary, head and neck, or esophagus, intermediate prevalence in breast, bladder, pancreas, or prostate cancer, and relatively infrequent mutations in melanoma, testis, or cervical cancers (53) (IARC TP53 database). These somatic mutations show a remarkable diversity in their locations in the coding sequence and in their types. In several instances, it has been possible to demonstrate

that mutations were the direct consequence of site-specific DNA damage by mutagens such as polycyclic aromatic hydrocarbons from tobacco smoke in lung cancers of smokers, or aflatoxin in liver cancer in regions where this mycotoxin is a common food contaminant. Furthermore, the TP53 gene can also be mutated in the germline, causing a Mendelian syndrome of predisposition to multiple cancers called Li-Fraumeni syndrome. It is assumed that TP53 mutations may occur at the rate of 1 in 2500 births, making it a common cause of familial predisposition to cancer (54).

The biological basis for frequent alteration of p53 in cancer is now relatively well understood. The p53 protein is a transcription factor with multiple roles in antiproliferative cell responses, inducing cell cycle arrest, apoptosis, senescence, DNA repair or differentiation, the type of response depending from both the initial stimulus and from the nature of the cell (Fig. 1). It has long been considered that p53 was a “latent” factor, that is, a factor virtually absent from normal cells and tissues. Indeed, although expressed ubiquitously, p53 is rapidly degraded by a complex, proteasome-dependent machinery. The main regulator of p53 stability is Hdm2 (the human homolog of the product of the Murine Double Minute 2 gene). Hdm2 operates as a p53 E3-ubiquitin ligase that

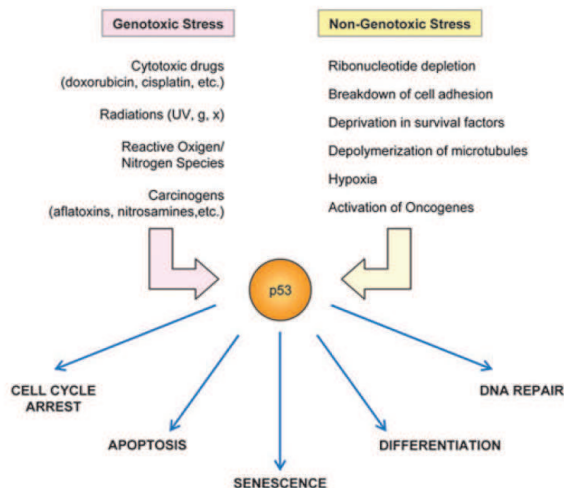


FIG. 1. p53, a sensor of multiple forms of stress. Signals that induce p53 activation are presented in two broad categories, genotoxic and nongenotoxic stress. After activation, p53 may induce a large range of suppressive responses, depending upon signal, cell type, and tissue context. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

targets p53 for rapid degradation by the proteasome, thus preventing its accumulation unless p53 is specifically induced by various activating signals. A number of other factors can also regulate p53 degradation, either by complementing or counteracting Hdm2 activity, or through distinct, Hdm2-independent, pathways (26, 45).

In response to a vast panel of genotoxic or nongenotoxic stress conditions, p53 becomes stabilized by multiple post-translational changes, escapes degradation, and accumulates in the nucleus, where it binds with high affinity to the regulatory regions of a large variety of target genes. Stresses that activate p53 include those that produce genotoxic damages (UV and γ -rays, chemicals, reactive oxygen species (ROS)), extreme physiological changes (hypoxia, depletion of microtubules or of ribonucleotides) as well as untimely or excessive growth signals (so-called "oncogenic stress", caused by constitutive activation of tyrosine kinase receptor or ras pathways) (Fig. 1). This large variety of inducing signals confers to p53 the properties of a multi-purpose, stress-induced suppressor. When activated in an acute way, p53 is an extremely potent factor that can lead cells to dramatic apoptosis in a matter of hours. However, when present at lower levels, p53 has a much more subtle range of effects that are now becoming to emerge.

This review focuses on understanding how p53 is regulated in this vast range of low-to-moderate stress conditions, and how cells may control p53 activity to avoid its untimely and counterproductive activation. These conditions are of particular importance because they represent changes that occur within the normal physiological range, as well as changes associated with normal developmental and aging processes. We develop the concept that the mechanisms responsible for the regulation of p53 in these conditions are at least partially distinct from those that drive rapid and acute p53 induction in response to high levels of DNA damage. In particular, we

propose that, in such "low stress" conditions, p53 activity is tightly regulated by two complementary mechanisms, redox control of p53 protein activity and transcriptional control of p53 expression through the production of p53 isoforms. While activation of p53 by stress generally results in the accumulation of the major 53 kDa peptide which has given its name to the protein, the patterns of protein expression in "low-stress" conditions are complex, with a wide family of products with electrophoretic mobility ranging from 33 to 53 kDa. In addition, the p53 protein is extremely sensitive to oxidation/reduction, changing its tertiary and quaternary structure according to its capacity to maintain a number of critical cysteines in a reduced form. In turn, p53 regulates a complex network of cellular pathways in which ROS are involved as effectors or second messengers (8, 21, 38). In this review, we summarize current knowledge on these "subinduction" levels of p53 and their regulation. Furthermore, we discuss how these regulatory mechanisms may participate in critical functions of p53 in physiological processes such as the control of energy metabolism and of senescence, two mechanisms in which ROS signaling plays a key role, as well as in the maintenance of stem cells pools.

Biological Significance of "Subinduction" Levels of p53

Early studies on mice with *TP53* alleles inactivated by homologous recombination (p53-null mice) led to the conclusion that p53 did not play any significant role in basal conditions in the absence of stress signals. These mice were found to develop normally and did not show any major physiological defect, although they were prone to the development of multiple early cancers (31, 40, 51). This view of p53 as "dispensable" for normal life has been challenged by a number of observations showing that p53 may exert important functions in other contexts than response to DNA damage (Fig. 2). First, it was found that inactivation of the p53 regulatory gene *MDM2* was embryonically lethal in mice. *MDM2*-null embryos were eliminated at an early preimplantation stage. This phenotype was however rescued in double knock-out mice lacking both *MDM2* and *TP53* alleles (33, 51). This observation suggests that p53 plays a critical, rate-limiting role during

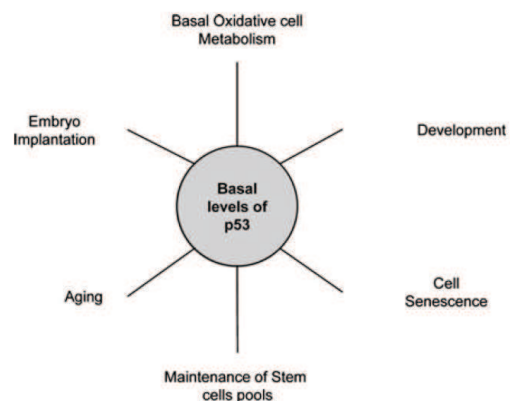


FIG. 2. Basal p53 activities. In addition to its role in growth suppression in response to acute stress signals, p53 also exerts pleiotropic effects when activated at low to moderate levels and thus play an important role in several physiological processes.

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early development in the absence of typical DNA-damaging stress and that this role must be kept under control by Mdm2 in order for normal development to occur. Second, recent studies have uncovered novel roles of low levels of p53 in the regulation of basal oxidative cell metabolism. The p53 protein regulates the transcription of a number of genes involved in the control of energy metabolism (reviewed in (10)). In particular, it represses glycolytic enzymes, downregulates glucose transport, and participates in the maintenance of oxidative phosphorylation. These activities may contribute to oppose the metabolic shift (Warburg effect) that characterizes cancer cells. Third, p53 has also emerged as an important regulator of cellular and organismal senescence. Mice with ultra-short telomeres (*Terc*^{-/-}) show a prematurely aging phenotype that can be rescued by inactivation of p53 (16). Even more significant, p53 acts as a barrier to the reprogramming of differentiated cells into induced pluripotent stem cells (46). This role of p53 is related to its function in response to DNA damage, since telomere erosion represents a form of DNA damage that activates p53. Furthermore, telomere erosion is a hallmark of aging which is promoted by increased production of ROS. Nevertheless, this mechanism operates in conditions that preserve cell viability, that is, conditions in which the levels of p53 are not dramatically increased to high levels that would result into the induction of massive apoptosis. A fourth physiological mechanism in which subinduction levels of p53 may play a role is reproduction. The leukemia inhibitory factor LIF is a transcriptional target of p53, the production of which at sufficient levels is required for the implantation of blastocysts or early embryos in the uterus (70). Close examination of reproduction patterns in p53-null mice have shown that female embryos have reduced pregnancy rates and litter sizes due to embryo implantation failure (27). In humans, a common single nucleotide polymorphism at codon 72 (R or P) in *TP53* shows geographic variations in allele frequency in relation to temperature (68). The R72 allele is most common in regions with cold winter temperatures and there is evidence that p53 carrying R72 is more potent in activating LIF transcription. This polymorphism may therefore represent an adaptation facilitating reproduction in unfavorable climates. Thus, it is becoming clear that p53 has much broader functions than induction of apoptosis when accumulated to high levels in response to acute DNA damage. Indeed, these recent observations suggest that low-to-moderate levels of p53 are required for the maintenance of many vital functions, including in particular the maintenance of stem cells pools and of tissue regeneration capacities.

The nature of the signals that activate p53 to such low-to-moderate levels is not clearly identified. An interesting hypothesis is that low (physiological) levels of oxidative stress may play a signaling role. There is growing evidence that p53, itself a redox-sensitive protein, controls a complex network of redox dependent reactions involved in the maintenance of cell homeostasis, of cell metabolism, and of cell senescence and tissue aging. Recent studies on the role of p53 isoforms provide strong clues for an essential role of basal p53 activity in the regulation of senescence and aging.

The p53 Isoform Network: Transcriptional Control of p53 Expression and Activity

Until recently, p53 was considered to be expressed as a single protein isoform of apparent molecular weight of

53 kDa, with variants corresponding to distinct polymorphic and/or post-translationally modified forms. However, in recent years, it has emerged that *TP53* has a complex expression pattern, with alternatively spliced forms, alternative initiation codons as well as transcripts initiated from different promoters (4, 12). The consequence of these variable expression patterns is that p53 can be expressed as multiple protein isoforms that differ from the canonical p53 by their proximal and/or distal domains. The biological and pathological relevance of these isoforms remain a matter of debate. Based on their expected functional domains, they may exert profound regulatory effects on p53 functions.

The p53 protein is composed of three main structural domains (Fig. 3A) (see reviews in Refs. 12, 26, 43, 45). The proximal, acidic N-terminus (residues 1–95) contains the major transactivation (TA-I) domain (1–42) and a secondary, weaker transactivation domain (TA-II) located around residues 43 to 73, which operates essentially as a modulator of the specificity of transactivation by the major domain. The TA-I domain also contains the binding site for Mdm2 (residues 17–29), the main regulator of p53 degradation (3). Further to these two domains lies a proline-rich region structured as a –SH3 binding domain, which provides a rigid link between the N-terminus and the DNA-binding core domain (DBD). The latter (residues 96–292) is made up of two anti-parallel beta-sheets linked together by flexible loops and helices, adopting the well-known “jelly roll” structure (11). The DNA-binding surface is made up of two parts, one defined by loops 2 and 3 that are bridged together by an atom of zinc and that bind to the minor groove of DNA, and the other made of a sheet-loop-helix motif that binds to the major groove of DNA (Fig. 3B). The distal part of the protein is made up of at least three subregions, the first forming a link that contains the main nuclear localization signal (residues 296–324), the second defining a complex oligomerization domain (OD; residues 325–365) and the third, in the C-terminus, representing a critical negative regulatory region. This extreme C-terminus is rich in basic residues. Both the acidic N-terminus and the basic C-terminus contain the majority of the post-translational regulatory sites (phosphorylation, acetylation, sumoylation, neddylation), whereas the central domain represents a more hydrophobic, tightly packed region which is relatively devoid of post-translational modifications.

Analysis of *TP53* gene expression identified up to ten isoforms affecting both N- and C-terminal domains of the p53 protein (Fig. 4) (35). Three C-terminus isoforms have been described (4). p53 α corresponds to the classical full-length p53, which contains the entire OD domains (4, 12). p53 β and p53 γ isoforms are produced by alternative splicing in intron 9, leading to truncated p53 proteins ending with distinct tails of respectively 10 and 15 new residues (Fig. 4C) (15). These three C-terminal isoforms could in theory be combined with three configurations of the N-terminal domain: TAp53, Δ 40p53, and Δ 133p53. TAp53 has the longest N-terminus, containing the entire sequence of the main TA domain, whereas Δ 40p53 and Δ 133p53 are shorter forms missing either the first 39 or 132 residues, respectively. In theory, these N- and C-terminal variants may combine with each other to generate up to 9 protein isoforms. A tenth isoform, Δ p53, has been described as the product of noncanonical alternative splicing between the exons 7 and 9 (63). So far, it is still not clear whether all of these isoforms are expressed in all tissues. It is most likely that their

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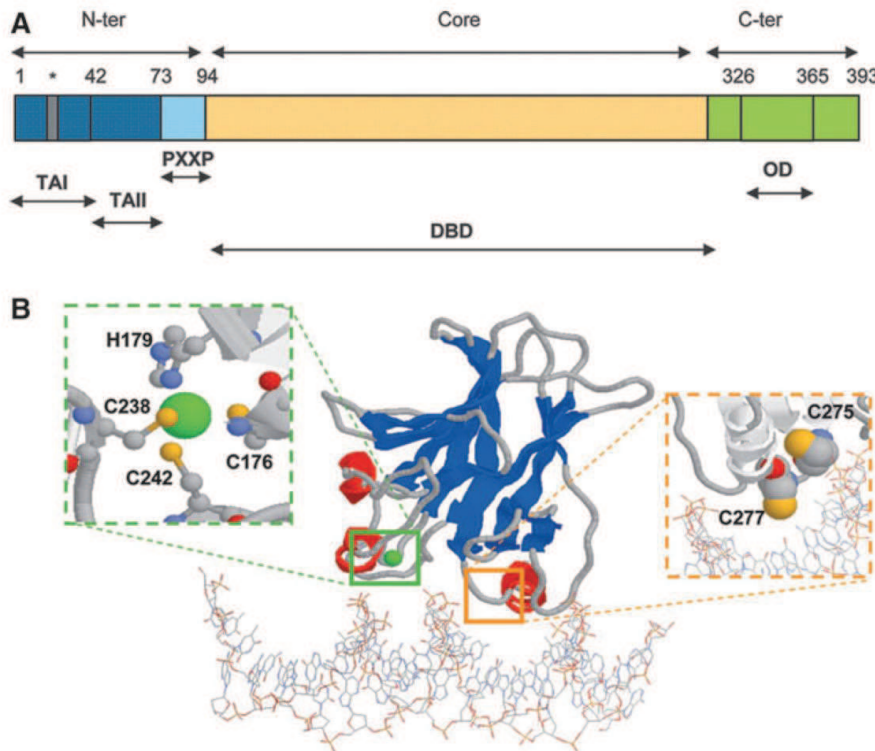


FIG. 3. Structure and redox-sensitive sites of human p53 protein. (A) Schematic view of the domain structure of human p53. The protein contains an N-terminal transactivation domain subdivided in a major subdomain (TAI) and a minor one (TAIL), a proline-rich domain (PXXP), a central DNA-binding domain (DBD), a C-terminal oligomerization domain, and a basic auto-regulatory region. Gray bar marked with an asterisk: Hdm2 binding site. (B) Structure of the DBD in complex with consensus DNA. A cartoon representation of the DNA-binding domain shows the helix (red)-loop (gray) structures binding in both major and minor groove of DNA. These loops are supported by a scaffold of beta-sheets (blue). The location of important redox-sensitive sites is shown, including the zinc (green)-binding cluster (Cys 176, 238, 242, and His 179).

and two cysteines (Cys 275 and 277), the latter binding to DNA in the major groove. Models were generated using RASMOL software based on data from Cho et al., 1994 (11). (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

expression is restricted to specific phases of cell proliferation, differentiation, senescence, or response to stress.

While the role of C-terminal isoforms is still far from being elucidated, recent results have provided insights on the role of N-terminal isoforms. It should be noted that these N-terminal isoforms present structural and functional similarities with the so-called ΔN isoforms of p63 and p73, the products of two *TP53* gene homologues that are involved in the differentiation of normal epithelial tissues, as well as in specific aspects of the regulation of neural and haematopoietic cells (12).

The $\Delta 133p53$ isoform

In contrast to the "canonical" form of p53, which is transcribed from a promoter P1 devoid of typical TATA-box and located upstream of the noncoding exon 1, the $\Delta 133p53$ isoform is expressed by internal initiation of transcription at an intragenic promoter P3, located in intron 4 (a P2 promoter, located in intron 1, has been shown to regulate an open-reading frame of unknown significance) (4, 44). Initiation at P3 produces a specific mRNA, p53I4. In this mRNA, the first AUG that can be used for initiation of translation corresponds to codon 133 of full-length p53. Thus, the corresponding protein isoform lacks the first 132 residues and is deleted from the entire TA domain, as well as from the first 30 residues of the DBD. Consistent with these structural features, it lacks DNA-binding capacity towards p53 response elements *in vitro* and counteracts the capacity of p53 to suppress cell growth. We have recently shown that human p53 can transactivate the

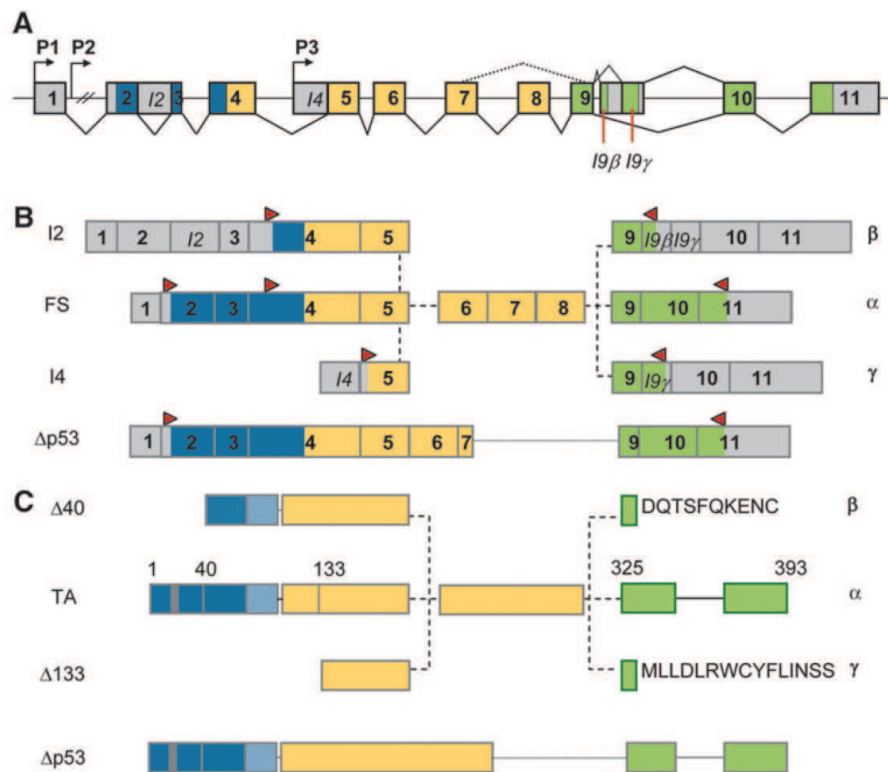
internal P3 promoter, thus regulating the expression of its $\Delta 133p53$ isoform (4,44). In turn, $\Delta 133p53$ can neutralize the suppressive activity of p53, suggesting a regulatory feedback mechanism by which p53 can modulate its own biological effects. This modulation may consist of selective interference with the capacity of p53 to transactivate target genes, thus contributing to shape the repertoire of p53-dependent responses in a manner that depends upon cell type and/or biological context. Similar conclusions have been drawn from studies in Zebrafish. The Zebrafish homolog of $\Delta 133p53$ is directly transactivated by full-length p53 in response to developmental as well as to DNA-damaging signals, resulting in enhanced expression of the isoform (9). This expression, in turn, could antagonize p53-induced apoptosis via activating bcl2L (closest to human Bcl-x(L)), a factor preventing mitochondrial permeabilization and release of ROS in the cytosol (9).

Although the exact role of $\Delta 133p53$ remains to be elucidated, a recent study has provided a clue for its involvement in the regulation of replicative senescence in normal fibroblasts (18). This study observed that replicative senescence of normal fibroblasts was accompanied by diminished expression of $\Delta 133p53$. Knocking-down $\Delta 133p53$ induced accelerated senescence, perhaps due to the fact that loss of $\Delta 133p53$ results in an untimely increase in basal p53 activity. In this study, rather than direct interaction and competition with p53, $\Delta 133p53$ appeared to repress miR34a, a microRNA transactivated by p53 and which in turn downregulates many genes required for cell proliferation and survival (Fig. 5). Since

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FIG. 4. Human p53 isoforms. (A) Intron/exon structure of the *TP53* gene showing the positions of P1, P2, and P3 promoters. p53P1 regulates the transcription of FSp53 (Full-Spliced) and p53I2 mRNA variants; p53P2, located in intron 1, controls the expression of an ORF of unknown function; p53P3 initiates the transcription in intron 4 to produce the p53I4 mRNA variant. Intron sizes are not to scale. Gray boxes: noncoding regions; blue boxes: regions encoding the N-terminal transactivation domain; orange boxes: regions encoding the central DBD; green boxes: regions encoding the C-terminal oligomerization domain. I2: intron 2 retained in p53I2 mRNA; I4: part of intron 4 retained in p53I4 mRNA; I9 β : part of intron 9 encoding p53 β ; I9 γ : part of intron 9 encoding p53 γ . (B) Structure of p53 mRNA splicing variants. All variants have in common a part of the region encoding the DBD and differ from their size and sequence of 5' and 3' ends. Domains are color-coded as in (A). Open arrow in right direction: start site of translation; open arrow in left direction: stop site of translation. (C) Structure of p53 protein isoforms. The canonical p53 (TAp53) combines TA N-terminus and α C-terminus. Δ 40p53 possesses a shorter N-terminus, lacking 39 amino acids and Δ 133p53 lacks the whole N-terminus plus part of the DBD. The α form conserves an entire C-terminal domain, whereas β and γ forms are missing several residues replaced by new amino acids. Δ p53 isoform lacks a large part of the DBD. Domains are color-coded as in (A). Dark gray bar: Hdm2 binding site. Based on Marcel and Hainaut, 2009, with modifications (43). (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).



ROS production plays critical role in the regulation of cell cycle and cell fate (7), it will be of particular importance to determine whether the Δ 133p53 isoform may modulate the redox networks controlled by p53.

The Δ 40p53 isoform

The Δ 40p53 isoform is produced by two different mechanisms using as template the "canonical" p53 mRNA synthesized from the P1 promoter. First, Δ 40p53 can be produced by internal initiation of translation at codon 40 using a completely spliced p53 mRNA (FSp53; fully spliced p53 mRNA) (13). FSp53 contains two internal ribosome entry sites (IRES, positions -1 and +39), used to initiate the translation of TAp53 or Δ 40p53 isoforms, respectively (62) (Figs. 4B and 4C). Second, Δ 40p53 may also be produced from an alternatively spliced mRNA that retains intron 2, p53I2. The retention of this short intron introduces several nonsense codons downstream of AUG 1, precluding the assembly of a full-length p53. However, initiation of translation at codon 40 (located in exon 4) allows for the assembly of a protein that lacks the first 39 residues and thus, the main TA domain (19) (Fig. 4B and 4C). Δ 40p53 appears to operate as a regulator of p53 activity in conditions when it is not activated in response

to genotoxic damage. Contrary to TAp53, Δ 40p53 does not accumulate after genotoxic stress. Due to the absence of the Hdm2-binding domain, Δ 40p53 does not interact with Hdm2 and therefore escapes the rapid degradation mechanism that regulates TAp53 level (13). Similar to TAp53, Δ 40p53 retains the capacity to bind DNA and may conserve a low but selective transactivation activity, as observed by luciferase assay on *p21^{WAF1}* promoter, a TAp53 target gene. However, due to the lack of the first 40 residues containing the major transactivation domain (TAD), the transcriptional activity of Δ 40p53 towards most p53 promoters is extremely weak (13). As shown by co-immunoprecipitation, Δ 40p53 interacts with TAp53 through their conserved OD domain. This interaction leads to the formation of mixed tetramers, the transactivation capacity of which depends upon their specific composition (Hafsi and Hainaut, unpublished data).

There is strong evidence for a biological role of Δ 40p53 in organismal aging and senescence. Studies by Maier and colleagues have investigated the effects of an N-terminally truncated p53 protein corresponding to Δ 40p53, expressed as a transgene in mice (42). Mice expressing the transgene had a profoundly altered growth rate, with a short size, a reduction in size and weight of many organs, and a premature aging phenotype with typical lordokyphosis and accelerated

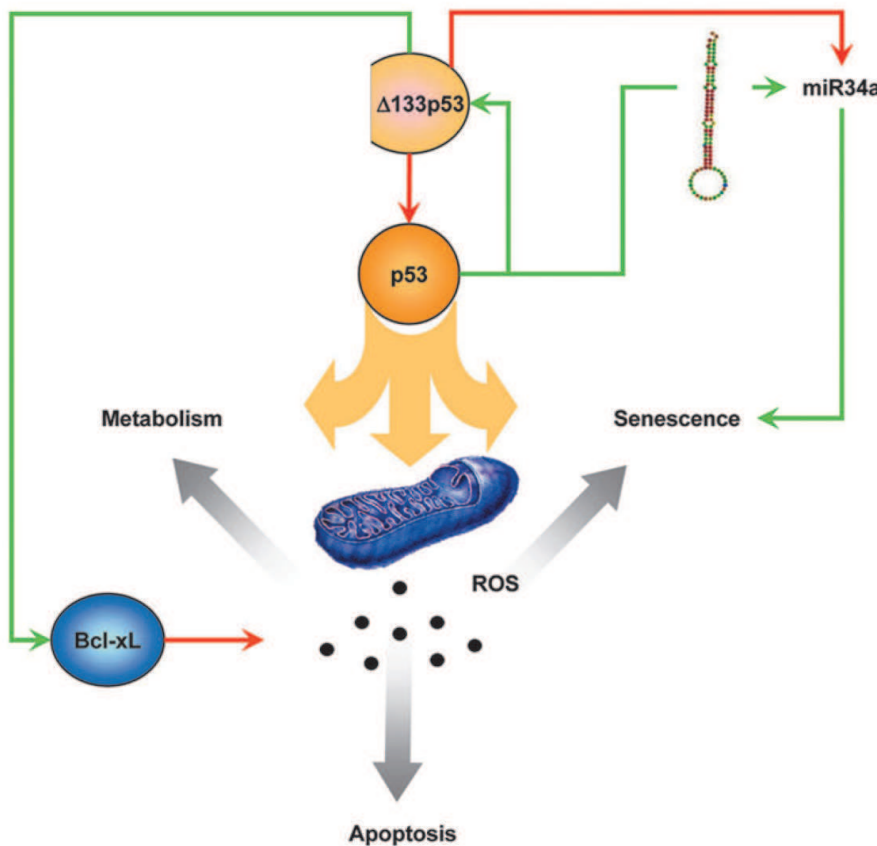


FIG. 5. Role of $\Delta 133p53$ in regulating senescence, apoptosis, and metabolism. A model integrating recent data on the role of $\Delta 133p53$ is proposed. $\Delta 133p53$ may act either directly by down-regulating mir34a or by inducing Bcl-xL. It may also act indirectly by binding to and inhibiting TAp53, thus perturbing its function as growth suppressor. We suggest that the effects of $\Delta 133p53$ converge to modulate the release of reactive oxygen species (ROS) by the mitochondria, with consequences on senescence, apoptosis, and metabolism. *Green lines:* positive effects (increased expression and/or activity); *Red lines:* negative effects (decreased expression and/or activity). (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

decrease in bone density. Interestingly, this phenotype was not observed when the transgene was expressed in a p53-null mouse background (42). Mice lacking p53 are prone to multiple early cancers and expression of $\Delta 40p53$ did not rescue that tumor-prone phenotype. Thus, the striking growth retardation and accelerated aging induced by $\Delta 40p53$ was strictly dependent upon the presence of a normal p53 gene. These observations are consistent with the notion that $\Delta 40p53$ is an important regulator of the suppressive and senescence-inducing functions of p53. The mechanisms by which $\Delta 40p53$ induces these effects is not completely understood (Fig. 6). Maier and colleagues have described that $\Delta 40p53$ transgenic mice have an altered, hyperactive IGF-I signaling pathway (42). Recently, the role of this pathway has been further documented by the demonstration that mice overexpressing the $\Delta 40p53$ isoform ($p44^{+/+}$ mice) display cognitive decline and synaptic impairment early in life (57).

At the molecular level, the first and most significant effect of overexpression of $\Delta 40p53$ may be to affect the stability of p53 and to modulate its basal level in many cells and tissues, resulting in altered control of basal p53 activity. This increased basal activity may lead to enhanced aging as a consequence of impairment of cell renewal and activation of cell death pathways. Accelerated aging has also been reported in mice co-expressing wild-type p53 and a deletion mutant lacking sequences encoded by the first six exons (73). As increased production of ROS is one of the hallmarks of the aging process, such aging phenotypes may be due to defects in the capacity of p53 to control ROS production, or to be

enhanced by suppressor activity in response to overproduction of ROS. Indeed, p53 deficiency has been shown to rescue several accelerated aging phenotypes in mouse models engineered with deletions of *Terc* or the metallo-proteinase *Zmpste24* (16, 76).

The p53 Redox Network: Sensing Redox Changes and Controlling Cell Metabolism

p53 as a redox- and metal-dependent transcription factor

The remarkable redox-sensitivity of the p53 protein first emerged as a very inconvenient trait for those researchers who were trying to produce and purify synthetic p53. The protein has a frustrating propensity to aggregate as soon as it is isolated from a healthy cellular environment. It even becomes rapidly oxidized in solution in contact with air, explaining the rapid disappearance of p53 activity from solutions kept for a few hours in a 4°C fridge.

p53 was initially identified as a redox-active transcription factor when it appeared that high affinity binding to consensus oligonucleotides *in vitro* required a strong reducing environment, equivalent to 10 mM dithiothreitol (DTT) (21, 22, 28). In contrast, treatment of p53 with oxidizing agents such as diamide completely prevented binding of p53 to specific DNA (23). These observations anticipated those deduced from the crystal structure of the DNA binding core domain of p53, which was described by Pavletitch and colleagues in 1993 in the form of a complex with DNA (11).

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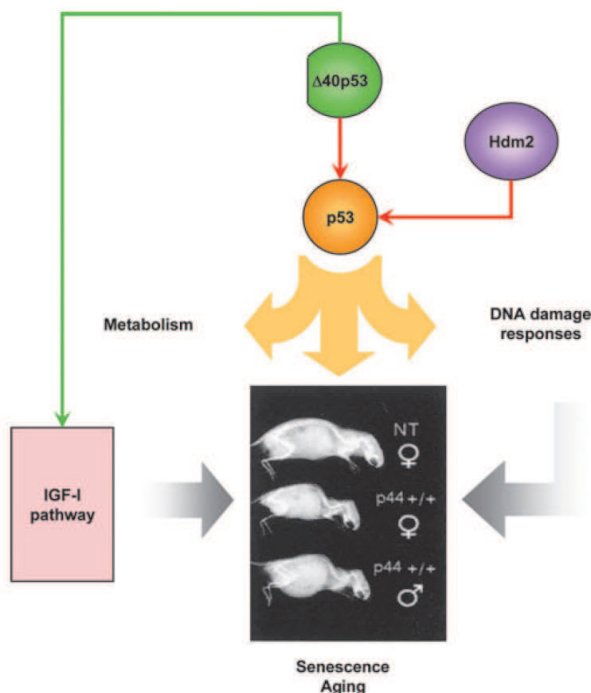


FIG. 6. Role of $\Delta 40p53$ in regulating aging and metabolism. A model integrating recent data on the role of $\Delta 40p53$ is proposed. This model proposes that $\Delta 40p53$ may have a direct effect on the IGF-I signaling pathway, thus modulating important growth and survival factors in many tissues. Alternatively, $\Delta 40p53$ may act as a regulator of p53 function by binding to the protein and affecting two of its properties, (1) its capacity to transactivate target genes, and (2) its capacity to bind Hdm2 and to undergo proteasome-mediated degradation. The lower panel, showing radiological analysis of senescent mice, is taken from Maier et al., 2004 (42). Arrows are color-coded as in Figure 5. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

The DBD of p53 contains 10 cysteines that cluster within the surface of the protein that makes direct contact with DNA (Fig. 3). Three of these cysteines, C176, C238, and C242, together with histidine H179, are involved in the binding of a divalent zinc ion (61). This zinc atom plays a structural role in bridging together loops L2 and L3 of the protein and in forming a stable structure interacting with the minor groove of DNA. Removal of zinc, or its replacement by other cations with high affinity for thiols such as cadmium (Cd^{2+}) or copper (Cu^{2+}), destabilizes p53 protein structure and disrupts DNA binding (49). Several nonzinc-binding cysteines may also be targets for redox regulation (61). C277 provides a hydrogen bond to DNA within the major groove and its oxidation prevents DNA binding. Three other cysteines are located within short distance of each other in that portion of the protein. Although their spatial arrangement does not appear to fit a tetrahedral model, it has been proposed that these residues may also be involved in binding metals (24, 79).

There is a complex interplay between zinc binding and redox regulation of p53. Zinc not only stabilizes the tertiary folding of p53 but also protects thiols from becoming oxidized

and forming disulfides that cross-link p53 either with itself or with other redox-active proteins. In the absence of zinc and of free thiols, p53 becomes oxidized into disulfide-linked aggregates. This intrinsic redox and metal sensitivity may provide a biochemical mechanism by which p53 DNA binding activity undergoes subtle variation in relation to changes of intracellular redox conditions (78, 79). Thus, p53 may behave as a "sensor" of redox changes within the cell. Such biochemical properties are not unique to p53. Two other transcription factors with a wide spectrum of target genes, AP1 and NF- κ B, share similar redox-dependent regulation (20, 80). Similarly to p53, NF- κ and AP1 are directly involved in controlling transcriptional programs in cells exposed to oxidizing agents.

In vivo sensitivity of p53 to oxidative stress

The biochemical properties summarized above suggest that p53 activity *in vivo* may be controlled by intracellular redox status and zinc bioavailability. Since oxidation of thiols can inactivate p53 by disrupting its wild-type conformation *in vitro*, it has been suggested that exposure to strong thiol oxidants *in vivo* may inactivate p53 function. In fact, this prediction has proven difficult to demonstrate since oxidative stress also induces DNA damage, which stabilizes and activates the p53 protein. This activation occurs at much lower levels of stress than those required to inactivate the p53 protein through direct oxidation. Thus, very high doses of H_2O_2 (1 mM) may lead to the inactivation of p53-dependent transcription, but such doses are cytotoxic and the physiological significance of this mechanism is not clear (55). Of greater physiological relevance are experiments using agents that induce depletion of glutathione (GSH), a critical factor in regulating cellular redox homeostasis, both as a direct ROS scavenger and as substrate for glutathione peroxidase (GPX), which contributes to remove H_2O_2 . Reduced (GSH) and oxidized (GSSG) glutathione have been shown to bind to p53 in cultured tumor cells (77). In basal conditions, the protein was only marginally glutathionylated but oxidant and DNA-damaging treatments greatly enhanced GSH modification. Mass spectrometry of GSH-modified p53 protein identified cysteines 124, 141, and 182, which are all located in the DNA-binding domain, as the main sites of glutathionylation, with C141 appearing to be the most reactive site (77). This residue is not involved in making direct contacts with target DNA but lies at the interface between p53 dimers, suggesting that its glutathionylation may decrease p53 activity by interfering with oligomerization.

Control of p53 activity by metal- and redox-dependent mechanisms

Inside cells, zinc availability and fluxes of ROS are tightly controlled by overlapping sets of effectors. ROS fluxes are regulated by electron transfer reactions between donor and acceptor molecules defining redox cycles and redox-signaling pathways. The main mechanism that controls the reduced status of thiol-containing proteins is the thioredoxin (TRX)-thioredoxin reductase (TRR) system (24). Zinc availability is controlled through metal transfer reactions mediated by metallothioneins (MT), a class of small cysteine-rich proteins that operate as intracellular buffers for Zn^{2+} and several other divalent metal ions (Cu^{2+} , Cd^{2+}). There is evidence that both

systems, TRX–TRR and MT, can regulate p53 redox status and biological activity *in vivo* (Fig. 7).

Trx is a protein of low molecular weight (12 kDa) present at high levels in the cytoplasm, nucleus, membrane, and mitochondria. Trx has two basic functions: it operates as hydrogen donor for ribonucleotide reductase (RNR) providing deoxyribonucleotides for DNA replication and as protein disulfide reductase for many oxidized proteins. Trx is itself reduced by TRR, using NADPH as source of reducing equivalents (52). Studies in yeasts and in human cells have shown that inhibition of TRR results in the accumulation of oxidized Trx, which in turn induces the formation of dis-

ulfides in p53 and inactivates DNA binding (56). However, in mammalian cells, there is scant evidence that Trx directly interacts with p53 and it is likely that its effect may be indirect (66, 74). Inhibition of TRR1 with siRNA resulted in accumulation and at least partial activation of p53, despite accumulation of oxidized Trx (66). Recent evidence suggests that suppression of TRX-1, the major isoform of Trx, induced premature senescence in normal human fibroblasts through a pathway that involved upregulation of both p53 and p16/ink4a pathways (82).

The main candidate as “intermediate” between Trx and p53 is APE1/Ref1, a bifunctional enzyme with two separate cat-

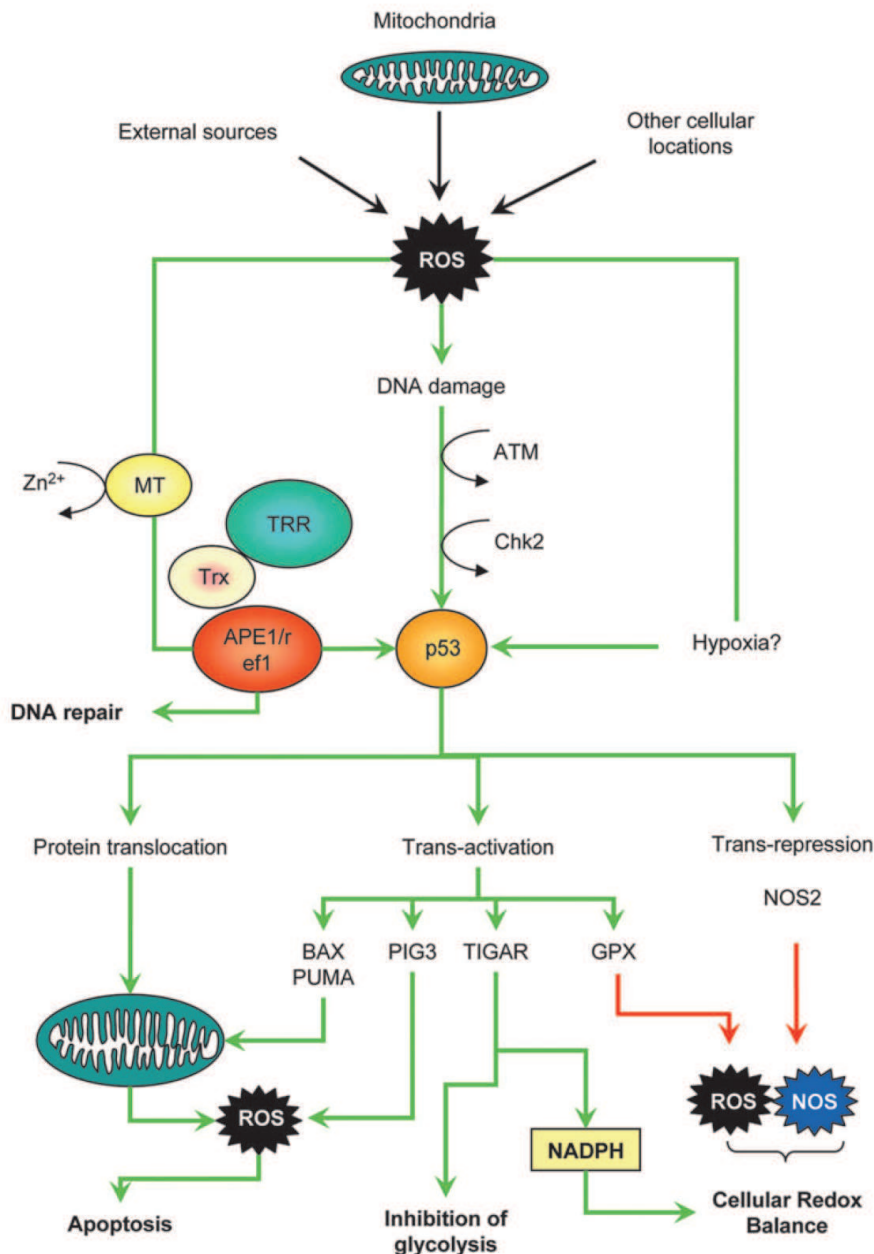


FIG. 7. An integrated model of the roles of p53 in ROS regulation and in the control of ROS-mediated processes. The upper part of the figure shows how ROS produced by several processes may contribute to activate p53. Three main pathways are proposed: (1) the DNA-damage pathway, in which ATM and Chk2, among other factors, signal to p53 the presence of ROS-induced lesions into DNA; (2) the redox-regulator pathway, in which changes in zinc binding and/or in thiol reduction may modulate the conformation and activity of p53 (MT, metallothioneins; TRR, thioredoxin reductase; Trx, thioredoxin). The bottom part shows the diversity of redox-dependent pathways controlled by p53, including (1) effects on the mitochondria; (2) effects on oxidative metabolism; (3) effects on intracellular redox balance. Arrows are color-coded as in Figures 5 and 6. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

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alytic domains, a thiol reductase which is recycled by Trx/TRR and an apurinic/apyrimidic endonuclease (2, 72) (Fig. 7). APE1/Ref1 provides the second major enzymatic step in base-excision repair (BER), the first one being the elimination of damaged bases by DNA glycosylases. The thiol reductase activity of APE1/Ref1 has been shown to control DNA binding by NF- κ B and AP1. APE1/Ref1 interacts with p53 and stimulates DNA binding *in vitro* (32). In mammalian cells, silencing of APE1/Ref1 accelerates the turnover of p53 and strongly decreases basal p53 levels (66). However, silencing of APE1/Ref1 has relatively little impact on p53 activation in response to DNA damage, leading to the hypothesis that this mechanism may essentially operate to control p53 protein activity in basal conditions. Through its bifunctional properties, APE1/Ref1 may provide a mechanism that keeps p53 function in check during DNA base-excision repair, preventing excessive activation of p53 that may lead to apoptosis in response to levels of DNA damage that can be corrected by the basal DNA repair machinery.

Metallothioneins (MT) are small proteins (7 kDa) containing about 30% of cysteines organized in two structural clusters capable of binding seven Zn²⁺ equivalents per MT molecules. Transfection of MT has been shown to regulate the Zn²⁺-dependent folding of p53 in cultured cells (49). Recent studies on the regulation of p53 by the Homeodomain-interacting protein kinase-2 (HIPK2) have shed light on the physiological significance of this regulation (59, 60). HIPK2 phosphorylates p53 at Ser46, a necessary step to induce p53-dependent apoptosis in response to severe DNA damage. Knockdown of HIPK2 inhibited Ser46 phosphorylation as well as acetylation of p53 on Lys382. In fact, HIPK2 appears to be required for the recruitment of p53 and co-activators of transcription onto promoters of pro-apoptotic genes. In addition to this inhibitory effect on p53 post-translational modifications, knockdown of HIPK2 also leads to p53 misfolding. This effect was due to enhanced expression of MT2a in response to HIPK2 knockdown and could be rescued either by supplementation with zinc or by silencing MT2a. These observations demonstrate that control of p53 folding by MT and zinc is tightly integrated with post-translational modification of p53 and in the regulation of the capacity of p53 to selectively activate different categories of promoters, such as, for example, the promoters of pro-apoptotic genes (Fig. 7). In this system, overexpression of MT2a appears to operate as a mechanism that prevents excessive p53 activation and thus protects cells against apoptotic responses (59).

Control of oxidative metabolism by p53

The p53 protein lies at the center of a vast network of pathways that use ROS and nitrogen oxygen species (NOS) as important signaling molecules (21, 38). In this network, p53 plays a dual role (Fig. 7), either as inducer of genes involved in protection against damage by ROS, or as regulator of genes whose products potentially generate ROS and whose action presumably contributes to p53-mediated cell death. Among genes that may contribute to the detoxification and protection against ROS, p53 has been shown to regulate glutathione peroxidase 1 (GPX1) (71), mitochondrial superoxide dismutase 2 (SOD2) (29), and aldehyde dehydrogenase 4 A1 (ALDH4A1) (81), which all encode products presumably

acting as antioxidants. In addition, p53 also represses the expression of inducible nitric oxide synthase 2 (NOS2), thus protecting cells against excess damage by NO and derivatives (17). Other potential antioxidant products include two p53-regulated sestrins, PA26 and Hi95 (encoded by SESN1 and SESN2, respectively), which are essential for regeneration of overoxidized peroxiredoxins (5, 6), the enzymes involved in the decomposition of hydrogen peroxide. Studies by Chumakov and colleagues have provided evidence that these potential antioxidant target genes are activated at low, quasi-basal levels of p53 and that loss of p53 function may sensitize cells to the damaging effects of low levels of ROS (64).

Conversely, p53 can operate as a trigger for the production of ROS and for ROS-dependent signaling. Candidate p53-target genes for such a role include *PIG3* (encoding a NADPH quinone oxidoreductase homolog) (58) and *FDXR*, encoding ferredoxin reductase, which transfers electrons from NADPH to cytochrome P450 via ferredoxin in mitochondria (30, 39). This increase in ROS production may participate in the initiation of apoptosis. Another ROS-dependent pro-apoptotic effect of p53 is the transcriptional activation of *BAX1* (50, 67) and *PUMA* (14), two factors that control mitochondrial permeability and facilitate the leakage of ROS from the mitochondria into the cytoplasm.

These various and apparently contradictory roles of p53 may all contribute to subtle effects on the regulation of energy metabolism, through which p53 may favor oxidative metabolism against glycolysis (reviewed in Ref. 10)). Figure 8 summarizes a number of recent observations on the role of p53 in energy metabolism. The role of p53 in downregulating glycolysis involves first the downregulation of glucose transporters at the plasma membrane through direct inhibition of *GLUT-1* and *GLUT-4* (65), and through indirect inhibition of *GLUT-3* by a mechanism involving NF- κ B (34). Next, p53 regulates the synthesis of two enzymes acting as rate-limiting factors in the glycolytic pathway. It induces *TIGAR* (TP53-induced glycolysis and apoptosis regulator), an enzyme with fructose bi-phosphatase activities that counteracts the activity of the glycolytic enzyme 6-phosphofructo-1 kinase (1, 37). In contrast, it downregulates phosphoglycerate mutase (*PGM*), an enzyme involved in the conversion of 3-phosphoglycerate into 2-phosphoglycerate during the late ATP-generating steps of glycolysis (36). This antiglycolytic activity is accompanied by the upregulation of enzymes that divert the metabolism of glucose to the pentose phosphate pathway (PPP), in particular glucose-6-phosphate dehydrogenase (41). In turn, hexokinase 2 (*HK2*), the rate-limiting enzyme generating glucose-6-phosphate, contains p53-response elements in its promoter and is another potential target for upregulation by p53 (47). The PPP is an important contributor of reducing equivalents in the form of NADPH, as well as an essential step for the biosynthesis of ribose and subsequent synthesis of nucleic acids.

In parallel with its capacity to limit glycolysis, p53 maintains and promotes oxidative phosphorylation through at least two mechanisms. First, it activates AIF (apoptosis inducing factor), a bifunctional protein with oxidoreductase function contributing to the assembly and function of Complex I of the respiratory chain (69, 75). The other function of AIF is extramitochondrial: under different apoptosis-inducing conditions, AIF translocates through the outer mitochondrial membrane to the cytosol and to the nucleus,

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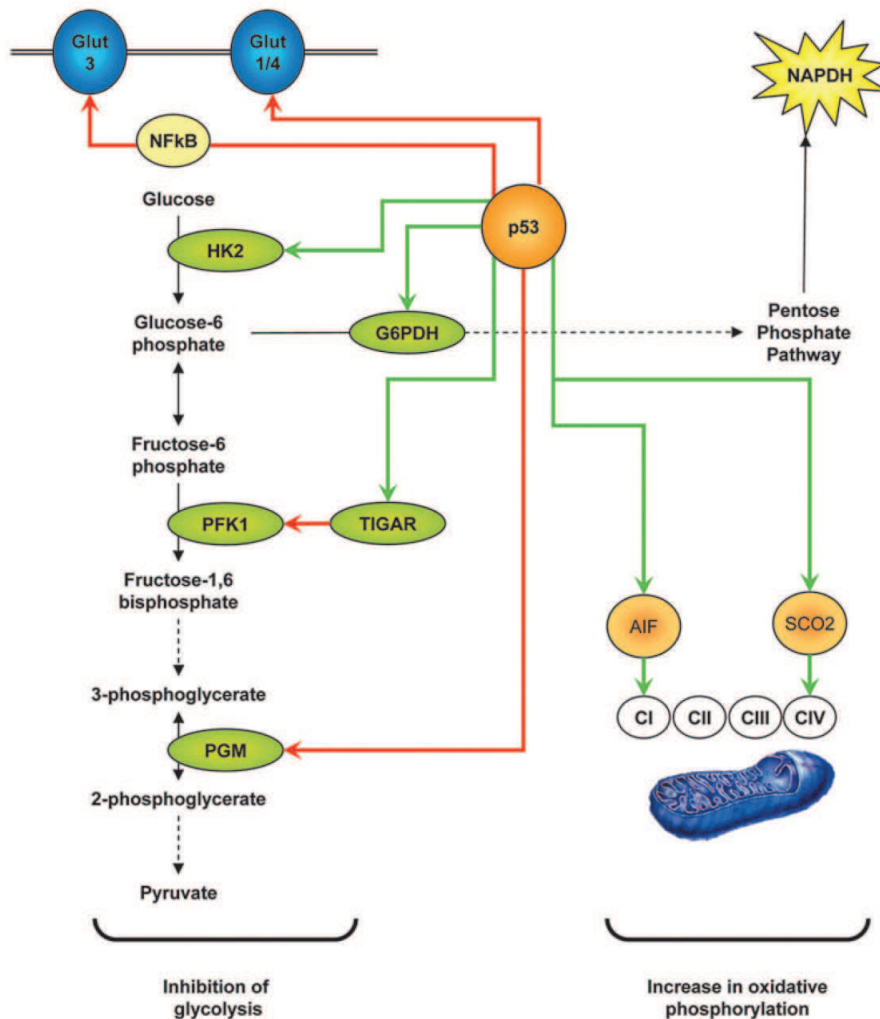


FIG. 8. p53 and regulation of metabolism. This figure summarizes recent data on the role of p53 as a transcriptional regulator of genes involved in the control of energy metabolism. *Left:* components of glucose transport (blue) and metabolism (green) regulated by p53. *Right:* components of mitochondrial oxidative phosphorylation chain regulated by p53 (orange). Arrows and lines are color coded as in Figures 5–7. Based on Cheung and Vousden, 2010, with modifications (10). (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

where it induces chromatin condensation as well as DNA fragmentation. Second, p53 transactivates SCO2 (synthesis of cytochrome c oxidase 2), a copper-dependent chaperone protein that is required for the assembly of cytochrome c oxidase (complex IV of the respiratory chain) (48).

Overall, these metabolic effects of p53 antagonize the Warburg effect that characterizes the metabolism of most cancer cells. The latter cells tend to become over-dependent upon glycolysis and to reduce their oxidative phosphorylation, oxygen-dependent metabolism. This effect is one of the biochemical adaptive mechanisms by which cancer cells survive in hypoxic and nutrient-deprived conditions. It is therefore possible that mutation of *TP53* and loss of p53 function contributes to the Warburg effect.

Conclusions: p53 as a Regulator of ROS in Stem Cell Maintenance

Until recently, studies on p53 have been mainly concerned by its role in the response to acute genotoxic stress induced by xenobiotics, radiations, or other exogenous DNA-damaging

substances. Under such exposures, most cells undergo rapid nuclear accumulation of p53, followed by a range of growth suppressive responses aimed at preventing the accumulation of genetic damage. There is now growing evidence that p53 activity is also involved in the response to levels of stress signals generated during normal physiological processes. The main biological evidence for such an effect comes from studies in mouse models showing that p53 deficiency rescues many engineered phenotypes of premature aging. This suggests that organismal aging, which is largely mediated by overproduction of reactive oxygen species, is at least in part mediated through p53 activation and inhibition of cell growth or renewal.

These observations shed a new light on p53 regulatory mechanisms that have been so far less well studied than those involved in the pathways of DNA damage signaling. Of particular relevance in this context is the central role of p53 at the core of a network of redox signaling mechanisms. Indeed, p53 is both a sensor of DNA damage induced by ROS, a direct sensor of ROS homeostasis through its intrinsic redox dependence, and a regulator of multiple ROS controlling

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mechanisms, either part of cellular antioxidant defense systems, or part of metabolic pathways involved in energy production. The model emerging from these observations is that small amounts of ROS generated during physiological processes may turn on p53 activity and trigger autoregulatory feedback mechanisms with either antioxidant response or, conversely, an escalating cycle of redox dysfunction leading to metabolic switch, mitochondrial release of ROS, genotoxic damage, and further activation of p53.

How cells handle the decision between antioxidant response and escalating cycles of redox dysfunction is a major focus for future research. One key mechanism in this respect may be the production of p53 isoform lacking the N-terminus. This hypothesis is essentially based on the observation that mice overexpressing the $\Delta 40$ p53 isoform have an accelerated aging phenotype similar to those induced by excess oxidative stress. This observation places N-terminally truncated forms of p53 in the position of operating as controllers of levels of p53 activation in response to endogenous oxidative stress, thus linking redox regulation, isoforms, and organismal senescence. Redox regulation plays essential role in the maintenance and renewal of normal stem cells. It is therefore possible that deletion of p53 function by mutation may result in perturbations of p53-dependent redox control, thus facilitating the formation and expansion of cancer stem cells.

Acknowledgments

Research on p53 isoforms at IARC is supported by la Ligue Nationale Contre le Cancer and by the French National Cancer Institute (INCa).

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Address correspondence to:

Dr. Pierre Hainaut

International Agency for Research on Cancer

150 cours Albert Thomas

69372 Lyon Cedex 08

France

E-mail: hainaut@iarc.fr

Date of first submission to ARS Central, November 9, 2010;
date of final revised submission, December 7, 2010; date of
acceptance, January 1, 2011.

Abbreviations Used

AIF = apoptosis inducing factor
BER = base-excision repair
DBD = DNA-binding domain
DTT = dithiothreitol
GPX = glutathione peroxidase
Hdm2 = human double minute-2
HIPK2 = homeodomain-interacting protein kinase-2
LIF = leukemia Inhibitory Factor
Mdm2 = murine double minute-2
MT = metallothioneins
NOS = nitrogen oxygen species
NOS2 = nitric oxide synthase 2
OD = oligomerization domain
PGM = phosphoglycerate mutase
PPP = pentose phosphate pathway
RNR = ribonucleotide reductase
ROS = reactive oxygen species
SCO2 = synthesis of cytochrome c oxidase 2
TA = transactivation
TIGAR = TP53-induced glycolysis and apoptosis
regulator
TRR = thioredoxin-reductase
TRX = thioredoxin

1.3 *TP53 and the Hallmarks of Cancer*

The tumour suppressor gene *TP53*, encoding the p53 phosphoprotein, plays a key role in the complex and powerful networks that counteract the effects of the Hallmarks of cancer. The diverse characteristics and capacities of p53 allow it to play critical roles in each of the Hallmarks of cancer. As a result, the p53 signalling pathway can be seen as a molecular device integrating the Hallmarks processes into a coherent biological program. A direct consequence of this role is that p53 function is at least partially disabled in most of cancers (Pfeifer and Hainaut, 2011). Taking into account the multiplicity of mechanisms other than mutation that may inactivate p53 functions (such as enhanced protein degradation through increased expression of cellular or viral proteins that target p53 for destruction by the proteasome), it may be considered that p53 function is inactivated, or somehow made deficient, in the vast majority of cancers. The fact that this deficiency may occur -or may show its effects- at any step in the carcinogenic process from pre-cancer condition to highly aggressive, metastatic lesions, makes it very complex to address the prognostic or predictive significance of measurable p53 alterations. Furthermore, such a sweeping role for a single molecule in such a mechanistically complex, multi-factorial disease raises the suspicion that p53 does not act as a frontline driver but as a kind of facilitator of carcinogenesis. In a recent review, P. Hainaut has developed the concept that the uniqueness of p53 as a target for inactivation in cancer may be due to the fact that this protein interferes in each of the ten Hallmarks of cancer processes, supporting the view that loss of p53 function not only make cells permissive to the acquisition of Hallmarks capabilities but also removes a critical brake that prevents the expression of Hallmarks capabilities. According to this model, p53 is not a specific component of any particular Hallmarks process but rather a molecular device that organizes the Hallmarks processes into a coherent biological program (Figure 9). In the section below, I am discussing this model based on the review by P. Hainaut (Hainaut, 2012).

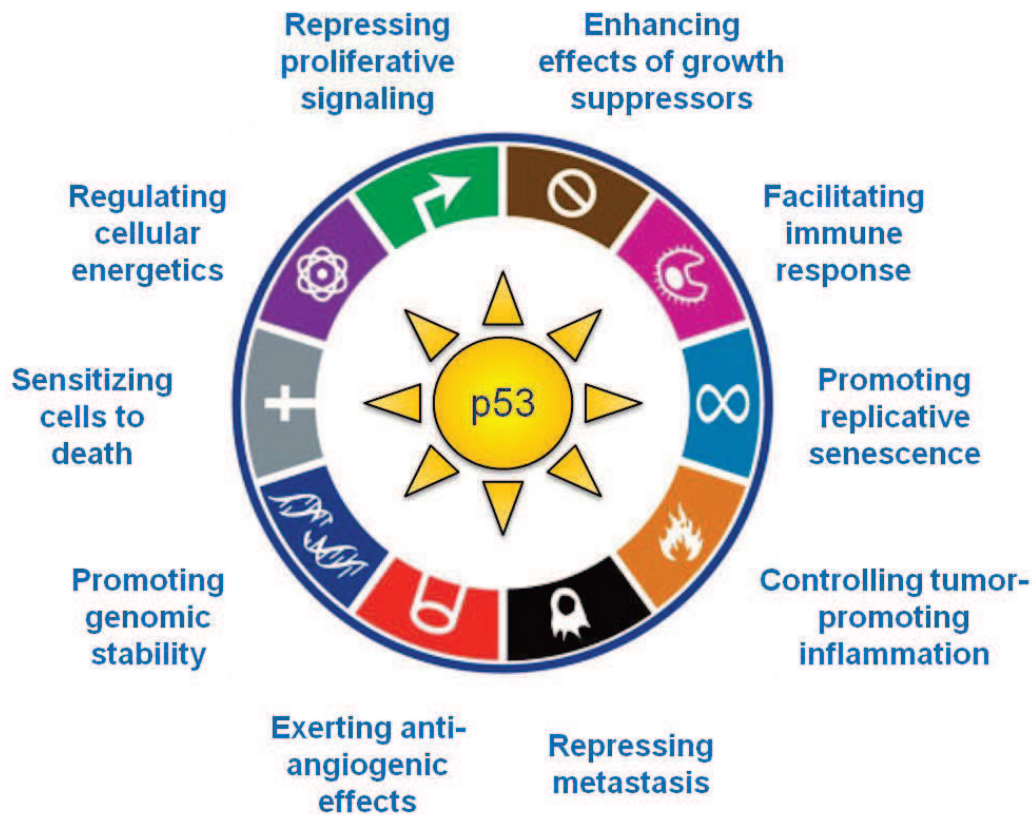


Figure 9. A p53-centric system in the “Hallmarks of cancer”

This model represents p53 as a central and multifunctional molecular device controlling each of the Hallmarks proposed by Hanahan and Weinberg (2011). Adapted from Hanahan and Weinberg (2011) and Hainaut (2012).

1.3.1 Repressing proliferative signalling

The most fundamental characteristic of cancer cells is their capability to sustain chronic proliferation. Unlike normal tissues, cancer cells can deregulate proliferative signalling through the production of growth-promoting signals, and through the pathways that regulate the progression through the cell cycle. The activation of the p53 protein and its network of genes sets in motion an elaborate process, which connect the p53 pathway to other signal transduction pathways to coordinate the cellular signals for growth and division. For instance, p53 regulates the transcription of *PTEN* (phosphatase and tensin homolog), the second most

frequently mutated gene in human cancer after p53 (Bostrom et al., 1998; Li et al., 1997; Suzuki et al., 1998). PTEN protein phosphatase can dephosphorylate protein substrates on serine, threonine and tyrosine residues (Myers et al., 1998). Induction of *PTEN* suppresses cell survival and proliferation by antagonizing the phosphoinositol-3-kinase (PI3K)/AKT signalling pathway (Maehama and Dixon, 1998).

In response to aberrant growth or oncogenic stress, p14^{ARF} protein is expressed. This protein is encoded by the alternative open reading frame of the *INK4a/CDKN2a* locus located on chromosome 9p21, which also expresses the cyclin kinase inhibitor p16 (Kamijo et al., 1997). p14^{ARF}, an activator of p53, interacts with Hdm2 and blocks its shuttling between the nucleus and cytoplasm via the nucleolus (Tao and Levine, 1999). Sequestration of Hdm2 in the nucleolus prevents it from binding and degrading p53, thus resulting in the accumulation and activation of p53.

The major effect of activated p53 is to block the cell cycle. Growth arrest is a reversible halt of the cell cycle that occurs at either the G1/S or G2/M barrier. Progress through cell cycle is thought to be mediated by sequential phosphorylation of the retinoblastoma (Rb) protein by G1 cyclin-dependent kinases, resulting in the release of active E2F transcription factor, which activates genes required for S phase transition (Kato et al., 1993; Mudryj et al., 1991). The critical mediator of the p53-mediated G1 arrest response is p21WAF1 (el-Deiry et al., 1993). Following activation by DNA damage, p53 induces the expression of *CDKN1A* (encoding p21WAF1), which is able to inhibit cdk2 and cyclin D/cdk4 complexes (LaBaer et al., 1997).

The second checkpoint corresponds to the G2/M phase. p21WAF1 also plays a role in mediating cell cycle arrest in the G2/M phase as it is induced after p53 over-expression (Agarwal et al., 1995). Another p53 target gene involved in the G2 arrest is GADD45, which interacts with cdc2 and inhibits its kinase activity, causing the dissociation of the cyclin B1/cdc2 complex (Wang et al., 1999). Moreover, through induction of 14-3-3 σ , p53 can also induce G2/M arrest in cells that have sustained DNA damage (Hermeking et al., 1997). The 14-3-3 σ kinase phosphorylates cdc25, preventing the activation of the cyclin B1/cdc2 complex (Peng et al., 1997).

The S-phase checkpoint involves three different modes of control: the replication control, the intra-S-phase checkpoint and the S/M checkpoint. p53 has been proposed to be

responsible of ensuring that cells do not enter mitosis with unreplicated DNA (Taylor et al., 1999). In addition, p53 has been shown to play a key role in the maintenance of the S-phase arrest induced by cdc7 down-regulation (Montagnoli et al., 2004), as well as in promoting chromatin condensation, a hallmark of mammalian cells that enter mitosis before completing DNA replication (Nghiem et al., 2002). Finally, the spindle assembly checkpoint ensures that cells do not enter anaphase until all chromosomes are aligned and attached to the microtubules of the mitotic spindle. Initially it was proposed that p53 was an important component of a spindle checkpoint in mice, as it is required during normal cell division (Cross et al., 1995). However, more recent evidence revealed that expression of p53 occurred after cells had exited mitosis and progressed to a G1-like state and that p53 plays a critical role in preventing aneuploidy by blocking endoreduplication of tetraploid cells that result from mitotic cells (Vogel et al., 2004).

1.3.2 Enhancing the effects of growth suppressors

Besides the hallmark capacity of inducing and sustaining growth-promoting signals, cancer cells must also evade powerful programs that negatively regulate cell proliferation. Many of these programs depend on tumour suppressor genes actions.

The growth suppressor *CDKN1A*, encoding the p21WAF1 cyclin kinase inhibitor, was the first one to be identified as a direct transcriptional target of p53 and is a central effector of the growth suppressive effect of p53 in response to intracellular stress signals such as DNA damage, levels of nucleotide pools or availability of oxygen (el-Deiry et al., 1993; Roninson, 2002). However, the effects of *p21WAF1* knockout in mice and its expression patterns in human cancer are consistent with a role for p21WAF1 as both a tumour suppressor and an oncogene (Roninson, 2002).

The cell-to-cell contacts formed by propagating cells in culture operate to suppress further cell proliferation. This “contact inhibition” is abrogated in a variety of cancer cells in culture, suggesting the existence of a comparable mechanism *in vivo* to maintain a normal tissue homeostasis. The mechanistic basis of contact inhibition is beginning to emerge. Merlin (also known as schwannomin), the product of the *NF2* (neurofibromatosis type 2) tumour suppressor gene, coordinates the processes of adherens junction stabilization and negative

regulation of epidermal growth factor receptor (EGFR) signalling by restraining the EGFR into a membrane compartment from which it can neither signal nor be internalized (Curto et al., 2007). Merlin also mediates contact inhibition of growth by suppressing recruitment of Rac to the plasma membrane (Okada et al., 2005). p53 stability and activity is increased by Merlin, through the inhibition of the Hdm2-mediated degradation of p53. Moreover, over-expression of Merlin leads to p53-dependent apoptosis in response to serum starvation or to a chemotherapeutic agent (Kim et al., 2004).

Another mechanism of contact inhibition involves p53 and the tumour suppressor *LKB1*, which organizes epithelial structure and polarity, and helps maintain tissue integrity. *LKB1* is genetically inactivated in patients with Peutz-Jeghers syndrome and somatically mutated in a variety of cancers. Over-expression of *LKB1* inhibits cell proliferation through the degradation of the c-myc oncogene protein (Liang et al., 2009). In contrast, knockdown of *LKB1* accelerates cell cycle progression through G1/S checkpoint and enhanced cell proliferation mediated at least in part by a decline of p53 and p16 growth suppressor pathways (Liang et al., 2010).

The pleiotropic cytokine, transforming growth factor- β (TGF- β), is one of the main regulators of anti-proliferative pathways. TGF- β has received much attention as a major inducer of Epithelium to Mesenchyme Transition (EMT) during embryogenesis, cancer progression and fibrosis (Wendt et al., 2009; Xu et al., 2009). TGF- β ligands bind to cognate serine/threonine kinase receptors leading, intracellularly, to phosphorylation and activation of the Smad family of signal transducers (Massague, 2000). A recent study has identified p53 as an *in vivo* relevant partner of Smad2 in the activation of multiple TGF- β target genes. Smad and p53 protein complexes bind on a target promoter and synergistically activate TGF- β induced transcription (Cordenonsi et al., 2003). Another p53-dependent mechanism involves the *SnoN* proto-oncogene product, which negatively regulates TGF- β signalling through its interactions with Smad2 and Smad3 complexes. The tumour suppressor role of SnoN results from its interaction with the promyelocytic leukaemia (PML) protein and the accumulation of SnoN in PML nuclear bodies, thus allowing SnoN to stabilize p53 and induce premature senescence (Figure 10) (Lamouille and Derynck, 2009; Pan et al., 2009).

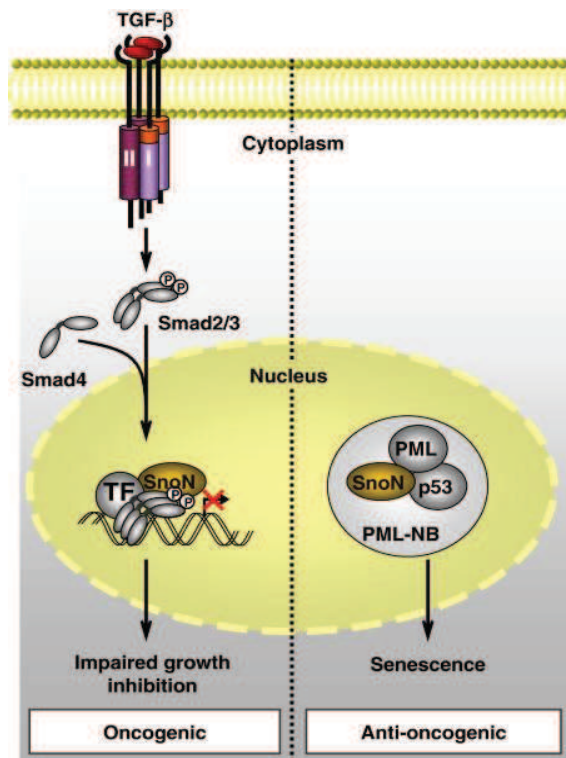


Figure 10. Oncogenic and anti-oncogenic roles of SnoN

In the nucleus, SnoN can bind the Smad complex with transcription factors (TF) and represses TGF- β -induced inhibition of proliferation, thus acting as an oncogene. Independently of the Smad interactions, SnoN can interact with the promyelocytic leukaemia (PML) protein in PML nuclear bodies (PML-NB), resulting in stabilization of p53. This leads to premature senescence and defines an anti-oncogenic role for SnoN (Lamouille and Derynck, 2009).

1.3.3 Sensitizing cells to death

In response to exposure to radiation or various chemotherapeutic drugs that induce double strand DNA breaks, p53 initiates an apoptotic program in a number of cell types. p53 controls the transcription of several classes of genes involved at different levels in apoptosis signalling. The execution of apoptosis involves two main pathways: the death receptor pathway (extrinsic) triggered by so called death receptors (DR) such as CD95/Fas and Killer/DR5 (Danial and Korsmeyer, 2004); and the mitochondrial pathway (intrinsic) where many components have been found to be p53 target genes as described below.

The intrinsic pathway is also termed the “mitochondrial pathway” as the mitochondria acts as the centrepiece (Figure 11A). APAF-1, the key component of the apoptosome, required for pro-caspase-9 recruitment and activation, has been reported to be a primary p53 response gene (Moroni et al., 2001). Also relevant for the pro-apoptotic p53 response appears to be its ability to regulate Bcl-2 family members, such as Bcl-2-associated protein X (Bax), and the BH3-only proteins: p53-upregulated modulator of apoptosis (Puma), Noxa and Bcl-2-interacting domain death agonist (Bid); or directly by transrepressing Bcl2 (Hoffman et al., 2002; Sugars et al., 2001). BH3-only proteins most likely trigger apoptosis by engaging multiple Bcl-2 pro-survival proteins in a stimulus- and possibly also cell-type-dependent manner, leading to Bax and/or Bcl-2 antagonist/killer (Bak) oligomerization, mitochondrial outer membrane permeabilization accompanied by cytochrome c release and subsequent cell death (Mikhailov et al., 2003; Willis et al., 2005).

As for APAF-1, p53-mediated induction of Bax is not critical for apoptosis initiation but may rather serve to amplify apoptosis signalling. However, there is solid evidence that induction of BH3-only proteins is essential for p53-induced apoptosis. For example, the BH3-only protein Bid, which is considered to amplify apoptosis signalling particularly by linking the DR pathway to the intrinsic pathway, was reported to be induced transcriptionally by p53 in response to γ -irradiation (Sax et al., 2002). Another example is Puma, whose mRNA and protein were both reported to be induced, in normal as well as in malignant cells, following DNA damage or in response to oncogenic stress; and this induction in response to DNA damage strictly depends on p53 (Nakano and Vousden, 2001). Moreover, Noxa (whose promoter region contains a functional p53-binding site (Oda et al., 2000)) and Puma appear to play an overlapping role in mediating cell death in response to DNA damage caused by cytotoxic drugs, such as etoposide or γ -irradiation (Villunger et al., 2003).

Induction of the extrinsic pathway, also known as the death receptor pathway, occurs following p53 transactivation of genes encoding Fas (also known as Apo-1 or CD95), TNF receptor-1 (TNFR-1), DR5/Killer and TNF-related apoptosis-inducing ligand (TRAIL) in the cell plasma membrane, leading to activation of pro-caspase 8 and apoptosis (Wu et al., 2000) (Figure 11B). p53 also represses the pathway regulating the bioavailability of survival factors (Figure 11C). It induces the expression of insulin-like growth factor binding protein 3 (*IGF-BP3*), which encodes a protein that binds and neutralizes IGF-1 and IGF2, preventing Bcl-2 and Bcl-XL activation (Buckbinder et al., 1995; Butt et al., 1999; Kooijman, 2006).

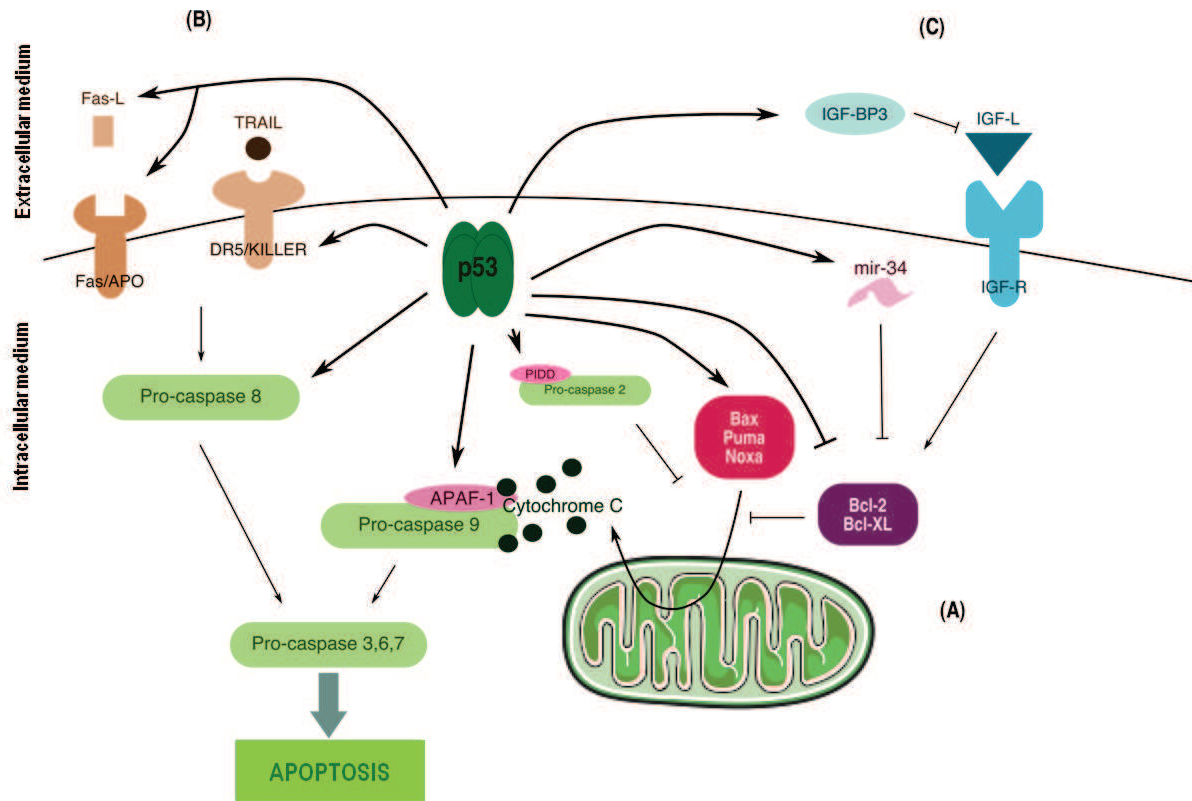


Figure 11. p53 and apoptosis

p53 can induce apoptosis via several pathways: the mitochondrial pathway (A), the death receptor pathway (B), and the pathway regulating the bioavailability of survival factors (C) (adapted from Marcel V, PhD Thesis, University of Lyon, 2010).

Autophagy is induced in response to various stress stimuli, including starvation, trophic factor deprivation, hypoxia, endoplasmic reticulum stress and oxidative stress (Levine and Abrams, 2008). Autophagy has diverse physiological functions, including stress adaptation, development, immunity and protection against neurodegeneration. It can also function as a tumour suppressor or cell-survival pathway as demonstrated by the fact that loss of autophagy genes leads to increased DNA damage, chromosomal instability and deregulated control of cell growth. This indicates a potential overlap in tumour suppressor-related autophagy effects and p53 activities. However, the p53 tumour suppressor, previously thought to positively regulate autophagy, may also inhibit it.

For example, genotoxic stresses caused by DNA damaging agents induce p53-dependent autophagy (Feng et al., 2005; Zeng and Kinsella, 2007). Similarly, forced expression of p14ARF or oncogenic activation of p53, induce autophagy in human cancer cells (Abida and

Gu, 2008). The mechanisms of p53-dependent induction of autophagy are still incompletely understood, but are thought to involve both transcription-independent functions (AMPK activation), as well as transcription-dependent functions (upregulation of mTOR inhibitors, *PTEN* and *TSC1*, or the p53-regulated autophagy and cell death gene, *DRAM*) (Crichton et al., 2007; Feng et al., 2005).

Paradoxically, elevated autophagy, often associated with the tumour microenvironment and/or treatment with cytotoxic agents, can also increase tumour cell survival and in this sense, autophagy is considered to be pro-oncogenic. The mysteries underlying p53 regulation of autophagy extend beyond the question of whether p53-mediated autophagy is pro-death or pro-survival. Tasdemir et al. directly challenge the notion that p53 is a positive regulator of autophagy by showing that chemical inhibition of p53 increases autophagy in both normal and transformed cells in a transcription-independent way (Tasdemir et al., 2008).

1.3.4 Suppressing replicative immortality through senescence

The senescence phenomenon was first described by Hayflick and Moorhead in human fibroblasts (Hayflick and Moorhead, 1961). When maintained in culture, normal cells undergo a phase of expansion through division until they enter senescence, which is characterized by large cell size, flat vacuolated morphology, inability to synthesize DNA, and the presence of the senescence-associated β -galactosidase (SA- β -gal) marker (Dimri, 2005). The telomeres, located at the end of chromosomes and composed of tandem repeats of 6 nucleotides (TTAGGG), determine how many rounds of division a cell can achieve before reaching replicative senescence (Harley et al., 1990; Podlevsky and Chen, 2012). Maintenance of telomere stability is required for cells to escape from replicative senescence and proliferate indefinitely. Telomerase is a cellular ribonucleoprotein reverse transcriptase which stabilizes telomere length by adding telomere repeats to the ends of the chromosomes, thus compensating for the continued erosion of telomeres. This enzymatic activity is almost absent in normal, post-mitotic cells but is expressed at high levels in 90% of immortalized and transformed human cells (Shay and Wright, 2001).

Recently, it has been shown that even brief reactivation of endogenous p53 in p53-deficient tumours can produce complete tumour regressions through induction of senescence

(Ventura et al., 2007; Xue et al., 2007). In these studies, the primary response to p53 reactivation was not apoptosis, but instead involved the induction of a cellular senescence program that was associated with differentiation and the upregulation of inflammatory cytokines. This program, although producing only cell cycle arrest *in vitro*, also triggered an innate immune response that targeted the tumour cells *in vivo*, thereby contributing to tumour clearance. This indicates that p53 loss can be required for the maintenance of aggressive carcinomas, and illustrates how the cellular senescence program can act together with the innate immune system to potentially limit tumour growth.

p53-dependent senescence may also be induced in cells with dysfunctional telomeres. Indeed, it was shown that p53 abrogation rescued the small size phenotype and the functionality of epidermal stem cells of telomerase-deficient mice with dysfunctional telomeres. This suggested the existence of a p53-dependent senescence response acting on stem/progenitor cells with dysfunctional telomeres that is actively limiting their contribution to tissue regeneration, thereby impinging on tissue fitness (Flores and Blasco, 2009).

1.3.5 Exerting anti-angiogenic effects

During tumour formation, a disruption in the balance between productions of pro- and anti-angiogenic molecules occurs allowing continued proliferation and growth of tumour cells (Bergers and Benjamin, 2003).

p53 is able to block this process either by activating anti-angiogenic molecules or by repressing pro-angiogenic ones. For example, p53 transcriptionally activates the alpha (II) collagen prolyl-4-hydroxylase (*alpha(II)PH*) gene, resulting in the extracellular release of anti-angiogenic fragments of collagen type 4 and 18 (Teodoro et al., 2006). In contrast, p53 significantly inhibited the transcription of vascular permeability factor/vascular endothelial growth factor (*VPF/VEGF*), which is considered to be the most important directly acting angiogenic protein, by regulating the transcriptional activity of *Sp1* and also by down-regulating the Src kinase activity (Pal et al., 2001). In addition, p53 inhibits angiogenesis by inducing the production of Arresten, a collagen-derived anti-angiogenic factor (CDAF) that is processed from $\alpha 1$ collagen IV (*COL4A1*). The p53 protein directly activates the transcription

of *COL4A1* and also increases the metalloproteinase-mediated release of Arresten, thus controlling the production of an important anti-angiogenic factor (Assadian et al., 2012).

1.3.6 Repressing invasion and metastasis

Another tumour suppressive activity of p53, which is still poorly understood, is its ability to modulate cell migration. It has been shown that p53 inhibits cdc42-induced filipodia formation (Gadea et al., 2002). In mouse embryonic fibroblast cells, absence of p53, or overexpression of its dominant negative isoform $\Delta 133p53$, induced rounded blebbing movements through over-activation of RhoA and ROCK (Rho-associated coil-containing protein kinase)-dependent translocation of RhoA to membrane blebbing structures. Thus, loss of p53 (as well as the expression of one p53 isoform, $\Delta 133p53\beta$) increased cell motility and may thus contribute to tumour invasiveness (Gadea et al., 2007).

Another example is the transrepression of focal adhesion kinase (*FAK*) gene by p53 (Golubovskaya et al., 2008). FAK is a non-receptor tyrosine kinase localized at the contact points between cells and their substratum (Burridge et al., 1992). FAK associates with integrin receptors and recruits other molecules to the site of this interaction thus forming a signalling complex that transmits signals from the extracellular matrix to the cell cytoskeleton (Mitra and Schlaepfer, 2006). FAK has been shown to play a role in proliferation and migration, but also in survival and angiogenesis, and has been proposed as a new potential therapeutic target in cancer (McLean et al., 2005; van Nimwegen and van de Water, 2007). Analysis of human FAK gene promoter has identified p53 binding sites (Golubovskaya et al., 2004) and more recently, it has been found that DNA-damaged-induced p53 showed an increased binding to FAK promoter resulting in the repression of FAK mRNA and protein expression (Golubovskaya et al., 2008).

Another important connection between p53 and metastasis involves the Twist1 protein, a regulator of embryogenesis. Twist1 has been shown to induce EMT and is over-expressed in a large fraction of human cancers (Ansieau et al., 2008). A common cancer-derived mutant p53 protein, p.R175H, up-regulates Twist1 expression in several cancer cell lines, suggesting that

increased Twist1 might occur in cancer cells as the consequence of gain-of-function mutations in *TP53* (Kogan-Sakin et al., 2011).

1.3.7 Promoting genetic and genomic stability

Among the main properties of p53 as a regulator of cell-cycle progression and apoptosis, it has been rapidly identified as a major candidate in maintaining genetic stability, a property that earned it the appellation of “guardian of the genome”. Indeed, by promoting cell cycle arrest and DNA repair in cells with genetic damage, p53 protects cells from acquiring further DNA defects that may initiate cancer or drive its progression.

Exposure to UV light, reactive oxygen species or to some drugs can lead to DNA damage such as modifications of the bases, DNA strand breaks or adducts formation. These damages are repaired by specific mechanisms, including nucleotide excision repair (NER), base exclusion repair (BER) and homologous recombination (HR) after double strand breaks. p53 has been shown to play a role in each of these mechanisms.

The NER pathway is the primary mechanism in cells for the removal of helix-distorting, replication-blocking DNA adducts induced by exogenous agents such as UV radiation and a variety of genotoxic chemicals (Sancar et al., 2004). NER can be regulated by transcriptional and post-transcriptional control of the XPA (*Xeroderma pigmentosum* group A) protein (Fan and Luo, 2010; Kang et al., 2010; Kang et al., 2011). Functionally, XPA is believed to play roles in verifying DNA damage, stabilizing repair intermediates, and recruiting other NER factors to the damaged site (Batty and Wood, 2000; Yang et al., 2006). Both ATM and ATR (ATM and RAD3-related) are protein kinases belonging to the phosphoinositide 3-kinase-like kinase (PIKK) family. These pathways are comprised of a series of DNA damage sensors, signal mediators and transducers, and downstream effectors (Branzei and Foiani, 2008; Sancar et al., 2004). p53, Checkpoint kinase-1 (Chk1), and MAPKAP Kinase-2 (MK2) are the three main downstream checkpoint proteins that can be directly or indirectly activated by ATR following UV irradiation (Helt et al., 2005; Reinhardt et al., 2007). The DNA damage-induced response of XPA nuclear import is significantly reduced in p53-deficient cells, suggesting that the ATR/p53-dependent checkpoint is required for the UV-induced XPA nuclear import and the removal of UV-DNA damage (Li et al., 2011a; Li et al., 2011b).

BER is the main pathway responsible for the repair of DNA damage caused by hydrolysis, oxygen free radicals, and simple alkylating agents. BER is initiated by the hydrolysis of the *N*-glycosylic bond linking a modified base to the deoxyribose, which excises the damaged base. As a result of glycosylase activity, apurinic or apyrimidinic (AP) sites are generated. The removal of AP sites is initiated by a second class of BER enzymes, the apurinic/apyrimidinic endonuclease redox factor 1 (APE1/Ref1). The resulting AP sites are cleaved by the AP endonuclease APE1/Ref1 and the resulting single-strands are processed by either short-patch (where a single nucleotide is replaced) or long-patch BER (Gatz and Wiesmuller, 2006; Seeberg et al., 1995). Since 1999, Rotter's group has provided accumulating evidence suggesting a direct involvement of p53 in BER (Offer et al., 2001; Offer et al., 1999). In these studies, an augmented BER activity was observed when p53 was over-expressed in cultured cells. Meanwhile, p53 was shown to interact with polymerase β indicating a direct role of p53 in BER (Zhou et al., 2001). Furthermore, APE1/Ref-1 also binds to p53, contributes to redox-dependent p53 protein stabilization, and enhances both DNA binding and p53 transactivation (Gaiddon et al., 1999; Jayaraman et al., 1997; Seemann and Hainaut, 2005). The p53 protein contributes to BER at several levels, both in a positive and in a negative fashion. Indeed, the initial step in BER is also subject to negative transcriptional regulation by p53. Following exposure to nitric oxide (NO) p53 downregulates 3-methyl adenine (3-MeAde) DNA glycosylase via transcriptional trans-repression (Zurer et al., 2004). Moreover, p53 induces *PPM1D* encoding protein phosphatase 1D (or Wip1), which interacts with Uracil DNA glycosylase 2 (Ung2) and suppresses BER. PPM1D, in turn, inhibits p53 activity through inactivating dephosphorylation of the p38 MAP kinase (Lu et al., 2004a; Lu et al., 2004b). In analogy to the Hdm2 feedback loop, these negative regulatory mechanisms executed by p53 may serve to return DNA repair to the deactivated state. The general message emerging from these observations is that p53 may enhance BER as part of its genome-stabilizing, tumour-suppressive activities. However, after activation of p53 in response to stress, high levels of p53 may repress BER to return DNA repair to the deactivated state and therefore participate to maintenance of basal DNA damage capacity. In conditions where p53 becomes activated to very high levels, such as for example in response to severe DNA damage, p53 may stop BER to accelerate apoptosis of cells that have accumulated DNA damage beyond repair capacity.

Mismatch repair (MMR) is the main mechanism that corrects DNA following DNA polymerase errors, removing mismatches in heteroduplex DNA during recombination and

preventing homologous recombination (HR). The identification of MutS homologue 2 (*MSH2*), MutL homologue 1 (*MLH1*), and postmeiotic segregation 2 (*PMS2*) as p53 target genes has linked p53 to MMR (Chen and Sadowski, 2005; Scherer et al., 2000; Warnick et al., 2001). Additionally, the p53 target genes *MSH2*, *MLH1* and *PMS2* are able to signal cell cycle arrest and apoptosis after certain types of DNA damage via p53 or its homologue p73 (Stojic et al., 2004).

p53 appears to be a major component of the DNA damage response pathway. Nevertheless, there is only limited evidence that impaired p53 function leads to an increased mutator phenotype, which is predisposing to different types of cancer, both familial and sporadic. Spontaneous tumours in a mouse model of Li-Fraumeni Syndrome (LFS) (*TP53*^{+/-} murine model) show only modest increase in mutation loads (Hill et al., 2006). In humans, there is a highly significant increase in copy number variations among carriers of germline *TP53* mutations with a familial cancer history (Shlien et al., 2008). However, as discussed in Palmero et al., *TP53* mutation carriers do not appear to be hyper-sensitive to mutagens although radio-sensitivity has been demonstrated in cultured cells and observed in some LFS patients who received radiation-based treatments (Palmero et al., 2010).

1.3.8 Controlling tumour-promoting inflammation

Inflammation is a complex biological response involving a multi-factorial network of chemical signals connecting immune system, vascular structures and tissue microenvironment designed to heal the afflicted tissue. During tissue injury associated with wounding, cell proliferation is enhanced while the tissue regenerates; cells produce signalling molecules, cytokines, growth factors, angiogenic factors, and reactive oxygen and nitrogen species (ROS and RNS). These species include hydrogen peroxide (H_2O_2), nitric oxide (NO^*), and reactive intermediates such as hydroxyl radicals (OH^*), superoxide ($O_2^{\cdot-}$), and peroxynitrite ($ONOO^-$) (de Moraes et al., 2007). It was in 1863 that Rudolf Virchow noted the presence of large amount of leucocytes in neoplastic tissues and made a connection between inflammation and cancer. He suggested that the “lymphoreticular infiltrate” reflected the origin of cancer at sites of chronic inflammation (Balkwill and Mantovani, 2001). Indeed, sustained cell proliferation in an environment rich in inflammatory cells, growth factors, activated stroma, and DNA-

damage-promoting agents, turns these protective responses into a cancer-promoting process (Coussens and Werb, 2002; Kundu and Surh, 2008; Wu and Zhou, 2009). Many aspects of chronic inflammation involve p53 in a direct or indirect way (Kamp et al., 2011). First, following DNA damage caused by generated ROS and RNS, p53 is activated and induces a growth suppressive response that contributes to preserve cell viability and genetic integrity (Hafsi and Hainaut, 2011). Second, p53 regulates the expression of genes encoding enzymes involved in the detoxification and protection against ROS. Among these genes, p53 has been shown to up-regulate cyclooxygenase 2 (*COX2*) (de Moraes et al., 2007), down-regulate mitochondrial superoxide dismutase 2 (*SOD2*) (Hussain et al., 2004), and activate glutathione peroxidase (*GPXI*) (Tan et al., 1999) and aldehyde dehydrogenase 4 A1 (*ALDH4A1*) (Yoon et al., 2004). Third, p53 represses the expression of inducible nitric oxide synthase 2 (*NOS2*), protecting cells against excess damage by NO and derivatives (Ambs et al., 1998; Forrester et al., 1996). It has also been demonstrated that *TP53* mutation is correlated with increased expression of *NOS2* and enhanced damage by nitrogen species in cancers developing in a context of chronic inflammation (Ambs et al., 1999; Vaninetti et al., 2008). Fourth, p53 entertains a complex network of functional cross-talks with NF- κ B, the main transcription factor involved in the regulation of inflammatory responses. For example, p53 and NF- κ B cooperate in up-regulating *COX2* expression, which provides a survival mechanism in chronically inflamed tissues (Benoit et al., 2006; de Moraes et al., 2007). Recently, Liu and colleagues demonstrated the involvement of p53 in the responses of inflammatory cells to lipopolysaccharide (LPS). Culture of mice LPS-stimulated neutrophils and macrophages with nutlin-3a, a specific inducer of p53 stabilization, attenuated NF- κ B DNA-binding activity and production of proinflammatory cytokines (Liu et al., 2009). In addition, a novel mechanism of p53 in the repression of NF- κ B by the glucocorticoid receptor has been described where p53 loss severely impaired glucocorticoid receptor target genes downstream NF- κ B signalling in a mouse model of LPS shock (Murphy et al., 2011). The functional cross-talk between p53 and NF- κ B may play an important role in regulating organismal senescence and life span: it seems that the efficiency of p53 signalling declines during aging whereas that of NF- κ B is clearly enhanced.

1.3.9 Regulating cell bioenergetics

Cancer cells consume large quantities of glucose and primarily use glycolysis for ATP production, even in the presence of adequate oxygen. This metabolic signature (aerobic glycolysis or the Warburg effect) enables cancer cells to direct glucose to biosynthesis, supporting their rapid growth and proliferation (Warburg, 1956a, b; Warburg et al., 1927). One of the most spectacular developments in the understanding of p53 functions is the discovery that it plays a fundamental role in cell bioenergetics. Loss of p53 activity may be pivotal for the Warburg effect (Gottlieb and Vousden, 2010; Hafsi and Hainaut, 2011). p53 appears to favour oxidative metabolism against glycolysis (Cheung and Vousden, 2010). This includes down-regulation of glucose transporters at the plasma membrane through direct inhibition of GLUT-1 and GLUT-4 (Schwartzberg-Bar-Yoseph et al., 2004), and indirect inhibition of GLUT-3 by a mechanism involving NF- κ B (Kawauchi et al., 2008). Further downstream, p53 regulates the synthesis of two rate limiting enzymes in the glycolytic pathway. It induces *TIGAR* (*TP53*-induced glycolysis and apoptosis regulator), an enzyme with fructose bi-phosphatase activities that counteracts the activity of 6-phosphofructo-1 kinase (Bensaad et al., 2006; Li and Jogl, 2009). In contrast, it down-regulates *PGM* (phosphoglycerate mutase), which converts 3-phosphoglycerate into 2-phosphoglycerate during the late ATP-generating steps of glycolysis (Kondoh et al., 2005). This antiglycolytic activity is accompanied by the upregulation of enzymes that divert the metabolism of glucose to the pentose phosphate pathway (PPP), a metabolic shunt that uses glucose for biosynthesis rather than energetic purposes. The p53 protein binds to glucose-6-phosphate dehydrogenase (G6PD), the first and rate-limiting enzyme of the PPP, and prevents the formation of the active dimer. Tumour-associated p53 mutants lack this G6PD-inhibitory activity (Jiang et al., 2011).

In parallel with its capacity to limit glycolysis, p53 maintains and promotes oxidative phosphorylation through at least two mechanisms. First, it activates *AIF*, encoding apoptosis inducing factor, a bifunctional protein with oxido-reductase function contributing to the assembly and function of complex I of the respiratory chain (Stambolsky et al., 2006; Vahsen et al., 2004). Second, p53 transactivates *SCO2* (encoding synthesis of cytochrome c oxidase 2), a copper-dependent chaperone protein that is required for the assembly of complex IV of the respiratory chain (Wanka et al., 2012). [Figure12](#) summarizes these recent observations on the role of p53 in energy metabolism.

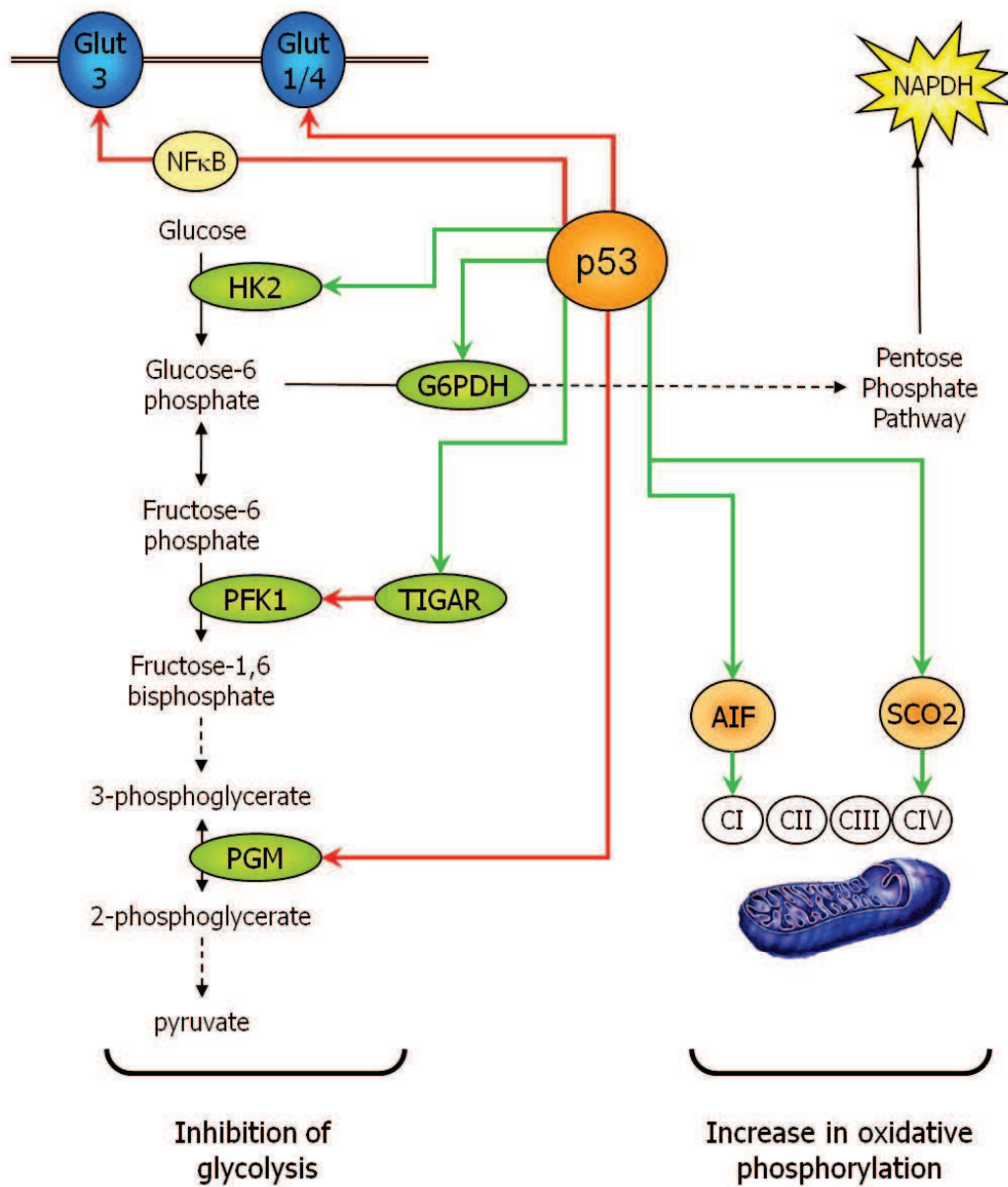


Figure 12. p53 and regulation of metabolism

p53 acts as a transcriptional regulator of genes involved in the control of energy metabolism. It participates to the inhibition of glycolysis (left) and the increase in oxidative phosphorylation (right). Green arrows: positive effects (increased expression and/or activity); red arrows: negative effects (decreased expression and/or activity) (Hafsi and Hainaut, 2011) (*see paragraph 1.2.4*).

1.3.10 Facilitating innate and adaptive immune response

The functions of p53 have been greatly extended beyond DNA repair and apoptosis. Recent studies suggest a novel role for p53 in the control of human Toll-like receptor (*TLR*) gene expression (Menendez et al., 2011). The *TLR* gene family mediates innate immunity to a wide variety of pathogenic agents through recognition of conserved pathogen-associated molecular motifs. The role of p53 in regulating *TLR* expression appears to be extremely diverse and complex. The promoters of most *TLR* family members contain both canonical and non-canonical p53 response elements, thus defining a potentially rich repertoire of dose- and context-dependent stress responses to p53 activation (Menendez et al., 2011).

In addition, several studies suggest a potential role of p53 in antiviral immunity. Mice lacking p53 have impaired and delayed antiviral response to influenza A virus (IAV), caused by disruption of both innate and adaptive immunity (Munoz-Fontela et al., 2011). In cells infected with hepatitis C virus (HCV), the HCV core protein induces the p53-dependent expression of *TAP1* (encoding transporter associated with antigen processing 1) and consecutive major histocompatibility complex (MHC) class I up-regulation, leading to a significant down-regulation of the cytotoxic activity of natural killer (NK) cells against HCV infected cells and facilitating the establishment of a chronic infection (Herzer et al., 2003).

Some other p53 activities might also directly contribute to help cancer cells to escape immune destruction. For instance, p53 represses ULBP2, a ligand for the natural killer cell immunoreceptor NKG2D, through miR-34a and miR-34c and loss of this function may contribute to eliminate an innate barrier against tumour development (Heinemann et al., 2012).

This non-exhaustive list of the multiple roles of p53 in the Hallmarks of Cancer show that these processes are deeply interconnected, representing various pathways linked in a large functional network. The broad implication of p53 supports the idea that it represents a unifying factor between Hallmarks processes. While acquisition of one or several Hallmarks capabilities drives cells towards cancer, loss of p53 disintegrates the coherence between the Hallmarks processes and removes molecular obstacles to malignancy. Thus, in cancer cells having lost p53 function, DNA damage can accumulate without inducing cell growth arrest, accompanied by a metabolic switch that confers energetic autonomy, bypassing replicative

senescence, activating embryonic developmental programs such as EMT, promoting tumour inflammation and avoiding immune destruction. In cells with normal p53 competence, transient activation of any of these capabilities will be compensated by p53-dependent down-regulation of other capabilities, thus maintaining homeostasis.

1.4 The TP53 family

For more than 20 years *TP53* has appeared to stand alone among oncogenes and tumour suppressor genes by not being a member of a multi-gene family. Until the late nineties, it was even unclear whether a true equivalent to *TP53* was present in organisms other than vertebrates. This view has considerably evolved over the past 15 years with the discovery of two homologues, *TP63* and *TP73*, which share strong structural, biochemical and biological analogies with *TP53*. Recently, an additional layer of complexity has emerged when the p53 isoforms were identified, providing another similarity that p53 and its homologues have in common.

1.4.1 TP53 homologues

1.4.1.1 p63 and p73 protein organization

1.4.1.1.1 Homologies with p53

TP63 (OMIM 602273) and *TP73* (OMIM 601990) genes are respectively located in positions 3q27.29 and 1p36 (Kaghad et al., 1997; Yang et al., 1998). They present the same intron/exon organization as p53, with a non-coding exon 1 and a long intron 1 (around 10kb in *TP53*). All family members have 3 structural domains that are essential for their function: an N-terminal transactivation domain (TAD), a DNA-binding domain (DBD) and an oligomerization domain (OD) (Figure 13). In addition, p63 and p73 have another protein/protein interaction domain at their C-terminus known as sterile alpha motif (SAM). The three main domains are well conserved and the highest level of homology is reached in the DBD (65% identity between p53 and p73, and 60% identity between p53 and p63), which suggests that the three proteins can bind to the same DNA sequences and transactivate the same promoters (Levrero et al., 2000).

This high structural homology reveals that p63 and p73 both acts as transcription factors like p53. They bind specifically to DNA onto conserved p53 response elements (p53REs) by using their DNA-binding domain (Kaghad et al., 1997; Shimada et al., 1999; Yang et al., 1998). The E3 ubiquitin ligase Hdm2 binds also to p73 but instead of promoting its degradation through poly-ubiquitination, it determines its re-localization to cytoplasm and

changes its transcriptional activity through attachment of the ubiquitin-like protein NEDD8 (Watson et al., 2006). Binding of Hdm2 to p63 has also been demonstrated but the functional outcome is still a matter of debate (Wang et al., 2001; Zdzalik et al., 2010). These proteins can undergo post-translational modifications. For example, PIAS-1 (the protein inhibitor of STAT-1) sumoylates p73, which inhibits its transcriptional activity and promotes exit from the G1 phase of the cell cycle (Munarriz et al., 2004). p300 and pCAF acetyl-transferases promote p63 and p73 acetylation and their transcriptional activity (Chae et al., 2012; MacPartlin et al., 2005) while deacetylases such as SIRT1 inhibit this function (Dai et al., 2007). Phosphorylation of p73 by the tyrosine kinase c-Abl or by p38 MAP kinase in response to DNA damage stabilizes and activates p73, while threonine phosphorylation by Cyclin/CDK complexes inhibits p73 transcriptional activity promoting progression through the cell cycle (Rufini et al., 2011; Sanchez-Prieto et al., 2002).

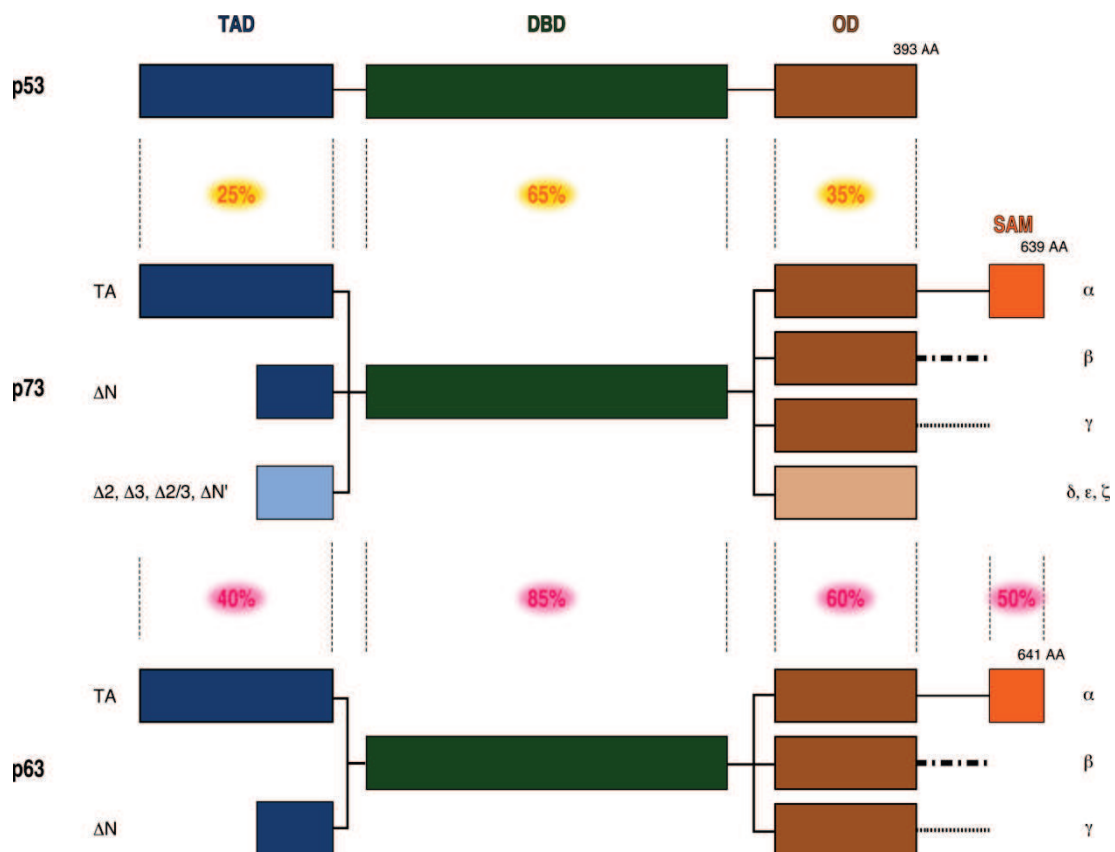


Figure 13. Homologies of the protein structures of TP53 family members

p53, p63 and p73 proteins contain a transactivation domain (TAD), a DNA-binding domain (DBD), and an oligomerization domain (OD). p63 and p73 proteins have another protein/protein interaction domain, the sterile alpha motif (SAM). The sequences of these domains are very similar (percentage in yellow: similarity between p53 and p63/p73; in pink: similarity between p63 and p73). The truncated domains of the different p63 and p73 isoforms are also indicated.

1.4.1.1.2 p63 and p73 isoforms

In the late nineties, the cloning of *TP63* and *TP73* revealed an elaborate pattern of mRNA expression resulting in several protein isoforms (Kaghad et al., 1997; Yang et al., 1998) (Figure 13). Both *TP63* and *TP73* genes can be transcribed from the proximal promoter P1 in exon 1, producing the TA forms which contain the entire TAD. An alternative promoter P2 located in the intron 3 will lead to the expression of N-terminally truncated isoforms (Δ Np63 and Δ Np73) (Ishimoto et al., 2002; Kaghad et al., 1997). Additional p73 isoforms truncated in their N-terminal domain have been described, resulting from alternative splicing of the mRNA produced from the P1 promoter: Δ Ex2p73 (missing exon 2), Δ Ex3p73 (missing exon 3), and Δ Ex2/3p73 (missing exons 2 and 3) (Ishimoto et al., 2002; Yang et al., 1998). These isoforms were found combined with several C-terminal forms generated by alternative splicing in exons 10 to 15 for *TP63* (producing five p63 isoforms: α to ϵ), and exons 10 to 14 for *TP73* (producing seven p73 isoforms: α to η) (Kaghad et al., 1997; Yang et al., 1998).

p53 family members are able to modulate the activity of each others. p53 and p73 can regulate the same response element in the *TP73* P2 promoter to induce Δ Np73 isoforms expression (Grob et al., 2001). Moreover, p53 positively regulates Δ Np63 promoter activity, while Δ Np63 α mediates the silencing of its own promoter thereby altering the pattern of p53-target gene expression (Harmes et al., 2003). Overall, p53 family members seem to be able to interplay with each other and to modulate their expression and activity in a finely-tuned manner.

1.4.1.2 p63 and p73 functions

1.4.1.2.1 Intrinsic functions

Although p53, p63 and p73 share structural and sequence similarities, p63 and p73 are rarely mutated in tumours, in contrast with p53. Instead, the *TP63* locus is amplified in 20-30% of squamous cell carcinomas of the head and neck, oesophagus or lung (Crook et al., 2000; Hibi et al., 2000; Taniere et al., 2001), and p73 is over-expressed in diverse types of cancers such as, bladder, prostate, liver and colorectal cancer (Rufini et al., 2011). While mice deficient for p53 function develop early, multiple cancers, knockdown of the entire *TP63* and

TP73 loci (targeting all isoforms) revealed the roles of p63 and p73 in epithelial differentiation and neuronal development, respectively (Mills et al., 1999; Yang et al., 2000).

p73^{-/-} mice showed increased apoptosis of sympathetic neurons in the superior cervical ganglion and reduced cortical thickness (Killick et al., 2011; Yang et al., 2000). Interestingly, when aged, p73^{+/-} mice develop an Alzheimer-like phenotype, with reduced motor and cognitive functions. The brains of these animals show atrophy and neuronal degeneration (Wetzel et al., 2008; Yang et al., 2000). The fact that no spontaneous tumours were observed could be due to the reduced lifespan of these p73^{-/-} mice. It later emerged that heterozygosity of p73 substantially shortened the animal's life span, and half of the p73^{+/-} cohort developed malignant tumours by 15 months of age. Importantly, these tumours show loss of heterozygosity of the remaining wild-type p73 allele, which is a typical hallmark of tumour suppressor inactivation in cancer. Moreover, survival of double heterozygote p53^{+/-}/p73^{+/-} mice is further reduced (from 10 to 6 months), due to an increased tumour burden and more aggressive metastatic disease. In many of the tumour specimens analyzed, the loss of heterozygosity of p73 was more frequent than the loss of the remaining p53 allele, revealing a potent tumour suppressor function for p73 (Flores et al., 2005). Specific TAp73 isoform KO mice showed an increased susceptibility to spontaneous and induced carcinogenesis, with more than 70% of the KO animals developing tumours, mainly lung adenocarcinomas (Tomasini et al., 2008). In contrast, there is no evidence of tumour development in Δ Np73^{-/-} mice, they are viable and fertile but display signs of neurodegeneration. Cells derived from Δ Np73^{-/-} mice were sensitized to DNA-damaging agents and showed an increase in p53-dependent apoptosis, suggesting a new role for Δ Np73 in inhibiting the molecular signal emanating from a DNA break to the DNA-damage response pathway (Wilhelm et al., 2010). Moreover, overexpression of oncogenic Ras influenced the TAp73/ Δ Np73 ratio in favour of Δ Np73, and the resulting downregulation of TAp73 and upregulation of Δ Np73 were important for Ras transforming activity (Beitzinger et al., 2008). Taken together, these findings support a tumour suppressive function for TAp73 and an oncogenic role for N-terminal truncated isoforms.

Differently from p73, p63 deletion does not seem to affect the central nervous system development (Holembowski et al., 2011). Total p63 KO mice show profound alterations of skin and other stratified epithelia as well as epithelial appendages including mammary and salivary glands, teeth and hair follicles. These animals also show truncated limbs and

craniofacial abnormalities as a consequence of an alteration of the apical ectodermic ridge and die soon after birth (Mills et al., 1999; Yang et al., 1999). Similarly heterozygous mutations of p63 in humans are responsible for a number of syndromes known as ectodermal dysplasias, characterized by defects of ectodermal structures such as hair, teeth, digits, sweat glands and nails (Celli et al., 1999). Two independent groups have generated these mice and have given a different interpretation of the phenotype. One group suggests that p63 is required for commitment and differentiation of epithelial precursors while the other proposes that it is essential for maintenance of the progenitor population (Mills et al., 1999; Yang et al., 1999). However, Δ Np63 facilitates the formation of the basal layer and TAp63 is able to drive the expression of proteins of the upper layers of the epidermis, suggesting that both models are not incompatible (Candi et al., 2006). The involvement of Δ Np63 in proliferation is supported by the observations in squamous cell carcinoma cell lines of head and neck cancer where *TP63* is amplified, inducing an overexpression of Δ Np63 (Hibi et al., 2000). In addition, Δ Np63 isoforms are commonly expressed as the major, if not the only, type of p63 isoform in epidermal stem cells (ESC) and in squamous cell progenitors (Medawar et al., 2008). In contrast, in squamous epithelia, TAp63 expression is restricted to differentiated cells (Nylander et al., 2002). Thus, this isoform may have an oncogenic potential. Moreover, after treatment with etoposide and doxorubicin of hepatocellular carcinoma cell lines, the expression of TAp63 isoforms was clearly induced and was accompanied by an upregulation of cell cycle arrest genes (Petitjean et al., 2005). TAp63 γ has recently been identified to play a role in NER (Liu et al., 2012). Overexpression of TAp63 γ significantly enhances NER of UV-induced DNA damage in H1299 and Saos-2 cells. TAp63 γ , but not TAp63 α/β and TAp73 $\alpha/\beta/\gamma$ isoforms, transcriptionally activates the expression of *DDB2*, *XPC* and *GADD45* through the regulation of the p53 binding elements in these genes.

1.4.1.2.2 Roles in the modulation of p53 activity

p63/p73 isoforms have opposite effects: Δ N forms are involved in cell proliferation and survival, while TA forms have a role in cell cycle arrest and apoptosis. In addition, Δ N isoforms act as dominant negatives on the TA isoforms (Nakagawa et al., 2002; Yang et al., 1998). The analogy between the *TP53* family members led to studies on the effects of Δ Np63 and Δ Np73 on p53. These two isoforms inhibit p53 transactivation activity and its ability to

induce apoptosis (Vossio et al., 2002). The dominant negative property of Δ Np63/ Δ Np73 isoforms could be exerted *via* their OD (Nakagawa et al., 2002), but this hypothesis is controversial (Ishimoto et al., 2002).

Interestingly, both p53 and TAp73 induce Δ Np73 creating an auto-regulatory feedback loop (Grob et al., 2001). By interfering with p53 and TAp73 the Δ N isoforms inhibit apoptosis and promote cell cycle progression. In response to DNA damage Δ Np73 isoforms are degraded thus allowing the TA forms to exert their effect. The role of Δ Np63 on p53 was studied in response to DNA damage. It has been shown that Δ Np63 is down-regulated following exposure to UV radiation in mouse or human keratinocytes, thus allowing p53 and TAp63 to induce cell cycle arrest or apoptosis (Liefer et al., 2000; Marchbank et al., 2003). The same decrease of expression after DNA damage was described for Δ Np73 isoform (Maisse et al., 2004).

p63 and p73 proteins not only modulate p53 suppressive functions, but also the oncogenic functions of p53 mutants. Studies show that mutant p53 (like p53R175H and p53R248W) can physically and functionally interact with p63 and p73 isoforms (Gaiddon et al., 2001; Strano et al., 2000). These findings define a network involving mutant p53 and the various spliced isoforms of p63 and p73 that may confer upon tumour cells a selective survival advantage.

1.5 The p53 isoforms

The p53 isoforms were first identified in early studies investigating p53 expression patterns. In 1984, Matlashewski et al. cloned an N-terminal variant of the human p53 mRNA, whereas in 1985, Rotter and co-workers detected an alternatively spliced C-terminal variant of mouse p53, latter isolated in human cells (Matlashewski et al., 1984; Wolf et al., 1985). However, the “p53 isoform” field has only really emerged in the past 10 years, when it became clear that *TP53* retained the elaborate patterns of isoform expression that characterizes its homologues, *TP63* and *TP73* (Marcel et al., 2011a).

1.5.1 Mechanisms of production of p53 isoforms

Like *TP63* and *TP73*, the human *TP53* gene encodes several p53 protein isoforms through conserved mechanisms (Khoury and Bourdon, 2010; Marcel et al., 2011a; Marcel and Hainaut, 2009). The main and most abundant p53 isoform is the canonical p53 protein, also termed TAp53 α , as it contains an entire TAD and the longest C-terminal domain (Figure 14). In addition to the TA forms, three Δ N forms have been identified that differ by their translation initiation site. This is used to designate them as Δ 40p53, Δ 133p53 and Δ 160p53. The four N-terminal p53 forms can be combined with three different C-terminal domains (α , β , γ). Recently, some cis- and trans-regulators have been identified as specific modulators of p53 isoform expression (Figure 14).

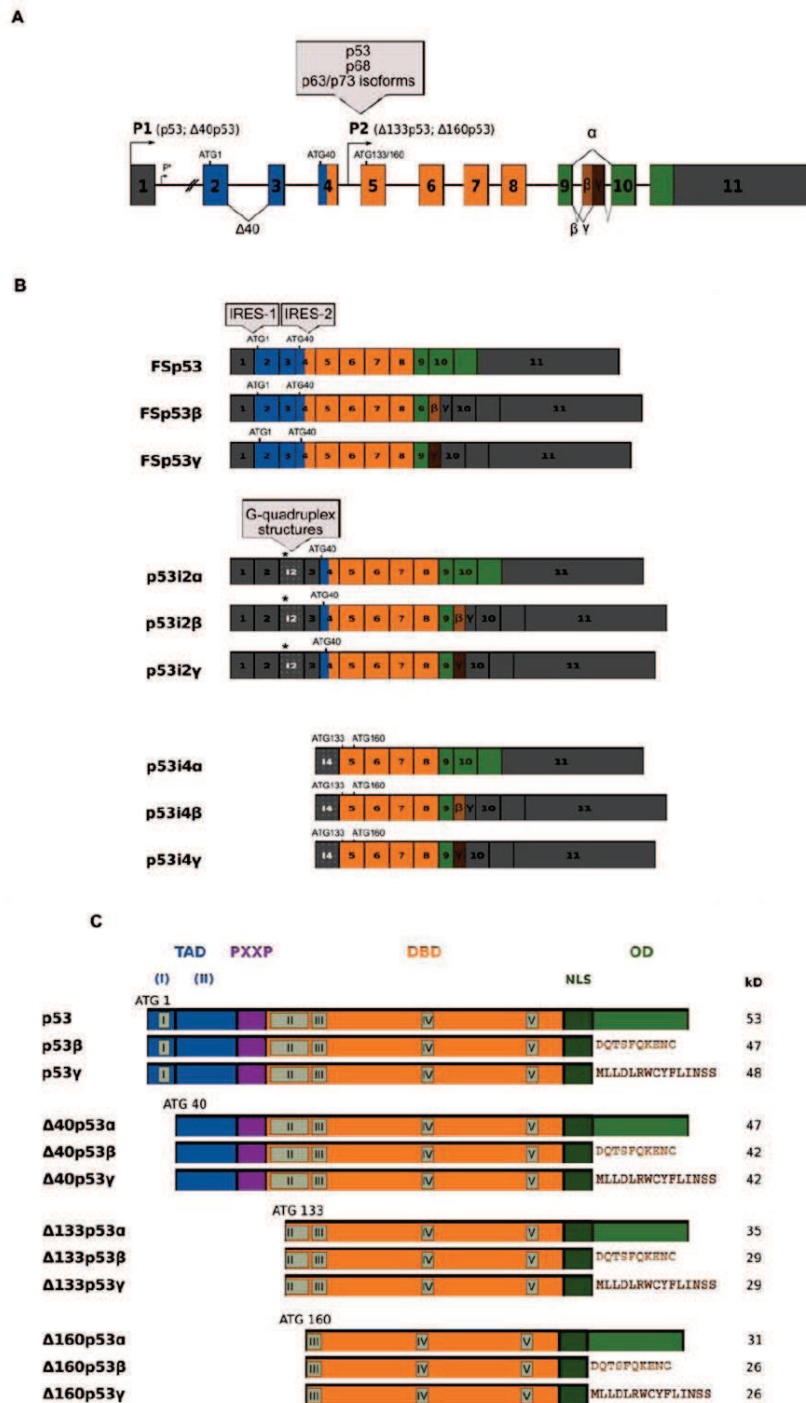


Figure 14. Representation of the structure of the human p53 isoforms

(A) Structure of the human *TP53* gene. The *TP53* gene contains 11 exons and expresses several isoforms owing to usage of alternative promoters (P1 and P2), translational initiation sites (ATG1/40/133/160) and splicing sites (^). (B) Human p53 mRNA variants. FSp53 (Fully-spliced p53); I2: intron 2; I4: intron 4; *: stop codons. (C) Human p53 protein isoforms. The canonical p53 protein contains a TAD (blue), a proline-rich domain (PXXP, purple), a DBD (orange) and an OD (green) that encompasses a NLS (green) and five regions conserved through evolution (I-V in grey boxes). At the C-terminal, the β and γ isoforms have different residues (brown). The theoretical molecular weight is indicated on the right. Coloured boxes: coding sequence; grey boxes: non-coding sequence (Marcel et al., 2011a).

1.5.1.1 $\Delta 40p53$ expression: two mechanisms for one protein

Compared to TAp53, the human N-terminal $\Delta 40p53$ forms lack the first 39 amino acids corresponding to the main TAD (Figure 14). Matlashewski et al. described for the first time an altered human p53 transcript arising by alternative splicing and retaining the entire intron 2 (Matlashewski et al., 1987). They later observed that the p53I2 mRNA contained three stop codons in-frame with the first start codon of exon 2, leading to a transcript starting to the next methionine in codon 40 (Ghosh et al., 2004). In addition to alternative splicing, $\Delta 40p53$ forms can be encoded from the fully-spliced p53 (FSp53) mRNA, which encodes the full-length p53 protein, through an internal initiation of translation at codon 40 (Courtois et al., 2002; Yin et al., 2002). Indeed, two internal ribosome entry sites (IRES) have been identified to regulate the translation of either p53 or $\Delta 40p53$ (Candeias et al., 2006; Ray et al., 2006). The relative contribution *in vivo* of these two mechanisms of expression of $\Delta 40p53$ still remains to be fully established.

Alternative splicing of intron 2 can be regulated through *G-quadruplex* structures located in intron 3 of p53 pre-mRNA (Marcel et al., 2011c). *G-quadruplexes* are three-dimensional structures, which result of the propensity of Guanine-rich sequences to fold into four-stranded, monovalent cation-dependent structures (Simonsson, 2001). Using reporter assays and RNA-*G-quadruplex* ligands, *G-quadruplex* structures appeared to promote the correct splice-out of intron 2, leading to the FSp53 mRNA. Disruption of the *G-quadruplex* structures however favoured the retention of intron 2 and thus p53I2 mRNA expression. This is the first evidence that the *TP53* sequence itself can modulate its own isoforms' expression through regulation of alternative splicing.

1.5.1.2 Production of $\Delta 133p53$ and $\Delta 160p53$

Like *TP63* and *TP73*, *TP53* contains an internal promoter that control the expression of two N-terminal isoforms (Bourdon et al., 2005). In addition to the proximal P1 promoter regulating p53 and $\Delta 40p53$ expression, an internal P2 promoter located between intron 1 and exon 5 regulates the transcription of p53 mRNAs initiated in intron 4 (p53I4) (Figure 14A and B).

Experimental studies showed that the translation of p53I4 mRNAs can be initiated at two different codons, AUG133 and AUG160, leading to the expression of $\Delta 133p53$ and $\Delta 160p53$, respectively, which lack the TAD and part of the DBD (Figure 14C) (Marcel et al., 2010a). Indeed, in the open reading frame of the human $\Delta 133p53$ mRNA, a second methionine is present at codon 160, which is contained within a Kozak's consensus motif conserved through evolution (Kozak, 1987; Marcel et al., 2010a). These observations evoke those of Courtois et al., describing the production of $\Delta 40p53$ isoform using AUG40 from p53 transcript (as described above) (Courtois et al., 2002).

Several studies have shown the capacity of p53 to regulate $\Delta 133p53$ expression (Aoubala et al., 2011; Marcel et al., 2010c). The internal P2 promoter contains p53REs located at the junction of exon 4/intron 4. p53 is able to bind these REs, resulting in an increased expression of $\Delta 133p53$ at both mRNA and protein levels. The expression of $\Delta 133p53$ can also be increased through the knockdown of p68, an RNA-helicase co-activator of p53-dependent transcription in response to DNA-damage (Moore et al., 2010).

1.5.1.3 C-terminal p53 isoforms

The alternative splicing of intron 9 of human *TP53* produces three different C-terminal p53 forms (α , β and γ) (Figure 14) (Bourdon et al., 2005; Flaman et al., 1996). Complete excision of intron 9 results in the expression of the α -forms corresponding to the classical p53 C-terminal domain. On the other hand, partial retention of intron 9 generates the β - or γ -forms, in which the OD is replaced by 10 or 15 new amino acids, respectively. However, the mechanisms that control the alternative splicing of intron 9 are unknown. The classical p53 C-terminal domain contains the main post-translational modification sites regulating p53 stability, such as the lysines residues ubiquitinated by Hdm2 (Meek and Anderson, 2009). Their absence in β - and γ -forms led to the investigation of the stability of the p53 β and p53 γ proteins by the Hdm2 ubiquitin proteasome pathway. To date, results remain controversial. For instance, whereas constitutively overexpressed FLAG-tagged p53 β or p53 γ do not appear to interact with Hdm2 or to be degraded in a proteasome-dependent manner (Graupner et al., 2009), Bourdon and co-workers observed that the endogenous p53 β protein can be degraded by the proteasome in an Hdm2-dependent manner (Camus et al., 2012).

1.5.2 Biological functions of p53 isoforms

1.5.2.1 $\Delta 40p53$ isoform

Based on their homologies with the p63 and p73 family members, the N-terminal p53 isoforms lacking the TAD (i.e., $\Delta 40p53$, $\Delta 133p53$ and $\Delta 160p53$) were expected to only act as dominant-negative regulators of p53 activity. Indeed, $\Delta 40p53\alpha$ expression down-regulated the p53-induced transactivation on reporter genes and counteracted p53-dependent growth suppression in colony formation assays (Courtois et al., 2002; Ghosh et al., 2004). In addition, in human diploid fibroblast WI38 cells, expression of endogenous $\Delta 40p53\alpha$ increased during the G1/S transition, in parallel with decreased expression of *p21WAF1* (Courtois et al., 2002). Furthermore, as $\Delta 40p53\alpha$ lacks the Hdm2-binding site, it escapes Hdm2-mediated degradation and does not accumulate in response to DNA damage, its expression persisting at low but stable amounts in many cell types (Courtois et al., 2002; Yin et al., 2002). These data support the notion that $\Delta 40p53\alpha$ inhibits basal p53 activities during cell cycle progression. However, whether $\Delta 40p53\alpha$ can exert p53-independent effects is still unknown. $\Delta 40p53\alpha$ lacks the first TAD, which has been shown to be dispensable for p53 transcriptional activity, and retains the second TAD, which can regulate gene expression (Brady et al., 2011; Ohki et al., 2007). Therefore, it is premature to consider $\Delta 40p53$ as a simple dominant negative inhibitor of p53.

A recent study by Ungewitter and Scrabble (2010) has shed a new and original light on the possible physiological role of $\Delta 40p53$ (Ungewitter and Scrabble, 2010). These authors reported that $\Delta 40p53$, in addition to being highly expressed in embryonic stem cells (ESCs), was the major p53 isoform during early stages of embryogenesis in the mouse. By altering the dose of $\Delta 40p53$ in ESCs, they identified a critical role for this isoform in maintaining the ESC state. Haploinsufficiency for $\Delta 40p53$ causes a loss of pluripotency in ESCs and acquisition of a cell cycle progression pattern, which is characteristic of somatic, differentiated cells, while increased dosage of $\Delta 40p53$ prolonged pluripotency and inhibited progression to a more differentiated state. At the molecular level, $\Delta 40p53$ appeared to control the switch from pluripotent ESCs to differentiated somatic cells by modulating the activity of full-length p53 at critical targets such as Nanog and the IGF-1 receptor (IGF-1R). These results are consistent with results in a mouse model expressing a $\Delta 40p53$ transgene, which suggested that $\Delta 40p53$

may operate as a regulator of IGF-I receptor signalling (Maier et al., 2004). These observations led Ungewitter and Maier to propose that the primary function of $\Delta 40p53$ in cells of the early embryo and stem cells, is to control their pluripotent status. However, it is unclear how $\Delta 40p53$ exert this effect through full-length p53, and how this particular activity is regulated during differentiation.

1.5.2.2 $\Delta 133p53$ isoforms

Compared with $\Delta 40p53\alpha$, the available data have clearly revealed that $\Delta 133p53$ controls p53 activity. In reporter assays, $\Delta 133p53\alpha$ acts as a dominant-negative inhibitor of p53 (Bourdon et al., 2005; Murray-Zmijewski et al., 2006). $\Delta 133p53\alpha$ does not bind to consensus p53REs *in vitro*, consistent with its partial lack of the DBD, and thus can also behave as a dominant mutant p53 protein (Marcel et al., 2010c). Nevertheless, instead of being a strict dominant inhibitor, $\Delta 133p53\alpha$ is instead a fine modulator of p53's suppressive activity as its expression determines cell fate in response to stress. Indeed, the induction of $\Delta 133p53\alpha$ expression inhibits p53-mediated apoptosis and G1 cell cycle arrest in response to doxorubicin treatment, without altering the p53-dependent G2 cell cycle arrest (Aoubala et al., 2011). These effects may be due to the ability of $\Delta 133p53\alpha$ to modulate gene expression in a promoter-dependent manner, as observed for *p21WAF1*, *Hdm2* and *Bcl-2* (Aoubala et al., 2011). Interestingly, $\Delta 133p53$ silencing has also been associated with replicative, but not oncogene-induced, senescence in normal human fibroblasts through transcriptional regulation of p53-target genes, including *p21WAF1* and miR-34a (Fujita et al., 2009). Overall, these results are consistent with an oncogenic capacity of $\Delta 133p53$.

In H1299 cells, co-expression of $\Delta 133p53\beta$ or $\Delta 133p53\gamma$ with p53 does not alter p53 transcriptional activity on the *p21WAF1* and *Bax* promoters or p53-mediated apoptosis (Bourdon et al., 2005; Murray-Zmijewski et al., 2006). $\Delta 133p53\beta$ and $\Delta 133p53\gamma$ are expressed in normal human tissues. However, several clinical studies have shown that $\Delta 133p53\beta$ and $\Delta 133p53\gamma$ are abnormally expressed in tumours, suggesting that they play a role in carcinogenesis (Bourdon et al., 2005; Song et al., 2009).

1.5.2.3 $\Delta 160p53$ isoforms

Endogenous expression of $\Delta 160p53$ protein was detected in U2OS, T47D, and K562 cells (Marcel et al., 2010a). Interestingly, the human erythroleukaemic K562 cells were described as devoid of p53 expression because of a base insertion at codon 135, leading to a frameshift and a premature stop codon, which prevents p53 protein expression (Neubauer et al., 1993). However, K562 cells express $\Delta 160p53\alpha$ and $\Delta 160p53\beta$ both at the mRNA and protein levels. Moreover, upon hemin-induced erythroid differentiation of K562 cells, only $\Delta 160p53\beta$ expression was reduced, while $\Delta 160p53\alpha$ expression was stable, suggesting that $\Delta 160p53\beta$ could play a role in erythroid differentiation.

1.5.2.4 C-terminal p53 isoforms

The biological functions of the C-terminal p53 isoforms (i.e., p53 β and p53 γ) remain poorly described and controversial. Bourdon et al. showed that, in the absence of stress, endogenous p53 β bound to the *Bax* and *p21WAF1* promoters, but only weakly to that of *Hdm2* (Bourdon et al., 2005). Moreover, in luciferase reporter assays in the absence of stress, the co-expression of p53 β and p53 enhanced the p53 transcriptional activity on the *p21WAF1* promoter but not on the *Bax* promoter, suggesting a promoter-dependent effect. These observations are consistent with the demonstration that p53 β cooperates with p53 to accelerate senescence in human fibroblasts (Fujita et al., 2009). By contrast, experimental studies failed to observe binding of FLAG-tagged p53 β or p53 γ onto p53RE consensus and to show a role of FLAG-tagged p53 β or p53 γ in p53-dependent apoptosis or senescence in cells constitutively overexpressing FLAG-tagged p53 β or p53 γ , and selected to grow in presence of neomycin (Graupner et al., 2009). Thus, there is still debate on whether p53 β or p53 γ exert their activities in an autonomous manner or through an interaction with p53. Furthermore, there is no evidence of distinct biological activities for p53 β or p53 γ . Overall, current experimental data on the biological roles of p53 isoforms are fragmented. Given that p53 isoforms differ from each other in the three functional domains (TAD, DBD and OD), their potential to modulate p53-dependent responses is expected to be diverse and cell type-dependent.

1.5.3 Lessons from animal models

Sequences homologous to p53/p63/p73 have been identified in vertebrates and even invertebrates. Surprisingly, the observation of the same pattern of expression of the human p53 family members into two classes of isoforms, TA and Δ N, is reported in animal models studies. The main three models studied to date are the drosophila, the zebrafish and the mouse, giving further insight into which of the several functions of p53 isoforms are of physiological or pathological relevance.

1.5.3.1 The *Drosophila* model

The diversification of the p53 gene family into three members occurred in vertebrates. Hence, invertebrates such as *Drosophila* contain a single p53-related gene, which contains two alternative promoters and encodes three protein isoforms: Dp53, containing 495 amino-acids and corresponding to the human full-length p53; Δ Np53, containing 385 amino-acids and considered as a general counterpart of the human N-terminal p53 forms as it is encoded by an mRNA transcribed from an internal promoter (i.e., human Δ 133) and contains a truncated TAD followed by a complete DBD and OD (i.e., human Δ 40); and Dp53 Δ C, encoded by a short transcript leading to a putative isoform bearing only the TAD (Bourdon et al., 2005; Brodsky et al., 2000; Jin et al., 2000; Ollmann et al., 2000) (Figure 15). The *Drosophila* p53 gene is activated by irradiation and exerts broad suppressive effects recapitulating those of the p53-family members, including regulation of apoptosis, aging, autophagy, differentiation and growth (Rutkowski et al., 2010). Historically, Δ Np53 was the first form identified and previously termed Dp53; hence most of the functional studies to date have focused on the role of this particular isoform. The subsequent identification of a form of *Drosophila* p53 matching the mammalian full-length (TA) p53 protein has led to a reassessment of this nomenclature, with the name Dp53 to designate the full-length protein isoform and Δ Np53 for the N-terminal truncated form.

Studies on the morphogenesis of imaginal discs have highlighted the role of Δ Np53 isoforms in the control of cell death (Brodsky et al., 2000; Brodsky et al., 2004; Fan et al., 2010; Ollmann et al., 2000). In this system, Δ Np53 controls apoptosis through the Reaper-Hid-Grim (RHG) cascade. Indeed, irradiated imaginal discs from flies with a mutant Dp53

gene show reduced apoptosis but normal cell-cycle arrest, suggesting a specific regulatory role of the Dp53 gene product in apoptosis (Brodsky et al., 2000; Brodsky et al., 2004). Apoptosis-induced proliferation requires *Drosophila* p53 and the caspase Dronc, and involves the release of mitogens such as Wingless (Wg) and Decapentaplegic (Dpp) that induce the growth of the surrounding tissues (Fan and Bergmann, 2008; Kondo et al., 2006; Wells et al., 2006). So far, the main isoform implicated in apoptosis appears to be DΔNp53, which directly regulates the *reaper* (*rpr*) gene expression, whose protein product activates caspases by inhibiting *Drosophila* Inhibitor of Apoptosis Protein (DIAP1) (Brodsky et al., 2000; Jin et al., 2000). Recently, Mollereau and co-workers found that both isoforms were capable of activating apoptosis, but that each induced distinct RHG family members (Rpr or Hid) to inhibit DIAP1. Furthermore, they observed that DΔNp53 induced *Wingless* (*wg*) expression and enhanced proliferation in the wing imaginal disc, suggesting that DΔNp53 promotes apoptosis-induced proliferation. In contrast to DΔNp53, Dp53 did not induce *wg* expression in the absence of the endogenous p53 gene (Dichtel-Danjoy et al., 2012). However, DΔNp53 also exerts effects through other pathways. In particular, DΔNp53 may activate either apoptotic or non-apoptotic responses in photoreceptor cells depending on the cell differentiation status (Fan et al., 2010; Peterson et al., 2002). Moreover, DΔNp53 appears to inhibit cellular differentiation in the retina independently of its apoptotic function (Fan et al., 2010).

The Dp53 gene product also exerts important roles in controlling lifespan in a sex- and stage-dependent manner. When overexpressed in adult flies, DΔNp53 limited lifespan in females and extended it in males. By contrast, when overexpressed during development, DΔNp53 exerted a similar dose-dependent effect on longevity in both sexes (Waskar et al., 2009). Conversely, inactivation of the Dp53 locus increased lifespan in females but had only minor effects in males. Similar phenotypes were observed in *Drosophila* expressing dominant-negative Dp53 mutant transgenes or overexpressing DΔNp53, suggesting that DΔNp53 interferes with Dp53 activity (Bauer et al., 2005). The role of *Drosophila* p53 has also been investigated in detail in other cellular functions such as DNA repair, cell cycle or aging (Biteau and Jasper, 2009; Brodsky et al., 2004; Jassim et al., 2003; Mandal et al., 2010). The study of the specific roles of each *Drosophila* p53 isoform, Dp53 and DΔNp53, will allow shedding new light on the roles of p53 isoforms in humans.

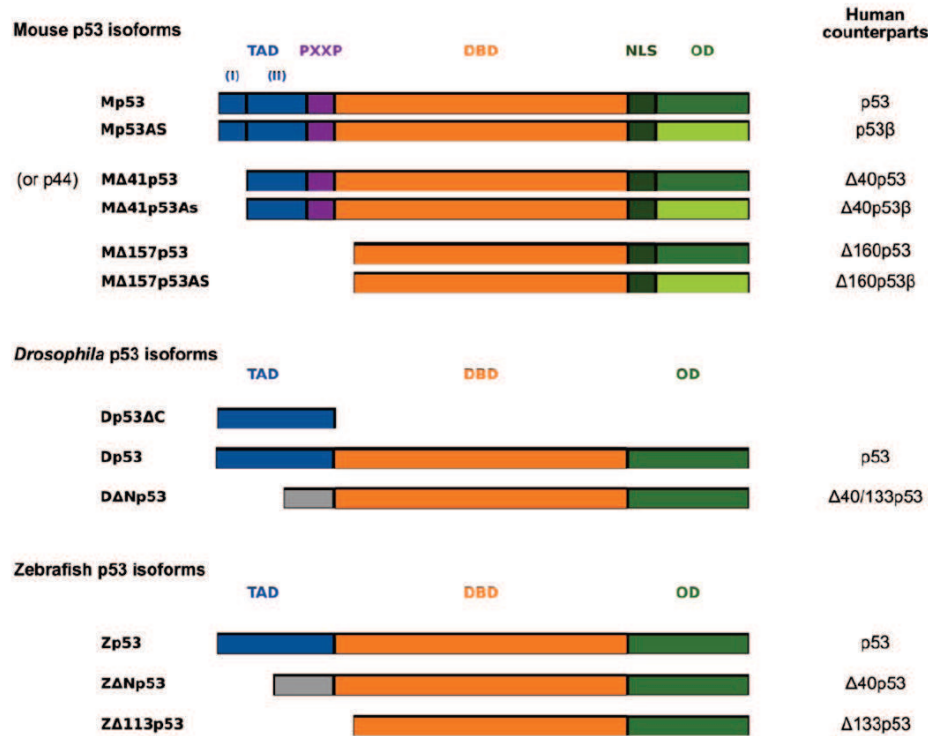


Figure 15. Structural organization of p53 isoforms in animal models

The three main domains (TAD, DBD and OD) present in human p53 are conserved in the mouse, drosophila and zebrafish. Only the mouse Mp53 contains the PXXP (purple) and NLS (green). AS: alternative splicing (green boxes); grey box: different residues compared with the full-length p53 protein (Marcel et al., 2011a).

1.5.3.2 The Zebrafish model

Zebrafish models have significantly contributed to our understanding of vertebrate development and, more recently, human disease. The growing number of genetic tools available in zebrafish research has resulted in the identification of many genes involved in developmental and disease processes. In particular, studies in the zebrafish have clarified roles of the p53 tumour suppressor in the formation of specific tumour types, as well as roles of p53 family members during embryonic development. The zebrafish p53 protein, Zp53, recapitulates the suppressive and pro-apoptotic functions of human p53 upon genotoxic stress (Storer and Zon, 2010). So far, only N-terminal Zp53 isoforms have been identified (Figure 15): Zp53, corresponding to the human p53 protein (Cheng et al., 1997); ZΔNp53, produced through an alternative splicing of intron 2 and thus similar to the human Δ40p53 forms (Davidson et al., 2010); and ZΔ113p53, produced by an internal promoter located within the zebrafish Zp53 gene that is regulated by Zp53 itself, and thus equivalent to the

human $\Delta 133p53$ isoform (Chen et al., 2005). In contrast to the human $\Delta 40p53$, AUG1 of Z Δ Np53 is located within the partial intronic sequence retained by alternative splicing (Davidson et al., 2010). Thus in Z Δ Np53, the 38 N-terminal residues containing the TAD are replaced by 33 residues derived from the intron 2 sequence. There is evidence that the Z Δ Np53 transcript accumulates in response to γ -ray irradiation (Davidson et al., 2010). In addition, ectopic expression of Z Δ Np53 resulted in a strong developmental phenotype with hypoplasia and malformation of the head, eyes and somites. This phenotype is dependent on the presence of Zp53, the two isoforms forming a protein complex through their ODs. Overexpression of an OD-mutant Z Δ Np53 or wild-type Z Δ Np53 in a mutant Zp53 background is phenotypically ineffective. These observations suggest that Z Δ Np53 exerts its effects by modulating the activity of Zp53 during zebrafish development.

Z Δ 113p53 was discovered in a different context. Chen and co-workers found that in zebrafish embryos, loss of the *Digestive organ Expansion Factor* gene (*Def*) led to defects in the morphogenesis of digestive organs. In a genome-wide screen, they identified a shorter form of Zp53 whose expression was upregulated in *Def*^{-/-} embryos (Chen et al., 2005). Upregulation of Z Δ 113p53 correlated with increased expression of p53-target genes involved in cell-cycle progression such as *cyclin-G1* and *p21WAF1*, whereas pro-apoptotic genes such as *Bax* and *Reprimo* were not activated. Furthermore, Z Δ 113p53 was found to selectively upregulate the *Bcl-2L* anti-apoptotic gene (Chen et al., 2009). This pattern of effects is consistent with the notion that Z Δ 113p53, like $\Delta 133p53$, operates as a modulator of p53 in selectively activating defined target genes.

1.5.3.3 The Mouse model

Six p53 isoforms have been described in mice, resulting from combination of three N-terminal p53 isoforms with two different C-terminal isoforms (Figure 15). The first report revealing that the mouse *TP53* gene codes for more than one functional protein was published by Rotter and colleagues in 1985, describing the presence of another p53 variant in transformed mouse fibroblasts (Wolf et al., 1985). This p53 variant was reported to be generated by alternative splicing using a cryptic 3' splicing site in intron 10, located 96 bp upstream of the regular 3' splicing site of exon 11, thereby naming it Mp53AS (alternatively

spliced) (Arai et al., 1986). A stop codon in the 96-bp insert from intron 10 leads to the production of a truncated p53 protein, in which the last 26 amino acids of mouse p53 are missing and are replaced by 17 new amino acids homologous to the human p53 β (Arai et al., 1986; Wolf et al., 1985). Later, M Δ 41p53 (also named p44) was isolated, the mouse counterpart of human Δ 40p53 forms (Mowat et al., 1985). In addition, Khoury et al. isolated a shorter N-terminal form produced by an internal promoter within the mouse p53 gene, M Δ 157p53, equivalent to the human Δ 160p53 form. Khoury et al. also showed that M Δ 41p53 and M Δ 157p53 can be expressed as a C-terminal AS variant. In stably transfected myeloid cells, Mp53AS is able to induce apoptosis, albeit with much slower kinetics (a 12-hour delay) when compared to that induced by p53, accompanied with a delay in *Bax* induction and *Bcl-2* repression (Almog et al., 1997). Indeed, Mp53AS is a less potent transactivator of *Bax* and *p21WAF1* promoters, although Mp53AS has a stronger affinity to p53REs (Almog et al., 2000). Interestingly, coexpression of Mp53 and Mp53AS in stably transfected myeloid cells or in transiently transfected p53-null H1299 cells results in an inhibition of p53 transcriptional activity and p53-mediated apoptosis (Almog et al., 2000). The p53AS inhibitory effect on p53-mediated apoptosis is Hdm2 dependent, as p53AS is able to induce higher levels of the Hdm2 protein than p53 (Almog et al., 2001).

In 2004, Maier et al. described a transgenic mouse overexpressing M Δ 41p53 (Maier et al., 2004). When expressed in a p53-null background, this isoform did not induce any particular phenotype. However, when expressed in a p53-competent background, an increased dosage of M Δ 41p53 led to reduced size, accelerated aging and a shorter lifespan associated with hypo-insulinemia and glucose insufficiency (Hinault et al., 2011; Maier et al., 2004; Pehar et al., 2010). These effects were attributed to the hyper-activation of the insulin-like growth factor (IGF)-signalling axis by M Δ 41p53, setting in motion a cascade that clamps unimpeded growth through p21WAF1 (Maier et al., 2004). Furthermore, these M Δ 41p53-overexpressing mice show cognitive decline and synaptic impairment early in life, also attributable to the hyper-activation of the IGF-1-signalling pathway (Pehar et al., 2010). These observations are consistent with studies performed *in vitro* and in the zebrafish model, indicating that Δ 40p53 isoforms may regulate growth suppression through modulation of p53 activity.

A premature aging phenotype was also reported in a non-physiological knock-in p53 mouse model expressing an M Δ 122p53 form, truncated for the first 122 residues (Slatter et

al., 2011). Although M Δ 122p53 has no physiological equivalent in mouse, it can be considered as an “intermediate” between the M Δ 41p53 and M Δ 157p53 isoforms, as it lacks the TAD and part of the DNA-binding domain. During adulthood, transgenic p53 ^{Δ 122p53/ Δ 122p53} mice showed premature aging symptoms, such as balding and arthritis, similar to that observed in the p53^{+/^m} mice (deletion exons 1-6) (Slatter et al., 2011; Tyner et al., 2002). In addition, earlier tumour onset and shortened lifespan were observed in p53^{+/ ^{Δ 122p53}} mice as compared with p53^{+/-} mice.

Overall, these results support the hypothesis that mouse N-terminal p53 forms may operate as dominant oncogenes to promote cell proliferation and inflammation. The studies summarized above highlight common themes in the p53 isoform field. First, overall patterns of isoform expression are well-conserved throughout evolution. Second, N-terminal isoforms have a major role as regulators of physiological processes related to development, aging, lifespan and, possibly, carcinogenesis. In this respect, two key mechanisms are emerging. The Δ 40p53 form exerts regulatory effects on signalling cascades controlled by p53, perhaps through direct interaction between the two isoforms. Conversely, isoforms corresponding to Δ 133p53 α modulate cell response by regulating gene expression in a p53-dependent and -independent manner. It should, however, be remembered that the current view of isoform activities remains fragmentary and that further studies are needed to better understand their roles and underlying mechanisms.

1.6 Functional inactivation of p53 in cancer

The regulation of p53 function is highly complex and occurs at many levels, thus involving a spectrum of determinants that activate specific components of the p53 network appropriate for the circumstances. Each of the stress responses tends to prevent cellular transformation and tumourigenesis by removing damaged cells from the proliferative cycle. It is thus obvious that inactivation of p53 by either mutation or deregulation of its pathway is commonly observed in human cancer. This part will summarise the principal mechanisms by which p53 function is altered in cancer, highlighting recent discoveries. Within this section p53-based anti-cancer therapeutic strategies developed to reactivate or restore p53 functions will be briefly presented.

1.6.1 Viruses

The small DNA tumour viruses, Polyoma virus, Simian Vacuolating Virus 40 (SV40), the Papilloma viruses and the human Adenoviruses, were first described during a period of intense virus discovery (1930-1960s) and shown to produce tumours in animals (reviewed in (Levine, 2009)). In each of these cases the viral DNA was shown to persist (commonly integrated into a host chromosome) and only a selected portion of this DNA was expressed as mRNA and proteins in these cancers (Botchan et al., 1974; Gallimore, 1974). For SV40, this region corresponded to the portion of the viral chromosome expressed early after infection and encoded two proteins, the SV40 large T-antigen and the small t-antigen (Huebner et al., 1963). For the Adenoviruses, this region corresponds to a subset of the early genes termed the E1A proteins and the E1B proteins composed of the E1B-55Kd and E1B-21Kd proteins (Ross et al., 1980a; Ross et al., 1980b). Subsequent experiments with the human papilloma viruses (HPV) types 16 and 18 uncovered a similar set of early proteins called the E6 and E7 proteins (Scheffner et al., 1994). The E6 and E7 proteins, encoded by the oncogenic HPV types 16 and 18, are viral products expressed in HPV-associated cancers. The viral encoded tumour antigens were identified and shown to be required to both establish the tumour and maintain the transformed cell phenotype (Howie et al., 2009; Pipas and Levine, 2001; Ross et al., 1980a).

The functions of these viral tumour antigens were explored and shown to have common features and mechanisms even though they appear to have evolved from diverse genes. The SV40 large T-antigen, the HPV E7 protein and the Adenovirus E1A protein were shown to bind to and inactivate the functions of the Retinoblastoma proteins in transformed cells (DeCaprio et al., 1988; Dyson et al., 1989; Whyte et al., 1988). This resulted in the activation of the E2F and DP transcription factors and the entry of cells into the S-phase of DNA synthesis which was required for viral DNA replication. These events triggered the activation of p53, which promotes apoptosis of these virus-infected cells limiting virus replication and tumour formation (Wu and Levine, 1994). These viruses responded by evolving and producing the SV40 large T- antigen, the HPV E6 protein and the Adenovirus E1B-55Kd protein, which bind to and inactivate the p53 functions in both the infected and transformed cells.

After initial observations that the SV40 large T-antigen was forming a complex with p53, it became later apparent that SV40 was in fact inactivating the functions of the p53 protein in tumours and transformed cells (May and May, 1999; Tegtmeier et al., 1977; Tegtmeier et al., 1975). Then, it was observed that an Adenovirus oncogene product, the E1B-55Kd protein, was able to form a protein complex with the p53 protein in virus infected and transformed cells (Sarnow et al., 1982). This viral protein also inactivates the functions of the p53 protein and adds to the ability of this virus to transform cells and produce tumours in animals. Several years later, it was demonstrated that the E6 oncogene products of HPV-16 and 18 (the tumourigenic human Papilloma viruses) were bound to the p53 protein in HPV transformed cells (Werness et al., 1990). The E6 protein recruited the E6-associated protein ligase (E6-AP) and this resulted in the poly-ubiquitination of the p53 protein and its rapid degradation in the proteasome of a transformed cell (Scheffner et al., 1990). This observation explains the rarity of *TP53* mutations in cervical cancers (Crook et al., 1992). Thus SV40, the Adenoviruses and the tumourigenic Papilloma viruses all targeted the same cellular protein for inactivation with their oncogene products and in so doing eliminated a tumour suppressor activity from these cells. Because the SV40 T-antigen, the Adenovirus E1B-55Kd and the HPV E6 proteins are completely unrelated in their amino acid sequences, this is an example of convergent evolution to satisfy a need that must be required for these viruses to replicate in cells.

1.6.2 TP53 mutations

Somatic mutations are the cornerstone of cancer. The development of cancer involves the contributions of many heritable genetic events as well as a large number of epigenetic changes, but what makes the turning point between untransformed and transformed cell irreversible is the acquisition of somatic mutations, conferring to cells a selective advantage for clonal proliferation.

The *TP53* gene is one of the most frequently altered genes, with over 50% of all the mutations described in human cancers (<http://www-p53.iarc.fr/>). Somatic *TP53* gene alterations are frequent in most human cancers (Figure 16) and germline *TP53* mutations predispose to a wide spectrum of early-onset cancers (Li-Fraumeni (LFS) and Li-Fraumeni-like syndromes (LFL)) (Hainaut and Hollstein, 2000; Petitjean et al., 2007) (Figure 17).

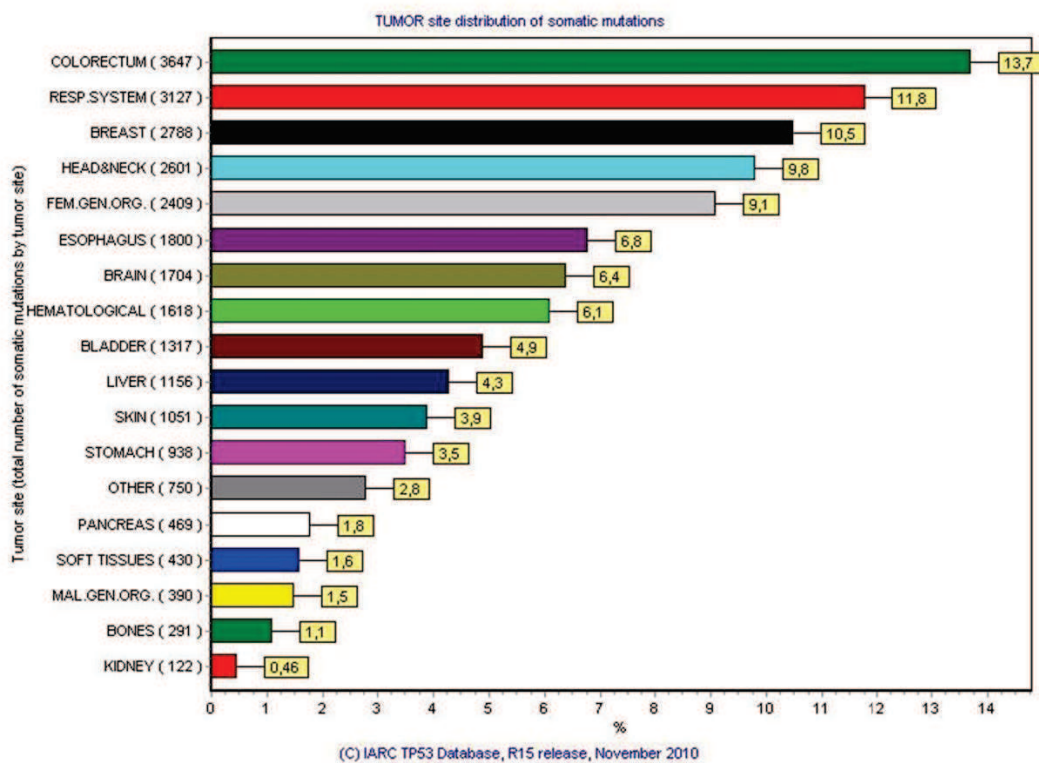


Figure 16. Tumour site distribution of somatic *TP53* mutations

<http://www-p53.iarc.fr/> (Petitjean et al., 2007)

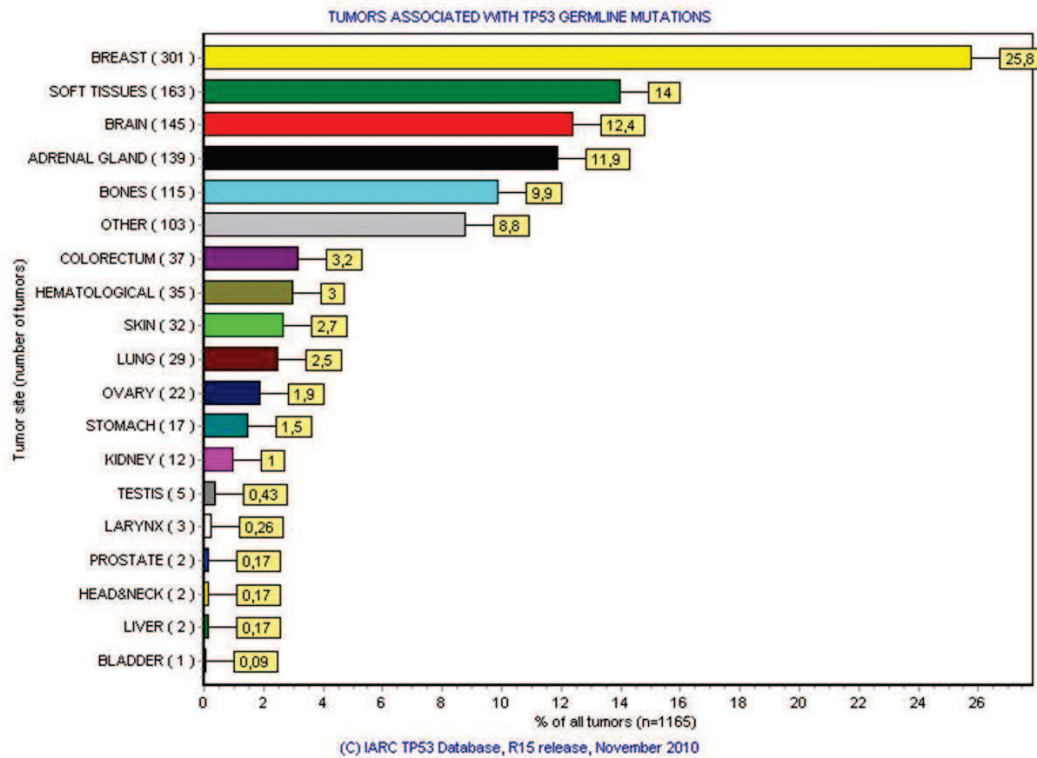


Figure 17. Tumour site distribution of germinal *TP53* mutations

<http://www-p53.iarc.fr/> (Petitjean et al., 2007)

Somatic *TP53* mutations occur in almost every type of cancer at rates from 38%-50% in ovarian, oesophageal, colorectal, head and neck, larynx and lung cancers to about 5% in primary leukemia, sarcoma, testicular cancer, malignant melanoma and cervical cancer (Olivier et al., 2010). The main modes of *TP53* inactivation are single-base substitution and loss of alleles. In contrast to other tumour suppressor genes that are mainly altered by truncating mutations, most *TP53* mutations are missense substitutions (75%) and cause single amino-acid changes at many different positions. Other alterations include frameshift insertions and deletions (9%), nonsense mutations (7%), silent mutations (5%) and other infrequent alterations (Olivier et al., 2002). Of note, 74% of *TP53* mutations fall within the DBD, more than 30% of which are at codons encoding residues that play essential structural and chemical roles in the contact with p53RE (Hainaut and Hollstein, 2000; Petitjean et al., 2007). These hotspot mutants have been classified as contact mutants, that retain the overall architecture of the DBD with loss of a critical DNA contact (i.e. R248 and R273); and structural mutants, which induce conformational changes in the DNA binding surface (i.e.

R175, G245, R249 and R282) (Joerger and Fersht, 2007b; Olivier et al., 2010). Outside the DBD, missense mutations represent only about 40%, the majority of mutations being nonsense or frameshift (Olivier et al., 2010). Mutants located in distant sites are presumed to inactivate p53 function by virtue of defects in protein conformation (Goh et al., 2011).

Experimental assays have been performed in yeast and human cells to measure various properties including: (1) transactivation activities (TA) of mutant proteins on reporter genes placed under the control of p53-RE; (2) induction of cell-cycle arrest or apoptosis; (3) dominant negative effects (DNE) over the wild-type protein inducing loss of function (LOF); (4) temperature sensitivity; (5) activities of mutant proteins that are independent and unrelated to the wild -type protein (gain of function (GOF)) (Figure 18).

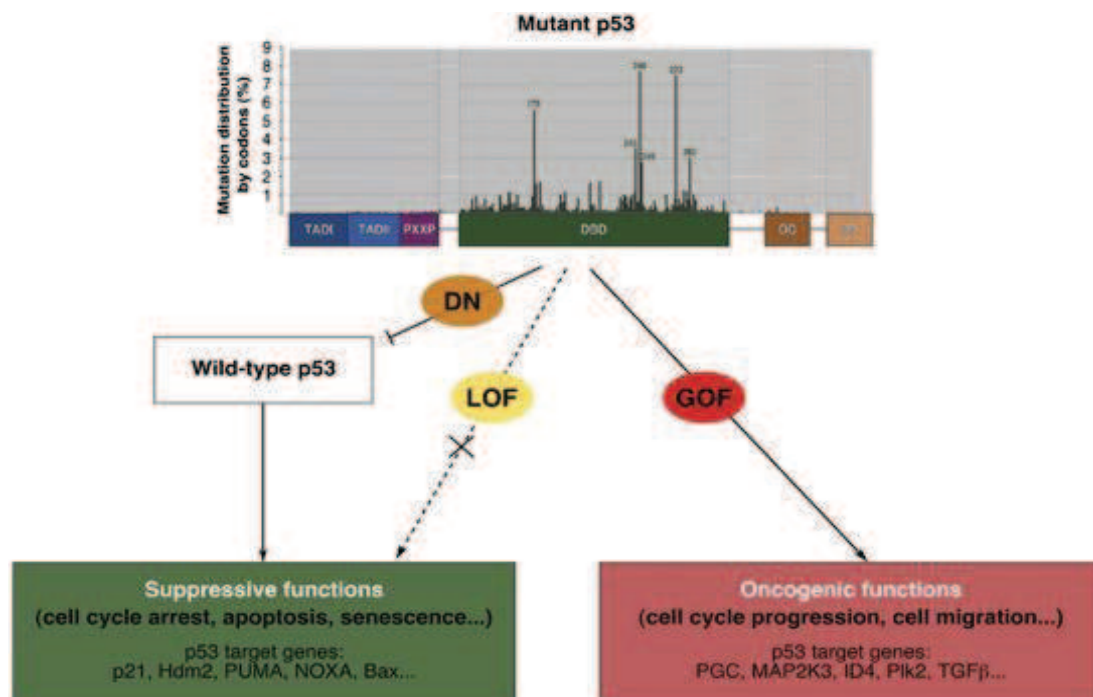


Figure 18. Properties of mutant p53 proteins

Missense mutations at hot-spot codons located in the DNA binding domain of p53 are the most frequent alterations in human cancers. Three types of p53 mutants have been described: dominant negative mutants (DN) inhibit the suppressive function of the residual wild-type p53 protein; loss of function mutants (LOF) lack suppressive function; while gain of function mutants (GOF) exhibit oncogenic properties through an array of mutant specific activities including aberrant protein interactions or gene regulation. TADI/II: transactivation domain I/II; PXXP: proline-rich domain; DBD: DNA binding domain; OD: oligomerization domain; BD: basic domain (Goldstein et al., 2011).

It has been hypothesized that reactivation of mutant p53 in tumours should trigger massive apoptosis and eliminate tumour cells. PRIMA-1 is a molecule found to be capable of restoring sequence-specific DNA binding and the active conformation to mutant p53 proteins *in vitro* and in living cells (Bykov et al., 2002b). PRIMA-1 rescued both DNA contact and structural p53 mutants. *In vivo* studies in mice revealed an antitumour effect with no apparent toxicity (Bykov et al., 2002a). Another way to reactivate p53 is by impairing the interaction Hdm2-p53. Overexpression of Hdm2, found in many human tumours, effectively impairs p53 function. Small-molecule antagonists of Hdm2, such as Nutlins, have been found to bind Hdm2 in the p53-binding pocket and activate the p53 pathway in cancer cells, leading to cell cycle arrest, apoptosis, and growth inhibition of human tumour xenografts in nude mice (Vassilev et al., 2004).

1.6.3 The p53/Hdm2/Hdm4 /p14ARF connexion

Abnormal accumulation of the Hdm2 protein is observed in many tumours, especially sarcomas (Onel and Cordon-Cardo, 2004). This accumulation can be due to amplification of the *Hdm2* gene, enhanced transcription of the gene or enhanced translation of its messenger RNA (Michael and Oren, 2002). A database listing the *Hdm2* gene amplifications showed that the overall frequency of Hdm2 amplification is 7% (Momand et al., 1998). The highest frequency is observed in soft tissue tumours (20%) and osteosarcomas have the second highest frequency of *Hdm2* gene amplification (16%), followed by oesophageal carcinomas (13%). In melanomas, where *TP53* gene is rarely mutated (95% wild-type), amplification of *Hdm2* is detected in 3-5% of the cases (Chin, 2003; Muthusamy et al., 2006).

In early studies, *Hdm2* was shown to be amplified and overexpressed in soft tissue sarcomas, and its increased level was found to be mutually exclusive with mutation of *TP53* (Oliner et al., 1992; Reifenberger et al., 1993). Although these tumours would be expected to no longer express p53, the opposite situation is generally observed, with a large number of tumours overexpressing both p53 and Hdm2. The reasons for this apparent paradox have not been elucidated. No formal exclusion between *TP53* gene mutation and Hdm2 accumulation has been clearly demonstrated.

Interestingly, a recent study has demonstrated an overexpression of *Hdm4* as an alternative way to inactivate p53 in human melanomas (Gembarska et al., 2012). In these tumours, Hdm4 is overexpressed in 65% of the cases, suggesting that Hdm4 has an important oncogenic role to alter p53 functions. Several efforts have focused on blocking Hdm2 as a strategy to reactivate p53 (Canner et al., 2009; Hedstrom et al., 2009; Issaeva et al., 2004; Shangary et al., 2008; Vassilev et al., 2004). One major limitation of Hdm2-specific therapy could be explained in tumour cells where Hdm2 expression is low and Hdm4 is high.

Alteration of upstream signals corresponds to inactivation of the *CDKN2* locus, which expresses both p14ARF protein, an activator of p53 in response to an oncogenic stress and the cyclin kinase inhibitor p16. The interaction of p14ARF with Hdm2 blocks Hdm2 shuttling between the nucleus and cytoplasm via the nucleolus (Iwakuma and Lozano, 2003). Sequestration of Hdm2 in the nucleolus thus results in activation of p53. Germline alterations (point mutations) and somatic alterations (point mutations and hypermethylation of the promoter) of this gene are frequent in malignant melanoma and impair the induction of p53 after an oncogenic stress (Chin et al., 1998).

1.6.4 Alternative pathways

Twist1 and Twist2 are closely related members of a family of basic helix-loop-helix (bHLH) transcription factors involved in gastrulation and mesoderm specification. Typically, Twist proteins regulate the expression of target genes by binding, as homo- or heterodimers, to E-box-containing promoters.

By investigating the role of Twist1 in the context of sarcomas, this study highlights an alternative mechanism of p53 inactivation and provides evidence that Twist1 promotes Hdm2-mediated degradation of p53 by directly interacting with its C-terminal regulatory domain and by interfering with key phosphorylations (Piccinin et al., 2012).

In inflammatory breast cancers or neuroblastomas, molecular and immunohistochemical analyses demonstrate accumulation of wild-type p53 in the cytoplasm of tumour cells, leading to functional inactivation of p53 (Moll et al., 1996). A new protein has been isolated, Parc (a Parkin-like ubiquitin ligase), that has been found to sequester p53 in the cytoplasm of cells in

the absence of any lesions (Nikolaev et al., 2003). Abnormal accumulation of this protein is observed in neuroblastomas cells and could therefore account for functional inactivation of p53 in this cancer.

1.6.5 TP53 polymorphisms

The *TP53* gene is highly polymorphic, with over 80% of known single-nucleotide polymorphisms (SNPs) located within introns or non-coding 5' and 3' sequences (Petitjean et al., 2007). Marcel et al. showed that *G-quadruplex* structures, formed in intron 3 and regulating intron 2 splicing (and thus $\Delta 40$ form expression), overlap a common polymorphism, *TP53* PIN3, which consists of a 16-bp duplication (A1, non-duplicated allele; A2, duplicated allele) (Lazar et al., 1993; Marcel et al., 2011c). Therefore, this polymorphism may modulate the structure and/or the stability of the *G-quadruplexes*, and affect $\Delta 40$ p53 expression.

Consistent with a functional effect, *TP53* PIN3 has recently been identified as a strong genetic modifier of germline *TP53* mutations. Indeed, carriers bearing A1A1 genotypes developed their first cancer on average 20 years earlier than carriers with an A1A2 genotype (Marcel et al., 2009). This effect was detected in a Brazilian cohort, in which the p.R337H mutation of partial penetrance is very common owing to a widespread founder effect (Garritano et al., 2010). It remains to be demonstrated whether a similar effect is observed in carriers of other *TP53* mutation types. Landi and co-workers have identified 11 different haplotypes defined by eight SNPs in a region from exon 3 to intron 4, overlapping part of the internal P2 promoter regulating $\Delta 133$ p53 and $\Delta 160$ p53 expression (Bellini et al., 2010). Using these different haplotypes as promoters to drive luciferase expression, they found significant differences in basal promoter activity that were confirmed by analysing endogenous $\Delta 133$ p53 expression in samples of normal colonic mucosa. Furthermore, *in silico* and *in vitro* DNA binding analyses suggested that a SNP in intron 4 (rs179287; C>T) affects proteins binding within the P2 promoter (Bellini et al., 2010). These results suggest that genetic polymorphisms may modulate basal $\Delta 133$ p53 expression. Whether these effects influence cancer risk remains to be demonstrated.

1.6.6 A role for p53 isoforms?

Given the fact that N-terminal p53 isoforms act as inhibitors of p53 transactivation, it can be expected that overexpression of certain isoforms may represent an alternative to a mutation in *TP53* for inactivating p53 in cancers, as observed in some small clinical studies (Avery-Kiejda et al., 2008; Bourdon et al., 2005; Machado-Silva et al., 2010).

There is emerging evidence that p53 isoform expression is deregulated in human cancers. In a study of 245 primary ovarian cancers, Hofstetter et al. observed that expression of p53 β was associated with serous and poorly differentiated cancers, and, when expressed together with the functional p53 protein, it was correlated with poor recurrence-free and overall survival (Hofstetter et al., 2010). By contrast, tumours expressing functional p53 and $\Delta 40$ p53 showed improved recurrence-free survival of patients compared with tumours expressing no $\Delta 40$ p53. Studies of patients with acute myeloid leukemia have shown that elevated expression of p53 β and/or p53 γ in blood cells was correlated with improved responses to chemotherapy (Anensen et al., 2006), which may predict decreased chemoresistance and improved overall survival. An effect of isoform expression has also been observed on breast cancer prognosis. In a cohort of 127 breast cancer patients, Thompson and co-workers reported that patients whose tumours expressed both mutant p53 and p53 γ mRNAs had a prognosis as good as patients whose tumours expressed a wild-type p53, suggesting that the expression of p53 γ may abrogate the poor prognosis commonly associated with *TP53* mutations (Bourdon et al., 2011).

2. Objectives

2 CONTEXT, OBJECTIVES AND APPROACHES

2.1 Context

For a long time after its discovery in 1979, p53 has been considered as an “orphan” protein, the product of a gene having no homolog in the genome of vertebrates. The discovery in 1997-98 of two distinct p53-related factors, p63 and p73, has led to re-think p53 as one member of a multi-gene family (Courtois et al., 2004b). Significantly, p63 and p73 were shown to retain a number of conserved features in their structure and expression patterns. In particular, these two proteins were found to be expressed as multiple isoforms due to alternative splicing, alternative promoter usage and alternative initiation of translation (Petitjean et al., 2005). These isoforms consisted in versions of p63 with defined truncations in the N-terminal domain (encoding the main transactivation domain) or the C-terminal domain (encoding a number of regulatory signals). The Δ Np63 and Δ Np73 isoforms were found to represent major isoforms of the protein, responsible for the bulk of protein expression in many cells. In contrast, TAp63 and TAp73 forms were found to be expressed at relatively low levels.

This observation attracted attention on the possibility that p53 may also retain a standard pattern of isoform expression. Studies published before the discovery of p63 and p73 had reported alternative forms of p53 in mouse and humans, although these forms appeared to have a restricted, and perhaps tissue-specific, expression patterns (Matlashewski et al., 1987; Wolf et al., 1985). The field of p53 isoforms started to emerge in 2002-2004 when it became apparent that many p53 isoforms could fit into the same N- or C-terminal truncation pattern as p63/p73 isoforms. Following the initial discovery of Δ 40p53 (initially called Δ Np53) (Courtois et al., 2002; Yin et al., 2002), a wide range of N- and C-terminal isoforms has been identified, raising the current isoform tally to at least 12 potential isoforms (Bourdon et al., 2005; Marcel and Hainaut, 2009). However, so far, the biochemical functions of each isoform, and its functional interaction with TAp53, have remained elusive.

Currently, two distinct N-terminal isoforms of p53 have been widely recognized, Δ 40p53 and Δ 133p53. Early studies have shown that each of them is impaired in its transcriptional

activity due to lack of the main transactivation domain (located in residues 1-42 of wild-type p53) (Aoubala et al., 2011; Courtois et al., 2002; Ghosh et al., 2004; Marcel et al., 2010c; Yin et al., 2002). Therefore, they have the potential to operate as dominant-negative inhibitors of p53 functions. However, there are so far very limited clues on how, when and where p53 isoforms exert their physiological functions. Moreover, whether expression of isoforms is deregulated in cancer has not been systematically addressed. Indeed, given their potential to inhibit p53 transactivation capacity, $\Delta 40$ p53 and $\Delta 133$ p53 may represent a plausible alternative mechanism to mutation for inactivation of p53 function.

Animal models of overexpression of p53 isoforms in vertebrates are available in the mouse ($\Delta 40$ p53) (Maier et al., 2004) and in the Zebrafish ($\Delta 40$ p53, $\Delta 133$ p53) (Davidson et al., 2010). These models have shown surprising results. Whereas the expression of N-terminal isoforms in a p53-null background has no clear phenotypic effect, expression in a background of wild-type TAp53 induced a phenotype of premature aging in the mouse and of perturbed development in Zebrafish. These observations suggested that N-terminal isoforms could exert strong effects by modulating the activity of wild-type TAp53.

2.2 Objectives

My Thesis is focused on the two N-terminal isoforms $\Delta 40p53$ and $\Delta 133p53$ and aims at better understanding their impact on overall p53 suppressor effects *in vitro* and in tumour tissues. Specifically, we have addressed the following objectives:

- To determine whether $\Delta 40p53$ interferes with p53 DNA-binding and transactivation capacity in intact cells, and to analyse how $\Delta 40p53$ may interfere with wild-type TAp53 activity, depending upon the relative levels of expression of each of these proteins;
- To analyse the mechanisms that regulate the expression of $\Delta 133p53$ and to assess the differences in the biochemical activities of $\Delta 40p53$ and $\Delta 133p53$;
- To analyse $\Delta 40p53$ and $\Delta 133p53$ expression in melanoma, a tumour type in which inactivation of *TP53* by mutation is infrequent, in order to determine whether isoform expression may represent a plausible alternative mechanism to inhibit p53 suppressor function.

2.3 Approaches

To address the first two of these points, we have used several well-defined cultured cancer cell lines that lack functional p53 protein and we have transfected p53 isoforms together with TAp53 in defined concentrations and conditions. We have then assessed the effects on p53 transactivation capacity and on DNA-binding activity. For $\Delta 133$ p53, we have further analysed the nature of the specific promoter regulating this isoform and we have shown that it contains p53-dependent Response Elements, suggesting that the two p53 isoforms, although having overlapping molecular and biochemical effects, might be regulated in response to very different signals.

To address the third point, we have turned to cutaneous melanoma, a highly metastatic tumour in which *TP53* mutation is rare and evidence for functional inactivation of wild-type p53 is building-up. We have analysed $\Delta 40$ p53 and $\Delta 133$ p53 expression levels in well-characterized lesions grouped in categories according to the extent of metastatic spread. Our results show enhanced isoform expression in most highly metastatic lesions, consistent with the hypothesis that isoform expression may play an active role in neutralizing p53 in melanoma.

Overall, our results contribute to the view that p53 isoform expression is not a side-effect of p53 regulation but is a potentially important mechanism for the fine-tuning of p53 functions. Furthermore, they indicate that deregulation of isoform expression may selectively contribute to cancer progression in tumours which retain wild-type p53 alleles.

3. Results

3 RESULTS

3.1 Effects of $\Delta 40p53$, an isoform of p53 lacking the N-terminus, on transactivation capacity of the tumour suppressor protein p53

Article No.1

Submitted to: BMC Cancer

3.1.1 Context

The p53 protein is expressed as multiple isoforms that differ in their N- and C-terminus due to alternative splicing, promoter or codon initiation usage (Marcel and Hainaut, 2009). $\Delta 40p53$ can be produced from two different mechanisms, initiation of translation at AUG 40 in fully spliced p53 mRNA or by alternative splicing retaining intron 2 (Courtois et al., 2002; Ghosh et al., 2004; Yin et al., 2002). Consequently, $\Delta 40p53$ lacks the first 39 residues containing the main transcriptional activation domain. It has been previously shown that $\Delta 40p53$ was unable to transactivate p53 target genes using luciferase reporter constructs, and that overexpression of $\Delta 40p53$ antagonized wild-type p53 transcriptional activity (Courtois et al., 2002). However, animal models overexpressing $\Delta 40p53$ in mouse or zebrafish have shown complex phenotypes suggestive of p53-dependent growth suppressive effects (Davidson et al., 2010; Maier et al., 2004; Pehar et al., 2010). These observations are not compatible with a simple role of $\Delta 40p53$ as an antagonist of wild-type p53, suggesting that when expressed in conditions where proteins are at roughly equivalent effect, $\Delta 40p53$ may modulate TAp53 activity in a subtle way.

The aim of this study is to analyse *in vitro* the effects of $\Delta 40p53$ on TAp53 transcriptional activity in conditions where both proteins are expressed at roughly equivalent levels.

3.1.2 Strategy

We performed co-transfections of constitutive (CMV-driven) expression vectors for TAp53 (full-length p53, corresponding to the α C-terminal isoform) and $\Delta 40p53$ (also as α C-

terminal isoform) in p53-null cell lines to identify the formation of hetero-tetramers between both isoforms. We have analysed the capacity of these hetero-oligomers to bind specifically to DNA, using an Electro-Mobility-Shift Assay (EMSA) with a ^{32}P -labeled p53 Response Element (p53RE) consensus oligonucleotide. We then measured in Saos-2 and H1299 cells the transcriptional activity of p53, using a β -galactosidase reporter gene assay, in presence of different amounts of $\Delta 40p53$. To precisely assess the inhibitory power of low levels of $\Delta 40p53$ on TAp53 activity, we have used a strategy previously developed by Chan et al. (2004) to investigate how many mutant p53 molecules were needed to inactivate a p53 tetramer (Chan et al., 2004). Finally, to determine how $\Delta 40p53$ may modulate the transcriptional activity of TAp53, we hypothesized that $\Delta 40p53$ could interfere with Hdm2-mediated degradation of mixed p53/ $\Delta 40p53$ oligomers. We thus co-transfected p53, $\Delta 40p53$ and Hdm2 expression vectors in Saos-2 cells and analysed p53 stability.

3.1.3 Results

We first analysed the intrinsic turnover of $\Delta 40p53$ in two cell lines that constitutively express detectable levels of this isoform, either together with TAp53 (HCT116) or alone (21PT). The breast cancer cell line 21PT contains an insertion of 1 base at codon 33 in *TP53*, leading to frameshift and to a premature stop downstream of the initiation codon at AUG 1. However, the sequence downstream of the internal initiation codon at AUG 40 is intact and usage of this codon results in the expression of $\Delta 40p53$. We show that, in both cell contexts, $\Delta 40p53$ has a slow turnover (several hours) as compared to the rapid turnover of TAp53, consistent with the notion that this isoform lacks the binding domain from Hdm2 and therefore escapes Hdm2-mediated degradation. Next, we show that $\Delta 40p53$ can form hetero-oligomers with p53. By EMSA and super-shifting experiments using an antibody to the N-terminus of TAp53 (DO-7), we showed that these hetero-oligomers could bind DNA in configurations compatible with the presence of 1, 2 or 3 monomers of $\Delta 40p53$ within a p53 tetramer. By co-transfection into two different p53-null cell lines, H1299 and Saos-2, we showed that $\Delta 40p53$ could modulate the transcriptional activity of TAp53 towards a generic p53-dependent β -galactosidase reporter gene. However, the amplitude and dose-dependence upon $\Delta 40p53$ of these effects differed between the two cell lines. In H1299 cells, co-

expression of the two proteins induced a decrease in transcription with an amplitude that depended upon the predicted composition of the hetero-tetramer and a dose-dependent variation that suggested that the inhibitory potential of $\Delta 40p53$ was stronger at low levels $\Delta 40p53/TAp53$ ratios than at high ratios (Figure 19). In Saos-2, a paradoxical effect was observed, with first a small but reproducible increase in activity for hetero-tetramers predicted to contain 1 or 2 monomers of $\Delta 40p53$, followed by a sharp decrease at higher $\Delta 40p53/TAp53$ ratios.

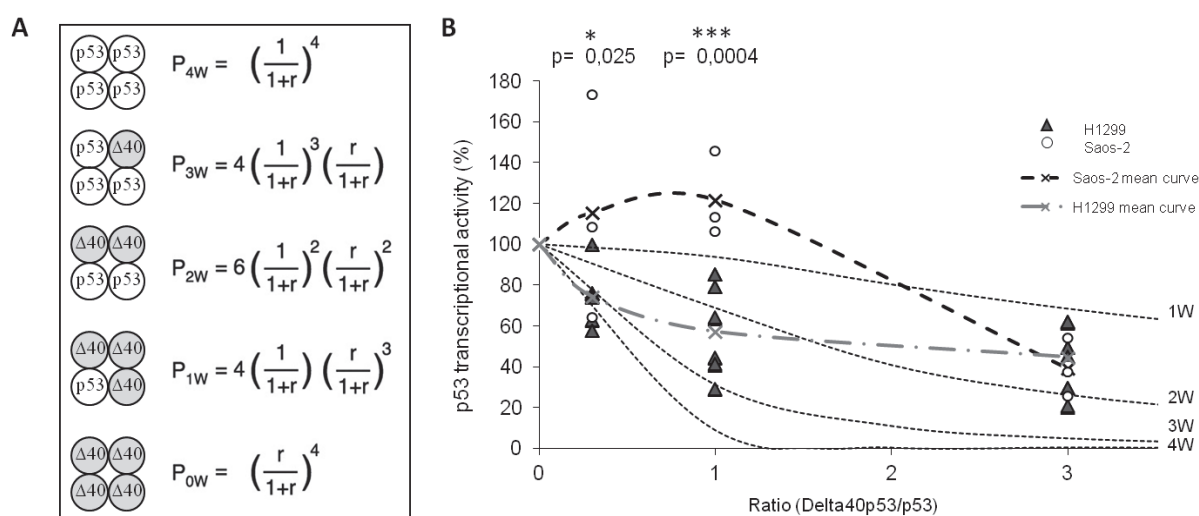


Figure 19. Predicted and observed inhibition capacity of $\Delta 40p53$

(A) Predicted composition and effects on transactivation capacity of different configurations of mixed $TAp53/\Delta 40p53$ hetero-oligomers, assuming a linear effect of $\Delta 40p53$. (B) The transcriptional activities obtained in the β -galactosidase experiments were plotted (large dotted lines) and compared to the predicted inhibition curves (small dotted lines). Grey triangles represent the different values obtained in three independent experiments done in triplicate in H1299 cells and grey crosses joined by dotted line, the average curve. White circles represent the different values obtained in three independent experiments done in Saos-2 cells and black crosses joined by dotted line, the average curve. * $P < 0.05$ and *** $P < 0.001$.

These results could not be interpreted solely on the basis of a dose-dependent titration of $TAp53$ transcriptional activity by $\Delta 40p53$. To determine whether $\Delta 40p53$ may also modulate transcriptional activity by interfering with Hdm2 binding, we co-transfected $TAp53$, $\Delta 40p53$ and Hdm2 in Saos-2 cells. Results show that expression of Hdm2 prevented the degradation of $p53$, suggesting that it interferes with the binding of Hdm2 to the hetero-tetramer. This

effect may account for an increased stability of hetero-oligomers that have incorporated 1 or 2 monomers of $\Delta 40p53$.

3.1.4 Conclusion

Our results confirm that $\Delta 40p53$ has an extended life-span and stability in cells as compared to TAp53. They also confirm previous findings that $\Delta 40p53$ could form mixed hetero-oligomers with TAp53, and further show that different configurations of these oligomers may bind to DNA. Thus, the effect of $\Delta 40p53$ on TAp53 cannot be interpreted as a simple competition for DNA-binding between two independent proteins. This effect consist into a more subtle impact of $\Delta 40p53$ on the activity of p53 tetramers, the strength of which may vary depending on how many $\Delta 40p53$ subunits are incorporated into the tetramers. This hypothesis was confirmed by analysing the effects of $\Delta 40p53$ on transactivation of a generic reported gene by TAp53 over a range of $\Delta 40p53$ /TAp53 ratios. In two different cell lines, the effects of $\Delta 40p53$ on TAp53 were not linear and were different from the predicted inhibition curves, suggesting that other factors than simple inhibition of transactivation by $\Delta 40p53$ were involved. Our results further show that, among such factors, interference with the binding of Hdm2 may have a critical role. Indeed, co-transfection of $\Delta 40p53$ in Saos-2 at least partially protected TAp53 against rapid degradation in the presence of ectopically expressed Hdm2.

Overall, these different effects suggest that $\Delta 40p53$ exerts subtle modulation on p53 activity, in particular in the range of expression levels where $\Delta 40p53$ is expressed at lower or equal levels to p53, which are generally observed in non-transfected cells. These modulation results from the combination of at least two effects resulting from the fact that $\Delta 40p53$ lacks residues 1-42 of TAp53, namely, escape from Hdm2-mediated degradation, which extends the lifespan of p53 oligomers and therefore transactivation capacity, and decreased transactivation due to decreased capacity of the hetero-oligomer to bind components of the transcription machinery. Altogether, our observations shed a new light on a possible role of $\Delta 40p53$ in the regulation of p53 activity and provide a model to explain the effects of $\Delta 40p53$ on TAp53 observed in animal models. Thus, $\Delta 40p53$ may either enhance or inhibit p53 function in a manner which is exquisitely regulated by the relative levels of each isoform and their interrelations with p53 regulatory systems such as Hdm2/Hdm4.

Submitted to BMC Cancer

Effects of Delta40p53, an isoform of p53 lacking the N-terminus, on transactivation capacity of the tumor suppressor protein p53.

Hind Hafsi¹, Daniela Santos-Silva¹, Stéphanie Courtois-Cox² and Pierre Hainaut^{1*}.

(1) International Agency for Research on Cancer and (2) Cancer Research Center, Lyon, France

*Current address:

International Prevention Research Institute, Lyon, France

*Address for correspondence:

Pierre Hainaut, PhD

International Prevention Research Institute

15 Chemin du Saquin

69130 Ecully, France

Tel : +33 4 72 17 11 86

Fax : +33 4 72 17 11 90

email : pierre.hainaut@i-pri.org

Key words : p53, isoforms, Mdm2, Delta40p53, oligomerization

Abstract

The p53 protein is expressed as multiple isoforms that differ in their N- and C-terminus due to alternative splicing, promoter or codon initiation usage. Delta40p53 lacks the first 39 residues containing the main transcriptional activation domain, resulting from initiation of translation at AUG +40 in fully spliced p53 mRNA or in a specific variant mRNA retaining intron 2. Overexpression of Delta40p53 antagonizes wild-type p53 *in vitro*. However, animal models of Delta40p53 in mouse or zebrafish have shown complex phenotypes suggestive of p53-dependent growth suppressive effects. Here we co-transfected p53 and Delta40p53 in p53-null cell lines Saos-2 and H1299 to show that Delta40p53 forms mixed oligomers with p53 that bind to DNA and modulate the transcription of a generic p53-dependent reporter gene. In H1299 cells, co-expression of the two proteins induced a decrease in transcription with an amplitude that depended upon the predicted composition of the hetero-tetramer. In Saos-2, a paradoxical effect was observed, with a small increase in activity for heterotetramers predicted to contain 1 or 2 monomers of Delta40p53 and a decrease at higher Delta40p53/p53 ratios. In this cell line, co-transfection of Delta40p53 prevented Hdm2-mediated degradation of p53, suggesting that it interferes with the binding of Hdm2 to the hetero-tetramer. These results provide a basis for growth suppressive effects in animal models co-expressing roughly similar levels of p53 and Delta40p53.

Introduction

The p53 tumour suppressor protein, encoded by *TP53* gene (OMIM 191170), integrates endogenous and exogenous signals to modulate cell fate in response to multiple forms of environmental and cellular stresses [1]. In response to such stresses, the p53 protein escapes from down-regulation by Hdm2/Hdmx which bind in the N-terminal region of p53 and acts as E3 ubiquitin ligase to induce p53 nuclear export and degradation by the proteasome [2, 3]. The resulting nuclear accumulation, combined with multiple steps of post-translational modifications, leads to the activation of sequence-specific DNA-binding to response elements located in a wide panel of target genes, resulting in transcriptional regulation of genes involved in cell-cycle arrest, differentiation, DNA repair, autophagy, apoptosis, senescence and control of oxidative metabolism [4, 5]. These multiple effects contribute to an extensive repertoire of anti-proliferative biological responses. The type and degree of responses depend upon which specific components of this repertoire are activated in a manner which differs according to tissue, cell type, metabolic context and nature of inducing stress [5, 6]. Given these multiple, complex effects, it is likely that p53 activity is under extremely tight control and that several, overlapping mechanisms may concur to set tissue- and cell-specific thresholds for p53 activation in response to different types of stimuli.

In recent years, the identification of isoforms of the p53 protein has provided a new mechanism which may contribute to the fine tuning of p53 activity. Isoforms are produced by alternative splicing, alternative promoter or codon initiation usage, or combinations thereof [7]. The resulting proteins differ from canonical, full-length p53 protein, by truncation of a variable portion of the N-terminus (DeltaN isoforms) and by alternative C-terminal portions (C-terminal isoforms). So far, up to 4 distinct N-terminal and 3 C-terminal variants have been identified, leading in theory to 12 isoforms (including full-length p53; [8]. These isoforms retain at least part of the DNA binding and oligomerization capabilities, but differ through regulatory domains in the N- and C-terminus,

supporting the notion that their main biological effect is to modulate p53 protein functions. However, the existence, expression patterns and detailed biological function of each particular isoform is still poorly documented.

The Delta40p53 isoform is a form of the protein that lacks the first 39 residues containing the main transactivation domain (residues 1-42), as well as major activating phosphorylation sites and the binding site for Hdm2, the main regulator of p53 degradation [9, 10]. Delta40p53 is produced by two complementary mechanisms, alternative codon initiation usage at AUG 40 in fully-spliced p53 mRNA, and alternative splicing that retains intron 2, which introduces stop codons downstream of the +1 AUG and leads to the synthesis of a truncated protein using AUG 40 in exon 4 as initiation codon [11, 12]. *In vitro* studies have shown that Delta40p53 interferes with p53 transcriptional activity, acting as concentration-dependent dominant inhibitor when artificially expressed in excess to full-length p53 [9]. Two animal models overexpressing Delta40p53 have been reported, one in the mouse [13, 14] and the other in Zebrafish [15]. Overexpression of a transgene encoding a p44 protein corresponding to Delta40p53 (MD41p53) did not induce any specific phenotype in p53-deficient mice. However, when expressed in a wild-type Trp53 background, increased dosage of MD41p53 led to reduced size, accelerated aging and a shorter lifespan associated with hypo-insulinemia and glucose insufficiency [13, 14]. Compatible effects were observed in Zebrafish, in which expression of Delta40p53 in a p53-null background did not lead to a specific phenotype although expression in a p53-competent background resulted in impaired growth and development [15]. Overall, these results suggest that Delta40p53 exerts its main biological effects by modulating the activity of full-length p53. Furthermore, they suggest that Delta40p53 exerts effects other than simple dominant-negative inhibition of p53.

In this study, we have used biochemical approaches to assess the effects of co-expression of Delta40p53 and full-length p53 at different ratio into p53-null human cancer cell lines and we have

analyzed the effects on p53 protein expression, DNA-binding capacity and transcriptional activity towards a p53-dependent reporter gene. Our results show that Delta40p53 exerts an inhibitory effect when expressed in excess over full-length p53. However, when expressed at levels inferior or equal to full-length p53, Delta40p53 appears to exert more complex effects, ranging from inhibition to activation of p53 transcriptional activity, depending upon cellular context. These results support the hypothesis that Delta40p53 is a critical regulator of wild-type p53 function and provide a basis to interpret the complex biological phenotypes induced by this isoform in animal models.

Materials and methods

Cell culture

Human breast cancer 21PT cells (gift of V. Band, [16]) were grown at 37°C in 5% CO₂ in MEM with Earle's Salts medium (PAA, Linz, Austria) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin-glutamine, 10 mg/ml Epidermal Growth Factor, 10 µM hydrocortisone and 1 µg/ml human insulin. Human colorectal HCT116 cells (gift of B. Vogelstein, [17]) were cultured at 37°C in 5% CO₂ in Mc Coy's 5A medium modified (Invitrogen, Carlsbad, CA, USA) supplemented with 10% of fetal bovine serum and 1% of penicillin-streptomycin-glutamine. Human lung carcinoma H1299 cells (p53-null) (ATCC N°CRL-5803) were grown at 37°C in 5% CO₂ in RPMI-1640 medium (PAA, Linz, Austria) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin-glutamine. Human osteosarcoma Saos-2 cells (p53-null) (ATCC N°HTB-85) were grown at 37°C in 5% CO₂ in Mc Coy's 5A medium modified (Invitrogen) supplemented with 15% fetal bovine serum and 1% penicillin-streptomycin-glutamine. Mouse embryonic fibroblast BALB/c 10.1 cells (p53-null) (ATCC N°CCL-163), were grown at 37°C in 5% CO₂ in Dulbecco's Modified Eagle's Medium (PAA) supplemented with 10% of fetal bovine serum and 1% of penicillin-streptomycin-glutamine.

Expression vectors, transfections and reporter gene assays

Expression vectors pcDNA3-TAp53(mut40) and pcDNA3-Delta40p53, based on the pcDNA3.1 vector (Invitrogen) containing the Cytomegalovirus (CMV) enhancer-promoter, which is constitutively active and drives high levels of mRNA expression in human cells, were described previously [9, 18]. The pcDNA3-TAp53 (mut40) plasmid contains a mutation at codon ATG 40 (ATG→TTG) to prevent Delta40p53 expression by internal initiation of translation. The pcDNA3-empty vector was used as negative control as well as adjustment vector in dose-dependent transfection experiments in order to keep the amount of transfected DNA to a constant level. Depending upon the cell lines used, transfections were performed using Eugene (Promega, Fitchburg, WI, USA) (H1299 and Saos-2 cells) or Lipofectamine 2000 (Invitrogen) (BALB/c 10.1 cells).

Cycloheximide treatment

In 60 mm dishes, 4×10^5 HCT116 p53^{+/+}, 21PT, Saos-2 and H1299 cells were treated with 50 $\mu\text{g/ml}$ of cycloheximide (Sigma Aldrich, St Louis, MO, USA). Saos-2 and H1299 cells were treated 24h after transfection of 0.25 $\mu\text{g/ml}$ pcDNA3-TAp53 (mut40) or pcDNA3-Delta40p53 plasmid.

Beta-galactosidase assay

In 6-well plates, 3×10^5 H1299 and Saos-2 cells were transfected in duplicate using Eugene transfection reagent with 0.5 $\mu\text{g/ml}$ of pRGCDeltaFosLacZ plasmid (pRGC), containing the Ribosomal Gene Cluster (RGC), which includes three p53-binding sites, upstream *beta-galactosidase* gene, and 2 $\mu\text{g/ml}$ of pcDNA3-expression vector (empty, -TAp53, -Delta40p53). Different ratios of the two pcDNA3-expression vectors TAp53/Delta40p53 were transfected, first by keeping constant the amount of Delta40p53 vector (0.15/0.5, 0.5/0.5 and 1.5/0.5 $\mu\text{g/ml}$) and second by varying only the amount of Delta40p53 vector (0.5/0.15, 0.5/0.5 and 0.5/1.5 $\mu\text{g/ml}$). The total amount of plasmid has been adjusted by adding pcDNA3-empty vector to reach

2.5 $\mu\text{g/ml}$ of total transfected DNA in each condition. In addition, cell transfection efficiency was assessed by transfecting 1 $\mu\text{g/ml}$ of a GFP-expressing vector. Twenty four hours post-transfection, cells were harvested and beta-galactosidase activity was measured using Beta-galactosidase Enzyme Assay System kit (Promega).

Western Blot

Proteins were extracted using RIPA-like buffer (50 mM Tris-HCl pH 7.4, 250mM NaCl, 0.1% SDS, 0.5% NP-40, 2 mM DTT, 1 mg/ml protease inhibitors [500 mM phenyl-methyl-sulfonyl-fluoride (PMSF), 0.5 mg/ml leupeptin, 2 mg/ml aprotinin, 1.4 mg/ml pepstatin A]) and 30 μg of cleared extracts were loaded on mini protean TGX 4-15% gels (Bio-Rad, Hercules, CA, USA) and transferred using TransBlot Turbo Transfer System (Bio-Rad). Membranes were hybridized with several antibodies: monoclonal anti-p53 DO-7 antibody, which binds the epitope at residues 19-26 and detects only TAp53 (DAKO, Cambridgeshire, UK); rabbit polyclonal anti-p53 CM1 antibody (Novocastra, Newcastle, UK) and monoclonal anti-p53 PAb1801 (Oncogene Research Products, Cambridge, MA, USA), which detect both TAp53 and Delta40p53; p21^{WAF1} antibody (Calbiochem, Billerica, MA, USA); Hdm2 (Calbiochem) and Ku80 (Abcam) or anti-actin (Santa Cruz, CA, USA) antibodies were used as a loading control. Secondary peroxidase coupled goat anti-mouse or - rabbit antibodies were used, followed ECL detection according to the manufacturer's instructions (Amersham Biosciences AB, Buckinghamshire, UK).

Co-immunoprecipitation assay

In 100 mm dishes, 2×10^6 BALB/c 10.1 cells were transfected using Lipofectamine 2000 with 2 $\mu\text{g/ml}$ of pcDNA3-expression vectors (empty, -TAp53, -Delta40p53). The same TAp53/Delta40p53 vector ratios (0.15/0.5, 0.5/0.5 and 1.5/0.5 $\mu\text{g/ml}$) as in the beta-galactosidase assays were used. The total amount of plasmid was adjusted by adding empty vector to reach 2 $\mu\text{g/ml}$ in each condition. Cells were lysed 24 h post-transfection by adding 250 μl of lysis buffer (10 mM Tris pH

7.5, 140 mM NaCl, 0.5% Triton, 2 mM DTT, 1 mM EDTA and 1 tablet of protease inhibitor cocktail/10 ml Complete Mini, Roche). Total protein extracts (500 μ g) were cleared by incubation (4°C-1 h) with 25 μ l of anti-mouse sepharose beads (Sigma) and then incubated (4°C-overnight) with p53 mouse monoclonal DO-7 antibody. Proteins/antibodies complexes were analyzed by Western blot using CM1 antibody.

Electromobility shift assay

EMSA were performed as described in Verhaegh et al., 1997 [19]. Briefly, nuclear cellular proteins of H1299-transfected cells were extracted and incubated with mixture containing 32 P-radio-labeling oligonucleotide with p53 response element consensus p53^{con} (5'-GGACATGCCCCGGGCATGTCC-3') [18]. Two antibodies were used to shift DNA:p53 complexes, the monoclonal PAb421 antibody recognizing p53 DBD and known to stabilize DNA:p53 complexes; and the monoclonal DO-7 antibody specific for the N-terminal portion of p53 protein, recognizing TAp53 but not Delta40p53.

Statistical analysis

Statistical analysis were performed using the software GraphPad Prism and *P*-values were indicated by (*) when *P* < 0.05 and (**) when *P* < 0.01.

Results

Intrinsic stability of Delta40p53 isoform

Figure 1A shows a schematic representation of TAp53 and Delta40p53 protein domains, illustrating the lack in Delta40p53 of the N-terminal residues motifs that contain the main transcriptional activation domain (TA, residues 1-42) as well as the amino-acid motif that binds Hdm2 (residues 17-29). Consistent with lack of targeting of Delta40p53 by Hdm2, this isoform is much more stable than full-length p53 [9, 10]. To estimate the difference in stability between p53 and Delta40p53,

we performed time-course western-blot analysis after cycloheximide translation block using PAb1801 (a monoclonal that recognizes residues 45-54, present in both isoforms) in breast cancer 21PT cells, which express only Delta40p53 due to an insertion mutation and frameshift at position 33 which leaves intact the open reading frame of Delta40p53, and in colorectal cancer HCT116 cells, which express wild-type TAp53 protein. Results show that, while in HCT116 p53 is significantly degraded after 15 min and becomes undetectable after 45 min, in 21PT Delta40p53 shows greater stability, with no apparent decrease in protein levels within 8 hrs and with the protein still detectable after 20 hrs (Figure 1B). Next, we transfected expression vectors for either full-length p53 or Delta40p53 in two p53-null cancer cell lines, H1299 and Saos-2. The stability of each protein was evaluated by Western Blot after cycloheximide translation block. Results show that in both cell types, Delta40p53 was more stable than TAp53 (Figure 1C). In addition, TAp53 appeared to be more stable in H1299 than in Saos-2 cells. The extended stability of TAp53 in H1299 cells is compatible with previously published data that these cells have low Hdm2 protein levels and activity (Marcel et al., 2010; xx). Overall, these results concur with previously published results by Yin et al., (2002), Courtois et al., (2002) and Gosh et al., (2004) [9-11] and support that Delta40p53 has an increased half life, which is consistent with the lack of Hdm2-binding site and thus escape from Hdm2-mediated degradation.

Complex formation between TAp53 and Delta40p53

Delta40p53 conserves the oligomerization domain of p53 and can, in principle, associate with TAp53 to form hetero-oligomers. To determine whether the two isoforms could complex when co-expressed in intact cells, and in which proportion, we transfected mouse BALB/c 10.1 cells (p53-null) with constant amounts of Delta40p53 and variable amounts of full-length p53. The proteins were co-immunoprecipitated with DO-7 (recognizing only full-length p53), followed by western blot analysis using the pan-isoform antibody CM1. Presence of Delta40p53 in the immunoprecipitates demonstrates complex formation, since DO-7 is not capable of directly immunoprecipitating

Delta40p53. Figure 2 shows that the two proteins could form complexes. When the two isoforms were present in equal amounts, these complexes contain equal amounts of both full-length p53 and Delta40p53. To further analyze whether hetero-oligomers of p53 and Delta40p53 may bind to DNA, we performed Electro-Mobility Gel Shift Assays (EMSA) using a synthetic p53 response element and extracts of H1299 cells transfected with increased levels of Delta40p53 in the presence of constant levels of TAp53 (Figure 3). Both proteins could bind consensus DNA in the presence of the monoclonal antibody PAb421, which is known to stabilize and super-shift p53:DNA complexes. In the absence of PAb421, low levels of DNA-binding were observed for p53, but not for Delta40p53. We then used DO-7 to perform super-shift experiments. Since this antibody recognizes an epitope present in p53 but not in Delta40p53, we reasoned that the amplitude of the super-shift induced by DO-7 would correlate with the proportion of p53 present in the hetero-oligomer. In the absence of Delta40p53, DO-7 induced a super-shift of p53:DNA complexes consistent with the binding of several molecules of DO-7 to the complex. Increasing the expression of Delta40p53 relative to p53 resulted in the formation of intermediate super-shifted species, consistent with the hypothesis that hetero-oligomers are formed by incorporating Delta40p53 which does not bind DO-7. Overall, these experiments demonstrate that the two forms of p53 can bind specifically to DNA and can form mixed hetero-oligomers containing both p53 and Delta40p53.

Effects of Delta40p53 on p53 transcriptional activity

Previous studies have shown that Delta40p53 was unable to transactivate reporter constructs containing p53 response elements derived from different p53 target genes, including p21WAF1, BAX, (others). Furthermore, expression of excess levels of Delta40p53 could antagonize p53 transcriptional activity, thus behaving as a potential dominant inhibitor of p53-dependent gene regulation. However, these experiments were performed in conditions where levels of Delta40p53 exceeded by far the levels of p53 itself [9, 11]. To address the effect of Delta40p53 when

expressed at low levels relative to TAp53, we co-transfected either H1299 or Saos-2 cells with TAp53, variable amounts of Delta40p53, and a beta-galactosidase p53 reporter gene. Results (Figure 4) show that Delta40p53 had no measurable transcriptional activity on its own but modulated the transcriptional activity of TAp53 with different effects in the two cell lines. In H1299, Delta40p53 appeared to inhibit TAp53 transcriptional activity in an incremental manner, depending upon the dosage of Delta40p53. In Saos-2 cells, inhibition of p53 transcriptional activity towards the beta-galactosidase reporter was observed only with higher amounts of Delta40p53, whereas the transcriptional activity was maintained or even slightly increased when Delta40p53 was expressed at lower levels.

Inhibition curves of p53 transcriptional activity by Delta40p53

The results above suggest that in Saos-2 cells, inhibition of TAp53 transcriptional activity by Delta40p53 does not follow a linear curve determined by oligomerization between p53 isoforms. To test this hypothesis, we have used the strategy developed by Chan et al. (2004) to investigate how many mutant p53 molecules were needed to inactivate a p53 tetramer [20]. Assuming that the probability of formation of tetramers containing 1, 2 or 3 subunits of Delta40p53 is dependent upon the relative levels of expression of each of the proteins, we have predicted the theoretical inhibition curves of TAp53 activity by Delta40p53 and we have compared these predictions to actual levels of expression of the TAp53-dependent beta-galactosidase reporter in H1299 and in Saos-2 cells (Figure 5). Results show that inhibition curves did not fulfill the theoretical model of dose-dependent hetero-tetramer formation. In addition, the curves were strikingly different between the two cell lines. In H1299 cells, the inhibition of TAp53 activity by Delta40p53 was very effective at low levels of Delta40p53, consistent with the prediction curve in which 1 subunit of Delta40p53 can inhibit a tetramer in which the 3 other subunits are TAp53. However, the effectiveness of this inhibition decreased with amounts of Delta40p53. When the two proteins were transfected at 1:1 ratio, the inhibition curve was roughly consistent with a pattern in which 2

subunits of Delta40p53 were required to inhibit one tetramer. At higher amounts of Delta40p53 relative to TAp53, the inhibition curve tended towards the pattern in which 1 subunit of TAp53 is sufficient to maintain transcriptional activity. In Saos-2 cells, expression of Delta40p53 relative to TAp53 at ratios below or equal 1:1 led to a small but reproducible increase of 20-25% in TAp53 transcriptional activity, while in the higher ratio of 3:1, the inhibition was as effective as in H1299 cells. Thus, Delta40p53 appears to have paradoxical effects on the transcriptional activity of p53, suggesting that in addition to interfering with p53 through oligomerization, Delta40p53 may also modify other parameters that determine p53-dependent gene expression in a cell-dependent manner.

Delta40p53 protects TAp53 from Hdm2-mediated degradation in Saos-2 cells

One mechanism by which Delta40p53 may influence the transcriptional activity of TAp53 is through modulation of p53 protein stability and, in particular, modulation of Hdm2-mediated degradation. Indeed, Delta40p53 is intrinsically more stable than TAp53 due to absence of Hdm2-binding capacity and it is possible that, depending upon cell context, the Delta40p53 isoform may confer an increased stability to mixed oligomers containing both TAp53 and Delta40p53. This hypothesis was tested by analysing the levels of TAp53 in Saos-2 co-transfected with TAp53, Delta40p53 and Hdm2. Figure 6 shows that co-transfection of TAp53 together with Hdm2 resulted in the almost complete disappearance of TAp53, in agreement with enhanced degradation of the protein. As expected, levels of Delta40p53 were not decreased by co-transfection with Hdm2. Co-Transfection of the three factors resulted in the maintenance of detectable levels of TAp53 with increased phosphorylation on serine 15 even in the presence of Hdm2, compatible with protection by Delta40p53 from Hdm2-dependent degradation. Thus, in Saos-2 cells, Delta40p53 appears to be capable of modulating the stability of TAp53, leading to the persistence in cells of higher levels of TAp53 bearing phospho-serine 15, a hallmark of p53 activation.

Discussion

Delta40p53 was the first identified N-terminal isoform of p53 and there is evidence from animal model systems that it exerts a strong regulatory effect on the biological activity of the p53 protein [13-15]. However, the nature and biological consequences of this effect are still poorly understood. In this study, we have used *in vitro* approaches to show that Delta40p53 can form mixed oligomers with TAp53 that can bind to specific DNA and modulate the capacity of p53 to activate a generic reporter gene driven by a standard p53 response element. This modulation did not follow a proportional dose-response according to the relative expression of TAp53 and Delta40p53. In H1299 cells, co-expression of the two proteins induced a decrease in the transcriptional activity, but the amplitude of the effect varied depending upon the predicted composition of the heterotetramer. In Saos-2, a paradoxical effect was observed, with a small increase in transcriptional activity for heterotetramers predicted to contain 1 or 2 monomers of Delta40p53, and a strong decrease for heterotetramers predicted to contain 3 monomers of Delta40p53. These different effects suggest that Delta40p53 may exert subtle modulation on p53 activity, in particular in the range of expression levels that are generally observed in non-transfected cells, where Delta40p53 is expressed at lower or, at the most, equal levels to TAp53. The differences between the two cell lines can be rationalized by considering that Delta40p53 may alter the dynamics of heterotetramers in two opposite ways. First, it may alter the binding to component of the basal transcription machinery, thus decreasing transcriptional activation. Second, it may interfere with the binding of Hdm2 to the heterotetramer, thus modulating its subsequent post-translational modification, its nuclear export and its degradation through the proteasome machinery. Supporting this view, we show that, in Saos-2 cells, Delta40p53 could at least partially protect TAp53 from Hdm2-mediated degradation. The differences between the two cell lines with respect to the effect of Delta40p53 on the transcriptional activity of TAp53 may be due to multiple cell-specific mechanisms, including levels of expression of Hdmx, which co-regulates p53 stability together with Hdm2, as well as Hdm2-independent effects. Of note, p53 monomers are resistant to Hdm2-

mediated degradation *in vitro* and in intact cells, despite the presence of Hdm2 binding sites on each p53 monomer. Dimerization, but not tetramerization, is required for Hdm2-dependent degradation. Our results suggest that, in order to be efficiently degraded, a complex of p53 isoforms should contain at least two Hdm2-binding domains, perhaps allowing for simultaneous interaction with both Hdm2 and Hdmx.

The observation that Delta40p53 may exert, in defined condition and cell context, a positive effect on p53 transcriptional activity, may account for some of the biological characteristics of animal models expressing this isoform. Both in the mouse expressing p44, an equivalent to Delta40p53, and in Zebrafish, expression of Delta40p53 alone in a p53-null background does not significantly affect the phenotype [13-15]. In contrast, dramatic effects are seen when both TAp53 and Delta40p53 are co-expressed, and these effects are consistent with an increase and modulation of p53-dependent suppressive effects rather than an inhibition of these effects. Mice co-expressing TAp53 and Delta40p53 do not show a tumor-prone phenotype attributable to inhibition of p53 activity [13, 14]. In contrast, they show increased organismal and tissular senescence attributable to decline in stem cell renewal and in general fitness. In Zebrafish, co-expression of the two proteins lead to developmental defects attributable to hypoplasia, malformation of the head, eyes and somites, a phenotype which is also compatible with enhanced, rather than decrease, p53 suppressive activity [15]. These effects are rescued by co-expression of a dominant-negative p53 mutant, suggesting that they are indeed dependent upon increased p53 suppressive activity. Overall, our observations shed a new light on a possible role of Delta40p53 in the regulation of p53 activity in stem and progenitor cells, contributing to either enhance or inhibit p53 function in a manner which is exquisitely regulated by the relative levels of each isoform and their interrelations with p53 regulatory systems such as Hdm2/Hdmx.

It is recognized that this *in vitro* study has a number of limitations. First, the effects have been studied using only one type of consensus p53 RE and reporter gene. Given the wide repertoire of

p53 RE and their capacity to respond in a different way to p53 activation, it is plausible that modulation of transactivation by $\Delta 40p53$ may have different effects on different p53 targets. Second, although implicating Hdm2 as an essential regulator of the activity of TAp53/ $\Delta 40p53$ hetero-oligomers, our study has not quantified variations in the binding of Hdm2 to different types of hetero-oligomers and has not clearly identified the consequences on p53 protein stability. Third, $\Delta 40p53$ is not the sole N-terminal isoform which may regulate p53 activity. In particular, $\Delta 133p53$ (which lacks the 40 N-terminal transactivation domain, the proline-rich domain and the proximal part of the DNA-binding domain) also retains the oligomerization domain and may interfere as third partner in hetero-oligomers containing TAp53 and $\Delta 40p53$. We have previously shown that, in contrast to $\Delta 40p53$, $\Delta 133p53$ does not bind to DNA and prevents TAp53 to recognize its cognate response element. Recent studies have proposed that $\Delta 133p53$ may play a role as enhancer of p53-dependent cell senescence [21]. Overall, our results add to the complex picture of interactions between p53 and its N-terminal isoforms to suggest that, in defined conditions, these isoforms may either enhance or decrease the basal level of p53 activity, thus contributing to set a threshold for p53-dependent responses to a wide variety of endogenous or exogenous stimuli.

Acknowledgements

This study was supported by a grant from Institut National du Cancer (INCa), France.

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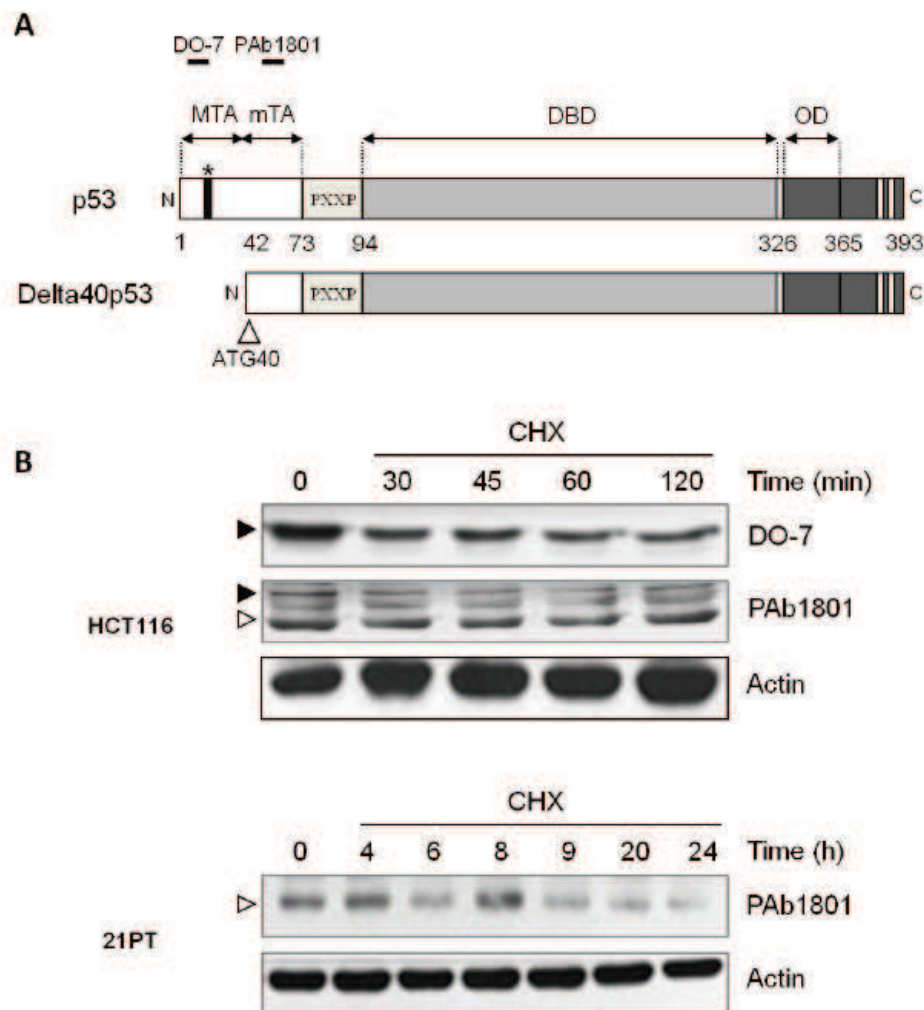


Figure 1: Intrinsic stability of p53 and $\Delta 40p53$ isoform

(A) Schematic representation of the domain structure of the p53 protein. The protein contains a transactivation domain (TA) with Hdm2-binding site (black bar), a proline-rich domain (PXXP), a central DNA-binding domain (DBD), a C-terminal oligomerization domain (OD) and nuclear localization signals (NLS, light grey bars). (B) Levels of expression of TAp53 and $\Delta 40p53$ detected with PAb1801 antibody in HCT116 p53^{+/+} and 21PT cells, after cycloheximide (CHX) treatment (50 μ g/ml) to block protein synthesis. Actin: loading control. (C) Levels of expression of transfected TAp53 and $\Delta 40p53$ expression plasmids (0,25 μ g/ml each) in Saos-2 and H1299 cells. Both cell lines were treated with CHX 24h post-transfection. TAp53 protein was detected with DO-7 antibody and $\Delta 40p53$ with PAb1801 antibody. Black arrow: TAp53, white arrow: $\Delta 40p53$.

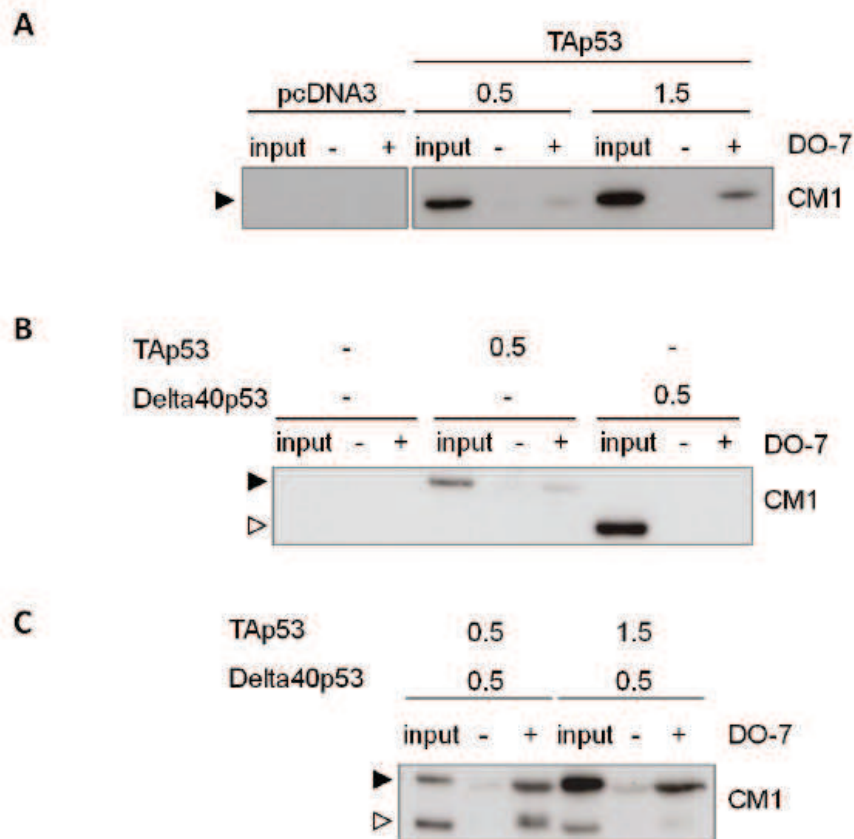


Figure 2: Formation of hetero-oligomers between TAp53 and $\Delta 40p53$

(A) Immunoprecipitation of TAp53 using DO-7 antibody. Immunoprecipitation was performed with DO-7 antibody (+) or without DO-7 antibody as a negative control (-) using increasing amounts of TAp53 transfected plasmid. Immunoprecipitated proteins were analysed by immunoblotting using CM1 polyclonal antibody. TAp53 immunoprecipitation is enhanced when the amount of transfected plasmid is increased. pcDNA3: empty vector; input: unprecipitated proteins issued from cell extract; black arrow: TAp53. (B) Immunoprecipitation of TAp53 or $\Delta 40p53$ using DO-7 antibody. Immunoprecipitation was performed using either TAp53 or $\Delta 40p53$ transfected plasmid. (C) Co-immunoprecipitation of TAp53 and $\Delta 40p53$ using different amount of transfected TAp53 plasmid. Same experiment of immunoprecipitation as described in (A) and (B), using co-transfection of both TAp53 (0.15; 0.5; 1.5 $\mu\text{g/ml}$) and $\Delta 40p53$ (0.5 $\mu\text{g/ml}$). In each condition, the two p53 isoforms interact but in particular when they are expressed in the same amount. Black arrow: TAp53; white arrow: $\Delta 40p53$.

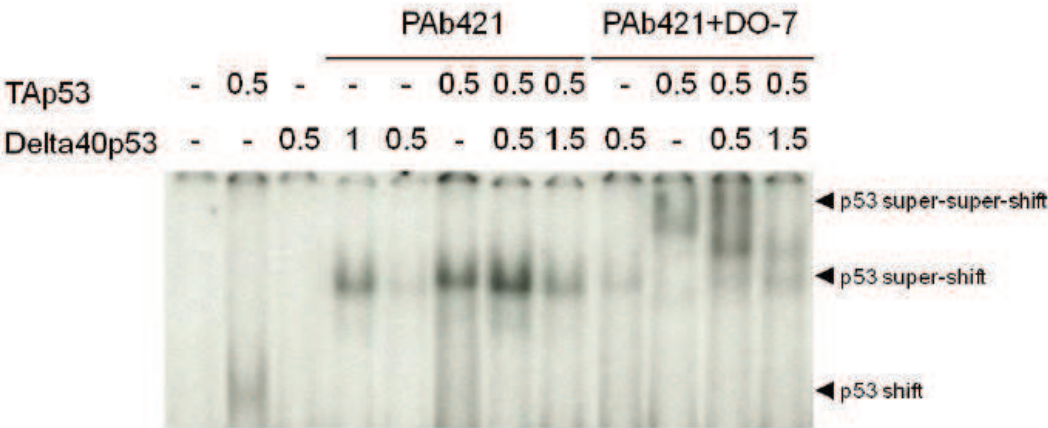


Figure 3: DNA-binding activity of TAp53 and $\Delta 40p53$

Electromobility shift assay (EMSA) measuring DNA-binding activity. p53-null H1299 cells were co-transfected with a constant amount of pcDNA3-TAp53 plasmid (0.5 $\mu\text{g/ml}$) in the presence of increasing amounts of pcDNA3- $\Delta 40p53$ plasmid (0.15, 0.5 and 1.5 $\mu\text{g/ml}$). Nuclear extracts were incubated with a ^{32}P -labeled p53^{con} oligonucleotide, with or without PAb421, which stabilizes and super-shifts p53:DNA complexes. DO-7 induces a super-super-shift of p53/DNA complexes. DNA:p53 shifts, DNA:p53:PAb421 super-shifts, DNA:p53:PAb421:DO-7 super-super-shifts are indicated by black arrows.

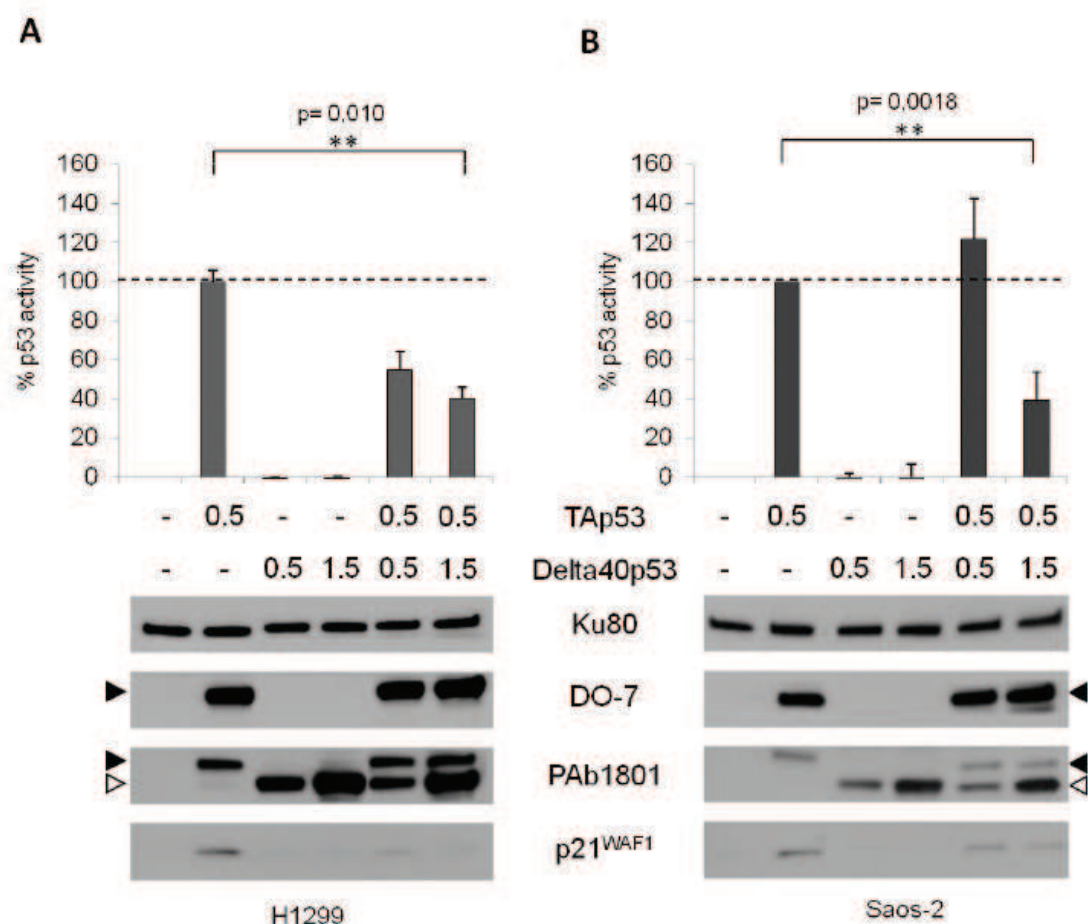


Figure 4: Effects of $\Delta 40p53$ on p53 transcriptional activity

(A) Inhibitory effect of $\Delta 40p53$ on transcriptional activity of increasing amount of Tap53 in H1299 cells. The transactivation activity of p53 isoforms was measured towards the p53 response element of RGC plasmid and plotted as relative β -galactosidase activity induced by Tap53 expressed alone at 0.5 μ g/ml (top panel). Tap53 and $\Delta 40p53$ protein expressions were analysed by immunoblotting using DO-7 and PAb1801 antibodies. The induction of p21^{WAF1} was also analyzed (bottom panel). $\Delta 40p53$ expression increased with the increase of amount of transfected plasmid and Tap53 was constantly expressed. (B) Same reporter assay experiment as defined in (A), but in Saos-2 cells. Each error-bar represents the standard deviation of three independent experiments, each performed in triplicate. Ku80: loading control. ** $P < 0.05$ calculated by t-test. Black arrow: Tap53; white arrow: $\Delta 40p53$.

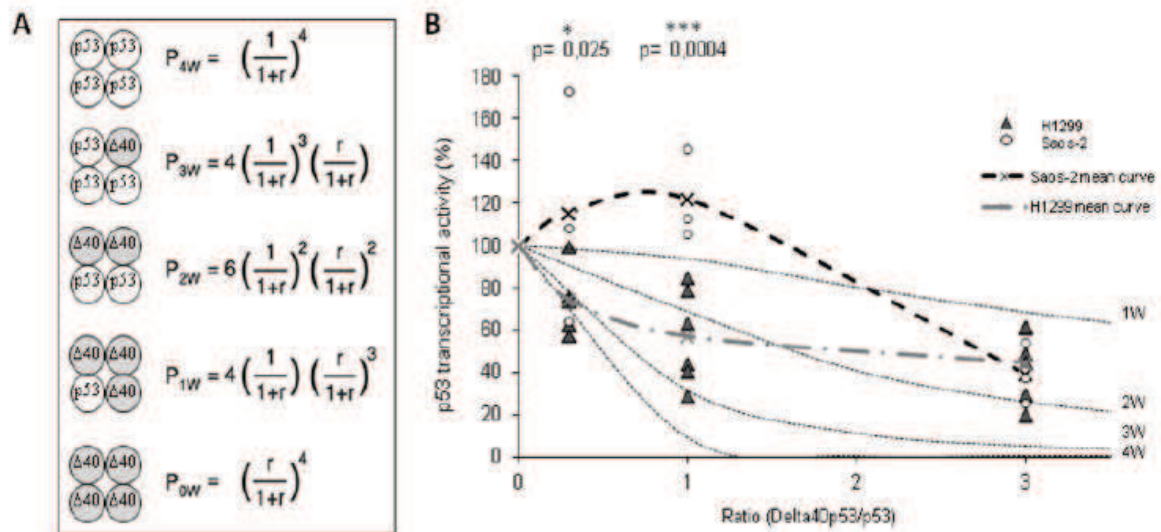


Figure 5: Predictive inhibition capacity of $\Delta 40p53$ isoform

(A) Mathematical approach to analyze probabilities of the tetramer formation, containing different numbers of TAp53 and $\Delta 40p53$ monomers (adapted from Chan et al. 2004). The equations defining the probabilities (P) of forming different tetramers are indicated, where r is the ratio (concentration of $\Delta 40p53$ /concentration of TAp53). p53 denotes TAp53 and $\Delta 40$, $\Delta 40p53$. (B) Prediction of TAp53/ $\Delta 40p53$ hetero-tetramer formation based on β -galactosidase assay results. The predicted inhibition curves (dotted lines) were calculated from the probability of forming the different tetramers shown in (A). The transcriptional activities obtained in the β -galactosidase experiment (Figure 4) were plotted. Grey triangles represent the different values obtained in three independent experiments done in triplicate in H1299 cells and grey crosses joined by dotted line, the average curve. White circles represent the different values obtained in three independent experiments done in Saos-2 cells and black crosses joined by dotted line, the average curve. * $P < 0.05$ and *** $P < 0.001$.

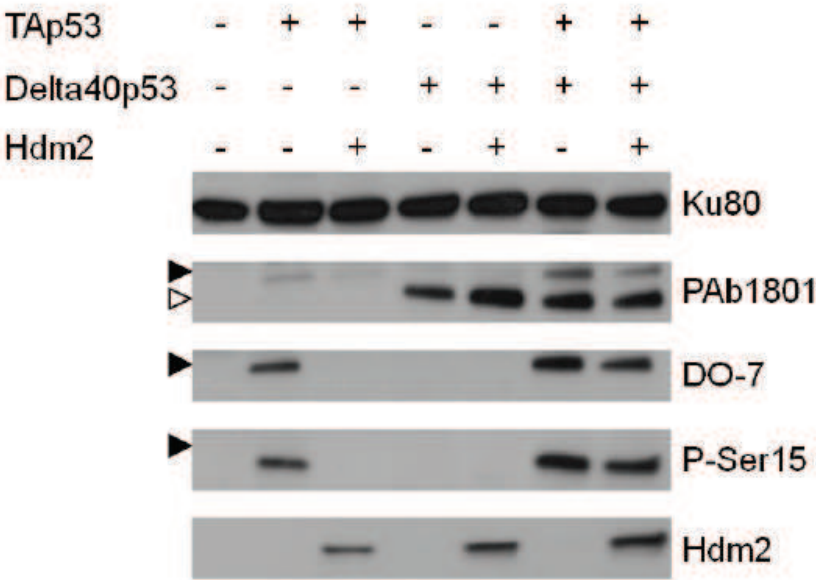


Figure 6: Delta40p53 protects TAp53 from Hdm2-mediated degradation in Saos-2 cells

TAp53 plasmid was co-transfected with Delta40p53 and Hdm2 in Saos-2 cells. The level of expression of the proteins was analysed by immunoblotting using DO-7 and PAb1801 antibodies for the detection of TAp53 and Delta40p53. The co-transfection of TAp53 together with Hdm2 results in the almost complete disappearance of TAp53, in agreement with enhanced degradation of the protein. As expected, levels of Delta40p53 are not decreased by co-transfection with Hdm2. Co-Transfection of the three factors results in the maintenance of detectable levels of TAp53 with increased phosphorylation on Serine 15 even in the presence of Hdm2.

3.2 p53 regulates the transcription of its $\Delta 133p53$ isoform through specific response elements contained within the TP53 P2 internal promoter

Article No.2

Oncogene, 29: 2691-2700, 2010

3.2.1 Context

The $\Delta 133p53$ isoform was discovered by JC Bourdon and collaborators in 2005, who first reported the existence of a p53 transcript initiated in the distal part of intron 4, independent from the main p53 transcript initiated upstream of exon 1 (Bourdon et al., 2005). These authors postulated that this transcript could drive the expression of an N-terminal truncated p53 by using as initiation codon an in-frame AUG located at position 133 in TAp53. Because of its regulation through an alternative internal promoter (P2 promoter), $\Delta 133p53$ shows a functional similarity with $\Delta Np63$, which is mainly expressed from an internal, intronic promoter regulated by both TAp63 and p53 (Grob et al., 2001; Harmes et al., 2003). The P2 promoter spans the region between intron 1 and exon 5 of *TP53* gene, covering around 1.5 kb (Bourdon et al., 2005). The resulting transcript, p53I4, contains the 3' end of intron 4 followed by all exons from 5 to 11. This p53I4 mRNA has been observed to be overexpressed in squamous cell carcinoma of the head and neck (Boldrup et al., 2007) and in breast cancers (Moore et al., 2010), suggesting a role for $\Delta 133p53$ isoform in tumourigenesis. In this study, we have used siRNA to the p53I4 mRNA to provide conclusive evidence for the existence of the $\Delta 133p53$ isoform and we have determined whether, similarly to $\Delta Np53$, this promoter could be regulated by TAp53. This work was carried out within collaboration with V. Marcel.

3.2.2 Strategy

To determine the role of p53 in $\Delta 133p53$ expression, we first over-expressed p53 in A549 cell lines. To examine whether p53 regulates p53I4 mRNA level and to demonstrate that this mRNA indeed supports the synthesis of $\Delta 133p53$, we silenced p53 expression in A549 cells

using a siRNA targeting exon 4 of *TP53* gene. Then, we assessed p53I4 mRNA expression in response to DNA damage induced by doxorubicin to investigate the possible regulation of $\Delta 133p53$ by p53 in response to stress. To investigate whether $\Delta 133p53$ isoform may modulate p53 DNA-binding activity, we performed an EMSA using a ^{32}P -labeled p53RE consensus oligonucleotide. We then performed a colony formation assay using p53-null H1299 cells transfected with constitutive expression vectors for p53 or $\Delta 133p53$ transfected vectors to determine whether $\Delta 133p53$ may inhibit p53 suppressive function. Next, we generated a luciferase reporter driven by the P2 promoter sequence identified by Bourdon et al. (2005) consisting in a 1.5 kb fragment from intron 1 to exon 5 of *TP53* gene, and by sequential deletions of this sequence, to determine whether p53 could transactivate the internal P2 promoter (Bourdon et al., 2005). This luciferase reporter was co-transfected in p53-null H1299 cells with various amounts of pcDNA3-p53 expression vector. Finally, chromatin immunoprecipitation (ChIP) and luciferase assays using P2 promoter deletion constructs were used to analyze the binding of p53 on different segments of the P2 promoter in A549 cells.

3.2.3 Results

3.2.3.1 Regulation of P2 promoter by TAp53

Induction of TAp53 in A549 cells (expressing wild-type p53) resulted in an increase in the levels of p53I4 mRNA and increased levels of a small-molecular weight variant of p53 protein, with an apparent molecular weight of 34 kDa. Usage of a siRNA to p53I4 decreased the levels of this 34 kDa species, identifying it as the $\Delta 133p53$ protein. Next, we identified putative p53 binding sequences in the P2 promoter. Using stepwise deletion mutants, we found that two of these sequences had a critical role in the activation of the promoter by p53. By ChIP experiments, TAp53 was found to bind to promoter fragment containing these sequences. We also showed that $\Delta 133p53$ did not bind specifically to a p53 consensus DNA sequence *in vitro*, but competed with wild-type p53 in specific DNA-binding assays. Finally, we found that $\Delta 133p53$ counteracted p53-dependent growth suppression in a clonogenic assay (Figure 20).

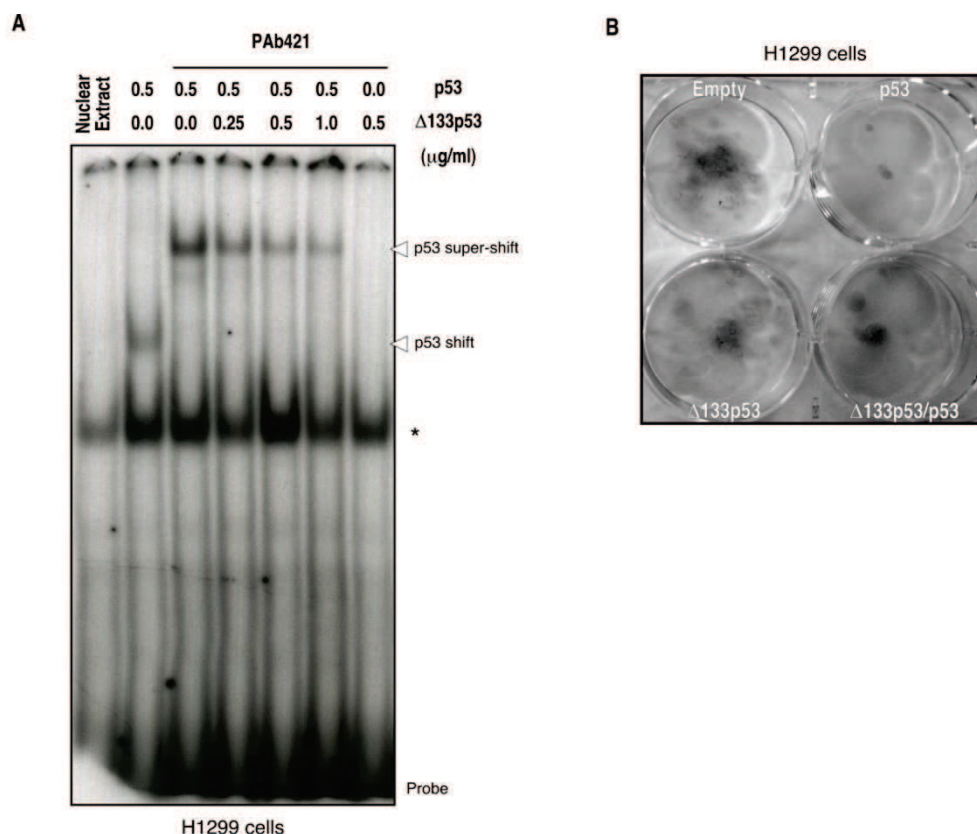


Figure 20. Effects of $\Delta 133p53$ on p53 biochemical and biological properties

(A) Electro-Mobility-Shift Assay (EMSA) measuring DNA-binding activity of $\Delta 133p53$. p53-null H1299 cells were co-transfected with a constant amount of pcDNA3-p53 vector (0.5 $\mu g/ml$) in the presence of increasing amounts of pcDNA3- $\Delta 133p53$ vector (0.25-1 $\mu g/ml$). Nuclear extracts were incubated with a ^{32}P -labeled p53RE oligonucleotide, with or without PAb421 antibody, which stabilizes and super-shifts p53:DNA complexes. DNA:p53 shifts and DNA:p53:PAb421 super-shifts are indicated by arrowheads. *: non-specific band. (B) Colony formation assays were performed using p53-null H1299 cells transfected with 0.5 $\mu g/ml$ of wild-type pcDNA3-p53 vector and/or - $\Delta 133p53$ vector during 14 days under 0.5 mg/ml of neomycin selection. As compared to wild-type p53, $\Delta 133p53$ did not suppress cell growth and partially reverted the suppression induced by wild-type p53.

3.2.3.2 Effect of $\Delta 40p53$ on P2 promoter activity

Since $\Delta 40p53$ had been shown to exert complex effects on the transcriptional activity of p53-dependent promoter, we investigated whether $\Delta 40p53$ could modulate the activation of the P2 promoter by TAp53. Both proteins were co-transfected into the p53-null cell line H1299 together with the P2-driven luciferase reporter. Results showed that $\Delta 40p53$ inhibited the activation of P2 promoter by TAp53, suggesting the existence of complex regulatory loops interconnecting TAp53, $\Delta 40p53$ and $\Delta 133p53$ (Figure 21).

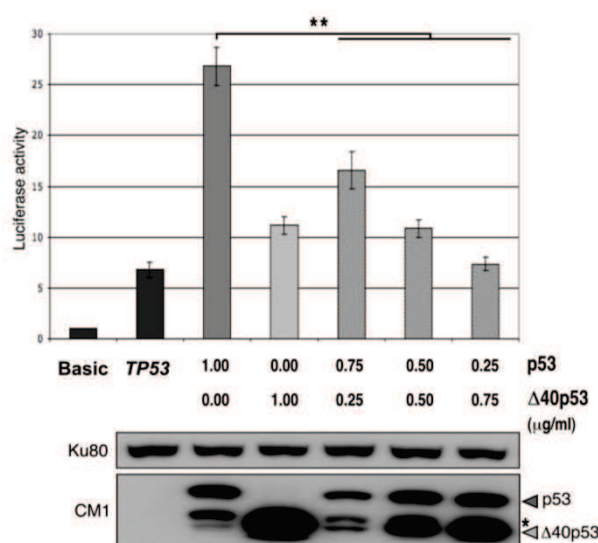


Figure 21. P2 promoter activity is down-regulated by $\Delta 40p53$

In p53-null H1299 cells, luciferase assays (upper panel) were performed using a luciferase reporter under the control of the 1.5 kb segment of *TP53* from intron 1 to exon 5, in the presence of both p53 and $\Delta 40p53$ in different ratio. The expression of both isoforms was verified by western blot with the p53 polyclonal antibody CM1 (lower panel). $\Delta 40p53$ decreases luciferase activity of P2 promoter. Basic: promoterless luciferase reporter; P2: luciferase reporter driven by *TP53* segment; Ku80: loading control. Black arrowhead: p53 protein; grey arrowhead: $\Delta 40p53$ protein; Ku80: loading control.

3.2.4 Conclusion

These results identify $\Delta 133p53$ protein as an independent isoform of p53, expressed from the internal promoter P2. By demonstrating that this promoter was regulated by TAp53 in response to genotoxic stress, we identify a novel auto-regulatory loop by which p53 may control the expression of an isoform potentially acting as an inhibitor of p53 suppressor activities. From a biological viewpoint, $\Delta 133p53$ does not appear to bind DNA and prevents TAp53 to form stable p53:DNA complexes. These observations are in agreement with the predicted structure of $\Delta 133p53$ that lacks a part of the DNA-binding domain and therefore has an unfolded DNA-binding domain. Its inhibitory effect on TAp53 may thus be due to a trans-conformational effect of $\Delta 133p53$ on TAp53 DNA-binding domain, similar to the one exerted by mutant p53 on wild-type p53 conformation and binding activity (Milner and Medcalf, 1991). Furthermore, $\Delta 40p53$ inhibited the induction of the P2 promoter by TAp53, suggesting that the three isoforms may have coordinated effects and may regulate each other. Overall, $\Delta 133p53$ is a novel target of p53 that may participate in a negative feedback loop controlling p53 function.



ORIGINAL ARTICLE

p53 regulates the transcription of its $\Delta 133p53$ isoform through specific response elements contained within the *TP53* P2 internal promoter

V Marcel, V Vijayakumar, L Fernández-Cuesta, H Hafsi, C Sagne, A Hautefeuille, M Olivier and P Hainaut

Molecular Carcinogenesis Group, International Agency for Research on Cancer, Lyon, Cedex, France

The tumor suppressor p53 protein is activated by genotoxic stress and regulates genes involved in senescence, apoptosis and cell-cycle arrest. Nine p53 isoforms have been described that may modulate suppressive functions of the canonical p53 protein. Among them, $\Delta 133p53$ lacks the 132 proximal residues and has been shown to modulate p53-induced apoptosis and cell-cycle arrest. $\Delta 133p53$ is expressed from a specific mRNA, p5314, driven by an alternative promoter P2 located between intron 1 and exon 5 of *TP53* gene. Here, we report that the P2 promoter is regulated in a p53-dependent manner. $\Delta 133p53$ expression is increased in response to DNA damage by doxorubicin in p53 wild-type cell lines, but not in p53-mutated cells. Chromatin immunoprecipitation and luciferase assays using P2 promoter deletion constructs indicate that p53 binds functional response elements located within the P2 promoter. We also show that $\Delta 133p53$ does not bind specifically to p53 consensus DNA sequence *in vitro*, but competes with wild-type p53 in specific DNA-binding assays. Finally, we report that $\Delta 133p53$ counteracts p53-dependent growth suppression in clonogenic assays. These observations indicate that $\Delta 133p53$ is a novel target of p53 that may participate in a negative feedback loop controlling p53 function.

Oncogene (2010) 29, 2691–2700; doi:10.1038/onc.2010.26; published online 1 March 2010

Keywords: p53; $\Delta 133p53$ isoform; promoter; transcriptional regulation; genotoxic stress

Introduction

The tumor suppressor *TP53* gene is one of the most frequently altered genes in human cancers (Petitjean *et al.*, 2007). The expression of its product, the p53 protein, is tightly controlled through protein interaction with the E3-ubiquitin ligase Hdm2 that induces p53 proteasome-dependent degradation (Haupt *et al.*, 1997;

Kubbutat *et al.*, 1997). Under stress, p53 is post-translationally modified, escapes to Hdm2-mediated degradation, accumulates in the nucleus and regulates the transcription of several target genes involved in growth suppressive responses including cell-cycle arrest, senescence and apoptosis. Among cell-cycle arrest genes, p21^{WAF1/CIP1} encodes a cyclin-dependent kinase inhibiting cyclin:CDK complexes at both G1/S and G2/M (el-Deiry *et al.*, 1993; Waldman *et al.*, 1995). p53-dependent apoptosis is mediated through several distinct pathways involving genes, such as *BAX* or *PUMA*, that induce mitochondrial apoptosis through caspase activation (Miyashita and Reed, 1995; Villunger *et al.*, 2003). A complex and critical question is to understand the molecular mechanisms by which p53 induces different types of suppressive responses, depending on the nature and intensity of the stress as well as on the cell and tissue characteristics (Vousden, 2006).

TP53 has been shown to produce nine isoforms (Bourdon *et al.*, 2005). These isoforms combine three different N-terminal transactivation domains (TADs) (the full-length N-terminus present in p53, $\Delta 40$ and $\Delta 133$) with three different C-terminal oligomerization domains (α , β and γ , generated by alternative splicing) (Marcel and Hainaut, 2009). The $\Delta 40p53$ and $\Delta 133p53$ isoforms lack, respectively, 39 residues, which carry the main TAD, or 132 residues, corresponding to the TAD, the proline-rich region and the proximal part of the DNA-binding domain (Figure 1a) (Courtois *et al.*, 2002; Bourdon *et al.*, 2005). $\Delta 40p53$ isoform binds p53 response elements (p53REs), but has no intrinsic transactivation capacity, resulting in the inhibition of p53 transcriptional and growth suppressive functions (Courtois *et al.*, 2002; Ghosh *et al.*, 2004). Recent studies have shown that $\Delta 133p53$ can also inhibit both p53 transcriptional activity in reporter assays and p53-dependent apoptosis (Bourdon *et al.*, 2005; Murray-Zmijewski *et al.*, 2006). Thus, p53 isoforms may regulate some aspects of p53 functions. The expression of both p53 and $\Delta 40p53$ is controlled by the P1 promoter located upstream of exon 1 (Figure 1a) (Tuck and Crawford, 1989; Ghosh *et al.*, 2004). A second promoter, P2, has been identified within *TP53* gene, in a region that spans the distal part of intron 1 to the proximal part of exon 5, covering about 1.5 kb (Bourdon *et al.*, 2005). The resulting transcript, p5314, carries the 3' end of intron 4 followed by all exons from

Correspondence: Dr P Hainaut, Molecular Carcinogenesis and Biomarkers Group, International Agency for Research on Cancer, 150 cours Albert Thomas, Lyon Cedex 08, 69372, France.
E-mail: hainaut@iarc.fr
Received 27 May 2009; revised 13 January 2010; accepted 19 January 2010; published online 1 March 2010

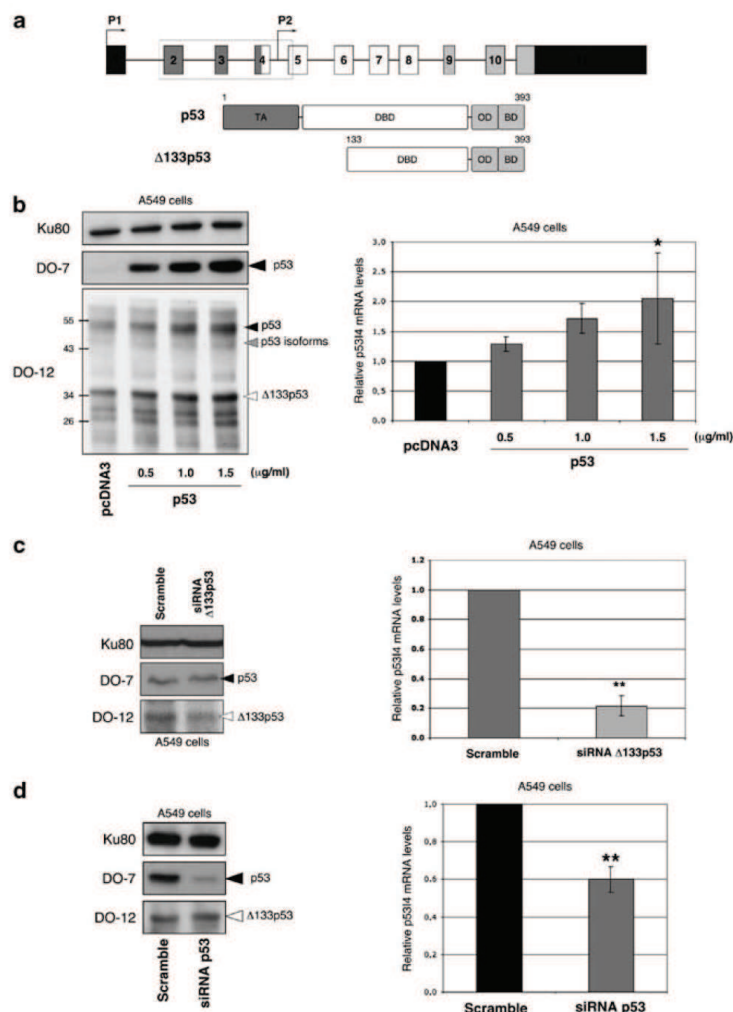


Figure 1 Correlation between p53 and $\Delta 133p53$ expression. (a) The *TP53* gene contains two promoters. The proximal P1 promoter regulates the expression of the p53 protein, which contains a transactivation domain (TAD), a DNA-binding domain (DBD), an oligomerization domain (OD) and a basic domain (BD). Fragment from intron 1 to exon 5 (box) contains the alternative P2 promoter that regulates the expression of the truncated $\Delta 133p53$ isoform. (b) Increased amounts of pcDNA3-p53 vector, expressing p53 cDNA, were transfected in A549 cells. p53 protein was detected by western blot 48 h post-transfection using DO-7 antibody (black arrowhead) and $\Delta 133p53$ using DO-12 antibody in the same extracts (white arrowhead) (left panel). p5314 mRNA levels were quantified by real-time PCR using a forward primer hybridizing within intron 4 (left panel). Increased expression of p53 was correlated with an increased expression of $\Delta 133p53$ at both protein and mRNA levels. Ku80: loading control; * $P < 0.05$; ** $P < 0.01$. (c) siRNA-targeting p5314 mRNA, which encodes $\Delta 133p53$ isoform, was introduced in A549 cells. The expression of p5314 mRNA (left panel) and expression of p53 and $\Delta 133p53$ proteins (right panel) were analyzed, respectively, by real-time PCR or western blot. siRNA treatment reduces the expression levels of both p5314 mRNA and $\Delta 133p53$ protein, whereas it not affects p53 expression levels. (d) In A549 cells, p53 expression was specifically knocked-down using a siRNA directed at exon 4 and $\Delta 133p53$ expression was analyzed at protein (left panel) and mRNA (right panel) levels. Decrease of p53 expression was correlated with a decreased expression of $\Delta 133p53$ at mRNA, but not at protein levels. Ku80: loading control. The DO-7 western blot was exposed for 1 min in (a) and for 10 min in (b).

5 to 11 and supports $\Delta 133p53$ synthesis. However, the regulation of the P2 promoter is still unknown.

TP53 belongs to a family that contains two other members, *TP63* and *TP73*, with strong functional and structural similarities (Kaghad *et al.*, 1997; Yang *et al.*, 1998). These two genes contain an internal promoter,

which regulates the production of N-truncated isoforms (Kaghad *et al.*, 1997; Ishimoto *et al.*, 2002). The p53 protein has been shown to bind the internal promoter of *TP63* and *TP73* and to act either as a negative or positive transcription factor (Grob *et al.*, 2001; Harnes *et al.*, 2003). These observations led us to investigate

whether the TP53 internal P2 promoter may share similar functional characteristics. We show that the P2 promoter of TP53 contains several p53REs, which can drive the expression of a reporter gene in a p53-dependent manner. Furthermore, we show that p53 binds P2 promoter in intact cells and regulates the expression of $\Delta 133p53$ in response to DNA damage. Finally, we provide evidence that $\Delta 133p53$ counteracts growth suppression by p53 in a clonogenic assay, suggesting a function of $\Delta 133p53$ in p53-regulatory loop.

Results

$\Delta 133p53$ expression correlates with p53 protein levels

To determine the function of p53 in $\Delta 133p53$ expression, we first overexpressed p53 in p53-expressing A549 cell lines using pcDNA3-p53 vector that contains p53 cDNA (Figure 1b). The p53 protein levels were verified using DO-7 antibody, which recognizes an epitope (18–23) present in the N-terminal domain of p53, but absent in $\Delta 133p53$ (Figure 1b, left panel). To detect $\Delta 133p53$ protein, we used DO-12 antibody, which recognizes a part of the DNA-binding domain common to both proteins (260–264), but allows to distinguish them on the basis of their different electrophoretic mobilities (Supplementary Figure 1a). To verify that the 34 kDa band corresponds to $\Delta 133p53$, we used a siRNA that specifically targets p5314 mRNA, encoding $\Delta 133p53$ protein. As shown in Figure 1c and in Supplementary Figure 1b, this siRNA significantly reduced both p5314 mRNA levels and the intensity of the 34 kDa band, while not affecting the levels of the canonical p53 protein. After p53 overexpression, an increase in $\Delta 133p53$ protein was observed that correlated with increased amounts of transfected pcDNA3-p53 vector and of expressed p53 protein (Figure 1b; Supplementary Figure 1a). A similar increase in p5314 mRNA was also detected in these conditions with a significant twofold increase in the presence of 1.5 μ g/ml of pcDNA3-p53-transfected vector (Figure 1b, right panel). Comparable effects were observed in another cell line expressing detectable basal levels of wild-type p53, MN1 (data not shown). These observations show that overexpression of p53 induces an increase in $\Delta 133p53$ isoform at both mRNA and protein levels.

To further show that p53 regulates p5314 mRNA levels, we silenced p53 expression in A549 cells. A siRNA-targeting exon 4 of TP53 gene was used to selectively eliminate full-length, but not p5314 transcript. At protein level, p53 silencing did not result in a detectable variation of $\Delta 133p53$ despite reducing p53 protein levels by >70% (Figure 1c, left panel). In contrast, at mRNA level, a significant decrease of 40% in p5314 was observed (Figure 1c, right panel). These data show that removal of p53 in A549 cells decreases basal levels of p5314 mRNA, but not of $\Delta 133p53$ protein. This suggests that p53 silencing affects $\Delta 133p53$ transcription, but that within the time course

of this experiment, the effect of this decrease is not detectable at the protein level, maybe because of its relatively long half-life.

$\Delta 133p53$ expression is modulated in a p53-dependent manner in response to stress

To investigate the possible regulation of $\Delta 133p53$ by p53 in response to stress, we assessed p5314 mRNA expression in response to DNA damage induced by doxorubicin in A459 cells (Figure 2a). Protein accumulation of p53 and Hdm2, a p53-target gene, was verified by western blot in response to two different doses of doxorubicin at 8 h of treatment. We observed a dose-dependent increase in $\Delta 133p53$ protein expression correlated with a concordant increase in p5314 mRNA, reaching a significant threefold increase at the highest dose. However, this dose-dependent increase of $\Delta 133p53$ is limited in cells transfected with a siRNA-targeting p5314 mRNA (Supplementary Figure 1c). These observations indicate that $\Delta 133p53$ is induced by p53 in response to DNA damage.

To assess the impact of p53 on $\Delta 133p53$ expression at basal levels, we next used the MN1 breast cancer cell line grown in estrogen-free conditions (Figure 2b). This condition has been shown to reduce basal levels of p53 in estrogen-dependent breast cancer cell lines (Hurd *et al.*, 1997). At protein level, estrogen deprivation resulted in a marked decrease in p53 and Hdm2 expression after long deprivation times. These variations were paralleled with matched variations in $\Delta 133p53$ expression, at both protein and mRNA levels. Using real-time PCR, the decrease after 12 days of deprivation (20% decrease) was statistically significant. Together, these observations indicate that treatments regulating basal levels of p53 protein, either positively or negatively, exert matched effects on $\Delta 133p53$ mRNA and protein levels.

$\Delta 133p53$ isoform interferes with p53 DNA-binding and growth suppression

It has been shown that $\Delta 133p53$ isoform can form hetero-oligomers with p53 protein and can inhibit p53-dependent transcription in luciferase assays (Murray-Zmijewski *et al.*, 2006; Chen *et al.*, 2009). To investigate whether $\Delta 133p53$ isoform may modulate p53 DNA-binding activity, we performed electro-mobility-shift assays (EMSA) using a ³²P-labeled p53RE consensus oligonucleotide (Figure 3a). Western blot confirmed the presence of the two proteins at expected levels (Supplementary Figure 2a). In the presence of p53 alone, a shift was observed, which was both super-shifted and stabilized by the addition of the monoclonal PAb421 antibody, as described earlier (Figure 3a) (Verhaegh *et al.*, 1997). In the presence of $\Delta 133p53$, no shift was observed, whereas in the presence of the two proteins, $\Delta 133p53$ seems to significantly decrease the super-shift generated by the formation of DNA:p53:PAb421 complex (Figure 3a; Supplementary Figure 2b). These observations indicate that $\Delta 133p53$ does not bind p53RE DNA on its own, which is consistent with the

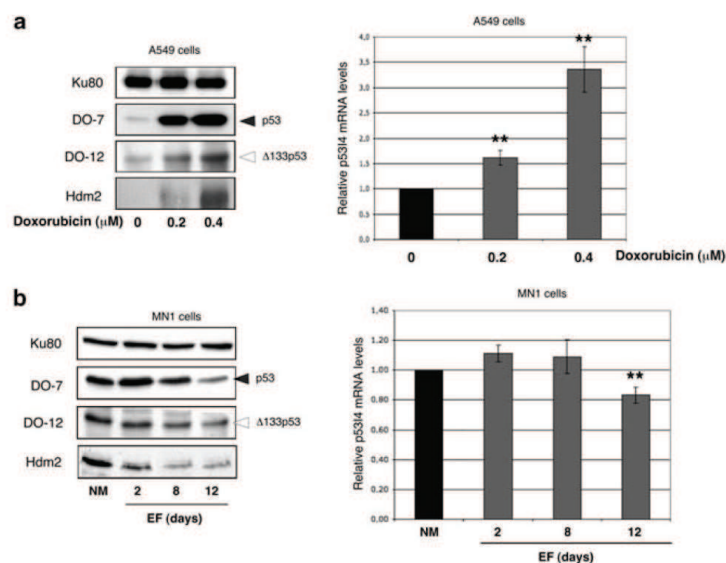


Figure 2 p53-dependent modulation of $\Delta 133p53$ expression. (a) A549 cells were treated during 8 h with the DNA-damaging drug doxorubicin. The induction of p53, $\Delta 133p53$ and Hdm2 was determined by western blot (left panel) and the expression of p5314 mRNA was analyzed using real-time PCR (right panel). Ku80: loading control; ** $P < 0.01$. (b) Wild-type p53-expressing MN1 cells were cultured for up to 12 days in estrogen-free medium (EF). p53, Hdm2 and $\Delta 133p53$ proteins as well as p5314 mRNA were analyzed as in (a). Modulation of p53 by estrogen deprivation correlated with $\Delta 133p53$ protein and p5314 mRNA variations. NM: normal medium.

notion that it lacks L1 loop of the DBD (residues 117–142) (Bourdon *et al.*, 2005). Furthermore, it reduces p53 DNA-binding activity, maybe by forming hetero-oligomers unable to bind p53RE DNA. To verify this hypothesis, EMSA were conducted using DO-7 antibody in addition to Pab421 (Supplementary Figure 2b). Indeed, hetero-oligomers containing $\Delta 133p53$, which lacks DO-7 epitope, would undergo an incomplete shift in the presence of DO-7 antibody. No such shift was detected, suggesting that p53: $\Delta 133p53$ complexes are unable to bind specifically to p53RE DNA. The above results suggest that $\Delta 133p53$ isoform may inhibit p53 transcriptional capacity by inhibiting its DNA-binding activity.

To determine whether such an effect may inhibit p53 suppressive function, a colony formation assay was performed using p53-null H1299 cells transfected with vectors expressing p53, $\Delta 133p53$ or both (Figure 3b). After 14 days of selection, cells transfected with empty vector were spread over the surface of the well. Conversely, expression of p53 drastically reduced colony formation, in agreement with its growth suppressive activity. When compared with empty vector, cells transfected with $\Delta 133p53$ tended to form less colonies; however, $\Delta 133p53$ did not exert a suppressive effect similar to the one of p53. When co-transfected at equal amounts, $\Delta 133p53$ at least partially prevented the suppressive effect of p53. These observations suggested that $\Delta 133p53$ may counteract p53 suppressive function.

p53 transactivates the internal P2 promoter of TP53 gene $\Delta 133p53$ expression at mRNA levels was correlated with the expression levels of p53 protein, suggesting that p53 transactivates the internal P2 promoter. To test this hypothesis, we generated a luciferase reporter driven by the P2 promoter sequence identified by Bourdon *et al.* (2005), consisting in a 1.5 kb fragment from intron 1 to exon 5 of TP53 gene. This luciferase reporter was co-transfected in p53-null H1299 cells with various amounts of pcDNA3-p53 expression vector (Figure 4a, right panel). The expression of exogenous p53 protein and of its target gene Hdm2 was verified by western blot (Figure 4a, left panel). The basal transcriptional activity of the P2 promoter in the absence of p53 was fivefold higher than the one of the Basic promoterless luciferase vector. In the presence of pcDNA3-p53 vector at concentrations above 0.25 μg/ml, luciferase activity was significantly increased by about sixfold over the basal transcriptional activity of the P2 promoter. The luciferase activity was concordant with p53 expression levels (Figure 4a, left panel) and showed a dose-dependent increase with amounts of transfected pcDNA3-p53 vectors between 25 and 500 ng/ml (Supplementary Figure 3a). These results indicate that the P2 promoter activity is regulated by p53.

It has been reported that $\Delta 40p53$ isoform lacks part of the TAD and has no transcriptional activity toward reporter system carrying p53REs (Courtois *et al.*, 2002; Ghosh *et al.*, 2004). Different amounts of $\Delta 40p53$ were transfected in the presence of the luciferase

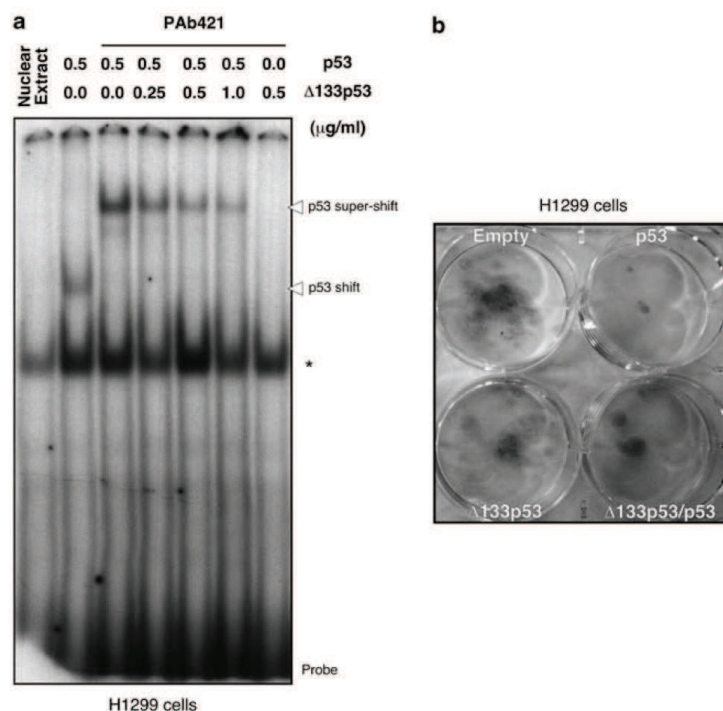


Figure 3 Effects of $\Delta 133p53$ on p53 biochemical and biological properties. (a) Electro-mobility-shift assays (EMSA) measuring DNA-binding activity. p53-null H1299 cells were co-transfected with a constant amount of pcDNA3-p53 vector (0.5 $\mu\text{g/ml}$) in the presence of increasing amounts of pcDNA3- $\Delta 133p53$ vector (0.25–1 $\mu\text{g/ml}$). Nuclear extracts were incubated with a ^{32}P -labeled p53RE oligonucleotide, with or without PAb421, which stabilizes and super-shifts p53:DNA complexes. DNA:p53 shifts and DNA:p53:PAb421 super-shift are indicated by arrowheads. *: non-specific band. (b) Colony formation assays were performed using p53-null H1299 cells transfected with 0.5 $\mu\text{g/ml}$ of wild-type pcDNA3-p53 vector and/or $\Delta 133p53$ vector during 14 days under 0.5 mg/ml of neomycin selection. As compared with wild-type p53, $\Delta 133p53$ did not suppress cell growth (60% of colonies compared with empty conditions) and partially reverted the suppression induced by wild-type p53 (10% in presence of p53 compared with 40% in presence of both p53 and $\Delta 133p53$).

reporter to investigate whether p53 TAD is required to modulate the P2 promoter activity (Supplementary Figure 3b). Compared with the significant increase in luciferase activity with pcDNA3-p53 vector, $\Delta 40p53$ alone has only minimal, non-significant effects on luciferase activity. These data suggest that p53 transcriptional activity is required to transactivate the P2 promoter.

p53REs are located within the P2 promoter

The p53 protein binds to response elements defined as repeats of a palindromic consensus sequence (5'-RRRCWWGYYY-3') (el-Deiry *et al.*, 1992). We used MatInspector software to identify putative p53RE by analyzing the fragment from intron 1 to exon 5 of *TP53* that corresponds to the P2 promoter (Cartharius *et al.*, 2005). This DNA fragment contains four putative p53REs (Figure 4b; Supplementary Figure 4a). The first p53RE, termed as RE-1, is located in the 5' end of intron 3. RE-2 is contained within exon 4, whereas the RE-3 and -4 are located 9 bp apart in intron 4 just upstream of the initiation start site of transcription. None of these REs shows a perfect match with the p53

consensus, the degree of matching varying from 60% (RE-3) to 85% (RE-2) (Supplementary Figure 4b).

To evaluate the contribution of these p53REs, we generated four deletion mutants of the promoter into the luciferase reporter (Figure 4b). In the absence of p53, these reporters showed differences in their basal transcriptional activity, suggesting that they may contain different regulatory elements in addition to p53 (Supplementary Figure 5a). These differences in basal activities have been normalized to evaluate the activation induced by p53. As shown in Figure 4b, p53 clearly stimulated the activity of the three P2 luciferase constructs (9–10-fold over Basic): P2 (containing RE-1 to RE-4); P2 Δ 1 (RE-1 to RE-4) and P2 Δ 2 (RE-2 to RE-4). However, it had only minor effects on P2 Δ 3 (RE-3/4) and P2 Δ 4 (no RE) (less than twofold over Basic). These results suggest that, among the four putative REs, RE-2 may have the major effect.

To investigate the function of RE-2 in greater details, we introduced mutations at critical bases of RE-2 by successive rounds of site-directed mutagenesis (Figure 4c). The resulting reporter constructs, termed as P2 Mut1 and Mut2, carry, respectively, 2 or 4 introduced mismatches with the consensus sequences. In the presence of the



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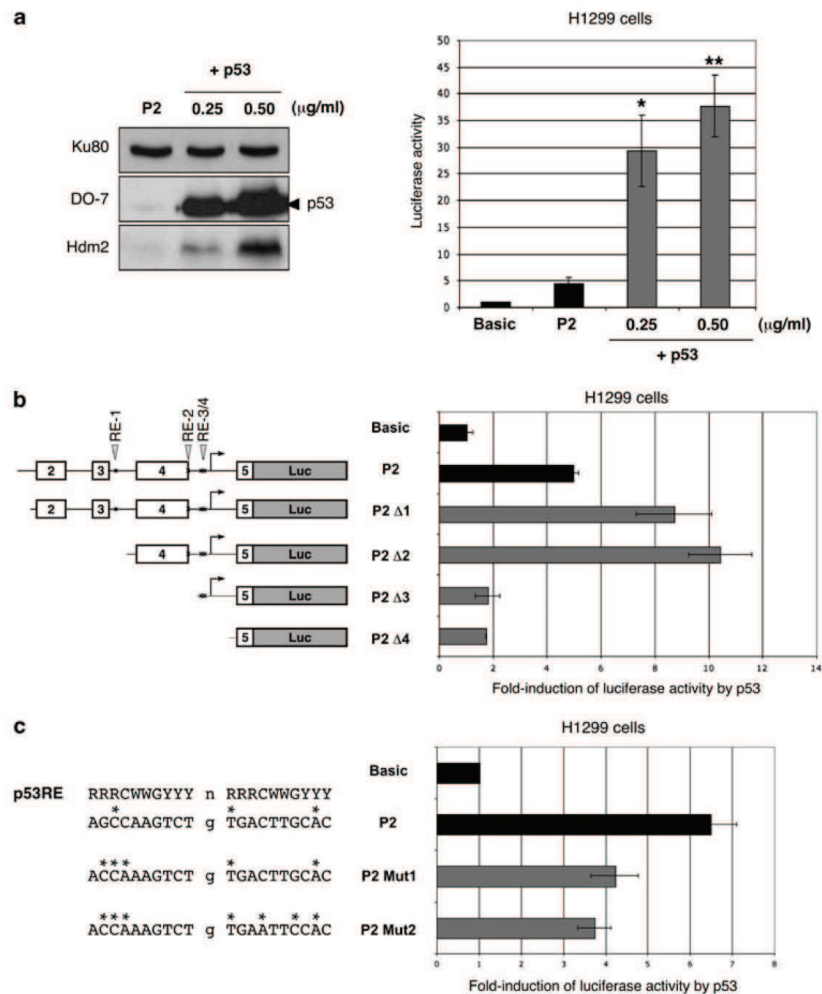


Figure 4 P2 promoter activity is regulated by p53. (a) In p53-null H1299 cells, luciferase assays (right panel) were performed using a luciferase reporter under the control of the 1.5 kb segment of *TP53* from intron 1 to exon 5, in the presence of increasing amounts of pcDNA3-p53, the expression of which was verified by western blot (left panel). Increase in luciferase activity was concordant with p53 protein levels. Basic: promoterless luciferase reporter; P2: luciferase reporter driven by *TP53* segment; Ku80: loading control; * $P < 0.05$; ** $P < 0.01$. (b) MatInspector software (Cartharius *et al.*, 2005) was used to predict four putative p53-binding sites (RE-1 to -4) in the promoter P2. Luciferase reporter deletion constructs were engineered as pictured and tested in the presence of p53. Mean fold induction was calculated as the ratio between luciferase activity of each construct in the presence of pcDNA3-empty or -p53 vector. p53 induced at least a fivefold increase of luciferase activity using P2, $\Delta 1$ or $\Delta 2$. In contrast, no induction was observed using $\Delta 3$ and $\Delta 4$. (c) Luciferase assays (right panel) were performed in cells co-transfected with pcDNA3-p53 vector and with either one of two plasmids carrying mutations in RE-2 (P2 Mut1 and P2 Mut2; left panel). Mutations in p53RE induced a 40% decrease of luciferase activity. p53RE: consensus sequence of p53RE; star: mismatch between p53RE and RE-2 in the promoter P2.

pcDNA3-empty vector, the three P2 plasmids exhibited the same luciferase activity, indicating no abolition of their basal transcriptional activities (Supplementary Figure 5b). In the presence of pcDNA3-p53 vector, the promoter activities of P2 Mut1 and Mut2 were reduced by about 40% as compared with wild-type P2 (Figure 4c). These observations suggest that RE-2 located in exon 4 participate in the p53-dependent regulation of P2 promoter, but does not account on its own for the full activation of P2 by p53.

p53 binds the internal promoter P2 of TP53 gene
We used chromatin immunoprecipitation (ChIP) to analyze the binding of p53 on different segments of the P2 promoter in A549 cells in basal conditions. The DO-7 antibody was used to immunoprecipitate p53 and non-immunized IgG were used as negative control (Figure 5). After ChIP, five PCR products were analyzed (Figure 5a). As controls, we used primers designed to amplify the p21^{WAF1/CIP1} promoter (positive

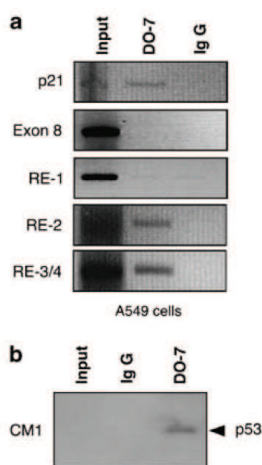


Figure 5 Binding of p53 on P2 promoter. (a) Chromatin immunoprecipitation assay (ChIP) for p53 protein onto P2 promoter in wild-type p53-expressing A549 cells in basal conditions. Purified DNA immunoprecipitated by p53 was analyzed by PCR using specific primers to amplify regions located in p21^{WAF1/CIP1} promoter (positive control), in exon 8 of *TP53* gene (negative control) and in three parts of promoter P2 containing the putative p53REs RE-1, RE-2 and RE-3/4. Input: ChIP positive control; IgG: ChIP negative control; DO-7: immunoprecipitation using DO-7 antibody. (b) In A549 cells, p53 was immunoprecipitated using DO-7 antibody and immunodetected using CM1. A band of low intensity was detected in DO-7 immunoprecipitated sample.

control) and exon 8 of *TP53* gene (negative control). ChIP with IgG did not generate any amplification. In contrast, ChIP with DO-7 antibody generated an amplification signal for p21^{WAF1/CIP1} promoter, but not for *TP53* exon 8. With P2 promoter fragments, no amplification was observed with primers targeting a DNA fragment containing RE-1 (Figure 5a). In contrast, amplifications were observed for fragments encompassing RE-2 and RE-3/4. These observations suggest that p53 physically binds several p53REs located between exon 4 and intron 4, but not in intron 3.

Mutant p53 does not induce $\Delta 133p53$ expression

To further assess the specificity of the wild-type p53 effect on the P2 promoter, we analyzed its regulation in response to DNA damage by doxorubicin in several cell lines expressing p53 with different functional status (Figure 6a). In wild-type p53 cells, doxorubicin treatment induced both p53 and $\Delta 133p53$ expression as expected. In contrast, in three cell lines expressing a non-functional p53 protein, treatment with doxorubicin had either no or only minimal effects on the expression of p53 and $\Delta 133p53$.

These observations were extended by analyzing the effects of several common 'hotspot' *TP53* mutants on the expression of the P2 luciferase reporter (Figure 6b). In contrast to wild-type p53, none of these mutants succeeded in activating the reporter. These observations

support the notion that only wild-type p53 with functional capacities transactivates the P2 promoter leading to $\Delta 133p53$ expression.

Discussion

Recently, it has been shown that *TP53* gene is expressed as several isoforms (Bourdon *et al.*, 2005). Among them, only $\Delta 133p53$ is regulated by an alternative promoter, P2, located within *TP53* (Bourdon *et al.*, 2005). Its overexpression has been observed in squamous cell carcinoma of the head and neck as well as in breast cancers, suggesting a function of $\Delta 133p53$ in tumorigenesis (Bourdon *et al.*, 2005; Boldrup *et al.*, 2007). However, the biological regulation and function of $\Delta 133p53$ are still unknown. Here, we have analyzed the P2 promoter and we show that its activity is regulated by p53. First, modulation of wild-type p53 expression induces $\Delta 133p53$ expression at both mRNA and protein levels. Second, the P2 promoter contains p53REs, which are involved in p53 responsiveness. Third, $\Delta 133p53$ prevents the binding of wild-type p53 to DNA segment containing its consensus sequence and inhibits p53 suppressive activity in a clonogenic assay. These data suggest that $\Delta 133p53$ may contribute to an auto-regulatory process by which p53 controls its own suppressor function.

At the time of $\Delta 133p53$ identification, several evidences supported the presence of an internal promoter involved in the production of $\Delta 133p53$ (Bourdon *et al.*, 2005). A transcript, p5314, initiated within intron 4, but exhibiting the same splicing pattern as full-length p53 mRNA downstream of exon 5, was observed in several human cell lines, in *Drosophila* and *Zebrafish* (Bourdon *et al.*, 2005; Chen *et al.*, 2005). In addition, a 1.5kb segment of *TP53* spanning from intron 1 (+10810) to exon 5 (+12396) conferred a sixfold induction to a luciferase reporter (Bourdon *et al.*, 2005). In this study, we confirm that the same *TP53* sequence possesses a promoter activity (fivefold induction of reporter) and we show that this segment confers responsiveness toward wild-type, but not mutant p53. Furthermore, using MatInspector software, we identified four putative p53REs upstream of the transcription initiation site. These p53RE have a degree of degeneration, which is in the same range as p53REs of many common p53-target genes (Menendez *et al.*, 2007). Using luciferase and ChIP experiments, we show that transcriptional activation by p53 is dependent on two contiguous regions containing p53REs: one in the 3'-end of exon 4 that correlates with the main p53 responsiveness and one in intron 4. These observations correlate with the one of Chen *et al.* (2009), who recently reported that the internal P2 promoter of *TP53* *Zebrafish* was induced by p53 through two close p53REs located in intron 4, just upstream the initiation site of transcription. These authors suggest that the two response elements operate in a cooperative manner. These data suggest that human $\Delta 133p53$ isoform and its *zebrafish*

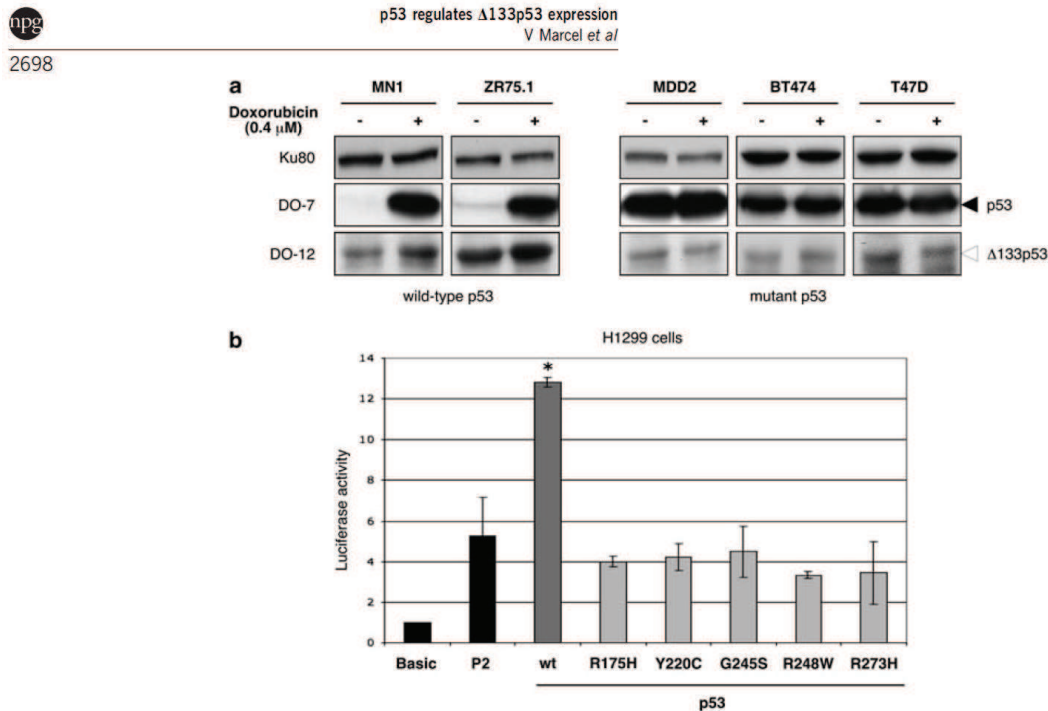


Figure 6 Effect of mutant p53 on $\Delta 133p53$ expression. **(a)** Breast cancer cells carrying different *TP53* mutation status were treated for 8 h with doxorubicin at 0.4 μ M. Protein extracts were analyzed by western blot to determine the expression of p53 (DO-7) and of $\Delta 133p53$ (DO-12). p53 mutant cells overexpress an inactive p53 protein. Ku80: loading control. **(b)** Luciferase assays were performed in the presence of different mutant p53 as indicated. As compared with wild-type p53, mutant p53 had no effect on P2 promoter activity. Wt: wild-type p53; * $P < 0.05$.

homolog are regulated by a conserved mechanism involving p53 protein.

In addition to *in vitro* data, we reported p53-dependent regulation of $\Delta 133p53$ isoform in cancer cell lines expressing wild-type, but not mutant p53. To detect $\Delta 133p53$, we have used the monoclonal antibody DO-12 that recognize a p53 epitope (260–264). This antibody has been described as the most appropriate to detect $\Delta 133p53$ among a panel of seven common p53 antibodies. Using siRNA-targeting p53i4 mRNA to specifically knock-down $\Delta 133p53$, we verified that the 34 kDa band indeed corresponds to $\Delta 133p53$ protein (Supplementary Figure 1b) (Bourdon, 2007). In response to DNA damage induced by doxorubicin, an increase in $\Delta 133p53$ expression followed the accumulation of p53 by about 8 h, a time-course compatible with that of products of other p53-regulated genes such as *Hdm2*, *BAX* or *p21^{WAF1/CIP1}* (Figure 2a and data not shown). In Zebrafish, a similar lag of 6 h has been observed between induction of p53 and $\Delta 113p53$ on ribosomal stress (Danilova *et al.*, 2008). The p53-dependent regulation of $\Delta 133p53$ expression in response to stress was also reported in response to γ -rays exposure (Chen *et al.*, 2009). Although several reports have described induction of $\Delta 133p53$ expression in response to stress, no data on basal activity of P2 promoter have been reported. Here, using siRNA and estrogen deprivation that both results in a reduction of

basal p53 expression, we observed a decrease in $\Delta 133p53$ expression at mRNA levels. These observations suggest that p53 has an important function in the maintenance of $\Delta 133p53$ expression at basal levels. Although p53 siRNA did not have a detectable effect on $\Delta 133p53$ protein expression after 48 h, this may be explained by the fact that $\Delta 133p53$ lacking the Hdm2-binding domain may escape Hdm2-mediated degradation, being thus more stable than p53 (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997).

In contrast to p53, $\Delta 133p53$ lacks the first 30 residues of the DNA-binding domain encoding the L1 loop and part of the loop-sheet-helix motif that form the structure binding to the major groove of DNA in p53RE (Cho *et al.*, 1994). As expected, we observed that this protein is unable to bind DNA containing the p53 consensus sequence using the same *in vitro* conditions than for p53. However, $\Delta 133p53$ could decrease the binding capacity of p53 on p53RE. As it has been described that p53 and $\Delta 133p53$ can form hetero-tetramers (Chen *et al.*, 2009), our data suggest that $\Delta 133p53$ tetramers lack sequence-specific DNA-binding capacity. The inhibition of p53-binding capacities by $\Delta 133p53$ is in agreement with the inhibitory effect of $\Delta 133p53$ on both p53 transcriptional activity and p53-dependent apoptosis (Bourdon *et al.*, 2005; Murray-Zmijewski *et al.*, 2006). Here, we provide support for long-term anti-suppressive effects of $\Delta 133p53$ by showing that co-expression of $\Delta 133p53$

and p53 could partially restore the proliferation capacity of H1299 cells in a clonogenic assay. However, despite the accumulation of evidences suggesting that $\Delta 133p53$ may counteract p53 suppressive function, there is still limited information available on the physiological function of $\Delta 133p53$. One of the problems is that $\Delta 133p53$ seems to be expressed at low levels as compared with p53, in particular after DNA damage. Recent results in Zebrafish shed a light on a possible mechanism of action (Chen *et al.*, 2009). They observed that the Zebrafish homologous of $\Delta 133p53$ could antagonize p53-induced apoptosis through activating Bcl-2L (homologous of the human Bcl-xL), and that knockdown of $\Delta 133p53$ enhanced p53-mediated apoptosis under stress conditions. These results are consistent with the ones reported here and indicate that small changes of $\Delta 133p53$ may contribute to a novel feedback pathway that modulates the p53 response.

In this study, we observed that the internal P2 promoter involved in $\Delta 133p53$ expression is regulated by p53. This feature is a familial trait also observed in the other TP53 gene family members, TP63 and TP73, which express N-truncated proteins that are regulated by internal promoters responsive to wild-type p53 (Grob *et al.*, 2001; Harmes *et al.*, 2003). These N-truncated proteins participate in auto-regulatory feedback loops that regulate p53 suppressive functions. This model seems to be relevant for $\Delta 133p53$ isoform as well. Thus, $\Delta 133p53$ is another player involved in these auto-regulatory feedback loops, which may selectively interfere with p53 capacity to transactivate target genes. It remains to be determined in which cell type and stress type context this regulation may have a major function.

Materials and methods

Cell culture

Two human cell lines expressing wild-type TP53 were employed: lung A459 and breast ZR75.1 cells. Human p53-null H1299 cells were used for luciferase assays and EMSA. Two human breast cell lines expressing mutant TP53, BT474 (E285K) and T47D (L194F) were used. Two isogenic breast cell lines derived from MCF-7 were employed, MN1 (stably transfected with an empty vector) and MDD2 (expressing a mini-peptide exerting negative effect on the endogenous wild-type p53) (Bacus *et al.*, 1996). Cells were treated for 8 h with doxorubicin (0.2 or 0.4 μ M). Breast MN1 cells were treated with medium deprived of both Red phenol and estradiol.

Vectors

Exogenous p53 isoforms (p53, $\Delta 40p53$ and $\Delta 133p53$) were produced using pcDNA3 vectors containing human cDNA of each isoforms (Courtois *et al.*, 2002). Five mutant pcDNA3-p53 were used (R175H, Y220C, G245S, R248W and R273H). The pcDNA3-empty vector was used as a negative control. Several luciferase reporter vectors were developed using pGL3-Basic, a promoterless luciferase plasmid (Promega, Fitchburg, WI, USA). The pGL3-P2 (or P2) vector contains the human P2 promoter (from intron 1 to exon 5) upstream of Luciferase gene. Four deleted and two mutant vectors were constructed using P2 vector (Supplementary Table 1). The pGL3-Basic was

used as negative control to standardize experiments and phRL-null plasmid as control of transfection efficiency to normalized experiments.

Transfection

The pcDNA3 expression vectors (1.5 μ g/ml completed with pcDNA3 empty) were transfected using 9 μ l of Eugene (Roche, Indianapolis, IN, USA) in A549 and H1299, harvested 48 h latter for overexpression and 24 h latter for EMSA assays. Silencing of p53 mRNA was performed in A549 cells seeded in antibiotic-free medium and directly transfected with 20 nm of either scramble or p53-targeted siRNAs (Eurogentec, Liege, Belgium) (Supplementary Table 1) using 12 μ l of Hyperfect (Qiagen, Huntsville, AL, USA). Cells were transfected twice at 24 h interval and harvested after 48 h of treatment. For luciferase assays using Eugene (Roche), 500 ng/ml of pGL3 plasmids were transfected with 10 ng/ml of phRL-null and with 1 μ g/ml of pcDNA3 expression vectors. Cells were harvested 48 h post-transfection and luciferase activity was analyzed using Dual Luciferase Reporter Assay System (Promega).

Real-time quantitative RT-PCR

Total RNA was extracted using Nucleospin RNA II kit (Machery-Nalgene, Duren, Germany) and reverse transcription was performed using random primers (Promega) and SuperScript II (Invitrogen, Carlsbad, CA, USA). RNA expression was analyzed by real-time quantitative RT-PCR using Brilliant SYBR Green Master Mix (Qiagen) and 0.4 μ M of primers (Supplementary Table 1). The p5314 mRNA expression level is normalized to that of Gapdh (lung cells) or of 28S (breast cells). The control of the experiment is used as the reference. All samples were analyzed in triplicates and two Q-PCRs were performed for each experiment.

Western blot

Proteins were extracted using RIPA-like buffer (50 mM Tris-HCl pH7.4, 250 mM NaCl, 0.1% SDS, 0.5% NP-40, 2 mM DTT, 1 mg/ml protease inhibitors) and were analyzed on a 10% SDS-polyacrylamide gel using several antibodies: DO-7 (monoclonal anti-p53 antibody specific of TAD; Dako, Cambridge-shire, UK); DO-12 (monoclonal anti-p53 antibody specific of DBD), which has been described as the most appropriate to detect $\Delta 133p53$ among a panel of seven common p53 antibodies (Bourdon, 2007); CM1 (polyclonal anti-p53 antibody; Novocastra, Newcastle, UK); Hdm2 (Abcam) and Ku80 (used as a loading control; Abcam). Detection was performed with the ECL kit (Amersham, Cambridge, MA, USA) by autoradiography.

Chromatin immunoprecipitation assay

In 100 mm plates, 3×10^6 A549 cells were seeded. The following day, 1% formaldehyde was added onto cells for 10 min at room temperature to cross-link proteins to DNA. Sonication was performed (twice: 10 \times (5 s on; 5 s off) at 21% amplitude) (Vibra cell 75041) and immunoprecipitation was carried out using 4 μ g of DO-7 antibody (anti-p53) and protein G-agarose beads (Invitrogen). DNA:p53 complexes were dissociated by reverse cross-linking using NaCl 200 mM and DNA was extracted by phenol/chloroform. Immunoprecipitated DNA was amplified by PCR (Supplementary Table 1).

Electro-mobility-shift assay

Nuclear cellular proteins of H1299-transfected cells were extracted and incubated with mixture containing 32 P-radio-



llabeling oligonucleotide with p53RE consensus as described earlier (Supplementary Table 1) (Verhaegh *et al.*, 1997). Two antibodies were used to shift DNA:p53 complex: the monoclonal PAb421 antibody recognizing p53 DBD and known to stabilize DNA:p53 complex; and the monoclonal DO-7 antibody specific of p53 protein.

Statistical analysis

Anova and Student's *t*-tests were performed using on-line tools (<http://faculty.vassar.edu/lowry/VassarStats.html>). In the histograms, black bars indicate the control used for statistics and *P*-values were indicated by (*) when *P*<0.05 and (**) when *P*<0.01.

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Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

VM is supported by la Ligue Nationale Contre le Cancer. LF-C is supported by The Association for International Cancer Research (AICR). This project is funded by la Ligue Régionale du Rhône Contre le Cancer.

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Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>)

3.3 Expression of $\Delta 40p53$ and $\Delta 133p53$, two isoforms of p53 lacking the N-terminus, in cutaneous melanoma

Article No.3

To be submitted to Virchow Archiv

3.3.1 Context

Due to their capacity to antagonize p53 when expressed in excess to TAp53, N-terminal isoforms $\Delta 40p53$ and $\Delta 133p53$ may provide a functional mechanism to inactivate p53 in tumours that do not contain mutant *TP53*. Indeed, the N-terminal isoforms, $\Delta 40p53$ and $\Delta 133p53$, which lack the first 39 ($\Delta 40p53$) or 132 ($\Delta 133p53$) residues, both inhibit growth suppression in a dominant-negative manner when expressed in significant excess as compared to TAp53.

Cutaneous melanomas are highly malignant tumours that retain wild-type *TP53* in over 90% of the cases, suggesting that p53 suppressive activity may be abrogated through alternative mechanisms (Chin et al., 2006; Tsao et al., 2012). Such alternative mechanisms have been demonstrated in cancers of the cervix, head and neck and in sarcomas, in which wild-type p53 is suppressed by targeting the protein for proteasome-mediated degradation through viral or cellular proteins acting as E3 ubiquitin ligases (Petitjean et al., 2007). In sarcomas, the main mechanism is amplification and over-expression of Hdm2, the main regulator of p53 protein stability (Onel and Cordon-Cardo, 2004). A recent study has shown that Hdm4, a RING finger protein which forms complexes with Hdm2, was up-regulated in a substantial proportion (65%) of stage I-IV metastatic melanomas (Gembarska et al., 2012). Hdm4 (also known as Hdmx) cooperates with Hdm2 to induce poly-ubiquitination and proteosomal degradation of p53 (Wang, 2011). In this study, we have analysed the expression of p53I2 mRNA (encoding $\Delta 40p53$) and p53I4 mRNA (encoding $\Delta 133p53$) isoforms to determine their potential involvement in inactivating p53 in cutaneous melanomas.

3.3.2 Strategy

Through collaboration with JC Marine, University of Ghent, Belgium, we have used mRNA extracted from short-term cultures of melanoma cells, and from stage I-IV melanomas which had previously been used to analyse Hdm4 expression levels by Gembarska et al. (2012) (Gembarska et al., 2012). A series of 40 cases was assembled, sub-grouped in 4 categories of 10 cases according to the extent of metastatic dissemination, with group 1: non-invasive melanomas (stage I-II), group 2: Regional Dermal (RD) melanomas; group 3: Nodal Metastatic (NM) melanomas and group 4: Distal Metastatic (DM) melanomas. The levels of expression of Fully-Spliced (FSp53), p53I2 and p53I4 mRNA were analysed by RT-PCR using GAPDH as internal standard and the ΔCt method to express the results. In cell lines, expression levels were further compared to the mean of levels in two primary cultures of normal melanocytes. In tumours, the mean ΔCt values in group 1 (non-invasive melanomas) were used as reference.

3.3.3 Results

RT-qPCR provides for a clear and unambiguous, quantitative assessment of steady-state levels of FSp53, p53I2 and p53I4 mRNA in melanoma cell lines and tissues. In cultured melanoma cell lines, enhanced expression of FSp53, p53I2 or p53I4 compared to primary melanocytes was detected in 53% of the cases (9/17). Expression of FSp53 and p53I2 was often enhanced in a coordinated way. In contrast, p53I4 was substantially enhanced in only one cell line and was not correlated with FSp53 or p53I2. Compared to primary stage I-II melanoma, enhanced expression of either p53I2 or p53I4 was detected in a substantial proportion of metastatic cases (70%; 21/30). When sub-grouped according to metastatic status, enhanced expression of either p53I2 or p53I4 was more frequent in nodal (NM) (9/10) and distant (DM) (8/10) melanomas than in regional dermal (RD) (4/10) lesions ($p=0.044$, Fisher-Exact) (Figure 22). Whereas enhanced expression of FSp53 mRNA was detected only in cases with enhanced expression of p53I2, p53I4 alone was increased in 6/30 lesions (20%).

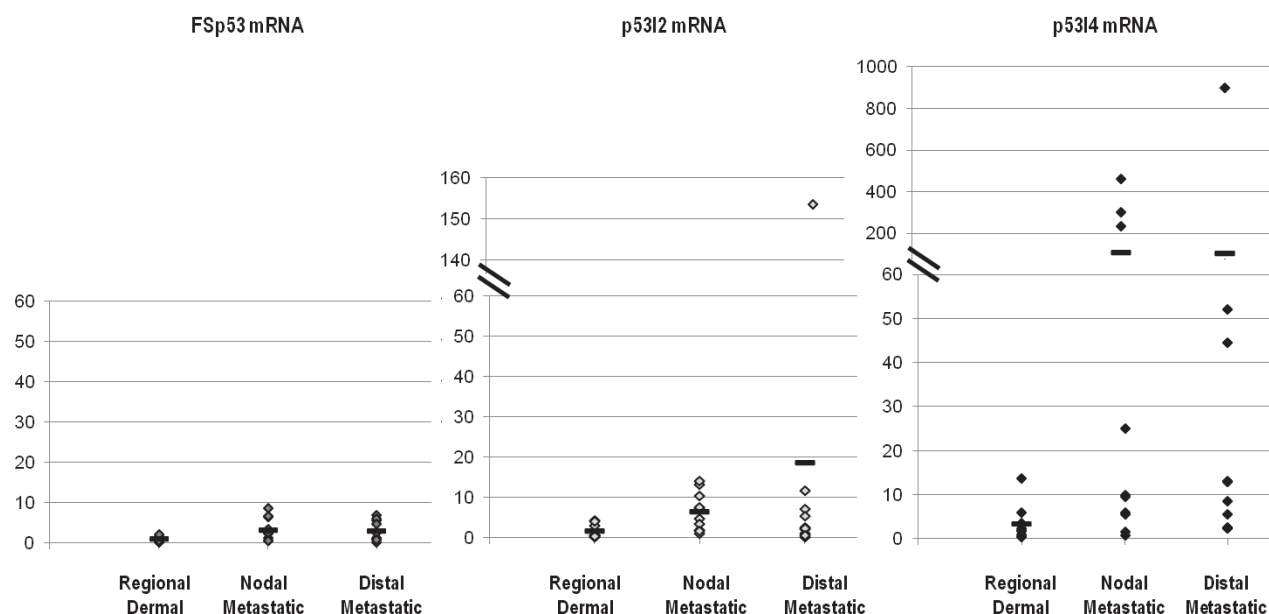


Figure 22. Representation of expression levels of FSp53, p53I2 and p53I4 mRNAs in melanoma tissues

qRT-PCR analysis were performed to determine the expression levels of FS-p53 (Fully-spliced p53, grey dots), p53I2 (retaining intron 2, white dots) and p53I4 (retaining intron 4, black dots). Bars indicate the average.

3.3.4 Conclusion

In this study, we have taken advantage of a pre-existing, well characterized series of melanoma cases sub-grouped according to metastatic status to analyse the levels of expression of mRNA encoding TAp53 (FSp53 mRNA), $\Delta 40p53$ (p53I2 mRNA) or $\Delta 133p53$ (p53I4 mRNA) using a qRT-PCR strategy. Our results show that levels of expression of these mRNA isoforms are increased in the majority of metastatic melanoma cases and was significantly higher and more frequent among melanoma with nodal (NM) or distant (DM) metastasis than among regional dermal (RD) or non-metastatic melanoma. Among the 3 NM and DM cases without enhanced p53I2 or p53I4 mRNA expression, one contained mutant *TP53* (p.R342X) whereas the mutation status of the two other cases was not available. FSp53 and p53I2 mRNA showed an overlapping pattern of enhanced expression, whereas p53I4 mRNA expression is enhanced in cases that show neither FSp53 nor p53I2 mRNA expression. These results are

compatible with the fact that FSp53 and p53I2 mRNA are alternative splicing variants expressed from the same promoter, whereas p53I4 mRNA is controlled by a distinct promoter located in intron 4. Thus, more than one mechanism may contribute to enhanced expression of p53 mRNA isoforms in metastatic melanoma. Overall, these results show that deregulation of expression of p53 mRNA encoding N-terminal truncated isoforms is common in nodal or distant metastatic melanoma lesions, suggesting that this phenomenon may contribute to functional inactivation of p53 during the spread of melanoma. Our study has strong limitations in the fact that it does not provide a direct assessment of p53 protein status. Indeed, thus far, there is no specific antibody that specifically recognizes $\Delta 40p53$ or $\Delta 133p53$ isoforms. Since their amino-acid sequence is a part of TAp53 sequence, isoforms are recognized by antibodies that also react with TAp53, making it cumbersome to analyse p53 isoform expression using histochemistry. Further studies will be needed to assess the levels of p53 protein isoforms and to understand the dynamics of interactions between TAp53 and p53 isoforms in melanoma cells that overexpress Mdm4 or not.

PRELIMINARY DRAFT MANUSCRIPT

to be submitted to Virchows Archiv

Expression of Delta40p53 and Delta133p53, two isoforms of p53 lacking the N-terminus, in cutaneous melanoma.

Hind Hafsi and Pierre Hainaut*

International Agency for Research on Cancer, Lyon, France

*Current address:

International Prevention Research Institute, Lyon, France

*Address for correspondence:

Pierre Hainaut, PhD

International Prevention Research Institute

15 Chemin du Saquin

69130 Ecully, France

Tel : +33 4 72 17 11 86

Fax : +33 4 72 17 11 90

email : pierre.hainaut@i-pri.org

Keywords : melanoma, metastasis, p53, isoforms, mRNA.

Abstract

Cutaneous melanomas are highly malignant tumours that retain wild-type *TP53* in over 90% of the cases, suggesting that p53 suppressive activity is abrogated through alternative mechanisms. We have used mRNA extracted from short-term cultures of melanoma cells and from stage I-IV melanomas to analyse the expression of FSp53 (Fully-Spliced) mRNA as well as p53I2 and p53I4 mRNA, encoding Delta40p53 and Delta133p53, respectively. These p53 isoforms lack the first 39 (Delta40p53) or 132 (Delta133p53) N-terminal residues and are unable to transactivate p53 target genes *in vitro*. In cultured cells, enhanced expression of FSp53, p53I2 or p53I4 compared to primary melanocytes was detected in 53% of the cases (9/17). Compared to primary stage I-II melanoma, enhanced expression of either p53I2 or p53I4 was detected in a substantial proportion of metastatic cases (70%; 21/30). When subgrouped according to metastatic status, enhanced expression of either p53I2 or p53I4 was more frequent in nodal (9/10) and distant (8/10) than in regional dermal (4/10) lesions ($p=0.044$, Fisher-Exact). Whereas enhanced expression of FSp53 mRNA was detected only in cases with enhanced expression of p53I2, p53I4 alone was increased in 6/30 lesions (20%). Thus, expression of mRNA encoding N-terminal p53 isoforms Delta40p53 and Delta133p53 increased during metastatic progression of cutaneous melanoma, suggesting a role for p53 isoforms in inactivating p53 suppressor functions.

Introduction

Cutaneous melanoma is the most lethal form of skin cancer and shows increasing incidence rates in most Western countries. This highly metastatic cancer is resistant to conventional therapy (Chin et al., 2006; Ibrahim and Haluska, 2009). Whereas the *TP53* gene is wild-type in about 95% of melanomas, activating mutations in the catalytic domain of the BRAF serine-threonine kinase are detectable in over 50% of the cases, with the most common mutation leading to substitution of valine by glutamic acid at position 600 (V600E). Other mutations occur in *NRAS* (in about 15% of melanoma), *MEK1* or *MEK2* (Chin et al., 2006; Tsao et al., 2012). Targeting the *BRAF* oncogene using the selective inhibitor vemurafenib (PLX4032) has shown promising results in Phase III trials (Bollag et al., 2012). However, most patients rapidly develop resistance, highlighting the need for combined therapy to improve the long-term effectiveness of such treatments.

In cancers with wild-type *TP53* alleles, the suppressive activity of the p53 protein is frequently switched-off by alternative mechanisms such as targeting p53 for proteasome-mediated degradation through viral or cellular proteins acting as E3 ubiquitin ligases (Petitjean et al., 2007). In melanoma, amplification of *MDM2*, encoding the main E3-ubiquitin ligase of p53, is detected in 3-5% of the cases (Muthusamy et al., 2006). A recent study has shown that another negative regulator of p53, Mdm4, was up-regulated in a substantial proportion (65%) of stage I-IV metastatic melanomas (Gembarska et al., 2012). Mdm4 (also known as Mdmx) cooperates with Mdm2 to induce polyubiquitination and proteosomal degradation of p53 (Wang, 2011). Inhibition of p53-Mdm4 interaction restored the pro-apoptotic functions of p53 in melanoma cells, resulting in increased sensitivity to cytotoxic treatments and to inhibitors of *BRAF* (V600E) oncogene, suggesting that enhanced Mdm4 expression antagonizes p53 suppressor functions (Gembarska et al., 2012).

Expression of p53 isoforms may represent another alternative mechanism for functional inactivation of p53 suppressor functions. These isoforms differ from full-length p53 (TAp53) by truncation of a variable portion of the N-terminus (DeltaN isoforms) and by alternative C-terminal portions (C-terminal isoforms) (Courtois et al., 2004a). The main N-terminal isoforms, Delta40p53 and Delta133p53, lack the first 39 and 132 residues, respectively, and are unable to transactivate p53 target genes (Bourdon et al., 2005; Marcel et al., 2010c). Delta40p53 is generated by two mechanisms, internal initiation at AUG 40 using fully-spliced p53 mRNA (Courtois et al., 2002; Yin et al., 2002), or alternative splicing of p53 pre-mRNA

that retains intron 2 to generate p53I2 mRNA, which introduces in-frame stop codons downstream of AUG 1 and leads to usage of AUG 40 as initiation codon (Ghosh et al., 2004). Delta133p53 is expressed from an alternative promoter within intron 4, leading to the expression of p53I4 mRNA that carries AUG 132 as initiation codon (Bourdon et al., 2005; Murray-Zmijewski et al., 2006). Both isoforms inhibit growth suppression in a dominant-negative manner when expressed in significant excess as compared to TAp53 (Aoubala et al., 2011; Courtois et al., 2002; Ghosh et al., 2004; Marcel et al., 2010b; Yin et al., 2002). Increased levels of mRNA encoding p53 isoforms have been reported in several cancers including breast and head and neck (Boldrup et al., 2007; Moore et al., 2010). In melanoma cell lines, Avery-Kiejda et al. have reported that small molecular weight variants of p53 were frequently, but not systematically overexpressed at both mRNA and protein levels (Avery-Kiejda et al., 2008). In contrast, these variants isoform were barely detectable in primary melanocytes. Furthermore, they show that ectopic expression of Delta40p53 in a melanoma cell line constitutively expressing low levels of this isoform inhibited the expression of two p53-dependent reporter genes (driven by the p53-response elements from *p21/WAF1* or *PUMA* promoters, respectively). Together, these observations suggest a role for enhanced expression of N-terminal isoforms in the functional inactivation of wild-type p53 in cancer cells (Marcel et al., 2011b).

In this study, we have used mRNA extracted short-term cultures of melanoma cell lines and from stage I-IV human cutaneous melanoma with well defined metastatic status to analyse the expression of p53, p53I2 (encoding Delta40p53 mRNA) and p53I4 (encoding Delta133p53 mRNA). These tumours have been previously analysed for their *BRAF* and *NRAS* mutation status and for enhanced expression of Mdm4 (Gembarska et al., 2012).

Patients, materials and methods

Patients and samples

All samples (melanoma tissues and cell lines) were kindly provided by Dr JC Marine, University of Ghent, Belgium. The panel of 17 short-term culture of melanoma cell lines has been described in Gembarska et al. (Gembarska et al., 2012). Patients and melanoma cases used for this study were recruited as part of the Melbourne Melanoma Project at the Peter McCallum Cancer Center, Melbourne, Australia (<http://melbournemelanomaproject.com/>). All

patients provided informed consent. From an initial group of 54 patients, a series of 40 fresh-frozen tissues consisting of 4 matched groups of 10 cases was assembled, including Primary Melanomas (PM ; stage I and II), Regional Dermal metastasis (RD, stage III), Nodal Metastatic lesions (NM, stage III) and Distant Metastatic lesions (DM, stage IV). The samples used in this study are the same as those previously analysed for Mdm4 expression at mRNA and protein levels by Gembarska et al., (2012), in which their characteristics are described in details (Gembarska et al., 2012). Briefly, melanoma patients were aged 19-82 years, with 46% of the cases over 60 years old. Pathological subtypes of primary tumours included mainly superficial spreading melanoma (46%), nodular melanoma (30%) and was unknown in 20% of the cases. *BRAF* V600E mutations were observed in 14/40 (35%) and *NRAS* mutations at Q61 in 6/40 cases (15%). There were no significant differences in the distribution of these mutations across the 4 sub-groups.

RNA extraction and quantitative Real-Time PCR (qRT-PCR)

RNA was extracted using the Rneasy minikit (Qiagen) according to the manufacturer's protocol and was quantified using a NanoDrop 1000 (Thermo Scientific). A total of 2 ug of RNA was reverse transcribed using the High-Capacity cDNA reverse transcription kit (Applied Biosystems). The same cDNA as for previously reported Mdm4 qRT-PCR were used for analysis of FSp53, p53I2 and p53I4 mRNA using Brilliant SYBR Green master mix (Qiagen) as fluorescent readout. Primers for each mRNA isoform are listed in Table 1. For each isoform, expression levels were normalized to GAPDH using the DeltaCT method. To evaluate expression in cell lines, the mean of DeltaCT values in two cultures of primary melanocytes was used as reference. In metastatic lesions, expression was assessed using as reference the mean of DeltaCT values for Primary Melanomas. Results for each metastatic melanoma sample were expressed using the DeltaDeltaCT method.

Statistical analysis

For each data point, results were expressed as means of DeltaDeltaCT values \pm standard deviation using data from at least 3 independent qRT-PCR replicates. These values were categorized in three groups defining low (mean DeltaDeltaCT value <0.3), normal (mean DeltaDeltaCT value between 0.3 and 3) or high expression (mean DeltaDeltaCT value >3).

The distribution of samples accross each expression group was analysed using the Freeman-Halton extension of the Fisher-Exact probability test for a two-rows by three-columns contingency table using the tools available at <http://vassarstats.net>.

Results

Three $p53$ mRNA isoforms (FSp53, p53I2 and p53I4) were analysed by qRT-PCR in a panel of 17 short-term cultures of melanoma cell lines and in 40 melanoma lesions categorized in 4 groups of 10 cases according to the extent of metastatic spread. Figure 1 shows a schematic representation of the transcripts analysed and the position of the primers used (sequences listed in Table 1). Figure 2 shows the levels of expression in melanoma cell lines using as reference the levels of expression in two primary cultures of normal melanocytes. It was considered that normal levels of expression encompassed a 3-fold variation relative to this control. Using this threshold, increased expression of FSp53 mRNA was observed in 8 cell lines, 5 of which also show enhanced expression of p53I2 mRNA. In contrast, increased expression of p53I4 mRNA was detected in only 1 cell line which did not show enhanced expression of FSp53 or p53I2 mRNA, and decreased expression was detected in 6 cell lines. These results suggest that changes in levels of expression of $p53$ mRNA isoforms occur among melanoma cell lines. Enhanced expression of p53I2 tended to be coordinated with enhanced expression of FSp53, while changes in expression of p53I4 mRNA appeared to be independent of p53I2 and/or FSp53. This observation is compatible with the notion that FSp53 and p53I2 are transcribed from the same, main $p53$ promoter whereas p53I4 mRNA is transcribed from a distinct, internal promoter located in intron 4 (see Figure 1).

Next, the same qRT-PCR strategy was used to analyse levels of $p53$ mRNA isoforms expression in cutaneous melanoma samples, sub-grouped according to the extent of metastatic spread. The mean of DeltaCT values in primary, stage I or II melanoma (PM) was used as reference. Compared to this reference, levels of expression of $p53$ mRNA isoforms were observed to be higher in Nodal (NM) or Distant (DM) metastatic lesions than in Regional Dermal (RD) lesions (Figure 3). Figure 4 shows the expression status of each RD, NM and DM cases in the form of a « heat map », in relation with their previously described BRAF, NRAS and TP53 mutation status. Of a total of 30 metastatic melanomas, 21 (70%)

showed increased expression of at least 3 fold in at least one of the p53 mRNA isoforms. Among a total of 20 NM and DM lesions, only 3 (sample numbers 28, 34, 37) showed no change or decrease in expression mRNA isoforms. Among these three cases, sample 28 contained a mutation in *TP53* (p.R342X) whereas the *TP53* mutation status of samples 34 and 37 was not available. Only one of the 21 samples with enhanced expression of p53 mRNA isoforms was found to contain a *TP53* mutation (sample 23, p.R273H). This sample showed a modest increase in only p53I2 mRNA. The differences between numbers of cases with enhanced p53I2 or p53I4 expression in NM or DM lesions compared to PM and RD lesions were significant ($P < 0.01$, Fisher-Exact). When comparing only metastatic lesions, enhanced expression of either p53I2 or p53I4 was more frequent in nodal (9/10) and distant (8/10) than in regional dermal (4/10) lesions ($p = 0.044$, Fisher-Exact).

Of the different mRNA isoforms, FSp53 mRNA was the least frequently enhanced (8/30 cases, all NM or DM) and was systematically associated with enhanced expression of p53I2 mRNA. The latter was detected in 14 cases (47%) and enhanced expression of p53I4 was detected in 18 cases (60%). Of note, enhanced expression of p53I4 mRNA alone was detected in 6 of the 20 metastatic melanoma samples (30%). Overall, these results suggest that expression of p53 mRNA isoforms encoding proteins with different N-terminal parts is often deregulated in melanoma in the absence of inactivating *TP53* mutation.

Discussion

Cutaneous melanoma is a highly metastatic form of cancer which is extremely difficult to treat using conventional cytotoxic protocols, suggesting that in these tumours the pathways of apoptotic response to DNA-damaging signals are extremely inefficient despite the fact that they contain, in their vast majority, wild-type *TP53* (Avery-Kiejda et al., 2008; Gembarska et al., 2012). This observation has led to suggest that alternative mechanisms were at work to inactivate p53 protein function in melanoma. Enhanced expression of isoforms of p53 lacking the N-terminus such as $\Delta 40p53$ and $\Delta 133p53$ may represent a plausible mechanism since these isoforms are unable to transactivate p53 target genes and have been shown to inhibit transactivation of such genes by wild-type p53 in a dominant-negative manner (Marcel et al., 2011b). In this study, we have taken advantage of an existing, well characterized series of melanoma cases sub-grouped according to metastatic status to analyse the levels of expression of mRNA encoding TAp53 (FSp53 mRNA), $\Delta 40p53$

(p53I2 mRNA) or Delta133p53 (p53I4 mRNA) using a qRT-PCR strategy. Our results show that levels of expression of these mRNA isoforms are increased in the majority of metastatic melanoma cases (70%). Moreover, enhanced expression was significantly higher and more frequent among melanoma with nodal (NM) or distant (DM) metastasis than among regional dermal (RD) or non-metastatic melanoma. In NM and DM combined, 17/20 tested cases (85%) showed enhanced expression of p53I2 and/or p53I4 mRNA. Among these cases, *TP53* mutation status was available of 9 cases and only one was found to be mutant (case 23, p.R273H). Among the 3 cases without enhanced p53I2 or p53I4 mRNA expression, one contained mutant *TP53* (p.R342X) whereas the mutation status of the two other cases was not available. FSp53 and p53I2 mRNA show an overlapping pattern of enhanced expression, whereas p53I4 mRNA expression is enhanced in cases that show neither FSp53 or p53I2 mRNA expression. These results are compatible with the fact that FSp53 and p53I2 mRNA are alternative splicing variants expressed from the same promoter, whereas p53I4 mRNA is controlled by a distinct promoter located in intron 4 (Aoubala et al., 2011; Bourdon et al., 2005; Marcel et al., 2010c). Thus, more than one mechanism may contribute to enhanced expression of p53 mRNA isoforms in metastatic melanoma. Overall, these results show that deregulation of expression of p53 mRNA encoding N-terminal truncated isoforms is common in nodal or distant metastatic melanoma lesions, suggesting that this phenomenon may contribute to functional inactivation of p53 during the spread of melanoma.

Small molecular weight variants of p53 have been reported to be frequently expressed in human melanoma cell lines but not in primary melanocytes (Avery-Kiejda et al., 2008). Using a combination of two-dimensional gel electrophoresis and western blotting, Avery-Kiejda et al. have shown that one of these small molecular weight isoform was compatible with p53beta, a C-Terminus variant generated by alternative splicing of exon 9 (Avery-Kiejda et al., 2008). Using RT-PCR, they also detected enhanced expression of p53I2, but not p53I4, in most melanoma cell lines. By transfection of expression vectors for p53beta or for Delta40p53 in a melanoma cell line with low constitutive p53 expression, they found that these two isoforms had opposite effects on the transactivation of p53-dependent reporter genes. Delta40p53 was shown to inhibit, whereas p53beta was shown to enhance, p53-dependent transcription (Avery-Kiejda et al., 2008). Our results on a different panel of melanoma cell lines are compatible with those of Avery-Kiejda et al., further supporting the notion that deregulated p53 mRNA expression is deregulated in melanoma (Avery-Kiejda et al., 2008).

The notion that p53 suppressor function may be functionally inactivated in melanoma in the absence of *TP53* mutation has been recently demonstrated in a recent study on the role of Mdm4 overexpression in cutaneous melanoma (Gembarska et al., 2012). This study reported that levels of expression of Mdm4, a critical co-regulator of p53 protein stability cooperating with Mdm2 to induce p53 protein degradation, was consistently overexpressed in a majority of malignant melanoma. Functional studies in cultured cells and in a mouse model of human melanoma revealed that expression of Mdm4 enhanced the survival and growth of melanoma cells by antagonizing p53 function. Inhibition of Mdm4-p53 interaction restored p53 function and increased responses to different forms of treatments including cytotoxic agents such as doxorubicin or specific inhibitor of activated *BRAF* oncogene, suggesting that restoring p53 may represent an interesting approach to enhance and extend the effects of targeted therapy.

Since the Mdm2-Mdm4 complex induce p53 degradation by binding to a motif in the N-terminal portion (residues 17-29), its action is expected to result in the elimination of TAp53 but not of the p53 isoforms lacking the N-terminus such as Delta40p53 or Delta133p53. Both of these isoforms have been shown to escape Mdm2-dependent degradation and to have an extended half-life as compared to TAp53 (Courtois et al., 2002; Marcel et al., 2010b). In initial studies, Delta40p53 was found to accumulate in response to overexpression of Mdm2 (Yin et al., 2002), perhaps as a result of accumulation of this degradation-resistant form of the protein. Therefore, overexpression of N-terminal isoforms may cooperate with Mdm4 overexpression to antagonize p53 suppressor function. Since the present study was conducted using the same melanoma specimens as those used to assess Mdm4 expression, we compared our data on p53 isoforms with those previously published on Mdm4 expression status (Gembarska et al., 2012). Overall, compared to mean expression levels in PM (stage I and II), Mdm4 mRNA levels were enhanced in 0/10 RD, 6/10 NM and 3/10 DM lesions. Among these 9 cases, 5 also showed enhanced FSp53 expression and all showed enhanced expression of either p53I2 and/or p53I4 mRNA. These results are compatible with the hypothesis that overexpression of Mdm4 synergizes with enhanced expression of p53 isoforms, Mdm4 inducing the elimination of TAp53 by degradation and N-terminal isoforms neutralizing the suppressor activity of residual p53 through dominant-negative inhibition.

Delta40p53 and Delta133p53 differ by their functional properties. While they both appear to retain the capacity to oligomerize with TAp53, Delta40p53 has an intact DNA-binding

domain (residues 96-306) and specifically binds to a p53 Response Element (RE) *in vitro*. In contrast, Delta133p53 lacks the proximal part of the DNA-binding domain, does not bind to specific DNA *in vitro*, and prevents TAp53 from binding to its cognate RE (Aoubala et al., 2011; Marcel et al., 2010b). Although the inhibitory capacity of these two isoform has not been systematically compared, Delta133p53 appears to have a stronger inhibitory effect than Delta40p53. Indeed, we have shown that, depending upon expression levels relative to TAp53, as well as cell context, Delta40p53 could exert either activating or inhibiting effects on TAp53. When expressed in excess compared to TAp53, its effect is however clearly antagonistic, preventing growth arrest induced by wild-type p53 *in vitro*.

Our study has strong limitations in the fact that it does not provide a direct assessment of p53 protein status. Indeed, thus far, there is no specific antibody that specifically recognizes Delta40p53 or Delta133p53 isoforms. Since their amino-acid sequence is a part of TAp53 sequence, isoforms are recognized by antibodies that also react with TAp53, making it cumbersome to analyse p53 isoform expression using histochemistry. In contrast, TAp53 can be identified through its differential reactivity with antibodies recognizing epitopes in the N-terminus of the protein, allowing for identification through differential western blotting. Another limitation is that our study does not address the expression status of the C-terminus isoforms p53beta and p53gamma, which may interfere with p53 functions through a different mechanism than inhibition of transactivation (Marcel et al., 2011b). Further studies will be needed to assess the levels of p53 protein isoforms and to understand the dynamics of interactions between TAp53 and p53 isoforms in melanoma cells that overexpress Mdm4 or not.

Acknowledgements

The authors are grateful to Dr JC Marine, University of Ghent, Belgium, for providing mRNA samples from primary melanoma and melanoma cell lines. This study was supported by a grant from Institut National du Cancer (INCa), France.

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Table 1. Primers used for qRT-PCR analysis of FSp53, p53I2 and p53I4 mRNA

mRNA	Forward primer (5' to 3')	Reverse primer (5' to 3')
FSp53	CCTATGGAACTACTTCCTG	AGGGGACTACGTGCAAGT
p53I2	ATGGGACTGACTTTCTGCTCT	
p53I4	TTCAACTCTGTCTCCTTCCT	CCAGACCATCGCTATCTGA
GAPDH	TCTCATGGTTCACACCCATGACGAACATG	AAGAAGATGCGGCTGACTGTCTGAGCCACAT

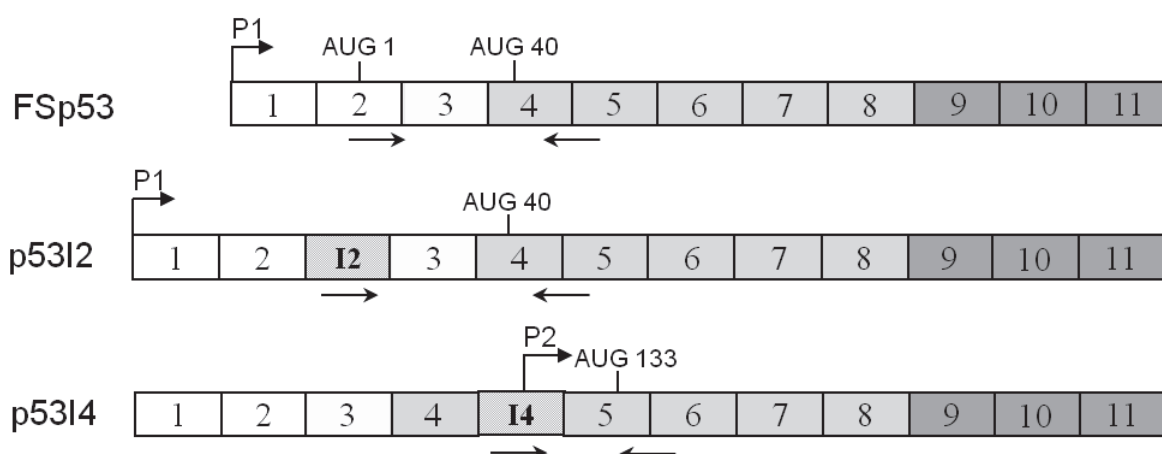


Figure 1. Structure of FS, p53I2 and p53I4 mRNA

A schematic representation of the *TP53* locus and of FSp53, p53I2 and p53I4 transcript is shown. The position of promoters for FSp53/p53I2 (P1) and for p53I4 (P2) are indicated on the locus map. Regions of the transcripts encoding different functional domains of the protein are indicated by different shades (white: transactivation domain; light grey: DNA-binding domain; dark grey: oligomerisation domain) and the position of different AUG codons is shown. Numbers correspond to p53 exons (1-11; exon 1 being non-coding). p53 intron 2 and intron 4 are in dotted boxes.

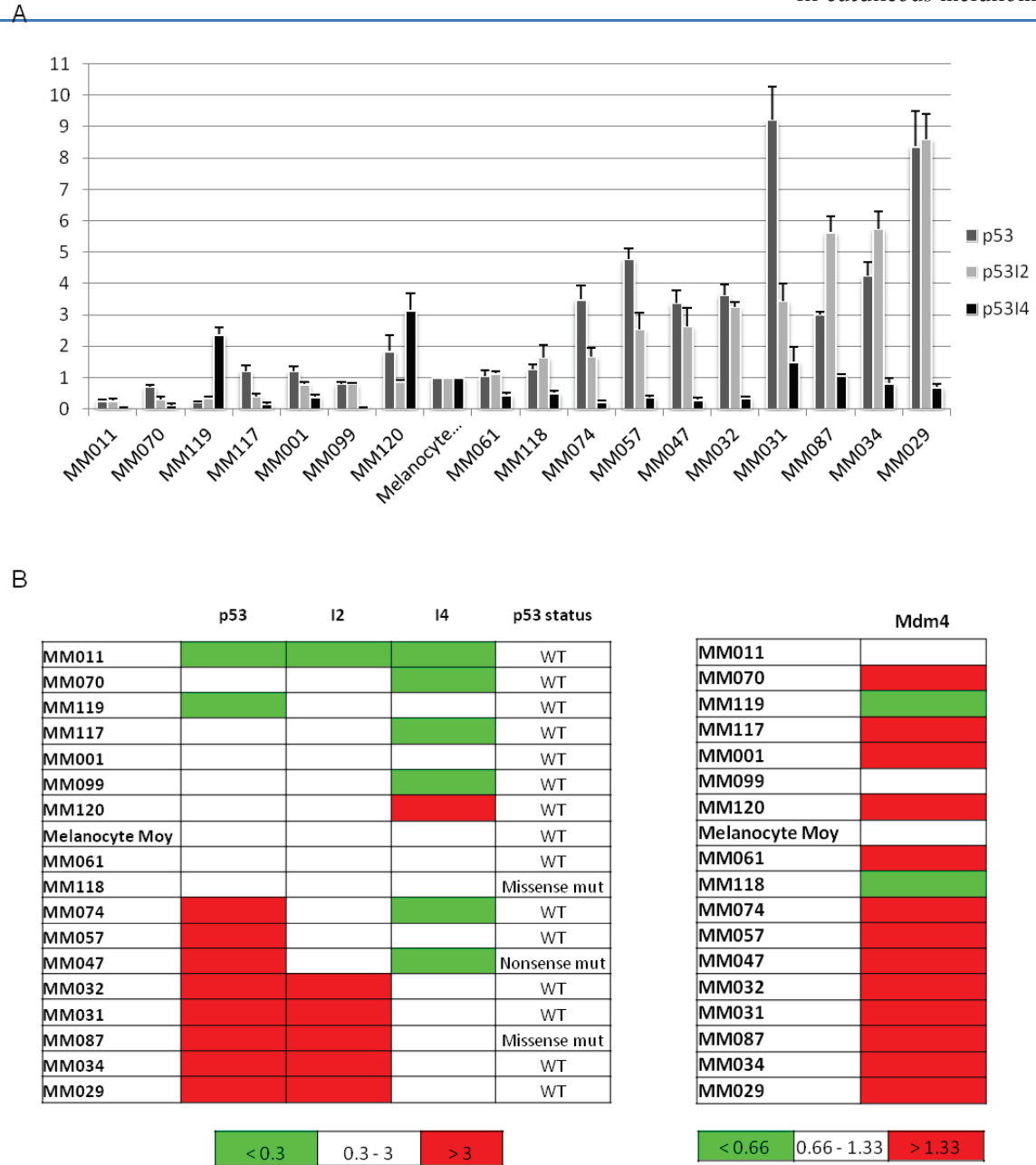


Figure 2. qRT-PCR analysis of FSp53, p53I2 and p53I4 mRNA in cultured melanoma cell lines

A: Histogram representation of average DeltaDeltaCT values (3 replicates) in melanoma cell lines using DeltaCT values in two primary melanocytes cultures as reference. Bars indicate s.d. Cell lines are arranged according to levels of p53I2 mRNA, from low (left) to high (right).

B: "Heat map" depicting expression levels as colors, showing samples with low (<0.3, green), normal (0.3-3, white) and high (>3, red) expression.

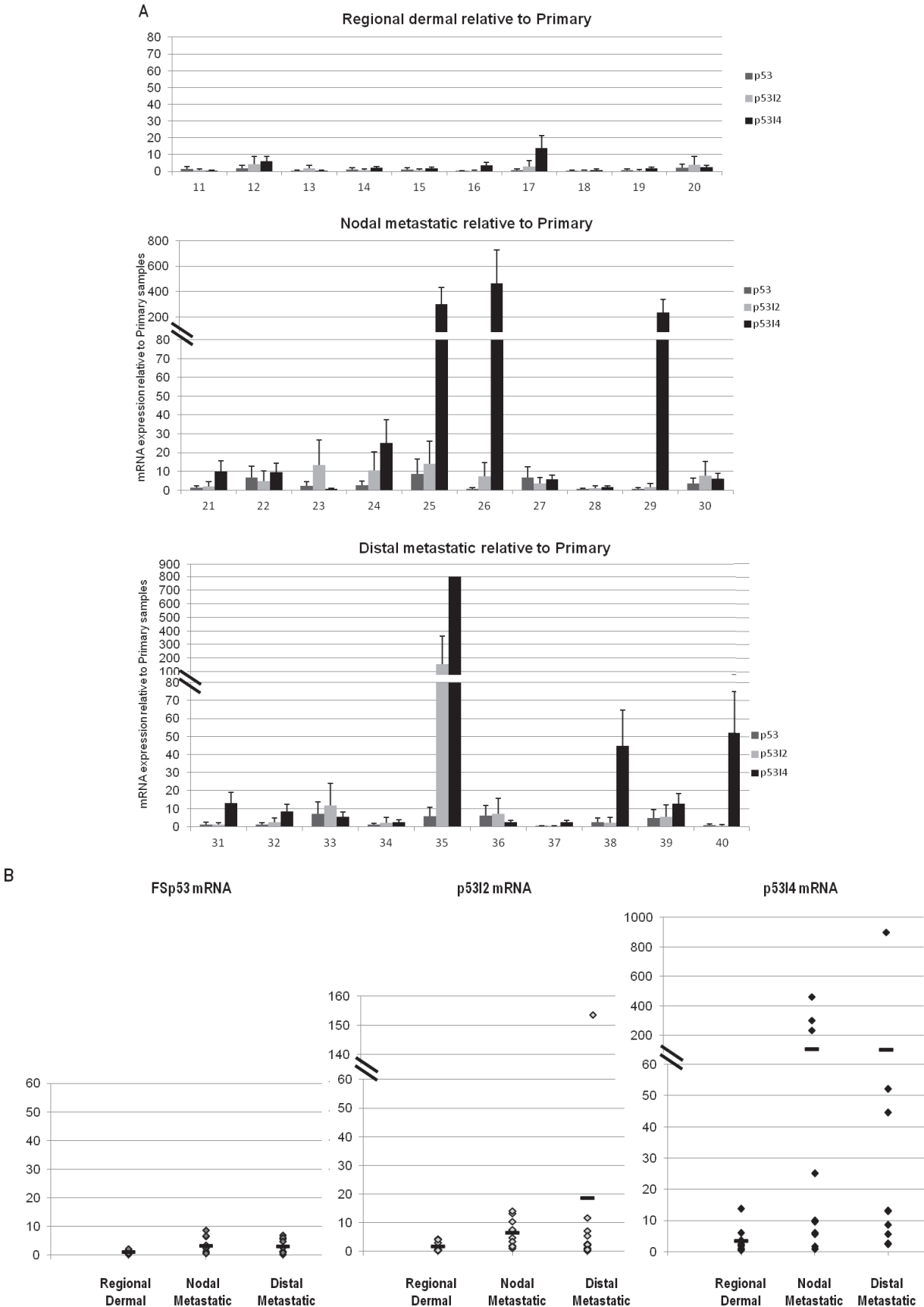


Figure 3

qRT-PCR analysis of FSp53, p53I2 and p53I4 mRNA in melanoma tissues

Expression levels are expressed as average DeltaDeltaCT values (3 replicates) in RD (Regional Dermal), NM (Nodal Metastatic) and DM (Distant metastatic) lesions using the mean values in Primary stage I-II melanomas (non-metastatic) as reference.

A: Histogram representation of expression levels in RD, NM and DM. Bars indicate s.d.

B: Levels of expression of each p53 isoform mRNA. Bars represent the mean value.

Reg Dermal/Primary	p53	p53l2	p53l4	BRAF	NRAS	TP53
11	N	N	N	V600E	WT	n.d.
12	N	H	H	WT	WT	WT
13	N	N	N	WT	Q61L	n.d.
14	N	N	N	WT	Q61R	n.d.
15	L	N	N	WT	WT	n.d.
16	N	L	H	WT	Q61K	WT
17	N	H	H	V600E	WT	n.d.
18	N	N	N	V601E	WT	n.d.
19	N	N	N	WT	WT	n.d.
20	N	H	N	WT	WT	n.d.

Nod Metast/Primary

21	N	N	H	V600E	WT	WT
22	H	H	H	WT	WT	n.d.
23	N	H	N	WT	WT	mut(c.818 G>A; p.R273H)
24	N	H	H	WT	WT	n.d.
25	H	H	H	WT	WT	n.d.
26	N	H	H	WT	WT	WT
27	H	H	H	WT	WT	n.d.
28	N	N	N	WT	Q61R	mut(c.1024 C>T; p.R342X)
29	N	N	H	WT	Q61K	n.d.
30	H	H	H	WT	WT	n.d.

Distant Metast/Primary

31	N	N	H	WT	WT	n.d.
32	N	N	H	V600E	WT	n.d.
33	H	H	H	V600E	WT	n.d.
34	N	N	N	WT	WT	n.d.
35	H	H	H	WT	WT	WT
36	H	H	N	V600E	WT	n.d.
37	L	L	N	V600E	WT	n.d.
38	N	N	H	WT	WT	n.d.
39	H	H	H	WT	WT	n.d.
40	N	N	H	V600E	WT	WT

	Low <0.3	Normal 0.3-3	High >3
RD/P			
p53	1	9	0
p53l2	1	6	3
p53l4	0	7	3
NMP			
p53	0	6	4
p53l2	0	3	7
p53l4	0	2	8
DMP			
p53	1	5	4
p53l2	1	5	4
p53l4	0	3	7

Figure 4

« Heat map » of expression levels of p53 isoform mRNA in melanoma

Expression levels are categorized in three groups (low (<0.3, green), normal (0.3-3, white) and high (>3, red) expression, with reference to mean value in non-metastatic melanomas). The gene mutation status of *BRAF*, *NRAS* and *TP53* is given (from (Gembarska et al., 2012)).

4. Discussion

4 DISCUSSION

The p53 tumour suppressor protein has an extremely complex pattern of regulation at transcriptional and post-translational levels, leading to describe p53 as a prototype of “massively regulated” transcription factor (Hollstein and Hainaut, 2010). As highlighted in our Introduction review, the discovery of p53 isoforms has added another layer of complexity to the many mechanisms that regulate p53 functions (Marcel and Hainaut, 2009). However, so far, there is limited understanding of the patterns of expression and of the functions of each of the potentially existing 12 p53 isoforms. In this Thesis, we have focused on the two major p53 N-terminal truncated isoforms, $\Delta 40p53$ and $\Delta 133p53$. We have analysed their patterns of interactions with wild-type TAp53 (the full-length form which retains an intact transactivation domain) and we have investigated whether their expression could be deregulated in melanoma, a cancer type in which *TP53* mutations are infrequent (Chin et al., 2006; Tsao et al., 2012). Our results show that both $\Delta 40p53$ and $\Delta 133p53$ entertain complex interactions with TAp53, with different effects despite the fact that the two isoforms have generic capacity to operate as dominant-negative inhibitors of p53.

At the onset of this project, $\Delta 40p53$ had been characterized (Courtois et al., 2002; Yin et al., 2002) but the existence of $\Delta 133p53$ was still a question of debate (Bourdon et al., 2005). From a structural viewpoint, $\Delta 40p53$ is a counterpart of $\Delta Np63$ and $\Delta Np73$ isoforms, which both lack a segment of the N-terminus that encompasses the main transactivation domain (Ishimoto et al., 2002; Kaghad et al., 1997). $\Delta 40p53$ corresponds to a form of p53 which is truncated at a point located between two clearly structurally defined domains, removing a segment of the protein containing binding sites for the basal transcriptional machinery, for p300/CBP acetylase and for the Hdm2/Hdm4 protein complex, the main regulator of p53 protein stability (Dornan et al., 2003; Ohki et al., 2007). $\Delta 40p53$ is predicted to retain a folded central DNA-binding domain and a C-terminal oligomerisation domain. In contrast, $\Delta 133p53$ represents a form of the protein which is truncated within the structurally coherent, globular DNA-binding domain. Removal of the first 132 residues encompasses the interaction domains listed for $\Delta 40p53$ and a number of additional domains, including the proline-rich domain containing SH3 binding sites for the c-Abl protein (Brown et al., 2000), the minor transactivation domain that selectively modulates the transcriptional activation of genes

involved in apoptosis and a beta-sheet motif that is an integral part of the DNA-binding domain (Cho et al., 1994). Together with a loop-helix motif encoded by residues 266 to 290, this beta-sheet constitutes the motif that binds in the major groove of target DNA (Cho et al., 1994). Thus, $\Delta 133p53$ is predicted to have a crippled and at least partially unfolded DNA-binding domain. Based on these considerations, it is expected that the two N-terminal isoforms may have different biological effects and regulation mechanisms.

4.1 $\Delta 40p53$: titrating TAp53 transactivation capacity while enhancing its stability

$\Delta 40p53$ is expressed in an apparently p53-independent manner, using the same pre-mRNA as TAp53 and either alternative splicing or alternative codon initiation usage (Marcel and Hainaut, 2009). Our results show that the resulting protein forms mixed oligomers with TAp53 which retain DNA-binding capacity. When expressed at excess levels, $\Delta 40p53$ competes with TAp53 for its incorporation into tetramers and inhibits the transactivation capacity towards a standard reporter gene. However, when expressed at lower levels in which TAp53 and $\Delta 40p53$ may exist in roughly equivalent amounts, $\Delta 40p53$ appears to have a paradoxical effect which depend upon cell context. In H1299 cells, we found that $\Delta 40p53$ exerted an inhibitory effect on transactivation. In contrast, in Saos-2 cells, $\Delta 40p53$ exerted a “two-phases” effect, with preserved and even enhanced p53 transcriptional activity at low levels of $\Delta 40p53$, followed by an inhibitory effect at higher levels. These results suggest that the interactions of $\Delta 40p53$ with TAp53 could not be simply rationalized to a competition between the two proteins for oligomerization and DNA-binding. Depending upon other factors, $\Delta 40p53$ may exert some kind of positive effect on TAp53 functions.

We reasoned that, in addition to the lack of a transactivation domain, a major difference between $\Delta 40p53$ and TAp53 was the lack in the former of the Hdm2 binding domain (residues 17-29) (Bottger et al., 1997). Compatible with this hypothesis, our analysis of $\Delta 40p53$ expression in two cell lines that constitutively express detectable amounts of this isoform, either alone or together with TAp53, detected that $\Delta 40p53$ was more stable than TAp53. This observation raised the question of knowing whether, in mixed oligomers containing both $\Delta 40p53$ and TAp53, the degradation behaviour would be driven by the TAp53 or the $\Delta 40p53$ isoform. By co-transfecting Hdm2 together with both isoforms, we have shown that, in Saos-2 cells, $\Delta 40p53$ could at least partially protect TAp53 from rapid degradation. This result suggests that, in certain conditions, $\Delta 40p53$ may cooperate with TAp53 in forming mixed complexes which retain stable expression of $\Delta 40p53$ together with transactivation capacity of TAp53 (Figure 23). This model provides a rationale to understand the effects in both Saos-2 and in H1299 cells. In H1299 cells, we speculate that $\Delta 40p53$ has only a small effect on TAp53 stability. Thus, the “dominant” effect even at low concentration

of $\Delta 40p53$ is the competitive inhibition of transactivation. With increasing concentrations of $\Delta 40p53$, however, this inhibition effect is partially compensated by the effect on stabilization and activity, leading to a progressive shift towards lower inhibition efficiency. In contrast, in Saos-2 cells, $\Delta 40p53$ can enhance stability and activity in a range of concentrations where the inhibitory effect on transactivation is, at best, limited. In such conditions, the biological effect is enhanced transactivation capacity, followed by rapid decrease at higher $\Delta 40p53$ concentrations. This model postulates that H1299 and Saos-2 cells differ in their intrinsic expression of factors that regulate p53 protein stability.

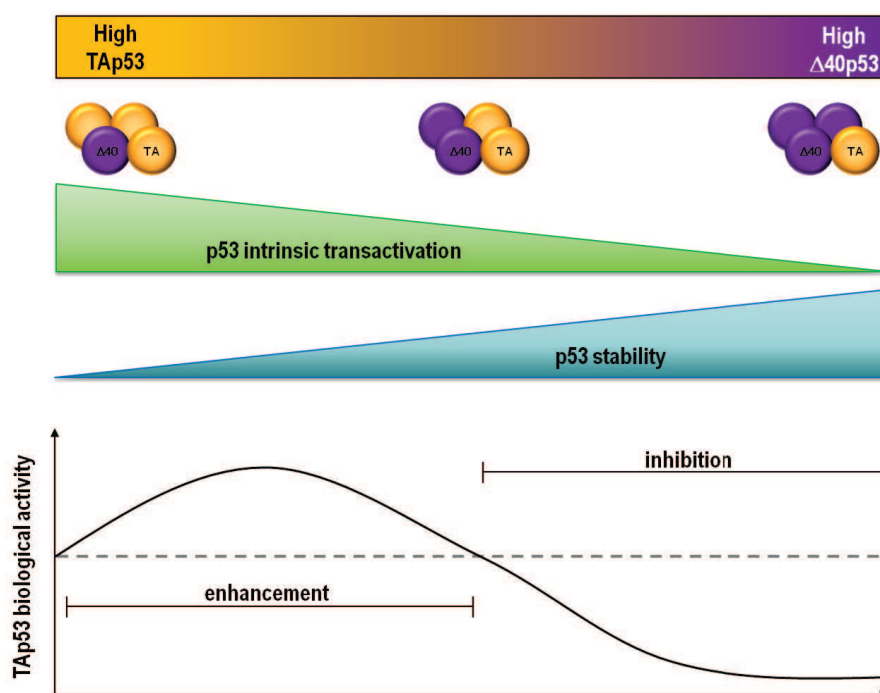


Figure 23. “Two-phases” model of regulation of TAp53 by $\Delta 40p53$

We postulate here that p53 biological response depends on the ratio $\Delta 40p53$ /TAp53 isoforms are expressed and the cellular context. In one hand, $\Delta 40p53$ has only a small effect on TAp53 stability, thus a dominant negative role on TAp53 transactivation. On the other hand, $\Delta 40p53$ can enhance stability and activity in a range of concentrations where $\Delta 40p53$ has only a limited inhibitory effect on TAp53.

While the molecular mechanisms leading to decreased transactivation are easy to understand, and supported by numerous published studies using N-terminal truncated p53 (Courtois et al., 2002; Ghosh et al., 2004; Yin et al., 2002), the mechanisms of enhanced transactivation are an open question. The N-terminus contains, in addition to the Hdm2-binding site, the binding sites for Histone Acetyl Transferases (HATs) such as p300/CBP and

for components of the basal transcriptional machinery. Thus, several scenarios can be considered. First, incorporation of $\Delta 40p53$ may decrease the binding of Hdm2/Hdm4 complexes, thus decreasing degradation and inducing the accumulation of the hetero-oligomer. Second, it may somehow modulate the interaction of the hetero-oligomer with HATs and with components of TBP. A third scenario is that post-translational modifications such as phosphorylation, acetylation and ubiquitination of p53 may depend upon the numbers of TA domains in the hetero-oligomer. Dissecting these mechanisms is a task of considerable complexity. Our results implicate a possible role for Hdm2, although we have not been able to detect a measurable change in steady-state p53 levels. Interestingly, a wealth of evidence show that TAp53 is not degraded by Hdm2 when in a monomeric form and that at least dimer formation is needed for targeting by Hdm2 and degradation (Kubbutat et al., 1997). These observations add weight to our hypothesis that inclusion of one or two subunits of $\Delta 40p53$ within a tetramer may affect its targeting by Hdm2/Hdm4.

4.2 $\Delta 133p53$: a novel p53 auto-regulatory loop implicating p53 isoforms

$\Delta 133p53$ is expressed using a different mRNA than TAp53 and $\Delta 40p53$. This mRNA is transcribed from a promoter overlapping with a region of the *TP53* gene extending from intron 1 to exon 5. The transcription start site is located in intron 4 and AUG 1+ corresponds to methionine codon at position 133 in exon 5. The existence of $\Delta 133p53$ was postulated by JC Bourdon and his collaborators in 2005, on the basis of their identification of a specific mRNA predicted to encode this protein (Bourdon et al., 2005). In 2010, in collaboration with Virginie Marcel, we provided a demonstration of the existence of this protein by showing that a siRNA targeted specifically to sequences derived from intron 4, which are present in $\Delta 133p53$ mRNA but not in TAp53 mRNA, reduced by 70% the expression of a p53 product of about 34 kDa, compatible with the predicted molecular weight of $\Delta 133p53$ (Marcel et al., 2010c).

The main mechanism of $\Delta Np63$ isoform expression is transcriptional activation through a self-regulated internal promoter. This observation prompted us to investigate whether p53 may also directly regulate its own internal P2 promoter. Close examination of promoter sequence coupled with promoter dissection experiments identified 4 potential p53 Response Elements (p53RE). Two of these elements lied in close vicinity to each other near the transcription start site. Their deletion reduced the level of promoter activity and abrogated its regulation by TAp53. Site-directed mutagenesis of critical residues within these p53RE decreased, but not totally abrogated, promoter activity, suggesting that this promoter retains activity even in a degenerated form. The fixation of p53 on a fragment of the promoter encompassing these two p53REs was confirmed by ChIP assay.

Consistent with its expected protein structure, we found that $\Delta 133p53$ did not bind to specific DNA *in vitro*. When co-expressed with TAp53, it counteracted DNA-binding. In contrast with $\Delta 40p53$, super-shifting experiments did not show evidence that mixed oligomers could bind to DNA. As it has been described that TAp53 and $\Delta 133p53$ can form hetero-oligomers (Chen et al., 2009), our DNA-binding data were consistent with the hypothesis that $\Delta 133p53$, when incorporated in a p53 oligomer, may scramble its conformation and prevent stable interaction with DNA. As a result, $\Delta 133p53$ is unable to transactivate target genes and, in a clonogenic assay, was shown to counteract TAp53-induced growth suppression when

expressed in excess as compared to TAp53. Overall, these results support the hypothesis that, although sharing some functional similarities with $\Delta 40p53$, $\Delta 133p53$ is involved in a distinct regulatory system and is exerting different effects on TAp53 than its sister N-terminal truncated isoform. While the expression of $\Delta 40p53$ is driven by the same main P1 p53 promoter as TAp53, the expression of $\Delta 133p53$ is driven by its own, distinct promoter, and is regulated by p53 itself. Thus, the regulation of $\Delta 133p53$ by p53 defines a novel auto-regulatory feedback loop in which p53 regulates the expression of an isoform acting as dominant-negative inhibitor of its own function (Figure 24). As shown in the result section, this feedback loop can reach further complexity by considering that excess levels of $\Delta 40p53$ may suppress the effect of TAp53 on the internal P2 promoter. On the other hand, it should be considered that $\Delta 133p53$ may bind to $\Delta 40p53$ and antagonize its effects by scrambling its capacity to bind to DNA. Thus, despite obeying to different regulatory mechanisms, the two N-terminal isoforms may entertain a form of mutual equilibrium.

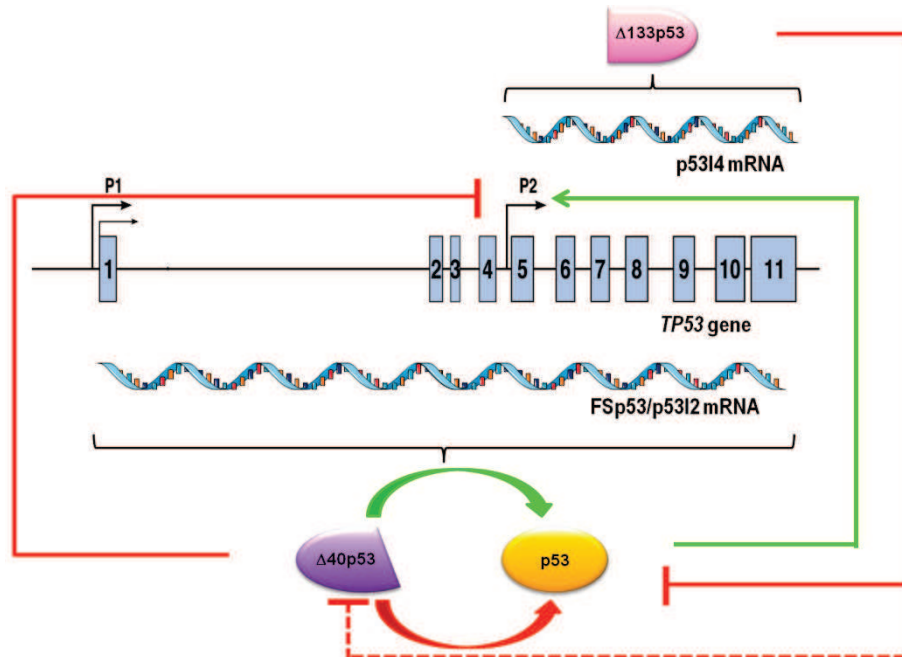


Figure 24. Auto-regulatory feedback loop of p53 and its isoforms

TAp53 and $\Delta 40p53$ are expressed from the P1 promoter, while $\Delta 133p53$ expression is driven by its own promoter P2. TAp53 is able to induce the expression of $\Delta 133p53$ on its own internal P2 promoter. In turn, $\Delta 133p53$ has the capacity to inhibit TAp53 activity, suggesting an auto-regulatory negative control of p53 on its own function. Moreover, we illustrate here that $\Delta 40p53$ has a suppressive effect on the TAp53 capacity to induce the P2 promoter. Finally, it should be considered that $\Delta 133p53$ may bind to $\Delta 40p53$ and antagonize its effects by scrambling its capacity to bind to DNA (dashed red line). Green arrows: positive effects (increased expression and/or activity). Red lines: negative effects (decreased expression and/or activity).

4.3 Interactions between TAp53 and N-terminal isoforms: physiological relevance

The physiological role of N-terminal p53 isoforms is far from clear. First, although small molecular weight isoforms are commonly detected in cultured cells and in tissues, they are generally expressed together with TAp53 and in amounts which are inferior or, at best, equal to basal TAp53 levels. Second, in contrast to TAp53, N-terminal isoforms do not accumulate in response to genotoxic stress or other forms of stress that activate p53 suppressor functions. Therefore, upon activation, TAp53 becomes the overwhelmingly dominant form of p53, accumulating at levels much higher than the basal levels of N-terminal p53 isoforms. Neither $\Delta 40\text{p}53$ nor $\Delta 133\text{p}53$ have been shown to transactivate gene expression on their own. In contrast, their biochemical capacity to antagonize TAp53 activity is well established. Together, these observations suggest that the main effect of N-terminal isoforms is to modulate the activity of basal TAp53 levels and perhaps control its capacity to selectively regulate different promoters in response to low-levels of stress signals.

In 2009, Virginie Marcel in our laboratory has proposed a model suggesting that N-terminal isoforms could operate as “buffers” to control basal p53 levels and activity in normal conditions (Marcel and Hainaut, 2009). The central assumption of this model is that cells are equipped with mechanisms that prevent inappropriate or untimely activation of p53 in response to low levels of stress signals such as those generated as by-product of physiological processes. For example, basal cell metabolism generates intracellular reactive oxygen species that are normally detoxified by redox regulatory systems but may also induce low levels of DNA damage which may activate p53 and therefore turn on cell-cycle arrest, senescence or apoptosis (Hafsi and Hainaut, 2011). To avoid turning these responses on as undesired consequence of normal metabolic processes, N-terminal p53 isoforms may provide a molecular “buffer” for TAp53 activity, setting thresholds above which TAp53 should accumulate in order to exert defined suppressive effects. According to this hypothesis, isoform levels may define incremental thresholds which set the limits for activation of p53 towards specific promoters, and thus for the induction of distinct physiological responses. For example, at intermediate levels of activation, TAp53 may partially override N-terminal isoforms to activate essentially cell-cycle arrest, DNA repair and growth regulatory responses that preserve cell survival. At high levels of activation, however, TAp53 may completely

override the “buffer” provided by N-Terminal isoforms to activate promoters that induce cell senescence or apoptosis and therefore, death or permanent growth arrest. Our results are compatible with this model and provide further refinements (Figure 25). First, they suggest that the buffer provided by N-terminal isoforms results from a complex interplay between the two N-terminal isoforms and TAp53 with multiple cross-talks as illustrated in Figure D2. Second, our results with $\Delta 40p53$ suggest that changes in the levels of this isoform might actually provide a dynamic system to set the levels of basal p53 activity.

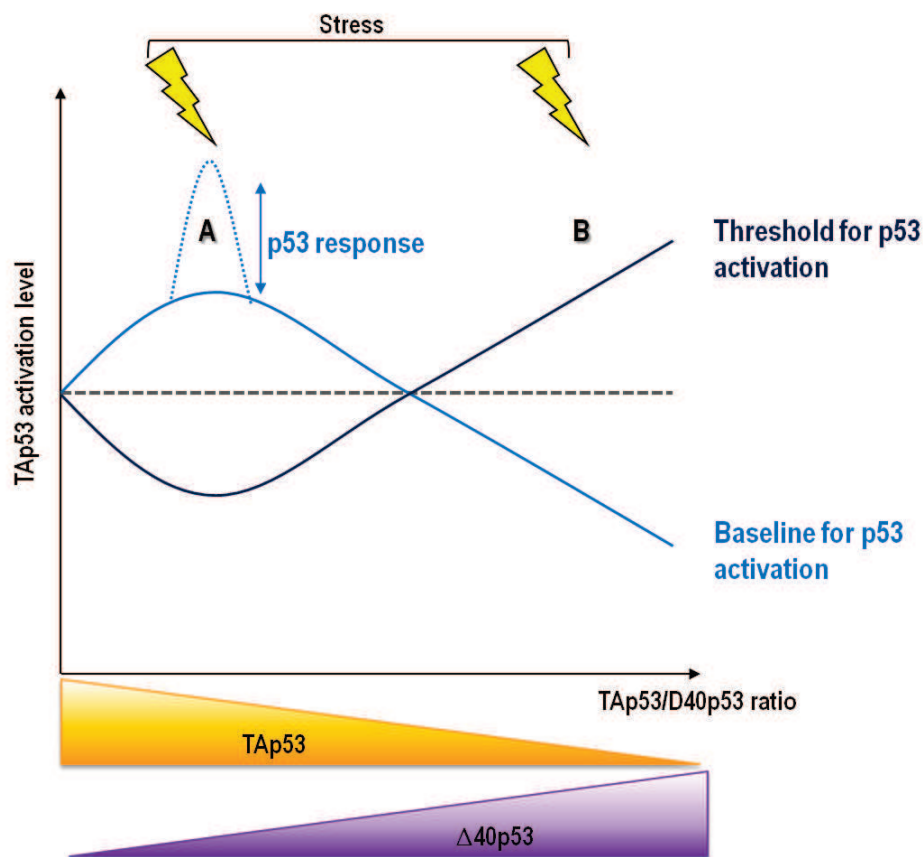


Figure 25. $\Delta 40p53$, a “buffer” regulating the p53 response

To avoid uncontrolled response of p53 to low levels of stress signals, $\Delta 40p53$ could act as a molecular “buffer” for p53 activation, setting thresholds above which p53 should accumulate and exert its functions (dark blue line). For example, at high level of activation (light blue line), p53 may override the “buffer” provided by $\Delta 40p53$ and induce target genes involved in cell cycle arrest or apoptosis (A) (dotted light blue line). On the other hand, at low to intermediate levels of activation, TAp53 may not be able to override the threshold provided by $\Delta 40p53$, resulting in the absence of p53 response (B).

One of the predictions of this model is that levels of $\Delta 40p53$ may be higher in cells that retain a high proliferative capacity such as stem cells and progenitor cells, thus setting up a high basal threshold for activation of TAp53. This would, in turn, preserve the

stem/progenitor status by preventing these cells from activating anti-proliferative responses such as differentiation, senescence and apoptosis. This prediction is compatible with results demonstrated by Ungewitter and Scrable (2010) (Ungewitter and Scrable, 2010). These authors have found that $\Delta 40p53$, in addition to being highly expressed in embryonic stem cells (ESCs), was the major p53 isoform during early stages of embryogenesis in the mouse. Furthermore, haploinsufficiency for $\Delta 40p53$ caused a loss of pluripotency in ESCs and acquisition of a cell cycle progression profile typical of differentiated cells (with longer G1 and G2 phases, in contrast with the rapid succession of S/M phases that characterizes ESCs). In contrast, increased dosage of $\Delta 40p53$ contributed to extend pluripotency and to inhibit the progression to a more differentiated state. However, in both cases, the activity of $\Delta 40p53$ was dependent upon the presence of TAp53. It is important to note that, in these experiments, haploinsufficiency for $\Delta 40p53$ actually promoted p53-dependent transcriptional control, with activation of the *p21^{WAF1}* promoter and suppression of promoters regulating genes characterizing the stem cell status such as Nanog. Increased dosage of $\Delta 40p53$ led to a transcriptional switch with increased expression of Nanog and decreased expression of *p21^{WAF1}*. These observations cannot be explained by a simple, dosage-dependent antagonistic effect of $\Delta 40p53$ on TAp53 activity. Rather, they suggest that when present at low levels relative to TAp53, $\Delta 40p53$ can activate basal p53 function. Conversely, when expressed at higher levels, $\Delta 40p53$ can titrate TAp53 function. These observations in ESCs are therefore fully compatible with our results and with our “two-phases” model of regulation of TAp53 by $\Delta 40p53$.

Our interpretation sheds new light on the effects of $\Delta 40p53$ in animal models. Two such models have been developed, in mouse (Maier et al., 2004) and in Zebrafish (Davidson et al., 2010). The mouse model is a transgenic model expressing an N-terminal truncated p53 matching $\Delta 40p53$ (p44) in either p53-null or in wild-type p53 background. In a p53-null background, no significant phenotype was observed. Expression of $\Delta 40p53$ in the absence of TAp53 (and of other p53 isoforms) appeared to have no effect on development, differentiation, physiological fitness or aging. These mice show the same susceptibility to early tumorigenesis as the parental, p53-null mice. In contrast, when $\Delta 40p53$ was expressed in a p53-competent background, a phenotype of early aging was detected. At 4 months of age, mice showed a physiological decline with loss of bone density, decreased skin renewal, hair graying and loss of memory. Further studies have shown that mice overexpressing $\Delta 40p53$ in

a p53-competent background developed a neurological deficiency reminiscent of Alzheimer's disease (Pehar et al., 2010). Interestingly, Western Blot analysis of p53 protein levels revealed an increase in TAp53 protein in p53-competent mice overexpressing $\Delta 40p53$ (Figure 26). This observation is compatible with our results in transfected cells showing that expression of $\Delta 40p53$ at low levels compared to TAp53 may promote the stabilization of the latter. Increased levels of basal p53 activity may largely explain the aging phenotype, which may result from the suppression of adult stem cells and the subsequent loss of tissue renewal capacity. Alternatively, mice overexpressing $\Delta 40p53$ may have constitutively high levels of oxidative stress that induces the stabilization of p53 through DNA damage. While such an increase in oxidative stress is not documented *in vivo*, we consider this possibility as unlikely since mice overexpressing $\Delta 40p53$ in a p53-null background do not show increased cancer risk as compared to the parental p53-null controls. Indeed, higher levels of oxidative stress would be expected to result in accelerated tumourigenesis in the absence of a functional p53 protein.

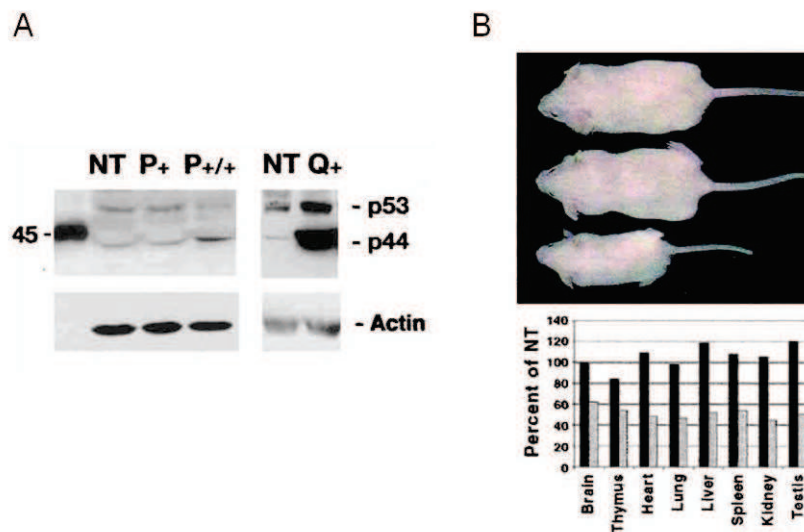


Figure 26. Overexpression of the $\Delta 40p53$ isoform of p53 in transgenic mice.

(A) $\Delta 40p53$ protein in transgenic and non transgenic (NT) mice. Western blots of 100 μ g thymus protein extracted from transgenic (P,Q) and NT (NT) tissues. The anti-p53 polyclonal antiserum CM5 detects both isoforms of p53, as indicated. TAp53 expression is increased in mice overexpressing $\Delta 40p53$. Biotinylated markers were run alongside the tissue extracts during SDS-PAGE; the marker corresponding to 45 kD is shown. (B) Effect of p44 on body size. Size of NT (top), hemizygous (middle), and homozygous (bottom) P mice at 3 months of age. Organ weights of representative hemizygous (black bars) and homozygous (stippled bars) transgenic mice are plotted as a percentage of NT weights. Transgenic animals were generated on a p53^{+/+} background. (Maier et al., 2004)

The Zebrafish model offers a biological parallel with the mouse model. After cloning of the Zebrafish $\Delta 40p53$ homolog, Rodeck and his collaborators have forced the expression of either human or Zebrafish $\Delta 40p53$ by micro-injecting their respective mRNAs into Zebrafish embryos (Davidson et al., 2010). They observed that ectopic expression of both species' $\Delta 40p53$ caused hypoplasia and malformation of the head, eyes and somites, yet partially counteracted the lethal effects caused by concomitant forced expression of TAp53. Moreover, TAp53 expression was required for the developmental aberrations caused by $\Delta 40p53$ expression. Strikingly, knockdown of p21WAF1 expression markedly reduced the severity of developmental malformations associated with forced expression of $\Delta 40p53$, suggesting that these malformations were indeed driven by an increase in basal p53 transcriptional capacity. Thus, in both animal models, the main effect of $\Delta 40p53$ appears to consist into increasing the basal level and activity of TAp53, rather than impairing p53-dependent growth suppression. We speculate that this effect is due to the fact that these organisms co-express TAp53 and $\Delta 40p53$ isoforms at respective levels which are compatible with increased stability and activity of TAp53 by interaction with $\Delta 40p53$.

The general expression patterns of p53 into TAp53 and N-terminal isoforms appears to be conserved in evolution. In *Drosophila*, two forms of p53 mRNA have been described, encoding respectively TAp53 (Dp53) and an isoform lacking the first 110 residues (D Δ Np53) (Bourdon et al., 2005; Jin et al., 2000; Ollmann et al., 2000). Studies by B Mollereau and his collaborators have shown that in p53-competent transgenic *Drosophila* overexpressing either Dp53 or D Δ Np53 in the imaginal discs, both isoforms could induce apoptosis through different pathways, whereas only D Δ Np53 could stimulate apoptosis-induced compensatory proliferation through the release of growth factors of the Wg (Wingless) family (Dichtel-Danjoy et al., 2012) (see [Appendix II](#)). It still unclear how these effects compare with those observed in mouse and zebrafish. Moreover, further studies are needed to determine whether the vertebrate orthologs can recapitulate p53 effects in *Drosophila* and vice versa.

To date, there is no animal model for $\Delta 133p53$. In Zebrafish, Davidson et al. have shown that the ortholog of $\Delta 133p53$ is a protein truncated for its first 113 residues ($\Delta 113p53$) (Davidson et al., 2010). Forced expression of TAp53 in Zebrafish embryos induced the expression of $\Delta 113p53$, in agreement with our *in vitro* results showing that TAp53 regulates the internal P2 promoter. In a p53-competent background, overexpression of $\Delta 40p53$ also

induced an increase in $\Delta 113p53$ mRNA, consistent with the idea that $\Delta 40p53$ could increase the basal activity of endogenous TAp53. However, forced expression of $\Delta 113p53$ could not reverse the malformation phenotype induced by $\Delta 40p53$, suggesting that in this context $\Delta 113p53$ is rather inefficient in antagonizing $\Delta 40p53$ effects.

4.4 Pathological relevance of N-terminal isoforms

Summarizing our results as well as the observations *in vivo* discussed above, we propose that expression of N-terminal isoforms may be critical for the maintenance of stem cell status. This, together with the fact that excess expression of N-terminal isoforms antagonizes p53 suppressor activity, suggests that overexpression of N-terminal isoforms may represent a mechanism for functional inactivation of p53 during tumourigenesis. Inactivation of p53 by somatic mutation of the *TP53* gene is common in human cancer and is generally associated with highly metastatic behaviour. A curious exception to this rule is cutaneous melanoma, in which *TP53* mutations are very rare (about 5% of the cases) despite a highly metastatic phenotype. It has long been proposed that, in melanoma, wild-type p53 protein may be inactivated by alternative mechanisms (Chin, 2003). Such mechanisms have been identified in several cancers, such as cervical and oral cancers in which the inhibition of p53 activity is caused by rapid degradation through Human Papilloma Virus (HPV) antigen E6 acting as component of an ubiquitin-ligase system (Dyson et al., 1989; Scheffner et al., 1993), and in sarcomas with amplifications of the *Hdm2* gene, also leading to increased degradation (Muthusamy et al., 2006; Reifemberger et al., 1993).

A previous report in melanoma cell lines has identified that most of them expressed small molecular weight variants of p53 (Avery-Kiejda et al., 2008). However, their precise identity remains obscure. At mRNA level, there is evidence that these cells express high levels of p53I2 (encoding $\Delta 40$ p53) and/or p53I4 (encoding $\Delta 133$ p53) mRNA. At protein level, expression of TAp53 β , a C-terminal isoform, has been documented while the expression of N-terminal isoforms has not been analysed in sufficient details. We have taken advantage of a collaboration with Chris Marine, University of Ghent, Belgium, to analyse the relative levels of p53I2 and p53I4 mRNA in primary melanoma sub-grouped according the extent of metastatic dissemination. This series of cases has been recently analysed for Hdm2 and Hdm4 expression by Gembarska et al., leading to the identification of Hdm4 overexpression as a plausible mechanism inducing rapid p53 turnover and inactivation in metastatic cutaneous melanoma (Gembarska et al., 2012). We found that expression of either p53I2 or p53I4 mRNA was substantially increased in 17/20 nodal and distal metastatic lesions. Only one of those had a *TP53* mutation, whereas 1 in 3 lesions with no significant change in p53I2 or p53I4 mRNA levels had a *TP53* mutation (the mutation status of the two other cases being unknown). The FS (Fully-Spliced) p53 mRNA encoding TAp53 was often up-regulated

together with p53I2, consistent with their common origin from the same pre-mRNA. Tumours with increased FSp53 levels tended to match those with increased Hdm4 levels (Gembarska et al., 2012). Overall, our results suggest that deregulation of wild-type p53 mRNA expression is a common occurrence in cutaneous melanoma. Moreover, levels of p53I2 and p53I4 seem to be correlated with nodal and distant metastatic behaviour. Thus, in melanoma cells, increased expression of Hdm4, $\Delta 40$ p53 and $\Delta 133$ p53 cooperate to reduce p53 protein activity and turn off p53 suppressor function (Figure 27).

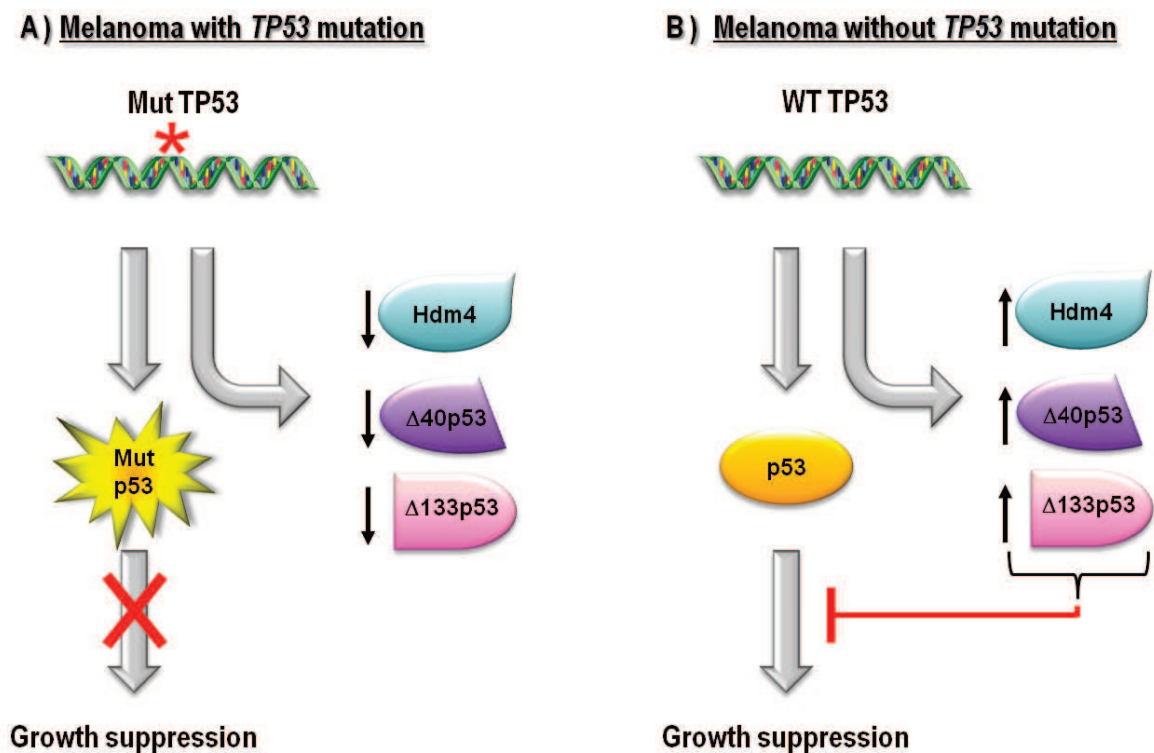


Figure 27. Alternative mechanisms for TP53 inactivation in human melanoma

TP53 is rarely mutated in human melanoma, suggesting alternative mechanisms to switch off p53 functions. When TP53 is mutated (A), mutant p53 inactivates p53 growth suppressive functions, and Hdm4, $\Delta 40$ p53 and $\Delta 133$ p53 expression levels are decreased. In contrast, when TP53 gene is not mutated (B), Hdm4, $\Delta 40$ p53 and $\Delta 133$ p53 have an increased expression, suggesting an interplay of these proteins to inhibit p53 activity and turn off p53 suppression functions.

It is striking to note that, in this cancer context, our model proposes that the main effect of N-terminal isoforms is to inactivate TAp53 functions, although we propose that in animal models, the main effect is to stabilize and increase basal p53 levels. This paradox can be resolved by considering that, in melanoma, N-terminal isoforms are expressed at much higher levels than TAp53, whereas in animal models they are expressed at roughly equivalent levels.

This speculation needs to be confirmed by systematic protein studies. Nevertheless, one of the factors that may tilt the balance towards a strong inhibiting effect of N-terminal isoforms is overexpression of Hdm4, which decreases p53 protein levels and therefore displaces the TAp53/N-terminal isoform ratio towards conditions in which p53 activity is strongly antagonized.

The notion that high levels of N-terminal p53 isoforms in melanoma are correlated with metastatic behaviour is interesting from a cancer stem cell perspective. Indeed, high levels of N-terminal isoforms may represent a “signature” for cancer cells which retain stem cell status and are prone to undergo Epithelial to Mesenchymal Transition (EMT), a critical process for dissemination of cancer cells to distant sites. Tumours with wild-type p53 but high levels of N-terminal isoforms may tend to have more stem cells, or stem-cell-like properties, than other tumours, thus providing a basis to explain their aggressiveness. Thus, restoring p53 activity may represent an elegant mechanism to prevent tumour cells to retain and accumulate highly malignant stem cells. This principle is supported by studies by JC Marine and collaborators (Gembaraska et al., 2012) who have shown that inhibition of Hdm2/Hdm4 complexes in melanoma cells could prevent progression in mouse xenografts. Whether a similar effect can be achieved by decreasing N-terminal isoforms or by blocking the formation of hetero-oligomers between TAp53 and N-terminal isoforms, remain to be demonstrated.

The isoform paradigm may apply to many other forms of aggressive cancer which retain wild-type *TP53*. Several reports have noted high levels of mRNA encoding different p53 isoforms in squamous cell carcinomas of the head and neck (Bolstrup et al., 2007) and of the breast (Moore et al., 2010) but it is not clear whether there is any correlation with the persistence of wild-type *TP53*. In this respect, our study is the most detailed to date although it has many shortcomings such as the lack of data at protein level. Another context in which N-terminal p53 isoforms may have an impact on tumour development is the Li-Fraumeni Syndrome, a disease of familial aggregation of multiple early cancers due to inheritance of a germline *TP53* mutation (Palmero et al., 2010). In subjects who inherit one copy of mutant *TP53*, levels of $\Delta 40p53$ (and perhaps $\Delta 133p53$) may be critical for determining the basal level of p53 activity and thus the efficiency of p53 responses to multiple stimuli during life. In 2009, Marcel et al. have shown that a common polymorphism located in intron 3 of p53 (a 16 bp motif present as one or two copies) was associated with early cancer risk in a Brazilian cohort of carriers of a specific germline *TP53* mutation, R337H (Marcel et al., 2009).

Individuals with two copies of the 16bp motif on the wild-type allele had a much reduced risk of early cancer (before age 30) compared to carriers of a wild-type allele with only one copy of the 16bp motif. *In vitro* studies have shown that this polymorphism falls into a segment of intron 3 that generates the formation of G-quadruplex structures in p53 pre-mRNA, and that these G-quadruplexes could regulate the splicing of the pre-mRNA to produce either FSp53 or p53I2 (Marcel et al., 2011c). Therefore, the level of $\Delta 40p53$ may vary according to *TP53* polymorphic status, providing a potent intragenic modifier of cancer risk in carriers of a germline *TP53* mutation.

5. Conclusions and perspectives

5 CONCLUSIONS AND PERSPECTIVES

In this Thesis, we claim as primary contributions (1) the demonstration that $\Delta 40p53$ can modulate TAp53 function with a bi-phasic effect that provides a basis to reconcile the apparent paradox between its biochemical (inhibitory) and physiological (apparently activating) effects on TAp53 and (2) the demonstration that mRNAs encoding N-terminal isoforms are often over-expressed in highly metastatic melanoma when compared to non-invasive forms, suggesting that N-terminal isoforms contribute to functionally inactivate p53. A secondary contribution is the analysis of the complex loops of interactions that link TAp53, $\Delta 40p53$ and $\Delta 133p53$ isoforms, leading to the concept that p53 function depends on the dynamic fluctuations of a protein network rather than on the expression of a single molecule. Our work has however major limitations. The main ones are (1) the absence of proof of effect for N-terminal isoforms in non-transformed, non-transfected experimental cell systems; (2) the lack of comparison between mRNA and protein expression studies in melanoma, and (3) the lack of consideration for other p53 isoforms such as C-terminal isoforms. Indeed, the mechanisms identified here are bound to be dependent upon the expression of C-terminal p53 isoforms, since they depend upon the formation of hetero-oligomers between N-terminal isoforms and TAp53. C-terminal isoforms differ from full-length p53 by a variable portion of the C-terminus. While the p53 β isoform retains dimerisation, but not tetramerization motifs, p53 γ has a truncation that encompasses the whole oligomerization domain. Thus, the rate of complex formation between isoforms may be critically dependent upon the status of C-terminal isoforms.

Our results are calling for further analysis of the role of p53 isoforms in the maintenance of stem cell status. Indeed, control of stemness may represent the main conserved biological activity of p53 family members. For example, $\Delta Np63$ isoforms are commonly expressed as the major, if not the only, type of p63 isoform in epidermal stem cells (ESC) and in squamous cell progenitors (Medawar et al., 2008). In contrast, in squamous epithelia, TAp63 expression is restricted to differentiated cells (Nylander et al., 2002). Knocking out p63 or $\Delta Np63$ alone results in impaired development of many tissues including skin, breast and prostate, and leads to major morphogenetic defects due to impaired epithelium to mesenchyme interactions. These defects result from the rapid exhaustion of pools of stem cells in these tissues, which

are unable to retain their stem status in the absence of $\Delta Np63$. The functions of $\Delta 40p53$ appear to have similarities with those of $\Delta Np63$. Further studies are needed to determine whether p53, p63 and perhaps p73 operate in parallel pathways of stem cell maintenance, or are all part of a complex, highly redundant regulatory network.

Finally, our results call to a reassessment of the roles of p53 in normal conditions and in stress responses. Since its identification as “guardian of the genome” in 1992 (Lane, 1992), the idea has prevailed that p53 had no significant function in cells unless it accumulates in response to a wide range of stress signals. Regulation through N-terminal isoforms sheds light on the fact that basal p53 levels are indeed important for the dynamics of stem cells and for their renewal. It follows that N-terminal isoforms of p53 might have a considerable impact not only on the expansion and dissemination of tumour stem cells, but also the pathogenesis of chronic diseases characterized by stem cell exhaustion as well as on normal aging. It would be very interesting to determine whether subtle changes in N-terminal isoform levels may contribute to increase the lifespan of organisms, or to improve the regeneration of aging tissues.

6. References

6 REFERENCES

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7. Appendices

7 APPENDICES

7.1 Appendix I: Biological functions of p53 isoforms through evolution: lessons from animal and cellular models

Cell Death and Differentiation (2011) 18, 1815–1824
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Review

Biological functions of p53 isoforms through evolution: lessons from animal and cellular models

V Marcel¹, M-L Dichtel-Danjoy², C Sagne^{3,4,5}, H Hafsi³, D Ma², S Ortiz-Cuaran³, M Olivier³, J Hall^{4,5}, B Mollereau², P Hainaut³ and J-C Bourdon^{*1}

The *TP53* tumour-suppressor gene is expressed as several protein isoforms generated by different mechanisms, including use of alternative promoters, splicing sites and translational initiation sites, that are conserved through evolution and within the *TP53* homologues, *TP63* and *TP73*. Although first described in the eighties, the importance of p53 isoforms in regulating the suppressive functions of p53 has only become evident in the last 10 years, by analogy with observations that p63 and p73 isoforms appeared indispensable to fully understand the biological functions of *TP63* and *TP73*. This review summarizes recent advances in the field of 'p53 isoforms', including new data on p63 and p73 isoforms. Details of the alternative mechanisms that produce p53 isoforms and *cis*- and *trans*-regulators identified are provided. The main focus is on their biological functions (apoptosis, cell cycle, aging and so on) in cellular and animal models, including mouse, zebrafish and *Drosophila*. Finally, the deregulation of p53 isoform expression in human cancers is reviewed. Based on these latest results, several developments are expected in the future: the identification of drugs modulating p53 isoform expression; the generation of animal models and the evaluation of the use of p53 isoform as biomarkers in human cancers.

Cell Death and Differentiation (2011) 18, 1815–1824; doi:10.1038/cdd.2011.120; published online 23 September 2011

Thirty years of research have shown that the p53 tumour-suppressor protein, encoded by *TP53* gene (OMIM 191170), integrates endogenous and exogenous signals to modulate cell fate to stress and cellular environments.¹ In addition, it has emerged that p53 is more than just a 'stress response' factor as it regulates embryo implementation.² The ability of p53 to integrate signals implies the existence of multiple and subtle levels of regulation. Over the years, transcriptional, translational and post-translational regulatory mechanisms have been uncovered.^{3,4} This biochemical diversity echoes the genetic diversity of the *TP53* locus, which contains multiple genetic polymorphisms defining over 100 distinct *TP53* haplotypes.⁵ Recently, an additional layer of regulatory mechanism has emerged through identification of p53 isoforms, which are physiological proteins expressed in normal cells from the *TP53* gene owing to the use of alternative promoters, splicing sites and/or translational initiation sites.^{6,7}

The p53 isoforms were first identified in early studies investigating p53 expression patterns. In 1984, Matlashewski *et al.* cloned an N-terminal variant of the human p53 mRNA, whereas in 1985, Rotter and co-workers detected an alternatively spliced C-terminal variant of mouse p53, latter isolated in human cells.^{8–10} However, the 'p53 isoform' field

has only really emerged in the past 10 years, when it became clear that *TP53* retained the elaborate patterns of isoform expression that characterizes its homologues, *TP63* and *TP73*. The rapid accumulation of descriptive, functional and clinical data on p53 isoforms has led to the emergence of a research community, which held its First International Meeting at the International Agency for Research on Cancer in Lyon, France, in September 2010.¹¹ This review provides a brief survey of the 'p53 isoforms' field at a time when it is emerging at the forefront of p53 research.

TP63 and *TP73*: the Isoform Paradigm

The two p53-related proteins, p63 and p73, share strong structural, biochemical and biological homologies.^{12,13} In particular, they bind specifically to DNA onto conserved p53 response elements (p53REs) by using their DNA-binding domain. In the late nineties, the cloning of *TP63* and *TP73* revealed an elaborate pattern of mRNA expression resulting in several protein isoforms.^{14,15} Several N-terminal forms, produced by the use of alternative promoters and/or alternative splicing (i.e., TA forms, which contains the transactivation domain (TAD), *versus* Δ N forms, produced from an

¹Centre for Oncology and Molecular Medicine, INSERM-European Associated Laboratory, University of Dundee, Ninewells Hospital, Dundee, Scotland DD1 9SY, UK;

²Apoptosis and Neurogenetics Group, Ecole Normale Supérieure de Lyon, Laboratory of Molecular Biology of the Cell, CNRS UMR5239, University of Lyon, UMS3444 Biosciences Lyon Gerland, 46 allée d'Italie, Lyon 69007, France; ³Molecular Carcinogenesis Group, International Agency for Research on Cancer, Lyon Cedex 08, France; ⁴INSERM U612, Bât. 110-112, Centre Universitaire, Orsay 91405, France and ⁵Institut Curie Centre de Recherche, Bât. 110-112, Centre Universitaire, Orsay 91405, France

*Corresponding author: J-C Bourdon, Centre for Oncology and Molecular Medicine, INSERM-European Associated Laboratory, University of Dundee (Mailbox 4), Ninewells Hospital, Medical School, Dundee, Scotland DD1 9SY, UK. Tel: +44 (0) 138 249 6401; Fax: +44 (0) 138 249 6363; E-mail: j.bourdon@dundee.ac.uk
Keywords: tumour suppressor; p53; p63; p73; p53 family; isoforms

Abbreviations: MEFs, mouse embryonic fibroblasts; TAD, transactivation domain; p53REs, p53 response elements
Received 19.5.11; revised 04.8.11; accepted 12.8.11; Edited by G Melino; published online 23.9.11



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p53 isoforms: from identification to biological functions
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internal promoter resulting in the presence of a different TAD), were found combined with several C-terminal forms generated by alternative splicing (five for p63: α to ϵ ; seven for p73: α to η).¹³ Far from being of minor component, the Δ Np63 and Δ Np73 forms are the major forms expressed in certain cell types.^{14,16} In mice, knockout of the entire *TP63* or *TP73* loci (targeting all isoforms) revealed the roles of p63 and p73 in epithelial differentiation and neuronal development, respectively,^{15,17} whereas no impact was observed in stem cell commitment.¹⁸ However, with the generation of isoform-specific knockout mice, a subtle interplay between the N-terminal isoforms has recently emerged, with the dynamic expression of N-terminal p63 or p73 isoforms appearing critical for maintaining the normal sequence of cell development (from stem to committed progenitors and then differentiated cells).^{19,20} Building on this idea, Aberdam and co-workers analysed the impact of Δ Np63 and TAp63 isoforms in cellular commitment. In murine embryonic stem cells, Δ Np63, but not TAp63, is highly expressed during epidermal commitment and is critical for the expression of the cytokeratins K14 and K5, two markers of keratinocyte proliferation, indicating that only Δ Np63 is required for the commitment of ectodermal into epidermal cells.^{21,22} Mills *et al.* observed that Δ Np63 α overexpression in mouse embryonic fibroblasts (MEFs) resulted in the bypass of Ras-mediated senescence and enhanced carcinoma development in mice, suggesting that Δ Np63 α inhibits senescence and therefore acts as an oncogene.²³ By contrast, overexpression of TAp63 forms in p53^{-/-} MEFs increased senescence and reduced tumour development *in vivo*, consistent with a p53-independent effect.²⁴ Studying mice deficient for specific p73 protein isoforms, Mak, Melino and co-workers revealed that, like p63, the different p73 isoforms had dual cellular roles. In particular, TAp73^{-/-} mice are spontaneously tumour-prone whereas Δ Np73^{-/-} cells show impaired tumour formation in nude mice.^{25,26} In addition to these cancer-related effects, knockout of either TAp73 or Δ Np73 isoforms resulted in isoform-specific defects in neurogenesis or neurodegeneration, respectively. Overall, these studies show that each N-terminal p63/p73 form has specific roles in regulating distinct cell differentiation pathways. Moreover, they demonstrate that the Δ N forms, by preventing senescence and maintaining progenitor cell status, may act as oncogenes, whereas TA forms, through their capacity to switch cells into a post-mitotic state, may act as tumour suppressors.

Generation of Human p53 Isoforms by Diverse Regulatory Mechanisms

Like *TP63* and *TP73*, the human *TP53* gene encodes several p53 protein isoforms through conserved mechanisms.^{6,7} The main and most abundant p53 isoform is the canonical p53 protein, also termed TAp53 α , as it contains an entire TAD and the longest C-terminal domain (Figure 1). In addition to the TA forms, three Δ N forms have been identified that differ by their translation initiation site. This is used to designate them as Δ 40p53, Δ 133p53 and Δ 160p53. The four N-terminal p53 forms can be combined with three different C-terminal domains (α , β , γ). Recently, some *cis*- and *trans*-regulators

have been identified as specific modulators of p53 isoform expression (Figure 1).

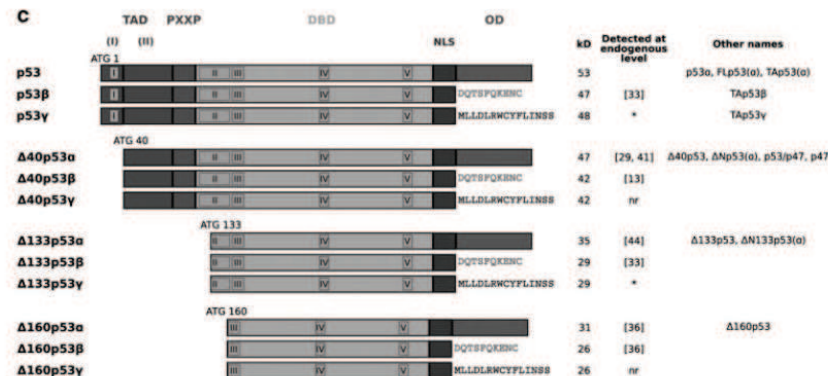
Δ 40p53 expression: one form, several mechanisms

Compared with p53, the human N-terminal Δ 40p53 forms lack the first 39 amino acids corresponding to the main TAD (Figure 1c). Matlashewski *et al.*²⁷ identified a p53 mRNA species retaining the entire intron-2 (p53I2), indicating that an alternative splicing event leading to the retention of the *TP53* intron-2 can occur (Figure 1b). They later observed that stop codons in intron-2 of the p53I2 mRNA prevent p53 expression from the first AUG.²⁸ However, p53I2-transfected cells were found to express a 45-kDa protein, undetectable using antibodies recognizing the p53-TAD epitopes (DO1 or DO7), that corresponds to Δ 40p53 initiated at a second AUG at codon-40, encompassed within a strong Kozal consensus.²⁹ Alternative splicing of intron-2 can be regulated through *G-quadruplex* structures located in intron-3 of the p53 pre-mRNA.³⁰ Using reporter assays and RNA-*G-quadruplex* ligands, it appears that *G-quadruplex* structures promote the correct splice-out of intron-2, leading to the fully spliced p53 (FSp53) mRNA encoding the full-length p53 protein; *G-quadruplex* disruption however favours the retention of intron-2 and thus p53I2 mRNA expression. This observation is the first clue that the *TP53* sequence itself can modulate its own isoforms' expression through regulation of alternative splicing.

In addition to alternative splicing, Δ 40p53 forms can be encoded from the FSp53 mRNA through an internal initiator of translation at codon-40.²⁹ Two internal ribosomal entry sequences (IRES) have been identified that regulate the translation of either p53 or Δ 40p53 (Figure 1b).^{31,32} However the relative contribution, *in vivo*, of each of these mechanisms which are producing Δ 40p53, remains to be fully established.

Production of Δ 133p53 and Δ 160p53 forms from the internal P2 promoter.

As for *TP63* and *TP73*, *TP53* contains an internal promoter that controls the expression of two N-terminal forms (Figure 1a).³³ In addition to the proximal P1 promoter regulating p53 and Δ 40p53 expression, an internal P2 promoter located between intron-1 and exon-5 regulates the transcription of p53 mRNAs initiated in intron-4 (p53I4) (Figure 1b).³³ This internal P2 promoter is different from the P⁺ promoter in *TP53* intron-1 identified by Reisman *et al.*^{34,35} that regulates the expression of an unrelated p53 transcript encoded by *TP53* intron-1. Site-directed mutagenesis and siRNA methods revealed that translation of p53I4 mRNAs can be initiated at two distinct codons, AUG133 and AUG160, leading to the expression of the Δ 133p53 and Δ 160p53 proteins, respectively, that lack the TAD and part of the DNA-binding domain (Figure 1c).³⁶ Surprisingly, Δ 160p53 forms are expressed in K562 cells which are considered as 'p53-null' cells. The *TP53* mutation in K562 cells results in a premature stop codon between AUG133 and AUG160, thus preventing the expression of the TA, Δ 40 and Δ 133 forms without compromising the Δ 160p53 reading frame. Thus, it should be kept in mind that some cells or tumours considered as 'p53-null' because of the presence of frameshift or nonsense mutations, may retain the capacity to express one or several p53 isoforms.^{29,36}



well as the different names of the isoforms used in the literature. The color reproduction of this figure is available at the *Cell Death and Differentiation* journal online

IP experiments demonstrate that the direct binding of p53 onto the p53REs results in an increased expression of both $\Delta 133\text{p53}$ mRNAs and the $\Delta 133\text{p53}$ protein. Some experimental evidence suggests that protein expression of

$\Delta 133p53\alpha$ can be induced through p53-independent mechanisms. In particular, the accumulation of the $\Delta 133p53\alpha$ protein was observed in response to the knockdown of p68, a DEAD Box helicase involved in multiple transcriptional regulatory processes, and more recently in response to the expression of some p63 or p73 isoforms.³⁹

The C-terminal spliced p53 forms, β and γ . The alternative splicing of intron-9 of human *TP53* produces three different C-terminal p53 forms (α , β and γ) (Figure 1a).^{8,33} Complete excision of intron-9 results in the expression of the α -forms corresponding to the classical p53 C-terminal domain (oligomerization domain, OD) (Figures 1b and c). On the other hand, partial retention of intron-9 generates the β - or γ -forms, in which the OD is replaced by 10 or 15 new amino acids, respectively. However, the mechanisms that control the alternative splicing of intron-9 are unknown.

The classical p53 C-terminal domain contains the main post-translational modification sites regulating p53 stability, such as the lysines residues ubiquitinated by Mdm2, an E3-ubiquitin ligase regulating p53 stability and activity.³ Their absence in β - and γ -forms led to the investigation as to whether the stability of the p53 β and p53 γ proteins is regulated by the Mdm2–ubiquitin–proteasome pathway. To date, results remain controversial. For instance, whereas constitutively overexpressed FLAG-tagged p53 β or p53 γ do not appear to interact with Mdm2 or to be degraded in a proteasome-dependent manner,⁴⁰ Bourdon and co-workers observed that the endogenous p53 β protein can be degraded by the proteasome in an Mdm2-dependent manner.

Biological Functions of Human p53 Isoforms

Based on early studies detailed below, the N-terminal isoforms lacking the TAD (i.e., $\Delta 40p53$, $\Delta 133p53$ and $\Delta 160p53$) were expected to only act as dominant-negative regulators of p53 activity. For instance, ectopic $\Delta 40p53\alpha$ expression downregulated the p53-induced transactivation on reporter genes and counteracted p53-dependent growth suppression in colony formation assays.^{28,29} In addition, in human diploid fibroblast WI38 cells, expression of endogenous $\Delta 40p53\alpha$ increased during the G₁/S transition, in parallel with decreased expression of p21.²⁹ Furthermore, as $\Delta 40p53\alpha$ lacks the Mdm2-binding site, it escapes Mdm2-mediated degradation and does not accumulate in response to DNA damage, its expression persisting at low but stable amounts in many cell types.^{29,41} These data support the notion that $\Delta 40p53\alpha$ inhibits basal p53 activities during cell-cycle progression. However, whether $\Delta 40p53\alpha$ can exert p53-independent effects is still unknown. Hainaut and co-workers observed that $\Delta 40p53\alpha$ contains an intact DNA-binding domain able to bind to p53REs *in vitro*. Thus, $\Delta 40p53\alpha$ may also exert intrinsic regulatory effects by competing with p53 for p53RE binding and thereby modulating their accessibility for other transcription factors, regulating the cell fate outcome depending upon cell type and cell context. In addition, $\Delta 40p53\alpha$ lacks the first TAD, which has been shown to be dispensable for p53 transcriptional activity, and retains the second TAD, which can regulate gene expression.^{42,43} Therefore, with our current knowledge, one should be

cautious in considering $\Delta 40p53$ as a simple dominant-negative inhibitor of p53.

Compared with $\Delta 40p53\alpha$, the available data have clearly revealed that $\Delta 133p53$ controls p53 activity. In reporter assays, $\Delta 133p53\alpha$ can also behave as a dominant-negative inhibitor of p53.^{13,33} $\Delta 133p53\alpha$ does not bind to consensus p53REs *in vitro*, consistent with its partial lack of the DNA-binding domain, and thus can also behave as a dominant mutant p53 protein.³⁸ Experimental studies suggested that, instead of being a strict dominant inhibitor, $\Delta 133p53\alpha$ is instead a fine modulator of p53's suppressive activity as its expression determines cell fate in response to stress. The knockdown of $\Delta 133p53\alpha$ expression promotes p53-mediated apoptosis and G₁ cell-cycle arrest in response to doxorubicin treatment, without altering the p53-dependent G₂ cell-cycle arrest.³⁷ These effects may be due to the ability of $\Delta 133p53\alpha$ to modulate gene expression in a promoter-dependent manner, as observed for *p21^{WAF1}*, *Mdm2* and *Bcl-2*.³⁷ Interestingly, $\Delta 133p53$ silencing has also been associated with replicative, but not oncogene-induced, senescence in normal human fibroblasts through transcriptional regulation of p53-target genes, including *p21^{WAF1}* and *mir-34a*.⁴⁴ Overall, these results are consistent with an oncogenic capacity of $\Delta 133p53$.

The biological functions of the C-terminal p53 isoforms (i.e., p53 β and p53 γ) remain poorly described and controversial. Bourdon *et al.*³³ showed that, in the absence of stress, endogenous p53 β bound to the *Bax* and *p21^{WAF1}* promoters, but only weakly to that of *Mdm2*. Moreover, in luciferase reporter assays in the absence of stress, the co-expression of p53 β and p53 enhanced the p53 transcriptional activity on the *p21^{WAF1}* promoter but not on the *Bax* promoter, suggesting a promoter-dependent effect. These observations are consistent with the demonstration that p53 β cooperates with p53 to accelerate senescence in human fibroblasts.⁴⁴ By contrast, experimental studies failed to observe binding of FLAG-tagged p53 β or p53 γ onto p53RE consensus and to show a role of FLAG-tagged p53 β or p53 γ in p53-dependent apoptosis or senescence in cells constitutively overexpressing FLAG-tagged p53 β or p53 γ , and selected to grow in presence of neomycin.⁴⁰ Thus, there is still debate on whether p53 β or p53 γ exert their activities in an autonomous manner or through an interaction with p53. Furthermore, there is no evidence of distinct biological activities for p53 β or p53 γ .

Overall, current experimental data on the biological roles of p53 isoforms are fragmented. Given that p53 isoforms differ from each other in the three functional domains (TAD, DNA-binding and OD), their potential to modulate p53-dependent responses is expected to be diverse and cell type-dependent. Further insight into which of these functions are of physiological or pathological relevance may come from animal model studies.

Animal Models: clues to the Physiological Significance of Isoforms

The simplest animal model to study p53 isoforms, *Drosophila melanogaster*. The diversification of the p53 gene family into three members occurred in vertebrates.

Thus, invertebrates such as *Drosophila* contain a single p53-related gene, which encodes three protein isoforms (Figure 2): Dp53, corresponding to the human full-length

p53; DΔNp53, a general counterpart of the human N-terminal p53 forms as it is encoded by an mRNA transcribed from an internal promoter (i.e., human Δ133) and contains a

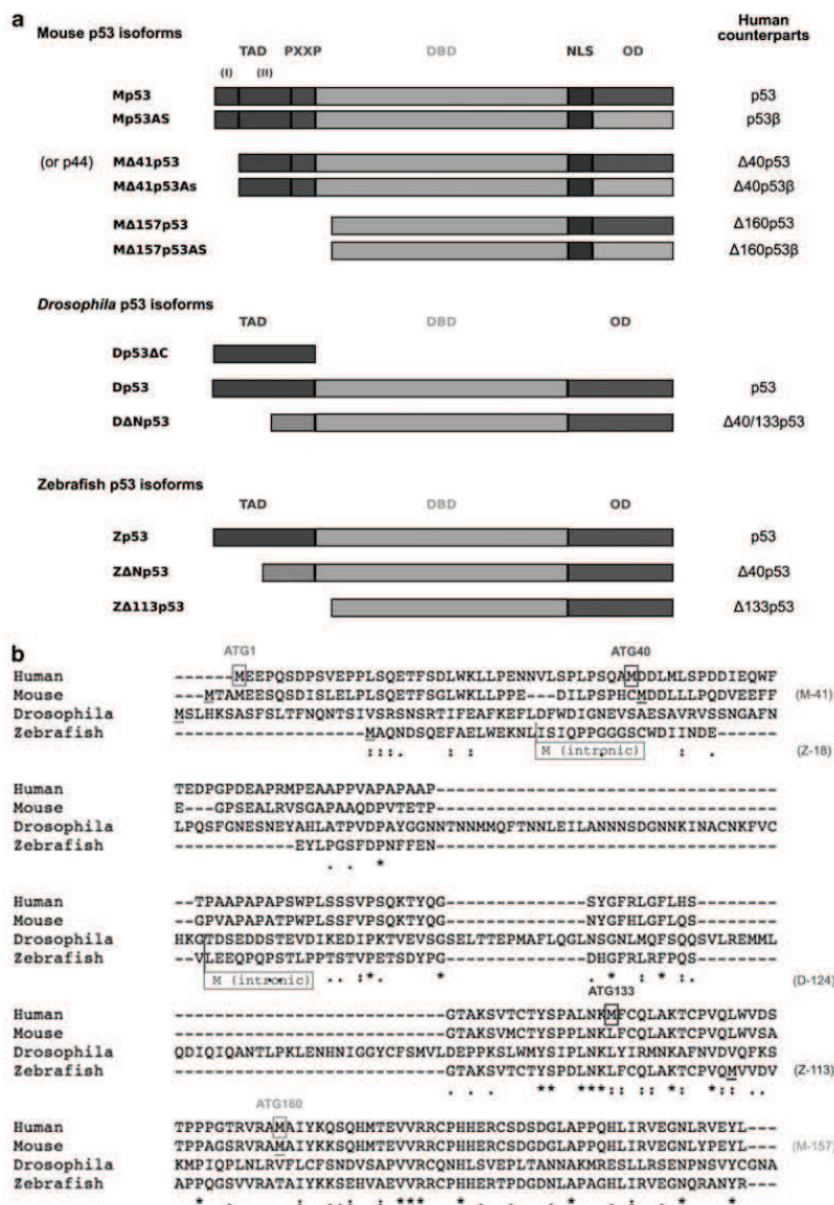


Figure 2 p53 isoforms in animal models. (a) Structural organization of p53 isoforms through evolution. Like humans, mouse, *Drosophila* and zebrafish express a full-length p53 protein, which conserves a TAD (blue), a DNA-binding domain (DBD, orange) and an OD (green). Only the mouse Mp53 protein presents a proline-rich domain (PXXP, purple) and a nuclear localization signal (NLS, green). In addition, all these animals express some p53 isoforms that have the same structural organization as the human p53 isoforms owing to the use of alternative promoters and splicing sites. M, mouse protein; D, *Drosophila* protein; Z, zebrafish protein; N-terminal p53 isoform identification, Δ forms; N-terminal p53 isoform denomination, codon number, when initiated ATG occurs in the coding sequence, or N, when initiation occurs in a non-coding sequence; C-terminal p53 isoform identification, AS (alternative splicing, green boxes); grey box, different residues compared with the full-length p53 protein. (b) Localization of translation initiation sites in animal p53 sequences. Red, ATG1 generating the full-length p53 protein; green, the methionine used to produce the homologues to the human Δ40p53 forms; blue, the methionine used to produce the homologues to the human Δ133p53 forms; orange, the methionine used to produce the homologues to the human Δ160p53 forms. The color reproduction of this figure is available at the *Cell Death and Differentiation* journal online

Table 1 Available p53 animal models

Species	Name	Modulation of p53 isoforms	Ref.
<i>Drosophila</i>		Overexpression of DΔNp53 in cells	45
		Overexpression of transgenic DΔNp53 under the control of tissue-specific promoters	47,49–53
		Overexpression of transgenic dominant-negative mutant DΔNp53	53,54
		Inactivation of <i>Dp53</i> locus	49,53
Zebrafish	Tg(Δ113p53:gfp)	Overexpression of transgenic ZΔ113p53-GFP morpholino (Δ113p53-MO)	61
		Overexpression of ectopic ZΔNp53	59
Mouse	MΔ41p53/MΔ41p53 wt/MΔ41p53	Overexpression of transgenic MΔ41p53	65
	MΔ122p53/MΔ122p53 wt/MΔ122p53 m/m wt/m	Overexpression of transgenic MΔ122p53	68
		'm' = deletion of exon 1–6	69

truncated TAD followed by a complete DBD and OD (i.e., human Δ40); and Dp53ΔC, encoded by a short transcript leading to a putative isoform bearing only the TAD.^{33,45–47} The *Drosophila* p53 gene is activated by irradiation and exerts broad suppressive effects recapitulating those of the p53-family members, including regulation of apoptosis, aging, autophagy, differentiation and growth.⁴⁸ Historically, DΔNp53 was the first form identified and previously termed Dp53; hence most of the functional studies to date have focused on the role of this particular isoform (Table 1).

Studies on the morphogenesis of imaginal discs have highlighted the role of DΔNp53 isoforms in the control of cell death.^{45,47,49,50} In this system, DΔNp53 controls apoptosis through the Reaper–Hid–Grim (RHG) cascade. Indeed, irradiated imaginal discs from flies with a mutant *Dp53* gene show reduced apoptosis but normal cell-cycle arrest, suggesting a specific regulatory role of the *Dp53* gene product in apoptosis.^{45,49} So far, the main isoform implicated in apoptosis appears to be DΔNp53, which directly regulates *reaper* (*rpr*) expression.⁴⁵ However, DΔNp53 also exerts effects through other pathways. In particular, DΔNp53 may activate either apoptotic or non-apoptotic responses in photoreceptor cells depending on the cell differentiation status.^{50–52} Moreover, DΔNp53 appears to inhibit cellular differentiation in the retina independently of its apoptotic function.⁵⁰

The *Dp53* gene product also exerts important roles in controlling lifespan in a sex- and stage-dependent manner. When overexpressed in adult flies (Table 1), DΔNp53 limited lifespan in females and extended it in males. By contrast, when overexpressed during development, DΔNp53 exerted a similar dose-dependent effect on longevity in both sexes.⁵³ Conversely, inactivation of the *Dp53* locus increased lifespan in females but had only minor effects in males. Similar phenotypes were observed in *Drosophila* expressing dominant-negative Dp53 mutant transgenes or overexpressing DΔNp53, suggesting that DΔNp53 interferes with Dp53 activity.⁵⁴ The role of *Drosophila* p53 has also been investigated in detail in other cellular functions such as DNA repair or compensatory proliferation.^{48,55,56} However, the specific roles of each *Drosophila* p53 isoform, Dp53 and DΔNp53, have not been studied in sufficient detail to understand the exact contributions of each isoform to development, stress responses or longevity.

Danio rerio, the historical model to study p53 isoforms. The zebrafish p53 protein, Zp53, recapitulates

the suppressive and pro-apoptotic functions of human p53 upon genotoxic stress.⁵⁷ So far, only N-terminal Zp53 isoforms have been identified (Figure 2): Zp53, corresponding to the human p53 protein;⁵⁸ ZΔNp53, produced through an alternative splicing of intron-2 and thus similar to the human Δ40p53 forms;⁵⁹ and ZΔ113p53, produced by an internal promoter located within the zebrafish *Zp53* gene that is regulated by Zp53 itself, and thus equivalent to the human Δ133p53 isoform.^{37,38,60} In contrast to the human Δ40p53, AUG1 of ZΔNp53 is located within the partial intronic sequence retained by alternative splicing.⁵⁹ Thus in ZΔNp53, the 38 N-terminal residues containing the TAD are replaced by 33 residues derived from the intron-2 sequence.

There is evidence that the ZΔNp53 transcript accumulates in response to γ-ray irradiation.⁵⁹ In addition, ectopic expression of ZΔNp53 resulted in a strong developmental phenotype with hypoplasia and malformation of the head, eyes and somites (Table 1). This phenotype is dependent upon the presence of Zp53, the two isoforms forming a protein complex through their ODs. Overexpression of an OD-mutant ZΔNp53 or wild-type ZΔNp53 in a mutant Zp53 background is phenotypically ineffective. These observations suggest that ZΔNp53 exerts its effects by modulating the activity of Zp53 during zebrafish development.

ZΔ113p53 was discovered in a different context (Table 1). Cheng and co-workers found that in zebrafish embryos, loss of the *Def* gene (Digestive organ Expansion Factor) led to defects in the morphogenesis of digestive organs. In a genome-wide screen, they identified a shorter form of Zp53 whose expression was upregulated in *def*^{−/−} embryos.⁶⁰ Upregulation of ZΔ113p53 correlated with increased expression of p53-target genes involved in cell-cycle progression such as *cyclin-G1* and *p21*^{WAF1}, whereas pro-apoptotic genes such as *Bax* and *Reprimo* were not activated. Furthermore, ZΔ113p53 was found to selectively upregulate the *Bcl-2L* anti-apoptotic gene.⁶¹ This pattern of effects is consistent with the notion that ZΔ113p53, like Δ133p53, operates as a modulator of p53 in selectively activating defined target genes.

Mouse, the next generation of animal models. Six p53 isoforms have been described in mice, resulting from combination of three N-terminal p53 isoforms with two different C-terminal isoforms (Figure 2). In addition to the full-length mouse Mp53, Rotter and co-workers identified an alternative p53 mRNA retaining part of intron-10 that encodes a shorter isoform with new residues in place of

the usual OD (Mp53AS), homologous to the human p53 β .^{10,62} Later, Mowat *et al.*⁶³ isolated M Δ 41p53, the mouse counterpart of human Δ 40p53 forms. In addition, Khoury *et al.* isolated a shorter N-terminal form produced by an internal promoter within the mouse p53 gene, M Δ 157p53, equivalent to the human Δ 160p53 form. Khoury *et al.* also showed that M Δ 41p53 and M Δ 157p53 can be expressed as a C-terminal AS variant. As for the human p53 isoforms, Rotter and co-workers reported that Mp53AS modulates Mp53-mediated apoptosis and Mp53 transcriptional activity in luciferase reporter assays.⁶⁴

In 2004, Maier *et al.*⁶⁵ described a transgenic mouse overexpressing M Δ 41p53 (Table 1). When expressed in a p53-null background, this isoform did not induce any particular phenotype. However, when expressed in a p53-competent background, an increased dosage of M Δ 41p53 led to reduced size, accelerated aging and a shorter lifespan associated with hypo-insulinemia and glucose insufficiency.^{65–67} These effects were attributed to the hyper-activation of the insulin-like growth factor (IGF)-signalling axis by M Δ 41p53, setting in motion a cascade that clamps unimpeded growth through p21.⁶⁵ Furthermore, these M Δ 41p53-overexpressing mice show cognitive decline and synaptic impairment early in life, also attributable to the hyper-activation of the IGF-1-signalling pathway.⁶⁷ These observations are consistent with studies performed *in vitro* and in the zebrafish model, indicating that Δ 40p53 isoforms may regulate growth suppression through modulation of p53 activity.

A premature aging phenotype was also reported in a non-physiological knock-in p53 mouse model expressing a 'M Δ 122p53' form, truncated for the first 122 residues (Table 1).⁶⁸ Although M Δ 122p53 has no physiological equivalent in mouse, it can be considered as an 'intermediate' between the M Δ 41p53 and M Δ 157p53 isoforms, as it lacks the TAD and part of the DNA-binding domain. During adulthood, transgenic p53 ^{Δ 122p53/ Δ 122p53} mice showed premature aging symptoms, such as balding and arthritis, similar to that observed in the p53^{+/-} mice (deletion exon 1–6).^{68,69} In addition, earlier tumour onset and shortened lifespan were observed in p53^{+/-}/ Δ 122p53 mice as compared with p53^{+/-} mice. Overall, these results support the hypothesis that mouse N-terminal p53 forms may operate as dominant oncogenes to promote cell proliferation and inflammation.

The studies summarized above highlight common themes in the 'p53 isoform' field. First, overall patterns of isoform

expression are well-conserved throughout evolution. Second, N-terminal isoforms have a major role as regulators of physiological processes related to development, aging, lifespan and, possibly, carcinogenesis. In this respect, two key mechanisms are emerging. The Δ 40p53 form exerts regulatory effects on signalling cascades controlled by p53, perhaps through direct interaction between the two isoforms. Conversely, isoforms corresponding to Δ 133p53 α modulate cell response by regulating gene expression in a p53-dependent and -independent manner. It should, however, be remembered that our current view of isoform activities remains fragmentary and that further studies are needed to better understand their roles and underlying mechanisms.

p53 Isoforms and Human Cancers

Genetic polymorphisms: effects on p53 isoform expression. The *TP53* gene is highly polymorphic, with over 80% of known single-nucleotide polymorphisms (SNPs) located within introns or non-coding 5' and 3' sequences.⁷⁰ Hainaut and co-workers showed that *G-quadruplex* structures, formed in intron-3 and regulating intron-2 splicing (and thus Δ 40 form expression), overlap a common polymorphism, *TP53* PIN3, which consists of a 16-bp duplication (A1, non-duplicated allele; A2, duplicated allele).^{30,71} This polymorphism may therefore modulate the structure and/or the stability of the *G-quadruplexes*, and affect Δ 40p53 expression (Table 2). Consistent with a functional effect, *TP53* PIN3 has recently been identified as a strong genetic modifier of germline *TP53* mutations. Indeed, carriers bearing A1A1 genotypes developed their first cancer on average 20 years earlier than carriers with an A1A2 genotype.⁷² This effect was detected in a Brazilian cohort, in which the p.R337H mutation of partial penetrance is very common owing to a widespread founder effect.⁵ It remains to be demonstrated whether a similar effect is observed in carriers of other *TP53* mutation types.

Landi and co-workers have identified 11 different haplotypes defined by eight SNPs in a region from exon-3 to intron-4, overlapping part of the internal P2 promoter regulating Δ 133p53 and Δ 160p53 expression.⁷³ Using these different haplotypes as promoters to drive *luciferase* expression, they found significant differences in basal promoter activity that were confirmed by analysing endogenous

Table 2 Role of p53 isoforms in human cancers

Cancer	Isoform	Observation	Ref.
Breast cancer	Δ 133p53 α p53 β p53 γ	Overexpression Loss of expression Loss of expression Prognosis of mutp53/p53 γ as good as wtp53	33 33,78 33,79
Acute myeloid leukaemia	p53 β /p53 γ	Increased expression in response to chemotherapy	78
Ovarian cancer	p53 β Δ 40p53	Serous and poorly differentiated tumours/worse recurrence-free and overall survival Improved recurrence-free survival	77 74
Li-Fraumeni syndrome	Δ 40p53	<i>G-quadruplex</i> structure overlapping <i>TP53</i> PIN3/early age at first cancer diagnosis in patients carrying A1A1 compared with patients carrying A1A2	72

$\Delta 133p53$ expression in samples of normal colonic mucosa. Furthermore, *in silico* and *in vitro* DNA binding analyses suggested that an SNP in intron-4 (rs179287; C > T) affects proteins binding within the P2 promoter.⁷³ These results suggest that genetic polymorphisms may modulate basal $\Delta 133p53$ expression. Whether these effects influence cancer risk remains to be demonstrated.

Mutations affecting the production of isoforms. Given the role of N-terminal p53 forms as inhibitors of p53 transactivation, it can be expected that overexpression of certain isoforms may represent an alternative to a mutation in *TP53* for inactivating p53 in cancers, as observed in some small clinical studies.^{33,74,75} Analysis of the IARC *TP53* database identified 1019 somatic cancer mutations that are predicted to disrupt the p53 coding sequence in the N-terminal region while leaving intact the sequence encoding at least $\Delta 160p53$. This represents 3.65% of all somatic mutations reported to date (version R15; www.p53.iarc.fr).⁷⁰ Therefore, rare and/or silent mutations in *TP53* may differentially affect the expression of p53 isoforms, as observed recently for mutations present in the IRES regulating the relative expression of p53 and $\Delta 40p53$ forms encoded by the full-length FSp53 mRNA.⁷⁶ With respect to C-terminal isoforms, Hofstetter *et al.*⁷⁷ have used a yeast-base functional assay and RT-PCR to show that mutations at splice sites of *TP53* intron-9 can lead to aberrant expression of p53 β mRNA in primary cultures of ovarian cancer cells. By contrast, somatic mutations at splice sites of introns 6 and 9 were found to generate spliced mutant p53 mRNAs (p53 ζ , p53 δ and p53 ϵ), distinct from the physiological isoforms. However, the role of such mutant isoforms in carcinogenesis is not known.

Isoform expression and cancer outcomes. There is emerging evidence that p53 isoform expression is deregulated in human cancers (Table 2). In a study of 245 primary ovarian cancers, Hofstetter *et al.*⁷⁷ observed that

expression of p53 β was associated with serous and poorly differentiated cancers, and, when expressed together with the functional p53 protein, it was correlated with poor recurrence-free and overall survival. By contrast, tumours expressing functional p53 and $\Delta 40p53$ showed improved recurrence-free survival of patients compared with tumours expressing no $\Delta 40p53$. Studies of patients with acute myeloid leukemia have shown that elevated expression of p53 β and/or p53 γ in blood cells was correlated with improved responses to chemotherapy,⁷⁸ which may predict decreased chemoresistance and improved overall survival. An effect of isoform expression has also been observed on breast cancer prognosis. In a cohort of 127 breast cancer patients, Thompson and co-workers reported that patients whose tumours expressed both mutant p53 and p53 γ mRNAs had a prognosis as good as patients whose tumours expressed a wild-type p53, suggesting that the expression of p53 γ may abrogate the poor prognosis commonly associated with *TP53* mutations.⁷⁹

Conclusion

The field of 'p53 isoforms' is still in its infancy, but the increasing number of genetic, biochemical and clinical studies have clearly established that p53 isoforms are fundamental and important components of the p53 pathway. Data obtained from animal and cellular models indicate that p53 isoforms regulate the cell fate in response to developmental defects and cell damages by differentially regulating gene expression, both in a p53-dependent and -independent manner. Furthermore, the current data suggest that p53 isoforms have roles in all biological activities regulated by p53 (Figure 3). Therefore, one can reasonably expect that the characterization of the biochemical and biological activities of p53 isoforms will impact on the fields of cancer, embryo development and aging.

Future experiments will be needed to gain further insight into how p53 isoforms modulate the different biological

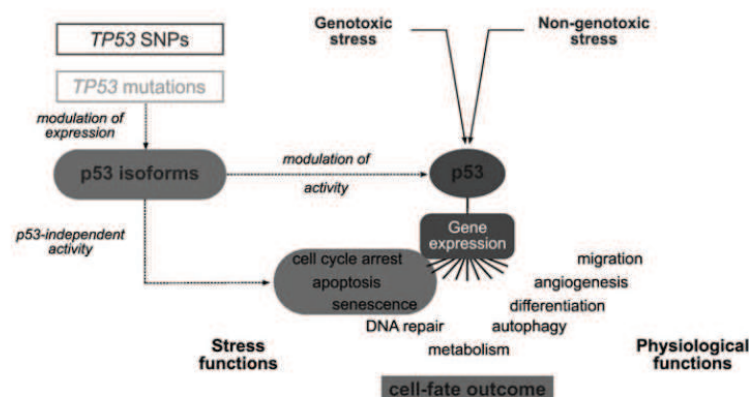


Figure 3 p53 isoforms in the p53 network. p53 integrates the different stress signals to adapt cell fate to the intensity and the nature of stress by regulating several biological functions to maintain genomic and cellular integrity. In addition, p53 controls physiological functions under basal conditions. Recent data suggest that p53 isoforms modulate p53-mediated cell fate outcome and may thus be key components of the p53-mediated decision not only in response to stress but also under basal conditions. It has also been reported that p53 isoforms have p53-independent activities and directly regulate cell-cycle arrest and apoptosis. In addition, genetic alterations, such as *TP53* SNPs and *TP53* mutations, affect the expression of p53 isoforms, which may result in tumorigenesis

activities. However, based on our current understanding of the p63, p73 and p53 isoforms, we have come to realize that the p53 pathway should no longer be considered as regulated by only the p53 protein, but by a set of p63, p73 and p53 isoforms that interplay with each other in regulating physiological functions. Further progress on this front will require the development of robust and standardized tools for the identification and quantification of p53, p63 and p73 protein isoform expression in experimental systems as well as in human tissues.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. We thank all the speakers for their participation in the 1st International p53 Isoforms Meeting, and Michelle Wisez for logistic help. The meeting was funded by IARC, the College of Medicine of the University of Dundee and the Laboratory of Molecular Biology of the Cell (UMR5239, CNRS at ENS de Lyon). Links: IARC TP53 database, <http://www-p53.iarc.fr/>; abstracts of the International p53 Isoforms Meeting, http://www-p53.iarc.fr/DownloadBook_p53isoforms2010_Final.pdf. VM is supported by a fellowship from the Breast Cancer Campaign; M-LD-D and BM by CNRS ATIP, Fondation pour la Recherche Médicale (FRM) and La Ligue contre le cancer; CS by a PhD grant from the French Ministry of Research; JH by INSERM and Institut Curie; PH and JH by l'Institut National contre le Cancer (INCa, France; Projet Libre 2009, 2009-192); and J-CB by Cancer Research UK.

Facts

- At least nine different mRNAs are expressed from the *TP53* gene encoding 12 distinct protein isoforms through the use of alternative promoters, splicing sites and internal initiation sites of translation.
- p53 isoforms regulate cell fate in response to developmental defects and cell damage by differentially regulating gene expression, both in a p53-dependent and -independent manner.
- The N-terminal p53 isoforms, $\Delta 40$ and $\Delta 133$, are not strict dominant-negative inhibitors of p53's suppressive function as they have p53-independent activities.
- The expression patterns and biological activities of p53 isoform are conserved through evolution (human, mouse, zebrafish and *Drosophila*).
- *TP53* SNPs and mutations alter the pattern of p53 isoform expression.
- p53 isoform expression is deregulated in human cancers and is associated with clinical prognosis.

Open Questions

- Development of standardized and robust tools is needed to systematically identify and quantify p53 isoforms in human and animal models.
- Characterization of the biochemical activities of p53 isoforms as well as their molecular targets.
- Is each p53 isoform specialized in a particular biological response? Have they redundant activities?
- Evaluation of the interplay between p53-, p63- and p73-family isoforms.
- Study the potential use of p53 isoform expression in human cancers as biomarkers and as therapeutic targets.

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7.2 Appendix II: *Drosophila* p53 isoforms differentially regulate apoptosis and apoptosis-induced proliferation

Cell Death and Differentiation (2012), 1–9
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Drosophila p53 isoforms differentially regulate apoptosis and apoptosis-induced proliferation

M-L Dichtel-Danjoy^{1,5}, D Ma^{1,5}, P Dourlen¹, G Chatelain¹, F Napoletano¹, M Robin¹, M Corbet¹, C Levet¹, H Hafsi², P Hainaut², HD Ryoo³, J-C Bourdon⁴ and B Mollereau^{*,1}

Irradiated or injured cells enter apoptosis, and in turn, promote proliferation of surrounding unaffected cells. In *Drosophila*, apoptotic cells have an active role in proliferation, where the caspase Dronc and p53 induce mitogen expression and growth in the surrounding tissues. The *Drosophila* p53 gene structure is conserved and encodes at least two protein isoforms: a full-length isoform (Dp53) and an N-terminally truncated isoform (DΔNp53). Historically, DΔNp53 was the first p53 isoform identified and was thought to be responsible for all p53 biological activities. It was shown that DΔNp53 induces apoptosis by inducing the expression of IAP antagonists, such as Reaper. Here we investigated the roles of Dp53 and DΔNp53 in apoptosis and apoptosis-induced proliferation. We found that both isoforms were capable of activating apoptosis, but that they each induced distinct IAP antagonists. Expression of DΔNp53 induced Wingless (Wg) expression and enhanced proliferation in both 'undead cells' and in 'genuine' apoptotic cells. In contrast to DΔNp53, Dp53 did not induce Wg expression in the absence of the endogenous p53 gene. Thus, we propose that DΔNp53 is the main isoform that regulates apoptosis-induced proliferation. Understanding the roles of *Drosophila* p53 isoforms in apoptosis and in apoptosis-induced proliferation may shed new light on the roles of p53 isoforms in humans, with important implications in cancer biology.

Cell Death and Differentiation advance online publication, 17 August 2012; doi:10.1038/cdd.2012.100

Epithelial tissues have the intrinsic capability to repair and regenerate, following irradiation or genetically induced cell death. However, how epithelial cells respond to injury and recover is not well understood. In the past few years, studies from metazoan models, such as *Drosophila*, forged the concept of apoptosis-induced proliferation, a process by which damaged cells entering apoptosis signal the surrounding unaffected cells to divide so as to recoup the tissue loss.^{1,2,3} Using *Drosophila* developing imaginal discs as a model, several groups have demonstrated that fly wing imaginal discs submitted to γ -irradiation or genetically induced cell death undergo apoptosis-induced proliferation.^{4,5} Apoptosis-induced proliferation requires *Drosophila* p53 and the caspase Dronc, and involves the release of mitogens such as Wingless (Wg) and Decapentaplegic (Dpp) that induce the growth of the surrounding tissues.^{6–8} Apoptosis-induced proliferation has also been observed in hydra, where dying cells express Wnt that is required for cell division.⁹ A recent study showed that when injected into mice, irradiated mouse embryonic fibroblasts can induce sustained growth of feeder tumour cells.¹⁰ Specifically, this study shows that caspase 3, the executioner of apoptosis, stimulates prostaglandin E2 expression and growth of surviving tumour cells. Other studies also demonstrate that mice deficient for the p53 inhibitor, MDM2, develop intestinal hyperplasia due to the

activation of the canonical Wnt and EGFR pathways.¹¹ Together, these results suggest that apoptosis-induced proliferation is a fundamental and conserved process by which epithelial tissues recover and regenerate after injury, and that p53 has an active role in both apoptosis and compensatory growth in mice and in *Drosophila*.

The p53 protein is the product of a well-known tumour suppressor gene, *TP53*. It is mutated in more than 50% of human cancers. Initial studies of p53 functions have highlighted its key role as a stress-induced factor, particularly in response to DNA damage. The results from decades of studies coined p53 as the 'guardian of the genome', as it induces DNA repair, cell cycle arrest or apoptosis after exposure to genotoxic stress,^{12,13} thus preventing the sequential accumulation of genetic alterations that underpins progression towards neoplasia. However, p53 is present in many lower eukaryotes, including *Drosophila*, where cancer is not a prevalent biological phenomenon. This paradox leads many to postulate that the tumour suppression function of p53 in vertebrates has probably evolved for some hitherto unappreciated primordial regulatory functions.^{14–16} However, the exact nature of such primordial functions has remained elusive.

Until recently, TP53 was thought to be expressed as a single major transcript. This view was radically transformed in

¹Group Apoptosis and Neurogenetics, Laboratory of Molecular Biology of the Cell, CNRS UMR5239, Ecole Normale Supérieure de Lyon, UMS3444 Biosciences Lyon Gerland, Université de Lyon, Lyon, France; ²International Agency for Research on Cancer, Lyon, France; ³Department of Cell Biology, New York University School of Medicine, New York, NY, USA and ⁴Department of Surgery and Molecular Oncology, European Associated Laboratory University of Dundee/Inserm U858, Dundee, UK
*Corresponding author: B Mollereau, Group Apoptosis and Neurogenetics, Laboratory of Molecular Biology of the Cell, CNRS UMR5239, Ecole Normale Supérieure de Lyon, UMS3444 Biosciences Lyon Gerland, Université de Lyon, 69007 Lyon, France. Tel: +33 4 72 72 81 63; Fax: +33 4 72 72 86 74; E-mail: bertrand.mollereau@ens-lyon.fr

⁵These authors contributed equally to this work.

Keywords: *Drosophila*; apoptosis; regeneration; p53; reaper; hid
Received 21.9.11; revised 12.6.12; accepted 10.7.12; Edited by M Oren

the last 15 years by the discovery of two vertebrate p53 paralogs, *TP63* and *TP73*. These two genes encode several protein isoforms with diverse functions in neuronal development, morphogenesis, immune response and responses to specific stress.¹⁷ Subsequent to the discovery of the ΔN isoforms of p63 and p73, studies have revealed that p53 express up to 12 protein isoforms generated by alternative splicing sites, codon initiation sites and internal promoter.^{18,19}

In invertebrate animals, only one gene represents the *TP53* gene family. It resembles *TP53* more than it resembles *TP63* and *TP73*. In *Drosophila*, the *p53* gene structure is highly conserved compared to its mammalian homologue. *Drosophila p53* (*Dp53*) gene structure was recently reviewed; it contains two alternative promoters and encodes three possible protein isoforms, *Dp53*, *D Δ Np53* and *Dp53 Δ C*.¹⁹ Experimental evidence only confirms the presence of the full-length *Dp53* and *D Δ Np53*. Therefore, in this study, we focused on *Dp53* full-length isoform corresponding to the human full-length (TA) p53 that includes a full transactivation domain and *D Δ Np53*, a general counterpart of the N-terminal truncated human p53 forms. *D Δ Np53* is encoded by an mRNA transcribed from an internal promoter like the N-terminally truncated human $\Delta 133p53$, but unlike $\Delta 133p53$, it contains a truncated trans-activation domain followed by a complete DNA-binding domain and an oligomerization domain such as that found in the human $\Delta 40Np53$.¹⁸ These findings raise the possibility that *Dp53* and *D Δ Np53* are the respective functional homologues of the human *TAp53* and $\Delta 40/\Delta 133p53$.

D Δ Np53 was the first p53 isoform identified in *Drosophila* and was thought to be the only p53 isoform for several years; it was therefore initially named *Dp53* or *Dmp53* in earlier publications.^{20–22} The subsequent identification of a form of *Drosophila p53* matching the mammalian full-length (TA) p53 protein has led to a reassessment of this nomenclature, with the name *Dp53* to designate the full-length protein isoform and *D Δ Np53* for the N-terminal truncated form. Studies on fly primordial germ cells, imaginal discs and adult photoreceptor cells have highlighted the role of *D Δ Np53* in regulating apoptosis.^{21,23–26} *D Δ Np53* induces apoptosis through the Reaper-Hid-Grim (RHG) cascade. It was proposed that *D Δ Np53* directly activates the expression of *reaper* (*rpr*), whose protein product activates caspases by inhibiting *DIAP1* (*Drosophila* inhibitor of apoptosis protein).^{21,22} In addition to its apoptotic function, the *Dp53* locus (*Dp53* and/or *D Δ Np53*) regulates several biological functions, such as cell cycle, DNA repair, aging and apoptosis-induced proliferation.^{3,27–30}

Here we have investigated the role of *Dp53* and *D Δ Np53* in apoptosis and apoptosis-induced proliferation. We found that both isoforms were capable of activating apoptosis, but that each induced distinct RHG family members to inhibit *DIAP1*. Strikingly, we observed that *D Δ Np53* induced *wg* expression and enhanced proliferation in the wing imaginal disc, suggesting that *D Δ Np53* promotes apoptosis-induced proliferation. In contrast to *D Δ Np53*, *Dp53* did not induce *wg* expression in the absence of the endogenous *p53* gene. Thus, we propose that *D Δ Np53* is the main p53 isoform that regulates apoptosis-induced proliferation. The physiological consequences of these dual functions of p53 isoforms on apoptosis and apoptosis-induced proliferation are discussed.

Results

Dp53 and D Δ Np53 activate distinct RHG genes to induce apoptosis. To study the respective functions of *Dp53* and *D Δ Np53*, we undertook a gain-of-function approach. We generated *UAS-Dp53* and *UAS-D Δ Np53* *Drosophila* transgenic lines for tissue-specific expression using the UAS/GAL4 system. To eliminate expression level variations due to position effects,³¹ we targeted individual *UAS-Dp53* and *UAS-D Δ Np53* insertions to the same chosen genomic region using the site-specific $\phi C31$ integrase system. *Dp53* and *D Δ Np53* cDNAs were expressed in the wing imaginal disc using the *MS1096* driver that is specific to the wing pouch and hinge areas (Figure 1 and Supplementary Figure 1).³² We observed robust production of *Dp53* in wing imaginal discs using an anti-*Dp53* antibody that recognizes the DNA-binding domain common to the *Dp53* and *D Δ Np53* isoforms (Figures 1a–c). Similar levels of *Dp53* and *D Δ Np53* isoforms were detected by western blot analysis (Figure 1g). We found that both isoforms induced caspase activation in wing imaginal discs, indicating that their expression leads to apoptosis (Figures 1d–f and Supplementary Figure S2).

To investigate the mechanisms by which *Dp53* and *D Δ Np53* overexpression lead to apoptosis, we examined *rpr* and *hid* expression (Figure 2). We used a *rpr*^{XRE}-lacZ (*rprZ*) reporter, which carries a 2.2-kb genomic region necessary for *rpr* induction in response to irradiation.²⁵ Although *Dp53* induced robust *rprZ* activation, *D Δ Np53* led to only a weak *rprZ* response in the wing imaginal discs (Figures 2b, c and g). To confirm this result, we tested how both *Dp53* isoforms can activate p53RE-GFPnl, another *rpr* activity reporter, which contains a 150-bp *rpr* enhancer sequence embedding a consensus p53-binding site.¹⁵ We found *Dp53* induced greater levels of GFP than *D Δ Np53* (Figures 2e and f). Furthermore, we found that *Dp53* production (*en>Dp53*) led to the formation of blisters in the adult wings (Supplementary Figure S3). Although we currently do not know what p53-related biological process is responsible for the blister formation, we found that the incidence of wing blisters was significantly reduced in an *rpr* mutant (Supplementary Figure S3 and Table S1a). Because of the pupal lethality induced by the expression of *D Δ Np53* (*en>D Δ Np53*), we could not test whether *rpr* mutant reduces wing blisters in this condition. Instead, we showed that *rpr* mutant partially suppressed pupal lethality induced by *D Δ Np53* (*en>D Δ Np53*), suggesting that the *D Δ Np53*-mediated phenotype involves *rpr* (Supplementary Table S1b). Next, we examined *hid* induction by *Dp53* and *D Δ Np53* using an anti-Hid antibody.⁴ However, as both *Dp53* isoforms lead to rapid elimination of apoptotic cells (data not shown), *Hid* expression was hard to detect. To overcome this difficulty, we examined the induction of *Hid* expression by *Dp53* isoforms with the *engrailed* driver in *drone*-null wing discs, where the cells were kept 'undead' (Figures 2h–j). The *engrailed* driver is expressed in the posterior part of the wing imaginal disc in a clearly delineated domain (Supplementary Figure S1a). We observed much stronger *Hid* staining in the *engrailed* domain where *D Δ Np53* was overexpressed compared with *Dp53*. Together, these experiments support that *rpr* is a primary target during *Dp53*-mediated apoptosis and suggest that *Dp53* is responsible for

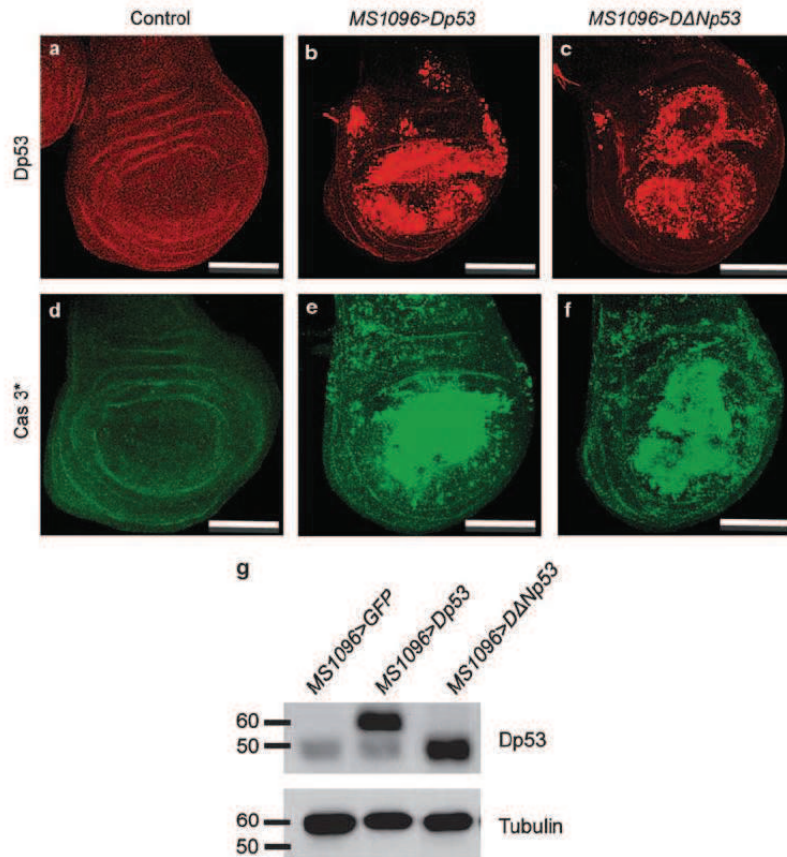


Figure 1 *Dp53* or *DΔNp53* expression induces caspase activation in wing imaginal discs. (a–f) Ectopic production of *Dp53* or *DΔNp53* using the *MS1096* driver. (a–c) *Dp53* and *DΔNp53* protein isoforms are detected by immunostaining with an anti-p53 antibody (25F4) directed against the common C terminus domain. (a) *MS1096>GFP* is used as a negative control. *Dp53* (b) and *DΔNp53* (c) are detected in the *MS1096* domain of expression. (d–f) Wing imaginal discs were stained using an anti-cleaved caspase 3 antibody (Cas 3*). Elevated levels of *Dp53* (*MS1096>Dp53* in e) or *DΔNp53* (*MS1096>DΔNp53* in f) induce strong caspase 3 staining in the *MS1096* domain. Caspase activation is not detected in control wing discs (*MS1096>LacZ* in d). (g) Western blot analysis of *Dp53* and *DΔNp53* in the wing imaginal discs using an anti-p53 antibody (C11) against the common C terminus domain. *MS1096>Dp53* and *MS1096>DΔNp53* show a band around 60 kDa and 50 kDa, respectively. The endogenous level of *DΔNp53* is detected in the wild-type control (*MS1096>GFP*). Tubulin is used as loading control. Scale bars are 100 μ m

damage-induced transcription of *rpr*. In contrast, *DΔNp53* is a poor activator of *rpr* and favors *hid*-mediated apoptosis.

***Dp53* and *DΔNp53* differentially regulate apoptosis-induced proliferation.** Johnston and colleagues⁷ have proposed that *Dp53* gene promotes the expression of mitogens, such as *Wg*, which is required for apoptosis-induced proliferation. However, the specific roles of the *Dp53* and *DΔNp53* isoforms in activating *Wg* have not been defined. To study the roles of *Dp53* and *DΔNp53* in apoptosis-induced proliferation, we examined *wg* expression and cell proliferation after *Dp53* or *DΔNp53* proteins were produced in the developing wing tissues (Figures 3 and 4). We first used the ‘undead cell’ model in which apoptosis is initiated by the expression of *Dp53* isoforms but its execution

is inhibited by expressing the inhibitor of caspase p35 (Figure 3). We found that *DΔNp53* induced strong and widespread *wg* expression associated with hyperproliferative tissue in a deformed wing disc (Figure 3d). In this context, we determined whether *wg* expression was induced in neighbouring unaffected cells, namely, in a cell-non-autonomous manner. We observed that *DΔNp53* induced *wg* expression both inside and outside of the *engrailed* domain of expression labelled with RFP (Figures 3d, d’ and d’'). This result indicates that *DΔNp53* induces *wg* expression both in a cell-autonomous and non-autonomous manner. In contrast to *DΔNp53*, when ectopically expressed, *Dp53* was only able to induce a moderate increase of *wg* that mainly resulted in a thickening of the *Wg* endogenous pattern of expression within the *engrailed* domain (Figure 3c). Moreover, *Wg*

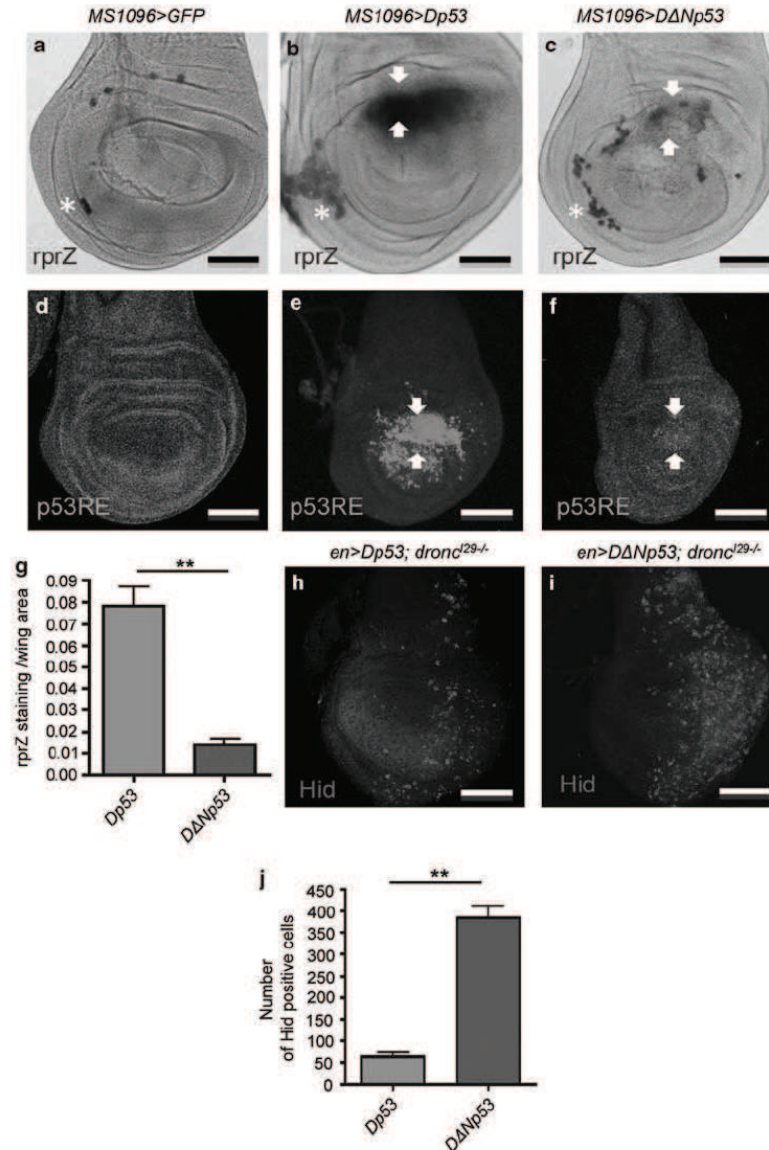


Figure 2 Dp53 and DΔNp53 use distinct RHG for apoptosis. (a–c) Wing imaginal discs carrying a $rpr^{XRE}/lacZ$ ($rprZ$) was stained for β -galactosidase activity. A strong induction of $rprZ$ is observed upon overproduction of Dp53 (MS1096>Dp53 in b; arrows). In contrast, weak $rprZ$ induction is observed upon overproduction of DΔNp53 (MS1096>DΔNp53 in c; arrows). No $rprZ$ induction is observed in the control disc (MS1096>GFP; a). White stars mark LacZ-positive phagocytes. (d–f) GFP fluorescence is observed in wing imaginal discs carrying $p53R-GFPnl$ s (p53 RE). Stronger GFP labelling is observed in Dp53 expression discs (e) compared with DΔNp53 expression discs (f), or in control wing discs (d). (g) Quantification of the $rprZ$ staining area relative to the total wing area. (h and i) Hid protein was visualized by immunostaining in 'undead cells' with an anti-Hid antibody in wing imaginal discs. Dp53 overproduction induces a mild hid expression (en>Dp53; dronc^{29-/-} in h). DΔNp53 induces a strong hid expression in the engrailed domain of expression (en>DΔNp53; dronc^{29-/-} in i). Scale bars are 100 μ m. (j) Quantification of Hid-positive cells per wing in h and i. * $P \leq 0.05$, ** $P \leq 0.01$, in Student's *t*-test

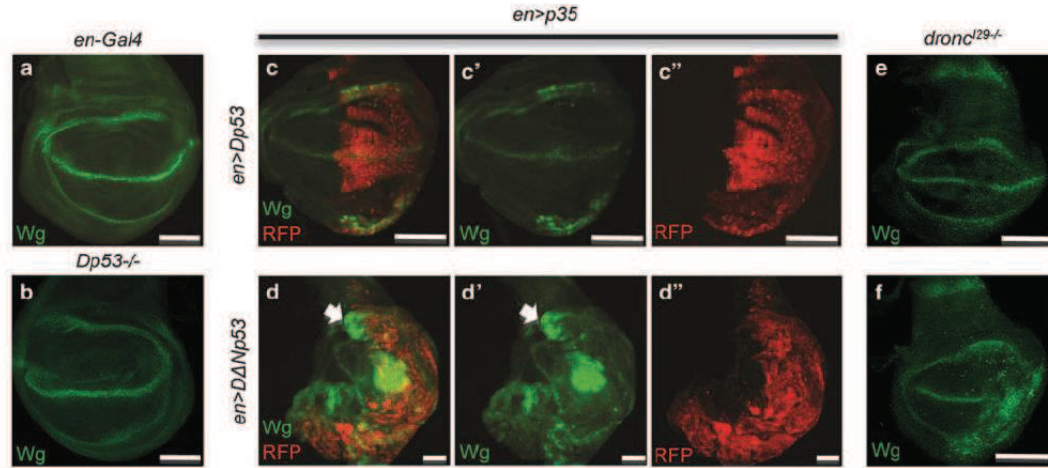


Figure 3 *D1Np53* induced *wg* expression in undead cells. (a–f) Wg protein was stained with an anti-Wg antibody (green). (a, b) The *wg* expression in wild-type (a) and in *p53*-null wing discs (b). (c and d) Double staining Wg (green) and RFP (red). Overproduction of *Dp53* or *DΔNp53* in wing imaginal discs expressing *p35* and RFP (*en>p35>RFP*). The *engrailed* domain expression is visualized by RFP. Wg (c' and d') and RFP (c'' and d'') single fluorescent channels are shown. (c, c' and c'') A mild induction of Wg is induced by *Dp53* overproduction (*en>Dp53*) resulting in broadening of the endogenous Wg expression pattern in the *engrailed* domain. (d, d' and d'') The overproduction of *DΔNp53* (*en>DΔNp53*) induces a strong and widespread induction of Wg inside and outside the *engrailed* domain of expression (arrow). (e and f) Overproduction of *Dp53* or *DΔNp53* in wing imaginal discs mutant for *dronc*^{29-/-}. (e) A mild induction of Wg is induced by *Dp53* overproduction (*en>Dp53; dronc*^{29-/-}) resulting in broadening of the endogenous Wg expression pattern in the *engrailed* domain. (f) The overproduction of *DΔNp53* (*en>DΔNp53; dronc*^{29-/-}) induces a strong and widespread induction of Wg. Scale bars are 100 μm

expression pattern was completely normal in *p53*-null wing disc, indicating that endogenous *p53* gene does not regulate *wg* expression (Figures 3a and b). Next, we examined whether the induction of *wg* by *Dp53* isoforms required *dronc*. We found that in *dronc* mutant wing discs, *DΔNp53* also induced stronger *wg* expression than *Dp53* (Figures 3e and f). From these results, we conclude that the regulation of *wg* expression by *Dp53* isoforms is *dronc* independent.

Next, we asked if the regulation of *wg* by *Dp53* isoforms can be detected in 'genuine' apoptotic cells. To achieve this goal, we used the strong MS1096 wing imaginal disc driver (Figure 4). As in the 'undead' cell model, we observed that *D1Np53* induced strong *wg* expression in 'genuine' apoptotic cells (Figure 4c). The increased level of *wg* expression was clearly detected in the dorsal part of the wing pouch region where the MS1096 is the strongest (Supplementary Figure S1b). The increased *wg* expression was associated with tissue accumulation and folding, suggesting hyperproliferation (Figures 4c and f). In contrast to *D1Np53*, *Dp53* expression did not alter the overall pattern of *wg* expression, but resulted in a thickening of the endogenous *wg* expression pattern (Figure 4b).

Next, we used a PCNA-EmGFP reporter that monitors E2f1 activity and EdU staining for cell proliferation.³³ We observed enhanced PCNA-EmGFP labelling in the presence of *DΔNp53*, indicating increase cell proliferation (Figure 4i). In contrast, PCNA-EmGFP was only weakly induced by *Dp53*, suggesting that *Dp53* induces little proliferation compared with *D1Np53* (Figures 4h and i). We also evaluated proliferation by EdU, a thymidine analogue that stains cells that have

transited to S phase (Figure 4j). Consistent with the PCNA-EmGFP assay result, the EdU staining revealed that *D1Np53* induces more proliferation than *Dp53*.

Drosophila p53 gene is proposed to act in a feedback loop to self-amplify and promote apoptosis-induced proliferation.^{7,34} Therefore, we wanted to examine how the endogenous *p53* gene contributes to the observed overexpression phenotype. We produced *DΔNp53* or *Dp53* in *p53*-null flies.³⁵ First, we found that elevated levels of *Dp53* or *DΔNp53* led to robust caspase activation, indicating that each isoform can induce apoptosis in the absence of the endogenous *p53* gene (Figures 5a and b). Next, we observed that *DΔNp53* retained the ability to increase *wg* expression in the *p53*-null flies (Figures 5d and d'). This suggests that *D1Np53* overexpression alone is sufficient to induce *wg* expression. In contrast, in *p53*-null wing discs, we observed that *Dp53* expression no longer induced any thickening of *wg* endogenous expression pattern. Rather, we observed a reduction of *wg* expression, which could be attributed to apoptosis of Wg-positive cells in this area (Figure 5c'). Together, these results show that *D1Np53*, but not *Dp53*, is the positive regulator of *wg* expression.

Discussion

The discovery of multiple *p53* isoforms raises the question of their functional specificity in the spectrum of *p53*-mediated biological responses. In *Drosophila*, as the first and only *p53* isoform identified in almost a decade, the truncated *DΔNp53* isoform was initially presumed responsible for all *p53* activities.

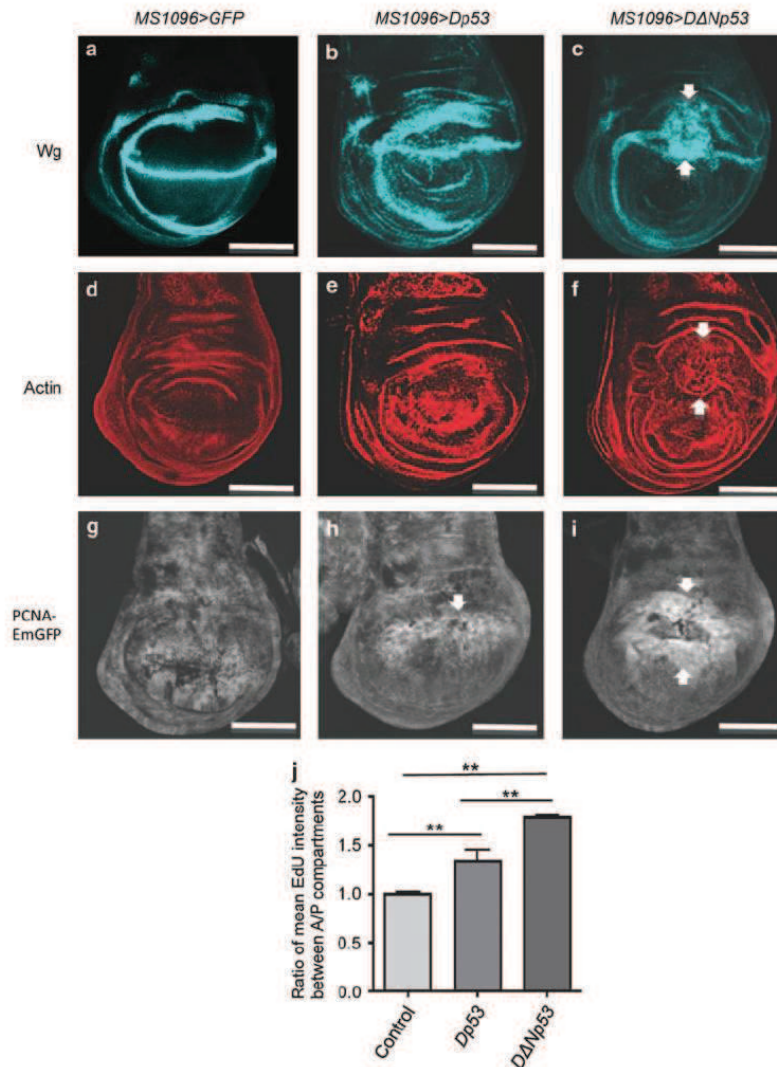


Figure 4 DΔNp53 induced *wg* expression and enhanced proliferation in wing imaginal discs. (a, d and g) Control wing imaginal discs (*MS1096>LacZ*). (b, e and h) Wing imaginal discs overproducing Dp53 (*MS1096>Dp53*). (c, f and i) Wing imaginal discs overproducing DΔNp53 (*MS1096>DΔNp53*). (a–c) Wg protein was stained with an anti-Wg antibody. (d–f) Actin was stained with phalloidin coupled with TRITC. (g–i) GFP fluorescence in wing imaginal discs carrying PCNA-EmGFP. (c) The overproduction of DΔNp53 leads to a strong increase of *wg* expression in the MS1096 domain. Increased *wg* expression by DΔNp53 is associated with tissue folding as visualized with actin staining (f) and with enhanced proliferation visualized with the PCNA-EmGFP reporter (i). Dp53 overproduction does not alter the overall Wg pattern but leads to an apparent thickening of the endogenous Wg domain (b). Dp53 overproduction does not induce tissue folding (e) and only induces some PCNA-EmGFP expression (h). Scale bars are 100 μm. (j) Quantification of EdU staining between the anterior and posterior compartments in control (*en>p35*) and wing discs overproducing Dp53 (*en>Dp53*) or DΔNp53 (*en>DΔNp53*). ***P* ≤ 0.01, in Student's *t* test

The identification of the full-length Dp53 isoform that contains a full N-terminal transactivation domain challenged this presumption. Here, using gain-of-function studies, we examined the role of these two isoforms in apoptosis and apoptosis-induced proliferation. We found that both Dp53

isoforms activate apoptosis but preferentially activate different DIAP antagonists (Rpr or Hid) for caspase activation (Figures 1, 2 and Supplementary Figure S2). We showed that DΔNp53 promotes *wg* expression and cell proliferation, independently of endogenous p53, whereas Dp53 is unable

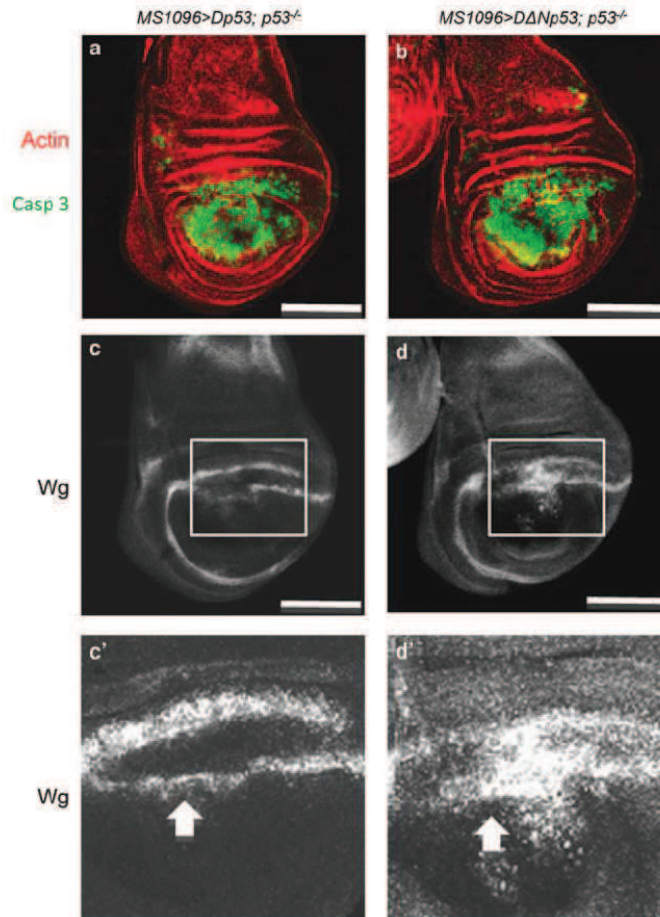


Figure 5 Dp53 and DΔNp53 differentially regulated *wg* expression. (a–d) Overexpression of Dp53 or DΔNp53 in *p53* mutant flies. Dp53 (a and c) (*MS1096>Dp53; p53^{-/-}*) and DΔNp53 (b and d) (*MS1096>DΔNp53; p53^{-/-}*) are expressed in *p53* mutant wing imaginal discs. (a and b) Overproduction of Dp53 or DΔNp53 leads to strong active caspase 3 staining (green). (c and c') The overproduction of Dp53 inhibits *wg* expression at the dorso-ventral boundary (arrow). (d and d') The overproduction of DΔNp53 induces *wg* expression (arrow). c' and d' are magnified views of the rectangles shown in c and d, respectively. Scale bars are 100 μm

to do so (Figures 3–5). We also found Dp53 to be primarily responsible for damage-induced transcriptional activation of *rpr*, whereas DΔNp53 is the *p53* isoform dedicated to promoting apoptosis-induced proliferation.

The landmark study of Abrams and colleagues²¹ showed that DΔNp53 binds a DNA damage response element in the *rpr* regulatory region, which is responsible for the induction of apoptosis in response to irradiation. Here we showed that in wing imaginal discs, Dp53 is a stronger inducer of *rpr* expression than DΔNp53 (Figure 2). Moreover, we showed that DΔNp53 strongly induced *hid* expression, whereas Dp53 was only a weak inducer. Together, these observations suggest that the transcriptional competence of DΔNp53 differs from that of Dp53, and is consistent with a previous

study showing that *hid* is transcriptionally induced by DΔNp53 in eye and wing imaginal discs.^{25,28,34} These results also suggest that some intrinsic ability to distinguish its activity for *rpr* and *hid* expressions is embedded in the N-terminus of the full length Dp53. Therefore, we propose that Dp53 is responsible for the damage-mediated activation of *rpr* for apoptosis, whereas DΔNp53 promotes apoptosis by inducing expression of *hid*. The physiological consequences of this functional segregation in apoptosis regulation by *p53* isoforms remain to be determined.

Previous works have shown that apoptotic cells secrete morphogens that induce proliferation of surrounding cells.^{4,36,37} Although more clearly detected in 'undead cells', mitogen gene expression and extra proliferation have also



been detected in genuine apoptotic cells.^{4,36,38} It was proposed that the initiator caspase Dronc leads to *Dp53* expression, which in turn activates mitogen gene expression,^{7,34} but the specific roles of *Dp53* and *DΔNp53* remain to be established. Here we showed that *DΔNp53* is a potent inducer of *wg* expression both in the 'undead cell' and genuine apoptotic cell models (Figures 3–5). Specifically, we showed that *DΔNp53* induced *wg* expression independently of *dronc* (Figure 3f). This indicates that *DΔNp53* acts downstream of the apoptotic pathway to induce proliferation via the expression of *wg*. Thus, like JNK,³⁹ *DΔNp53* promotes proliferation independently of the apoptotic cascade. Further analysis will be required to determine the relationship between JNK and p53 isoforms in the induction of proliferation.

Wells *et al.*⁷ proposed that in the apoptosis-induced proliferation process, there is a feedback loop that activates *wg* expression in 'undead cells' via Dronc and *Dp53*. Our results are consistent with such a feedback mechanism in which *Dp53* and *DΔNp53* induce apoptosis via *rpr* and *hid*, which in turn amplifies *DΔNp53* via Dronc to promote *wg* expression. Our results also suggest that the feedback loop not only functions in 'undead cells' but also in genuine apoptotic cells. Together, we propose that p53 isoforms act both upstream and downstream of the apoptotic pathway to promote *wg* expression and proliferation.

Our results show that *DΔNp53* is a potent inducer of *wg* expression in both wild-type and *p53*-null wing discs. In contrast, *Dp53* only weakly increased *wg* expression in wild-type but not in *p53*-null flies (Figures 3–5). Therefore, the weak induction of *wg* expression by *Dp53* in wild-type disc is likely dependent on the endogenous *p53* gene. Further investigations will be required to determine if *DΔNp53* is the only p53 isoform regulating *wg* expression or if another isoform such as *Dp53ΔC* or the one encoded by the recently annotated p53-RD transcript (Flybase) contribute as well to the regulation of *wg* expression.

One of the most intensely debated questions regarding *Drosophila* ΔNp53 isoforms is whether they have their own biological activity or exert a dominant negative activity on p53.^{40–42} The fact that *DΔNp53* induced *Wg* expression independently of endogenous *p53* gene indicates that *DΔNp53* does not require *p53* for this function. In vertebrate studies, zebrafish Δ113p53 and human Δ133p53 do not act exclusively in a dominant-negative manner toward p53 but differentially regulate p53 target gene expression to modulate p53 function.^{41,42} Similarly, our results show that *Drosophila* p53 isoforms have the capacity to use distinct targets to orchestrate their biological functions; we have shown that *Dp53* promotes *rpr* expression, whereas *DΔNp53* activates *Hid* and *Wg* expression in wing epithelium (Figures 2–5). Overall, we propose that balancing apoptosis and apoptosis-induced proliferation may represent one primordial function of the TP53 gene family, and that this function requires the expression of *Dp53* and *DΔNp53* isoforms in a tightly controlled manner. In vertebrate, this primordial functional capacity may be differently exploited by TP53, TP63 and TP73 to regulate specific aspects of death/proliferation in the equilibrium, depending upon tissues and physiological contexts.

Material and Methods

UAS-Dp53 and UAS-DΔNp53 transgenic lines. *Dp53* and *DΔNp53* cDNAs were cloned (Kpn1/Xba1) into a pUAST-w + attB transgenic fly vector. Best Gene, Inc. (Chino Hills, CA, USA) generated transgenic lines using φC31 integrase-mediated transgenesis. Vector DNA was injected in embryos carrying attP docking sites (strain 9736 at 53B2 and strain 9750 at 65B2). *W*⁺ embryos were selected and for establishing stable transgenic fly stocks.

Fly stocks. The following transgenic and mutant fly stocks were used: *MS1096-Gal4*, *en-Gal4*, *uas-lacZ* and *uas-RFP* (Bloomington stock); *uas-GFP*⁴³ *rpr*^{XRE}-*lacZ* (*rprZ*) and *dronc*²⁹ (kind gifts from A. Bergmann²⁵); *p53R-GFP*¹⁵ (*p53* RE; a generous gift from J. Abrams¹⁵), *rpr87*⁴⁴ deficiency (3L)H99 (*Df*(3L)H99, referred to as H99⁴⁵), *PCNA-EmGFP*³³ and *p53-null* (*p53* [5A-1-4]).³⁵ The following genetic combinations were used to express transgenes in wing imaginal discs: (1) *MS1096-Gal4, uas-GFP* (*MS1096 > GFP*), (2) *MS1096-Gal4, uas-Dp53* (*MS1096 > Dp53*), (3) *MS1096-Gal4, uas-DΔNp53* (*MS1096 > DΔNp53*), (4) *en-Gal4, uas-Dp53* (*en > Dp53*), and (5) *en-Gal4, uas-DΔNp53* (*en > DΔNp53*). Flies were raised under standard conditions at 25 °C.

Additional information can be found in the supplemental information.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. This work was supported by grant from the CNRS (ATIP) and Ligue contre le cancer (Comités de Savoie and Puy-de-Dôme) to BM and NIH R01GM079425 to HDR. Bench fees were funded by a Marie Curie Fellowship ERG (PERG03-GA-2008-230812) to MLDD. FN was supported by a fellowship of the Association Française contre les Myopathies. Work on p53 isoforms by PH and his team is supported by a grant from the French National Cancer Institute (INCa). This work was made possible by the DROSO-TOOLS and PLATIM facilities of the UMS3444, Biosciences, Lyon, France. We thank Virginie Marcel for critical reading of the manuscript. We also thank Carmen Garrido for technical help, and our colleagues and Bloomington centre for fly stocks and reagents.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)

7.3 Appendix III: Pharmacological rescue of p53 in cancer cells: the soloist meets the PRIMA donna

Cell Cycle 10:6, 1-6; March 15, 2011; © 2011 Landes Bioscience

CELL CYCLE NEWS & VIEWS

Cell Cycle News & Views

Pharmacological rescue of p53 in cancer cells: The soloist meets the PRIMA donna

Comment on: Bao W, et al. Cell Cycle 2011; 10:301–307

Hind Hafi and Pierre Hainaut*; International Agency for Research on Cancer; Lyon, France; *Email: hainaut@iarc.fr; DOI: 10.4161/cc.10.6.15120

In the realm of p53 research, “refolding” p53 has long been the equivalent of the alchemist’s quest of turning lead into gold. Missense mutations in *TP53* are common in human cancers, identifying p53 as a soloist in the orchestra of cell growth control. Quite often, these mutations cause the accumulation of a mutant, misfolded protein in the nucleus. This makes it an attractive target for therapeutic drugs since, in theory, re-folding of this accumulated p53 may result in high levels of active p53 sending cancer cells to apoptosis. Thus, over the past 10 years, the chase has been on for small molecules that rescue mutant p53 to an active conformational state. This is by no means a simple task. In contrast to drugs inhibiting activated oncogenes (which often target enzymatic activities), drugs re-activating suppressors should somehow bring back together different domains of a misfolded protein. The identification of PRIMA-1 by Wiman and colleagues in 2002 has represented a breakthrough in this direction.¹ In a recent study published in *Cell Cycle*, Strömblad and collaborators now demonstrate that this drug also rescues inactive, wild-type p53 in melanoma cells, extending the potential therapeutic applications of p53-refolding pharmacological strategies.²

PRIMA stands for “P53-Reactivation and Induction of Massive Apoptosis.” Faithful to

its name, it has been identified in a screen for drugs that induce apoptosis in cells expressing mutant p53 but not in their p53-null counterparts. PRIMA-1, a quinuclidine with a molecular mass of 185, rescues DNA binding activity in vitro for a large panel of mutant p53 proteins. Its methylated analog, PRIMA-1^{Met}, has even greater potency, leading to its development as a candidate therapeutic drug under the code name APR246. The therapeutic concept is that PRIMA-1^{Met} may selectively rescue mutant p53 and induce apoptosis in cancer cells, leaving wild-type p53 in normal cells mostly unaffected. However, so far there is little information on how PRIMA-1^{Met} affects p53 in cancer cells with no mutation. Indeed, in some cancer cells, p53 protein activity may be disrupted by other mechanisms with functional consequences equivalent to mutation. How such a functionally disrupted p53 may react to PRIMA-1^{Met} is unknown. Malignant melanoma is a typical example of aggressive cancer in which *TP53* is rarely mutated. In 2004, Bao and Strömblad showed that in melanoma cells, inactivation of wild-type p53 was mediated through α V integrin signaling.³ In their recent study, Strömblad and collaborators have used a three-dimensional collagen gel to culture primary melanoma cells. They show that PRIMA-1^{Met} induces

apoptosis through p53-mediated transactivation of the pro-apoptotic genes *APAF-1* and *PUMA*. Furthermore, treatment of mice carrying xenografts of human melanoma cells with PRIMA-1^{Met} (100 mg/kg) strongly suppresses tumor growth, an effect which is annihilated by silencing p53. Together, these observations provide evidence that PRIMA-1^{Met} can rescue inactive wild-type p53 in melanoma as well as mutant p53 in a wide panel of other cancers.

At first sight, that PRIMA-1^{Met} activates wild-type p53 appears to contradict claims that the drug is specific for mutant p53. However, there are good biochemical reasons to propose that the drug operates as an all-around rescuer of inactive p53, independently of its mutation status. In 2010, Lambert et al. showed that PRIMA-1 (the parent drug of PRIMA-1^{Met}) reactivated mutant p53 by binding to its core domain.⁴ Specifically, they showed that upon entering cells the drug was decomposed to form a metabolite, methylene quinuclidinone (MQ), that binds reactive thiols in mutant p53 and prevents them from forming inter- and intramolecular disulfide bonds. Indeed, p53 conformation is known to be exquisitely sensitive to oxidation-reduction.⁵ Correct p53 folding is dependent upon very precise redox equilibrium in vitro and in vivo. When binding thiols, PRIMA-1 may prevent misfolded

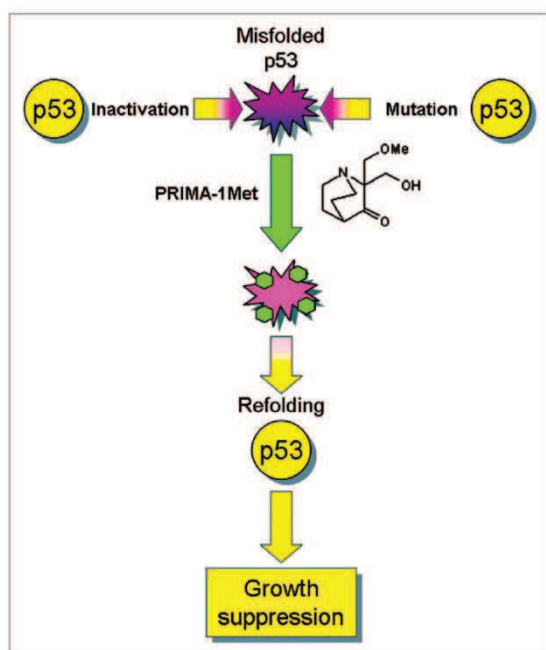


Figure 1. Rescuing misfolded p53 with PRIMA-1^{Met}. In cancer cells, the p53 protein is often inactivated by misfolding due to missense mutation in the DNA binding domain (in most common cancers) or to alternative mechanisms of inactivation that may lead to the accumulation of an inactive protein (e.g., in melanoma). By binding misfolded p53, PRIMA-1^{Met} prevents the protein from becoming cross-linked by disulfides and protects its capacity to fold into an active form capable of exerting suppressive effects.

p53 from forming aggregates, thus preserving its capacity to spontaneously fold into wild-type (or quasi-wild-type) conformation with DNA binding activity (Fig. 1). The fact that PRIMA-1^{Met} activates wild-type p53 in melanoma suggests that in these cells, p53 is in an unfolded state that resembles mutant p53, perhaps as the result of a perturbation of redox metabolism. This mechanism may explain the low prevalence of *TP53* mutation in melanoma: there would be no need to mutate *TP53*, since the inactivation job is carried out through another, yet unknown, pathway. In the meantime, the results of Bao et al. suggest that PRIMA-1^{Met} may exert therapeutic effects against cancers which retain inactive, wild-type p53, opening new grounds for the therapeutic usage of this drug in effective, low-toxicity treatment strategies targeting p53.

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ABSTRACT:

The p53 tumour suppressor protein has a highly complex pattern of regulation at transcriptional and post-translational levels. The discovery of p53 isoforms has added another layer of complexity to the mechanisms that regulate p53 functions. Indeed, p53 is expressed as 12 isoforms that differ in their N- and C-terminus due to alternative splicing, promoter or codon initiation usage. So far, there is limited understanding of the patterns of expression and of the functions of each of these isoforms.

In this Thesis, we have focused on the two major p53 N-terminal isoforms, $\Delta 40p53$ and $\Delta 133p53$. We have analysed their patterns of interactions with the full-length p53 and we have investigated whether their expression could be deregulated in melanoma, a cancer type in which *TP53* mutations are rare. Our results show that $\Delta 40p53$ can modulate p53 function with a bi-phasic effect, acting as a repressor or activator of p53 to control its levels and activity. Moreover, we demonstrate that the internal P2 promoter produces $\Delta 133p53$ and is regulated by p53 in response to genotoxic stress, identifying a novel auto-regulatory loop by which p53 may control the expression of an isoform acting as an inhibitor of p53 activities. Finally, we show that mRNAs encoding N-terminal isoforms are often over-expressed in highly metastatic melanoma when compared to non-invasive forms, suggesting that N-terminal isoforms contribute to functionally inactivate p53.

Thus, we propose that $\Delta 40p53$ and $\Delta 133p53$ modulate p53 functions within dynamic fluctuations of a protein network. Hence, p53 isoforms may have a major role in basal p53 activities as well as in the functional inactivation of p53 in cancer cells.

TITRE en français :

Isoformes du domaine N-terminal du suppresseur de tumeur p53: effets sur l'activité transcriptionnelle de p53 et expression dans les mélanomes cutanés

RESUME en français :

La protéine suppresseur de tumeur p53 est soumise à de complexes régulations transcriptionnelles et post-traductionnelles. La découverte d'isoformes de p53 a introduit un degré de complexité supplémentaire aux mécanismes de régulation des fonctions de p53. On dénombre à ce jour douze isoformes qui diffèrent de p53 dans leurs domaines N- et C-terminal. Cependant, les modes d'expression et de fonction de ces isoformes restent à être clarifiés.

Dans cette thèse, nous nous sommes intéressés aux deux isoformes $\Delta 40p53$ et $\Delta 133p53$, en analysant leur interaction avec p53 et en mesurant leur expression dans les mélanomes, un type de cancer où p53 est très rarement mutée. Nous montrons que $\Delta 40p53$ peut moduler l'activité de p53 avec un effet bi-phasique, tantôt activateur ou répresseur du niveau d'expression et des fonctions de p53. $\Delta 133p53$ est produite par un promoteur P2 localisé dans le gène *TP53*. Nous avons montré qu'en réponse à un stress génotoxique, l'expression de $\Delta 133p53$ est régulée par p53, qui se lie au promoteur P2. Ceci suggère une boucle d'auto-régulation par p53, qui est capable de contrôler l'expression d'une isoforme inhibant ses propres fonctions. Enfin, les isoformes $\Delta 40p53$ et $\Delta 133p53$ sont surexprimées dans les tumeurs métastatiques de mélanomes comparées aux tumeurs non-invasives, suggérant à ces isoformes un rôle dans l'inactivation de p53 dans les cancers.

Ainsi, $\Delta 40p53$ et $\Delta 133p53$ interagissent avec p53 de façon complexe, avec des effets plus contrastés que la simple inhibition de l'activité suppressive de p53. Les isoformes de p53 jouent ainsi un rôle majeur dans les activités basales de p53, ainsi que dans l'inactivation fonctionnelle de p53 dans les cancers.

DISCIPLINE :

Oncologie et Biologie moléculaire

MOTS-CLES :

protéine suppresseur de tumeur p53, isoformes, régulation transcriptionnelle, inactivation de p53, mélanomes

INTITULE ET ADRESSE DE L'U.F.R. OU DU LABORATOIRE :

Centre International de Recherche sur le Cancer – Groupe de Mécanismes Moléculaires et Biomarqueurs
150 cours Albert Thomas 69372 Lyon Cedex 08